Selection of the initiator tRNA by *Escherichia coli* initiation factors

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We have developed a new technique, called 'toeprinting,' which has allowed a study of the tRNA-binding properties of *Escherichia coli* translation initiation complexes. In response to natural mRNAs, the initiator tRNA and a variety of elongator tRNAs bind to the same tRNA-binding site on the 30S ribosomal subunit as long as a cognate codon is present near the Shine and Dalgarno sequence. The selection of the initiator tRNA in 30S initiation complexes is accomplished by initiation factors IF2 and IF3. 70S ribosomes accept both initiator tRNA and elongator tRNAs on natural mRNAs, much like 30S ribosomal subunits; IF3 and IF2 do not, however, select the initiator tRNA on 70S initiation complexes unless the initiation factor IF1 is present.

[Key Words: Initiation factors; ribosomes; ribosome-binding sites; tRNA]

Received June 29, 1989; revised version accepted September 19, 1989.

The process of translation initiation in *Escherichia coli* has been the subject of extensive experimentation over the last 25 years. Some recent reviews have focused on the mRNA requirements for rapid initiation at a particular ribosome-binding site (Gold et al. 1981; Stormo 1986; Gold and Stormo 1987; Gold 1988). The role of various sequence elements within a mRNA is now clear, particularly the Shine and Dalgarno domain and the initiation codon. Other reviews have focused on the non-mRNA components used for translation initiation on most mRNAs; these include the 30S ribosomal subunit, charged and formylated initiator tRNA, three initiation factors, and GTP (Maitra et al. 1982; Kozak 1983; Gualerzi et al. 1986; Hershey 1987). A consensus pathway for translation initiation in *E. coli* includes the formation of a 30S initiation complex intermediate, in which the initiator tRNA is bound to the 30S subunit (which is bound, itself, on the mRNA at the ribosome-binding site). The initiator tRNA is base-paired with the initiation codon (Guthrie and Nomura 1968; Gualerzi and Pon 1981; Hershey 1987). The initiation factors IF1, IF2, and IF3 (as well as GTP) catalyze 30S initiation complex formation (Wintermeyer and Gualerzi 1983). During or after the joining of the 50S subunit to the 30S initiation complex, the initiation factors are released, GTP is cleaved, and a 70S initiation complex is formed.

The precise mechanism by which each component acts is not well understood. Evidence has been presented that the 30S ribosomal subunit is competent for mRNA binding and initiation site recognition on an mRNA without the participation of any other component (Van Duin et al. 1980). Furthermore, initiator tRNA can be

bound into such 30S binary complexes in the absence of initiation factors (Nomura and Lowry 1967), and even elongator tRNA can be bound (Hartz et al. 1988). Polynucleotide-directed protein synthesis can start with the initiator tRNA or elongator tRNAs and can proceed in the absence of initiation factors (Wahba et al. 1969; Hershey 1987). In contrast, natural mRNA-directed protein synthesis always starts with the initiator tRNA and requires initiation factors. Thus, the initiation factors might be involved in the selection of the initiator tRNA (Gold 1988). Some portion of the literature on initiation factors points toward this simple view: Initiation factors IF2 and IF3 could provide the selective recognition of the initiator tRNA (Jay and Kaempfer 1974; Pon and Gualerzi 1974; Risuleo et al. 1976; Van der Hofstad et al. 1977). However, the literature includes suggestions of other roles for the initiation factors. Initiation factors IF3 and, to a lesser extent, IF1, have been implicated in the 70S dissociation (Subramanian and Davis 1970; Kaempfer 1972; Godefroy-Colburn et al. 1975). IF3 has also been implicated in the binding of the 30S subunit to the mRNA (Wahba et al. 1969; Suttle and Ravel 1974; Hershey 1987). Some suggestions that the 30S particle has an intrinsic high affinity site for the initiator tRNA would make the selection of initiator tRNA by the initiation factors superfluous (Bretscher and Marcker 1966; Nomura and Lowry 1967).

No experiments have been published in which initiation factors can be observed directly selecting the initiator tRNA over elongator tRNAs. The experiments reported here use a new method for inspecting ribosomes bound to mRNAs at translation initiation regions. The method, called extension inhibition, or toeprinting (Hartz et al. 1988), involves nothing more than cDNA synthesis by reverse transcriptase on a template mRNA on which a ribosome, together with a tRNA, is bound.

