Selective stimulation of translation of leaderless mRNA by initiation factor 2: evolutionary implications for translation

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Translation initiation in bacteria involves a stochastic binding mechanism in which the 30S ribosomal subunit first binds either to mRNA or to initiator tRNA, fMet-tRNA^{Met}. Leaderless λ cI mRNA did not form a binary complex with 30S ribosomes, which argues against the view that ribosomal recruitment signals other than a 5'-terminal start codon are essential for translation initiation of these mRNAs. We show that, in *Escherichia coli*, translation initiation factor 2 (IF2) selectively stimulates translation of λ cI mRNA *in vivo* and *in vitro*. These experiments suggest that the start codon of leaderless mRNAs is recognized by a 30S–fMet-tRNA^{Met}–IF2 complex, an intermediate equivalent to that obligatorily formed during translation initiation in eukaryotes. We further show that leaderless λ cI mRNA is faithfully translated *in vitro* in both archaeobacterial and eukaryotic translation systems. This suggests that translation of leaderless mRNAs reflects a fundamental capability of the translational apparatus of all three domains of life and lends support to the hypothesis that the translation initiation pathway is universally conserved.

Keywords: evolution/IF2/initiator tRNA/leaderless mRNA/translation initiation

Introduction

Formation of the bacterial translation initiation complex involves interactions between the 30S subunit, fMet-tRNA^{Met} and mRNA. Three initiation factors, IF1, IF2 and IF3, affect the kinetics of formation of the ternary complex by promoting selection of the correct start codon of the mRNA (for reviews see Gold, 1988; Gualerzi and Pon, 1990; Gualerzi *et al.*, 2000).

IF2 selects fMet-tRNA^{Met} by virtue of the formylated α -amino group (Hartz *et al.*, 1989; RajBhandary and Chow, 1995; Schmitt *et al.*, 1996), and stimulates its positioning in the ribosomal P-site by accelerating the codon–anticodon interaction (Pon *et al.*, 1985; Gualerzi and Wintermeyer, 1986; La Teana *et al.*, 1996).

IF1 occupies the ribosomal A-site (Moazed *et al.*, 1995); according to the molecular mimicry hypothesis

(Brock *et al.*, 1998) it may, with IF2, form a complex that mimics the aminoacyl-tRNA–EF-Tu–GTP complex. Alone or within this complex, IF1 could occlude access of the latter ternary complex to the ribosome until the initiation steps are completed.

IF3 is known to act as a fidelity factor in selection of the initiator tRNA by preventing binding of tRNAs other than initiator tRNA to the ribosomal P-site and discriminating against unusual start codons, such as AUU (Brombach and Pon, 1987; Butler *et al.*, 1987; Hartz *et al.*, 1989; La Teana *et al.*, 1993; Sacerdot *et al.*, 1996; Sussman *et al.*, 1996). These IF3 activities are probably the result of a conformational change imposed on the 30S subunit (Pon and Gualerzi, 1974; de Cock *et al.*, 1999).

Most of our knowledge about the mechanism of translation initiation stems from work carried out on canonical mRNAs comprising a 5' untranslated region (UTR), which generally contains a Shine–Dalgarno (SD) sequence. Nevertheless, leaderless mRNAs starting directly with a 5'-terminal AUG occur frequently in bacteria and other organisms (Janssen, 1993); it is not known whether the sequence of events characterizing translation initiation of 'leadered' mRNAs also applies to leaderless mRNAs. Several lines of evidence indicate that the initiation codon is the only constant, necessary and sufficient element of the translation initiation region (TIR) of both 'leadered' and leaderless mRNAs (Wu and Janssen, 1996, 1997). In fact, the 5'-UTR, including the SD sequence, is not essential for translation of canonical mRNAs (Calogero *et al.*, 1988; Melançon *et al.*, 1990), whereas the 5'-terminal start codon AUG is the only essential recognition element allowing translation of leaderless mRNAs (Wu and Janssen, 1996; Winzeler and Shapiro, 1997; van Etten and Janssen, 1998). We have recently shown that IF3 discriminates against 5'-terminal start codons and that high levels of this factor decrease the translational efficiency of a leaderless *tetR*–*lacZ* construct *in vivo* (Tedin *et al.*, 1999). We interpreted these results as showing that, in the absence of the SD–anti-SD interaction, the ternary complex between leaderless mRNA, fMet-tRNA^{Met} and the 30S subunit cannot withstand the destabilization caused by IF3 (Moll *et al.*, 1998; Tedin *et al.*, 1999).