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The position of reverse transcriptase terminations depends on the exact position of the mRNA with respect to the bound ribosome which, in turn, depends on the tRNA species bound in the ribosome. Our data show that ribosomes recognize translational initiation regions but do not discriminate strongly between tRNAs. However, the initiator tRNA can be selected over other tRNAs by the initiation factors.

Results

Principle of the toeprinting method

A ternary complex consisting of the 30S ribosomal subunit, tRNA, and mRNA stops reverse transcriptase that started cDNA synthesis downstream of the ribosome-binding site (Fig. 1; Hartz et al. 1988). The premature reverse transcriptase stop, called a toeprint, is located easily on a sequencing gel. Toeprinting does not call for an extensive purification of the RNA because a 5' ³²P-labeled primer can provide the assay specificity. Furthermore, we can easily monitor the bound tRNA species (Hartz et al. 1988). The strength of the toeprint versus the extent to which reverse transcriptase reads through to the 5' end of the mRNA is a quantitative measure of the formation and stability of the initiation complexes (Winter et al. 1987; McPheeters et al. 1988; Bläsi et al. 1989; D. Hartz et al., in prep.).

Toeprint signals from many different ribosome-binding sites

In our first report of toeprinting (Hartz et al. 1988), we noticed that gene 32 mRNA (whose initiation codon is followed by a phenylalanine codon) yielded a toeprint with either initiator tRNA or tRNA^{Phe}. The toeprints appeared 15 nucleotides 3' to the first nucleotide of their respective codons. If the P site on the 30S particle can bind different tRNAs and if the initiation scheme outlined above is correct, the position of a toeprint would logically depend on cognate codon/anticodon pairing between the bound tRNA and the mRNA. We asked whether the +15 distance between cognate codon and toeprint position is general and over what range codons are inspectable near the Shine and Dalgarno sequence during initiation. Toeprinting with 30S subunits and fMet-tRNA^{Met} works with a wide variety of mRNAs

Figure 1. Model for the toeprinting method. Reverse transcriptase terminates cDNA synthesis when it encounters a 30S particle and tRNA bound on the mRNA at the ribosome-binding site.

from T4-infected cells and also with transcripts prepared in vitro with T7 RNA polymerase (Fig. 2, lanes 3; Table 1). In most cases, reverse transcriptase stops +15 downstream from the first nucleotide of the actual initiation codon. In a several cases, toeprints occur at both +15 and +16, and in one case, an additional weak toeprint signal occurs at +14 (*orfD*). The T4 gene 1 mRNA was unique in that only a single stop at +17 was observed. The toeprint position at +15 with gene 32 mRNA is reproducible with reverse transcriptases from two different sources—avian myeloblastosis and Moloney murine leukemia virus (AMV and MMLV)—and even with the Klenow fragment of *E. coli* DNA polymerase I when single-stranded DNA is used as a mRNA (data not shown). The data support the idea, as discussed in Hartz et al. (1988), that these enzymes synthesize cDNA right up to the edge of the ribosome in the ternary complex and that the distance between the ribosome's downstream edge and the first base of the initiation codon approximates 13–14 nucleotides.

When crude mRNA preparations are used as tem-

Figure 2. Detection of ternary complexes on various T4 mRNAs [crude mRNA] by toeprinting analysis. Reactions contained neither 30S nor tRNA (lanes 1), 1 μM 30S subunits (lanes 2), and 1 μM 30S subunits plus 4.4 μM fMet-tRNA^{Met} (lanes 3). Preincubation was for 5 min at 37°C before AMV reverse transcriptase was added. (Lanes 4) Sequencing lanes (A lanes).

[illegible]

To prove that the weak toeprints seen in Figure 2 (lanes 2; crude mRNA preparations plus 30S, no initiator tRNA addition) are caused by endogenous tRNAs and to find out which elongator tRNAs are capable of ternary complex formation, we used the pRS170 in vitro transcript, which contains the gene 32 ribosome-binding site. When total *E. coli* tRNA (0.5 μ g) plus 30S subunits (0.2 μ M) were added to toeprinting reactions, exactly the same stops were seen as for gene 32 in vivo RNA in Figure 2, lane 2 (data not shown). We also tested single, uncharged tRNA species. Besides tRNA^{Met}, a variety of elongator tRNAs give toeprints if cognate codons are in the neighborhood of the real initiation codon. On the pRS170 transcript, tRNA^{Met}_m, tRNA^{Cys}, tRNA^{Val-1}, tRNA^{Val-2}, tRNA^{Phe}, tRNA^{Lys}, and tRNA^{Arg-1} all resulted in toeprints of variable strength, +15 from the