In the present study, we have examined the effect of IF2 on the recruitment of ribosomes to leaderless mRNAs. Our results demonstrate that ternary complex formation at 5'-terminal AUGs is stimulated by IF2 and that, in the presence of elevated concentrations of IF2, a leaderless mRNA can efficiently compete for translation with an mRNA containing a canonical ribosome binding site (*rbs*). Our results also suggest that the selection of the start codon of leaderless mRNAs is accomplished by ribosomes carrying the initiator tRNA bound in their P-site in the presence of IF2, through a mechanism resembling the

cl34SD mRNA: 5'- GUCUAGAAAUAUUUUGUUUAACUUUAAGAAGGAGAUUAUACAU AUGAGCACAAAAAGAAACCAUUAACACAAGAGC -3'

SD Met

-40 -20 +1 +20

cl34 mRNA: 5'- AUGAGCACAAAAAGAAACCAUUAACACAAGAGC -3'

Met

+1 +20

4102

Fig. 1. Rate of relative toeprints obtained with leaderless *cI* mRNA (A), *cISD* mRNA (B) and *ompA* mRNA (C) in the presence (filled symbols) and absence (open symbols) of IF2. The kinetic toeprinting reactions contained 2 pmol of 30S subunits, 5 pmol of IF2 (when present), 0.8 pmol of fMet-tRNA^{Met} and 1 mM GTP, and were pre-incubated for 20 s before the mRNA(s) was added for the times (given in seconds) indicated. The toeprint reaction was then started with MMLV as described in Materials and methods.

‘leadered’ as well as on leaderless mRNAs. However, since a 30S–initiator tRNA intermediary complex seemed to be instrumental for recognition of the start codon of a leaderless mRNA (see Table I), we expected the IF2 effect to be more pronounced for leaderless mRNAs. Ternary complex formation in the presence of IF2 was studied by kinetic toeprinting assays on leaderless λ *cI* mRNA, on *cISD* mRNA containing the 5′-terminal extension comprising the translational initiation region derived from phage T7 gene 10 (see Table I), and on *Escherichia coli* *ompA* mRNA containing a canonical *rbs*. Briefly, since a

30S subunit–initiator tRNA complex bound at the *rbs* can stop the progress of a downstream primed reverse transcriptase, toeprinting experiments are suited to test the efficiency of ternary complex formation at a given start codon (Hartz *et al.*, 1988). In the experiment shown in Figure 1, fMet-tRNA^{Met} was incubated for 20 s with a 2.5-fold stoichiometric excess of 30S subunits, and, when present, with IF2. Then, either *cI*, *cISD* or *ompA* mRNA was added, the incubation was continued for various lengths of time (see Figure 1) and the samples were subjected to toeprinting analysis. Figure 1 shows the increase in the relative toeprints [toeprint signal/(read-through signal + toeprint signal)] obtained for either mRNA in the presence or absence of IF2 plotted against incubation time. The relative toeprint signal obtained at 10 s was set to 1. The presence of IF2 resulted in an increase in ternary complex formation with both ‘leadered’ and leaderless mRNAs. However, the increase in ternary complex formation was more pronounced for leaderless mRNA. The presence of IF2 stimulated ternary complex formation on the leaderless mRNA ~2.1-fold, whereas only 1.5- and 1.7-fold stimulation was observed for the *cISD* and *ompA* mRNA, respectively (Figure 1).

The competition of leaderless λ *cI* mRNA with *ompA* mRNA for ribosomes in an *in vitro* translation system depends on IF2

We repeatedly found using *in vitro* translation assays that leaderless *cI* mRNA cannot efficiently compete with SD-containing ‘leadered’ mRNAs for a limiting amount of ribosomes (data not shown). In the following *in vitro* experiments we used *ompA* mRNA containing a canonical *rbs* rather than *cISD* mRNA as a control because the *ompA* product, outer membrane protein A (OmpA), has an electrophoretic mobility different from that of CI protein. To quantify the effect of IF2, a defined *E. coli* *in vitro* translation system was utilized in which either *cI* mRNA (Figure 2A and B) or *ompA* mRNA (Figure 2C and D) was translated in the presence of increasing molar IF2:ribosome ratios. The increase in this ratio resulted in a concomitant increase in *cI* mRNA translation. Relative to the translation rate in the absence of exogenously added IF2, the increase was ~4.5-fold at an IF2:ribosome molar ratio of 1:1 (Figure 2B). Under the same conditions, only 1.4-fold stimulation of *ompA* mRNA translation was observed (Figure 2C and D). In addition, we measured the rate of CI and OmpA synthesis as a function of increasing IF2:ribosome molar ratios using an *in vitro* translation system programmed with both *cI* and *ompA* mRNAs. When all three IFs were present at an IF:ribosome molar ratio of 0.2, translation of *cI* mRNA was hardly detected (Figure 2E) but, in contrast to that of *ompA* mRNA, translation of *cI* mRNA increased steadily with an increase in the IF2:ribosome ratio and reached a plateau when IF2 was in a 2.5-fold molar excess over ribosomes (Figure 2E and F). The maximum level of *cI* mRNA translation corresponded to an ~5-fold stimulation over the translation rate obtained at an IF:ribosome ratio of 0.2 (Figure 2F). These translation competition assays demonstrated that the translational efficiency of the leaderless mRNA is selectively increased by IF2.

Fig. 2. Selective stimulation of CI synthesis by IF2. (A–D) *In vitro* translation of equimolar amounts of *cI* and *ompA* mRNA, respectively, with an *E. coli* S100 extract as described in Materials and methods. The different ratios of IF2 to added 70S ribosomes are indicated above the panels. The ratio of IF1 or IF3 to 70S was 0.2:1 in all reactions. The samples were loaded on a 12% SDS–polyacrylamide gel and the rate of CI and OmpA synthesis at different molar ratios of IF2 to 70S ribosomes was calculated by ImageQuant analysis (B and D). In the absence of exogenously added IF2 (A and C, lane 1), the CI and OmpA synthesis rate was set to 1. (E and F) *In vitro* translation competition assay with *cI* and *ompA* mRNA at different molar IF2:70S ribosome ratios. The conditions were as in (A–D), except that the *in vitro* translation system was programmed with equimolar amounts of the two mRNAs. The CI and OmpA synthesis rate is set to 1 at a 0.2:1 molar ratio of IF1, IF2 or IF3 to ribosomes (E, lane 2).

Influence of IF2 on the competition for 30S subunits between a 5′-terminal AUG and an internal AUG preceded by a canonical SD sequence

To study further the influence of IF2 on the choice of start codon by ribosomes (i.e. 5′-terminal versus internal), a kinetic toeprinting experiment was performed on *ompA*Δ117 mRNA, which contains a 5′-terminal AUG codon and a downstream AUG preceded by the canonical SD sequence of *ompA* mRNA (Figure 3A). The ribosomal choice of either AUG triplet as a start codon is mutually exclusive on this mRNA (Moll *et al.*, 1998). The 30S subunits were pre-incubated for 20 s in a 2.5-fold stoichiometric excess over fMet-tRNA^{fMet} and, when present, of IF2. Then, *ompA*Δ117 mRNA was added and incubation was continued for various lengths of time (see Figure 3B). Finally, the samples were subjected to toeprinting analysis. The electrophoretic pattern of the products obtained by reverse transcription (Figure 3B) and their quantification (Figure 3C) showed that, in the absence of IF2, the ternary complex readily formed at the internal AUG codon (AUGi) and increased steadily with incubation time. In contrast, after 30 s incubation, hardly any toeprint signal was visible for the 5′-terminal AUG (AUG1), and even after 80 s the intensity of the signal for AUG1 was only 20% of that obtained for AUGi. However, a different picture was observed in the presence of IF2. Under these conditions, the 5′-terminal AUG1 was able to compete efficiently with AUGi, its toeprint signal being ~40% of that for AUGi at 20 s and reaching almost the same intensity after 80 s. These data demonstrated that IF2 shifted the 30S subunits to form a ternary complex at AUG1 at the expense of AUGi. Taken together with the

results shown in Figure 2E and F, these data support the view that, in the presence of IF2, a 5′-terminal AUG can efficiently compete for 30S subunits with a canonical initiation site.

Increased intracellular IF2 concentrations stimulate translation of a leaderless *ci-lacZ* mRNA

We next asked whether an artificially increased intracellular concentration of IF2 would result in a selective advantage for the translation of a leaderless mRNA *in vivo*. *Escherichia coli* strain UB89-1 harboring pKTplacCI, a plasmid encoding a leaderless *ci-lacZ* gene, was co-transformed with *pinfB*, which carries the *infB* (IF2) gene under the control of the λ p_L promoter. As shown in Figure 4A and C, upon induction of *infB* overexpression, the rate of translation of the *ci-lacZ* gene increased ~2.5-fold above the basal level. In the control strain transformed with the parental plasmid pLc2833, the *ci-lacZ* gene was expressed with approximately the same efficiency at 28 and 42°C. In contrast, the expression of *E. coli ompA* mRNA (Figure 4B) and other control mRNAs (not shown) that contained a canonical *rbs* did not significantly increase with higher concentrations of IF2.