seq. T-lane

30S only

30S + tRNA_f^{Met}

30S + tRNA_m^{Met}

30S + tRNA^{Cys}

30S + tRNA^{Val-1}

30S + tRNA^{Val-2}

30S + tRNA^{Phe}

30S + tRNA^{Leu-5}

30S + tRNA^{Lys}

30S + tRNA^{Arg-1}

I 2 3 4 5 6 7 8 9 10 11

Val

Cys

Val-1

Lys

Met

Phe

Arg

SD-AUAUAAAAAUGUUUAAACGUA

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cluding the strongest ones, is observed within a narrower range of ~7–10 nucleotides from the Shine and Dalgarno sequence (Fig. 3; Table 1). The toeprints of $\text{tRNA}_{\text{Met}}^{\text{Met}}$, $\text{tRNA}_{\text{Cys}}^{\text{Cys}}$, and $\text{tRNA}_{\text{Phe}}^{\text{Phe}}$ are as intense as the toeprint from $\text{tRNA}_{\text{Met}}^{\text{Met}}$, indicating that about equal amounts of complexes had formed. Weaker toeprints are formed by the other elongator tRNAs. This finding corresponds exactly to the previous result with crude gene 32 RNA (Fig. 2, gene 32, lane 2). Of the endogenous elongator tRNAs, $\text{tRNA}_{\text{Cys}}^{\text{Cys}}$ and $\text{tRNA}_{\text{Phe}}^{\text{Phe}}$ compete well with the $\text{tRNA}_{\text{Met}}^{\text{Met}}$ for the ribosomal P site. Good competition with the $\text{tRNA}_{\text{Met}}^{\text{Met}}$ is also observed on the crude mRNAs of gene 69, gene 45, gene 1, and *regA* (Fig. 2, lanes 2). Weak competition of the elongator tRNAs with the $\text{tRNA}_{\text{Met}}^{\text{Met}}$ is observed on the crude mRNAs of *rIBB*, *orf1*, and *orfD* (Fig. 2, lanes 2) and on the pRS270 in vitro transcript with added tRNA (data not shown). Thus, elongator tRNAs compete well with $\text{tRNA}_{\text{Met}}^{\text{Met}}$ for the 30S P site, in contrast to the notion that the P site has the highest affinity for initiator tRNA and discriminates against any elongator tRNA [Bretscher and Marcker 1966; Nomura and Lowry 1967]. A summary of the toeprint data on all mRNAs with endogenous or pure tRNAs is shown in Table 1, which is discussed fully below.

Comparison of $\text{A22}_{\text{Met}}^{\text{Met}}$, $\text{tRNA}_{\text{Met}}^{\text{Met}}$, and $\text{fMet-tRNA}_{\text{Met}}^{\text{Met}}$ in 30S ternary complexes

In the experiments presented so far, both $\text{tRNA}_{\text{Met}}^{\text{Met}}$ (Fig. 3) and $\text{fMet-tRNA}_{\text{Met}}^{\text{Met}}$ (Fig. 2) have been used. Both initiator tRNA species are able to form ternary complexes. Because either might be used in competition experiments with elongator tRNAs, we compared their binding characteristics on 30S complexes. At a constant, saturating 30S subunit level, increasing amounts of tRNA were added to toeprinting reactions containing the pRS170 transcript (Fig. 4). The relative toeprints were determined by densitometry from the autoradiographs (see Materials and methods). The relative toeprints do not reach 100% at apparently saturating concentrations of tRNA. This could be due to several causes; the complex formation has not gone to completion because the equilibrium is not reached yet (we know that this is correct, because longer preincubation increases the relative toeprint) and/or the equilibrium does not strongly favor the ternary complex. Alternatively, the reverse transcriptase might displace some of the complexes or wait for complex dissociation that is followed by rapid extension. The binding curves were adjusted to 100% binding range so that a best fit of our data with a bimolecular binding curve could be obtained (Fig. 5). The binding constants of $\text{tRNA}_{\text{Met}}^{\text{Met}}$ ($K_a = 7.4 \times 10^7/\text{M}$ and $\text{fMet-tRNA}_{\text{Met}}^{\text{Met}}$ ($K_a = 1.0 \times 10^8/\text{M}$) are not significantly different. Much weaker binding was observed for a synthetic anticodon stem and loop fragment of the initiator tRNA 'A22 $_{\text{Met}}^{\text{Met}}$ ' ($K_a = 1.8 \times 10^6/\text{M}$; Figs. 4–6).