In vitro translation of λ *ci* and *E. coli ompA* mRNA in systems derived from *Sulfolobus solfataricus* and rabbit reticulocytes

The results presented above strongly support the view that, in *E. coli*, a leaderless mRNA is recognized by a 30S–fMet-tRNA^{fMet} complex formed in the presence of IF2, through a mechanism resembling the obligatory translation initiation pathway typical of eukaryotes. It is interesting to note that leaderless mRNAs are quite

Fig. 3. Selection of the 5'-terminal and the internal AUG on *ompA* Δ 117 mRNA in the presence of IF2. **(A)** Depiction of the *ompA* Δ 117 mRNA. The start codon(s) and the authentic SD sequence of *ompA* mRNA are indicated by bars. For *ompA* Δ 117, two toeprint signals are obtained, which result from formation of a ternary complex over AUG1 (nucleotide -20 to -18) and AUGi (nucleotide 1-3), which represents the authentic start codon of *ompA* mRNA. The positions of the toeprint signals are indicated by filled (AUG1) and open circles (AUGi). **(B)** Kinetic toeprints on *ompA* Δ 117 mRNA in the presence and absence of IF2. The final concentration of 30S subunits, IF2, fMet-tRNA^{Met} and *ompA* Δ 117 mRNA was 0.5, 1, 0.2 and 0.005 pmol/ μ l, respectively. Lane 1, primer extension in the absence of 30S subunits and fMet-tRNA^{Met}. Lanes 2-6, kinetic toeprint analysis in the absence of IF2. Lanes 7-11, kinetic toeprint analysis in the presence of IF2. 30S subunits, fMet-tRNA^{Met}, IF2 and mRNA were incubated for different times (given in seconds) as indicated at the top. Lane 12, G sequencing reaction. The toeprinting signals obtained for AUG1 and AUGi are indicated by a filled and open circle, respectively. **(C)** Relative toeprints on *ompA* Δ 117 mRNA for AUG1 and AUGi. The relative toeprints at either start codon obtained in the absence of IF2 were normalized to that of AUG1 and AUGi obtained in the presence of IF2 after 80 s of incubation.

common in archaeobacteria (Bult *et al.*, 1996; Sensen *et al.*, 1996) and occur in the lower eukaryote *Giardia lamblia* (Adam, 1991). To investigate whether the capability to translate leaderless mRNAs is a fundamental property of all translation systems, we asked whether the λ cI mRNA

can be translated in heterologous *in vitro* translation systems derived from the archaeobacterium *S. solfataricus* and from rabbit reticulocytes. The translation systems were programmed with equimolar amounts of cI mRNA and *ompA* mRNA. In both systems the cI mRNA was translated much more efficiently than *ompA* mRNA (Figure 5). After normalization for the number of methionines present in the CI and OmpA proteins, the translational efficiency of cI mRNA was estimated to be 6- and 6.5-fold higher than that of *ompA* mRNA in the archaeobacterial and eukaryotic system, respectively. To ensure that translation of cI mRNA started at the 5'-terminal AUG in either translation system, the AUG start codon of cI mRNA was changed to CUG and the corresponding mRNA was translated *in vitro* in either system. As shown in Figure 5 (lanes 3, 6 and 9), in the absence of a 5'-terminal AUG codon the band corresponding to the full-length CI protein disappeared. The additional product with a molecular weight lower than that of CI synthesized by the reticulocyte lysate programmed with cI mRNA (Figure 5, lanes 5 and 6) originates from an in-frame AUG at codon 43 (S.Grill, unpublished results), which is preceded by a G at position -3 and followed by a G at position +4; these are the major determinants of start codon selection in higher eukaryotes (Kozak, 1997).

Discussion

The data presented in this study strongly support a model in which translation of leaderless mRNA in bacteria occurs through a mechanism in which the initiation site of the mRNA is recognized by a 30S-initiator tRNA complex assisted by IF2 (Figure 6B). This mechanism is analogous to the obligatory initiation pathway in eukaryotes (Figure 6C). When compared with an mRNA containing a canonical 5'-UTR, a negligible amount of 30S-mRNA complex was formed with cI34 mRNA in the absence of initiator tRNA (see Table I). We conclude, therefore, that the 5'-terminal AUG is the only element of this leaderless mRNA that is recognized by the ribosome. Consistent with this observation is the lack of physical evidence that *cis* elements contribute to ribosomal recognition of leaderless mRNA (Resch *et al.*, 1995, 1996; Tedin *et al.*, 1997; Moll *et al.*, 1998; Bläsi *et al.*, 1999; O'Connor *et al.*, 1999; La Teana *et al.*, 2000). Further support for this notion comes from the studies of van Etten and Janssen (1998), who demonstrated that the translational efficiency of mRNAs deprived of their canonical 5'-UTR (which included the SD sequence) depended on the presence of an AUG start codon.

The formation of two binary complexes (30S-mRNA and 30S-fMet-tRNA^{Met}), which are intermediates in the random pathway leading first to the formation of a pre-ternary complex and subsequently to a 30S initiation complex, is supported by kinetic analyses (for reviews see Gualerzi and Pon, 1990; RajBhandary and Chow, 1995; Gualerzi *et al.*, 2000). The unstable pre-ternary complex intermediate has been demonstrated in the presence of an excess of ribosomal protein S15 (Philippe *et al.*, 1993). Binary complexes of 30S with mRNAs have been detected for mRNAs having SD sequences in their 5'-UTR (Hartz *et al.*, 1991) as well as for mRNAs containing a 5'-UTR devoid of complementarity to the 16S rRNA (Calogero

et al., 1988). The evidence for the formation of the 30S-fMet-tRNA^{Met} complex is also compelling (Jay and Kaempfer, 1974; Wu *et al.*, 1996). The intrinsic randomness of the order of mRNA-fMet-tRNA^{Met} binding to the 30S ribosomal subunit during 30S initiation complex formation does not necessarily mean that both pathways are equally probable. In fact, depending on the nature of the mRNA, on the cellular concentration of the two ligands and, possibly, on the variation of environmental parameters, translation of individual mRNAs may preferentially proceed through the initial formation of an mRNA-30S complex followed by the binding of fMet-tRNA^{Met} or vice versa. In addition, the extent to which translation of different model and natural mRNAs depends on IF2 seems to vary (La Teana *et al.*, 1993; Pediconi *et al.*, 1995).

We have shown that increased concentrations of IF2 resulted in an increase in the translational efficiency of a leaderless mRNA *in vitro* (Figure 2) and *in vivo* (Figure 4)

relative to that of *ompA* mRNA containing an internal AUG triplet preceded by a canonical SD sequence. The experimentally generated transient increase in IF2 concentration resembles that which is known to occur *in vivo* during the cold adaptation that follows cold shock in *E.coli* (Jones *et al.*, 1987). It is interesting to note that, under cold-shock conditions, the ratio of leaderless *tetR-lacZ* mRNA to *lacZ* mRNA translation increased (K.Stiefel and U.Bläsi, unpublished data). The IF2-dependent stimulation of ternary complex formation on *cI* mRNA (see Figure 1) was less pronounced than the IF2 effect observed

Fig. 5. Translation of equimolar amounts of *cI* and *ompA* mRNA in the *E.coli* S30, rabbit reticulocyte and *S.solfataricus* *in vitro* translation systems. Samples from the *in vitro* translation reactions were analyzed on a 12% SDS-polyacrylamide gel. Translation of *ompA* mRNA with the *E.coli* S30 extract, reticulocyte system and *S.solfataricus* system is shown in lanes 1, 4 and 7, respectively. Translation of *cI* mRNA with the *E.coli* S30 extract, reticulocyte system and *S.solfataricus* system is shown in lanes 2, 5 and 8, respectively. Translation of *cI*_{AUG1→CUG} mRNA with the *E.coli* S30 extract, reticulocyte system and *S.solfataricus* system is shown in lanes 3, 6 and 9, respectively. The positions of the bands corresponding to *cI* and *OmpA* are marked. The band marked with a filled circle in lane 5 results from an internal translation event in *cI* mRNA (see text).

Fig. 4. *In vivo* expression of *cI-lacZ* and *ompA* in the presence of elevated concentrations of IF2. (A) Increased intracellular concentrations of IF2 stimulate *cI-lacZ* expression. β -galactosidase measurements were performed at various times after heat induction of the *infB* gene. White and black bars represent the β -galactosidase synthesis obtained with strain UB89-1 (pKTplacI/pinfB) at 28 and 43°C, respectively. The β -galactosidase activity obtained with strain UB89-1 (pKTplacI/plc2833) at 28 and 43°C is indicated by gray and hatched bars, respectively. The cultures were grown at 28°C and shifted to 43°C at an OD₆₀₀ of 0.3. The β -galactosidase values are given in Miller units. (B) Determination of OmpA synthesis at basal and increased concentrations of IF2. Aliquots of strain UB89-1 (pKTplacI/pinfB) were withdrawn at 28 or 43°C at the indicated OD values; 0.1 OD₆₀₀ units of total cellular protein were loaded on to a 12% SDS-polyacrylamide gel for a given OD₆₀₀ value. OmpA synthesis was determined by quantitative immunoblotting as described in Materials and methods. Black and white bars correspond to OmpA synthesis at 43 and 28°C, respectively. (C) Plasmid pinfB-directed synthesis of IF2. -, no induction of the plasmid-borne *infB* gene; +, heat induction of the *infB* gene at an OD₆₀₀ of 0.3. As described in (B), aliquots of strain UB89-1 (pKTplacI/pinfB) were withdrawn at OD₆₀₀ values of 0.3, 0.4, 0.6 and 0.8. For a given OD, 0.1 OD₆₀₀ unit of total cellular protein was subjected to SDS-PAGE and subsequent quantitative immunoblotting as described in Material and methods. Only the relevant section of the immunoblot showing the IF2-specific band(s) is depicted.

Fig. 6. Translational initiation pathways in prokaryotes and eukaryotes. (A) Recruitment of the prokaryotic ribosome by an mRNA containing a canonical *rbs* through the SD–anti-SD interaction in the absence of P-site-bound initiator tRNA. (B) Recognition of leaderless mRNA by a prokaryotic 30S ribosome–initiator tRNA complex. IF2 is depicted by a closed circle. (C) Recognition of the cap complex by an eukaryotic 40S ribosome–initiator tRNA complex (reviewed by Gallie, 1998). The cap-binding complex eIF4F, consisting of eIF4E (crescent), eIF4G (oval), eIF4A (circle) and eIF4B (small oval); eIF3 (black square) and eIF2 (closed circle) are also depicted.

in the *cI* mRNA *in vitro* translation reaction (Figure 2A). The two assays differ in that, in the toeprint assay, the ternary complex has to withstand the elongating reverse transcriptase, whereas in the *in vitro* translation reaction, the stability of the ternary complex is modulated by the presence of the other initiation factors. It is conceivable that IF2, besides stimulating the formation of the 30S–fMet-tRNA^{Met} binary complex, also increases the stability of the 30S initiation complex in the translation reaction. This effect may not be as obvious in the toeprinting assays because it can be diminished by the elongating reverse transcriptase.

In the *in vitro* experiments presented in this study (Table I; Figures 1 and 3), we used 30S ribosomes to assess ternary complex formation on leaderless mRNAs. However, several lines of evidence suggest that translation of leaderless mRNAs may also be accomplished by 70S ribosomes (Balakin *et al.*, 1992; Resch *et al.*, 1996; Moll, 2000). Filter binding studies revealed that 70S monosomes, like 30S ribosomes (see Table I), do not form a binary complex with leaderless *cI* mRNA (Moll, 2000). Accordingly, binding of 70S ribosomes to different leaderless mRNAs strictly requires the presence of initiator tRNA (Moll, 2000). Thus, translation initiation of leaderless mRNAs requires a preformed ribosome–initiator tRNA complex, regardless of whether it is carried out by 30S or 70S ribosomes.

Translation in the kingdom Bacteria is accomplished by a different set of factors from that in Eukarya. However, homology searches performed by Kyrpides and Woese (1998a,b) have revealed that translation factors from all kingdoms share significant analogy, leading these authors to conclude that the rudiments of the present translational components (i.e. initiation factors) and mechanisms may have been present at the universal ancestor stage. Among these factors is the bacterial type IF2, which has been found in the kingdoms Archaea and Eukarya. Leaderless mRNAs are found in all three kingdoms. The results shown in Figure 5, and the faithful translation of leaderless *rro* mRNA derived from the lactococcal phage *rI1* in the same translation systems as used in this study (P.Londei, unpublished data), support the notion that, regardless of their origin, mRNAs with a 5′-terminal start codon can be translated by all three systems.

The translational behavior of the leaderless *cI* mRNA contrasted with that of the leadered *ompA* mRNA, which was poorly translated in both the archaeal and eukaryotic cell-free systems. The inefficient translation of *ompA* mRNA in the reticulocyte lysate is readily explained by the absence of a eukaryotic consensus sequence (Kozak, 1987, 1989) around the start codon and by the presence of extensive secondary structures in its 5′-UTR (Rosenbaum *et al.*, 1993). According to the manufacturer, the reticulocyte lysate does not add a 7-methyl cap to the

in vitro transcribed mRNA, which would be required for binding of the cap complex eIF4F comprising the helicase activity eIF4A.

Despite the near identity of the anti-SD sequence of *S.solfataricus* and *E.coli* 16S rRNA (Olsen *et al.*, 1985), *ompA* translation was barely detectable in the *S.solfataricus* system (Figure 5, lane 7). However, since the *S.solfataricus* system operates at 73°C, it seems possible that the four-base complementarity of the *ompA* SD with the *S.solfataricus* anti-SD sequence is not sufficient for effective translation initiation. On the other hand, *ompA* translation depends on ribosomal protein S1 (Tedin *et al.*, 1997), a homolog of which has not been found among the 30S ribosomal proteins of *S.solfataricus* (Londei *et al.*, 1983).

Ribosomal protein S1 stimulates translation initiation in Gram-negative bacteria and in the low G+C branch of Gram-positive bacteria (Boni *et al.*, 1982, 1991; Roberts and Rabinowitz, 1989). However, translation of leaderless mRNAs is independent of S1 (Tedin *et al.*, 1997), which may suggest that these mRNAs were present before the SD-anti-SD interaction and the S1 interaction developed. It is also worth noting that a leaderless mRNA derived from *G.lamblia* is translated in the absence of a 5' cap structure and a consensus sequence surrounding the initiation AUG (Hughes and Andrews, 1997). It is tempting to speculate that, at the universal ancestor stage, mRNA recognition may have been achieved by a ribosome-initiator tRNA complex independently of ribosome recruitment signals. Hence, today's leaderless mRNAs might be viewed as remnants of ancestral mRNAs.

Materials and methods

Bacterial strains and construction of plasmids

Escherichia coli strain UB89-1 (Bläsi *et al.*, 1990) was grown in Luria-Bertani (LB) medium (Miller, 1972) supplemented with kanamycin (30 µg/ml), ampicillin (120 µg/ml) or tetracycline (20 µg/ml) where appropriate to maintain selection of plasmids. Growth of the liquid cultures was monitored photometrically by measuring the optical density at 600 nm.

The plasmid *pinfB*, provided by Dr C.Pon, University of Camerino, harbors the *infB* gene under transcriptional control of the λp_L promoter. The parental plasmid pLc2833 has been described previously (Remaut *et al.*, 1981). Plasmid pKTplacI was constructed by inserting a PCR fragment containing the *lac* promoter (from nucleotide -60 to +1) from plasmid pUHE21-2 (Lanzer and Bujard, 1988) and the first 189 nucleotides of the λ *cI* gene into the *Xba*I and *Sma*I sites of plasmid pKT35 (Resch *et al.*, 1995). In plasmid pKTplacI, the *lac* promoter is abutted to the start codon of the *cI* gene via an *Nco*I site, which ensured that transcription of *cI* mRNA starts at the adenosine of the start codon. Plasmid pAXL7 harbors the full-length *cI* gene under transcriptional control of a T7 promoter (Tedin *et al.*, 1997). Plasmid pKS0235 (Ried *et al.*, 1994) served as a source for wild-type *ompA* mRNA and was provided by Dr R.Koebnik, MPI Tübingen, Tübingen, Germany.

Filter-binding assay

Filter-binding assays were performed using a Schleicher and Schuell SRC 072/0 Minifold II slot blot apparatus. The *cI*34SD and *cI*34 mRNAs were both obtained by PCR technology and T7 RNA polymerase-directed mRNA synthesis. Both mRNAs were labeled with [α -³²P]CTP. In a reaction volume of 50 µl, 0.5 pmol of ³²P-labeled mRNA was incubated with 30S ribosomes at a molar ratio of 1:10 (see Table I). The fMet-tRNA^{Met} was added in a 2-fold excess over 30S ribosomes. The reactions were incubated for 10 min at 37°C. The samples were added to the filter under vacuum and washed with 25 volumes of VD+ reaction buffer (Tedin *et al.*, 1997). The filters were exposed to a Molecular Dynamics

PhosphorImager screen to quantify the retained mRNA. The total c.p.m. values were corrected for the number of Cs in each mRNA.

β -galactosidase assays

The β -galactosidase activities were determined as described by Miller (1972). Triplicate aliquots of each culture were taken at different optical densities during logarithmic growth.

Quantitative immunoblotting

Samples were withdrawn from the cultures at different OD₆₀₀ values as described in the legend to Figure 4C. Care was taken that, for a given OD₆₀₀, the same amount of cellular protein was loaded on 12% SDS-polyacrylamide gels. The proteins were then transferred electrophoretically to a nitrocellulose sheet (pore size 0.2 µm) using standard procedures. OmpA was detected with anti-OmpA antibodies, provided by Dr U.Henning, MPI Tübingen, followed by visualization of the OmpA immunocomplex with the ECL kit of Amersham Life Science according to the manufacturer's instructions. The intensity of the resulting bands was determined by ImageQuant analysis. The cellular level of OmpA before the temperature shift (OD₆₀₀ = 0.3) was set to 1. The cellular level of OmpA after the shift and in the non-induced culture was normalized to that of OmpA at an OD₆₀₀ of 0.3. The cellular level of IF2 at 28 and 42°C was examined by quantitative immunoblotting with anti-IF2 antibodies. The secondary antibody was labeled with alkaline phosphatase. IF2-specific bands were visualized with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium.

In vitro translation

For full-length synthesis of *cI* and *ompA* mRNA, plasmids pAXL7 and pKS0235 were digested with *Pvu*II and *Hind*III, respectively. The linearized plasmids served as templates for *in vitro* transcription reactions with T7 RNA polymerase.

In vitro translation of *cI* and *ompA* mRNAs with *E.coli* S100 extracts was performed essentially as described by Tedin *et al.* (1997). Mix I contained 16.6 mM MgOAc, 80 mM NH₄Cl, 30 mM Tris-HCl pH 7.7, 3.3 mM dithiothreitol, 1.6 mg/ml *E.coli* tRNA, 0.2 mM citrovorum, 16.6 mM KCl, 0.33 mM amino acids (without lysine), 66.6 mM [¹⁴C]lysine, 3.3 mM ATP, 0.66 mM GTP, 16.6 mM phosphoenolpyruvate and 0.04 mg/ml pyruvate kinase. Mix II contained S100 extracts, 3.3 pmol/µl IF-free *E.coli* 70S ribosomes, 0.66 pmol/µl IF1, 0.66 pmol/µl IF3, 2 mM Tris-HCl pH 7.7, 60 mM NH₄Cl and 10 mM MgOAc. Purified *E.coli* IF2 was added to Mix II at different IF:70S ratios (no IF2, or 0.2:1, 0.4:1, 1:1, 2.5:1 ratios of IF2:70S). The translation mixture contained, in a final volume of 25 µl, 18 µl of Mix I, 6 µl of Mix II (20 pmol of 70S, 4 pmol of IF1 and 4 pmol of IF3) and 10 pmol of mRNA. The translation reactions were started by adding mRNA and incubated at 37°C for 30 min.

The *in vitro* translation reactions with *S.solfataricus* extracts were performed according to Condo *et al.* (1999). The samples contained, in a final volume of 50 µl, 10 mM KCl, 20 mM Tris-HCl pH 7.0, 18 mM MgOAc, 7 mM β -mercaptoethanol, 3 mM ATP, 1 mM GTP, 5 µg of bulk *S.solfataricus* tRNA, 20 µM amino acids (without methionine), 2 µl of [³⁵S]methionine (1200 Ci/mmol), 20 µl of *S.solfataricus* S30 extract (pre-incubated for 10 min at 73°C) and 10 pmol of mRNA. The samples were incubated at 73°C for 40 min.

The rabbit reticulocyte lysate was obtained from Promega. The reactions, which were set up according to the manufacturer's instructions, contained 50 mM KOAc, 1 mM MgOAc and 5 pmol of mRNA in a final volume of 25 µl. The mixtures were incubated at 30°C for 60 min.

The labeled proteins were separated on 12% SDS-polyacrylamide gels. The gels were dried under vacuum and exposed to a Molecular Dynamics PhosphorImager screen for quantification.

Primer extension inhibition analysis (toeprinting)

IF-depleted 30S subunits were prepared as previously described (Spedding, 1990). They were judged as pure when 23S rRNA was absent from the preparations. Charged initiator tRNA was provided by Drs W.Wintermeyer and M.V.Rodnina, University of Witten/Herdecke.

The 189 nucleotide long *cI* mRNA (see Figure 1) was prepared exactly as described in Resch *et al.* (1996). The *cI*SD (see Figure 1) mRNA, which comprises the same RNA sequence as *cI* mRNA except for the 5'-terminal extension (see Table I), was obtained by means of PCR technology using a plasmid containing the full-length *cI* gene abutted with its 5'-terminal start codon to the translation initiation region of phage T7 gene 10, followed by transcription with T7 RNA polymerase as described previously (Resch *et al.*, 1996). Primer O8 (Moll *et al.*, 1998) was used for the toeprinting reactions with both mRNAs.

Plasmid pUH100 (Lundberg *et al.*, 1990) was used as a source of wild-type *ompA* mRNA (see Figure 1). The plasmid was digested with *HincII* and T7 RNA polymerase-generated run-off transcripts were used in the toeprinting reactions together with the *AvaII* primer (Moll *et al.*, 1998).

The *ompA*Δ117 mRNA (see Figure 3) was prepared from a PCR template (Tedin *et al.*, 1997), and the *AvaII* primer (Moll *et al.*, 1998) was used for toeprinting.

Toeprinting assays were performed essentially as described by Hartz *et al.* (1988). For the kinetic toeprinting reactions, 2 pmol of 30S subunits, 5 pmol of IF2 (when present), 0.8 pmol of fMet-tRNA^{Met} and 1 mM GTP were pre-incubated for 20 s in a 10 μl reaction before the mRNAs were added at a final concentration of 0.005 pmol/μl. The incubation times varied (see legends to Figures 1 and 3B). The Moloney murine leukemia virus reverse transcription reaction was then performed at 37°C for 3 min. The relative toeprints shown in Figures 1 and 3C were calculated as described by Hartz *et al.* (1991).

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