Selection of the initiator tRNA on 30S complexes by IF3

Because many different tRNAs can bind to the 30S sub-

Figure 4. Dependence of ternary complex formation on tRNA concentration. At constant 30S subunit concentration of 0.2 μM , increasing amounts of the initiator tRNA fragment A22 $_{\text{Met}}^{\text{Met}}$ (Fig. 6), $\text{tRNA}_{\text{Met}}^{\text{Met}}$ or $\text{fMet-tRNA}_{\text{Met}}^{\text{Met}}$ were added at the concentrations (μM) indicated above the corresponding lanes. Preincubations were for 10 min at 37°C.

unit in response to a cognate codon within the ribosome-binding site (Figs. 2 and 3), there should be a mechanism in vivo that restricts the access of elongator tRNAs to the P site. IF3 has been shown to selectively destabilize polynucleotide-primed 30S complexes formed with elongator tRNAs (Pon and Gualerzi 1974; Risuleo et al. 1976). Therefore, we included purified IF3 in toeprinting reactions that contained both initiator tRNA and elongator tRNAs. An equimolar mixture of $\text{tRNA}_{\text{Met}}^{\text{Met}}$, $\text{tRNA}_{\text{Cys}}^{\text{Cys}}$, and $\text{tRNA}_{\text{Phe}}^{\text{Phe}}$ produces toeprints of equal strength at +15 from their cognate codons on the pRS170 transcript (Fig. 7). As increasing amounts of IF3 are added, the $\text{tRNA}_{\text{Met}}^{\text{Met}}$ toeprint prevails over the other two. At higher IF3 levels (IF3/30S ratio >1), even the $\text{tRNA}_{\text{Met}}^{\text{Met}}$ toeprint is diminished. Increased $\text{tRNA}_{\text{Met}}^{\text{Met}}$ levels do not restore the diminished toeprint, so the kinetic effects of IF3 cannot be a result of an interaction of IF3 with free tRNA (data not shown). $\text{tRNA}_{\text{Met}}^{\text{Met}}$ is as well selected as $\text{fMet-tRNA}_{\text{Met}}^{\text{Met}}$ in the competition reaction (Fig. 7, lanes 8 and 9). Finally, IF3 selects $\text{tRNA}_{\text{Met}}^{\text{Met}}$ in mixed tRNA incubations even after the complexes have formed (Fig. 7, lane 10). IF3 must play an important role in the initiator tRNA selection, and the recognition signals for IF3 action are located in the RNA part of the tRNA. The recognition signal cannot be just the codon–

Figure 5. Semilogarithmic plot of scanning data from Fig. 4. The curves shown are the best fits to a theoretical binding curve. Saturation was assumed to be 100% for a relative toeprint of 40% for fMet-tRNA_{fMet} (●), 42% for tRNA_{fMet} (□), and 53% for A22_{fMet} (○), respectively.

anticodon interaction because tRNA_{fMet} behaves like an elongator tRNA with respect to IF3.

Then we tried A22_{fMet} in competition with tRNA_{Cys} and tRNA_{Phe} (Fig. 8). As expected from its weaker binding constant, A22_{fMet} does not compete efficiently with the two elongator tRNAs. Nevertheless, the fragment is selected at high IF3 concentration (IF3/30S ratio = 4; Fig. 8). To further elucidate the mechanism of IF3, we studied its influence on ternary complex formation in toeprinting reactions with single tRNA_{fMet} species; elongator tRNA_{fMet} and initiator tRNA_{fMet} behave differently with respect to IF3 (Fig. 9A,B). At a 1 : 1 ratio of IF3/30S, the tRNA_{fMet} toeprint is increased, whereas the tRNA_{fMet} toeprint is slightly decreased. At high IF3 concentration (IF3/30S ratio = 4) tRNA_{fMet} binding is diminished, whereas tRNA_{fMet} binding is not detectable. The A22_{fMet} fragment behaves like tRNA_{fMet} and might thus contain the critical features of the initiator tRNA for the discrimination by IF3. The elongator tRNA_{Phe} behaves similarly to tRNA_{fMet} (data not shown).

Selection of the initiator tRNA on 30S complexes by IF2

Initiation factor IF2 stimulates the binding of fMet-tRNA_{fMet} into 30S initiation complexes (Dubnoff et al. 1972; Vermeer et al. 1973; Jay and Kaempfer 1974), but IF2 also stimulates the binding of several elongator tRNAs into polynucleotide-primed 30S particles (Cannon et al. 1986). To determine whether IF2 could select the initiator tRNA over elongator tRNAs on 30S complexes, we added IF2 with an equimolar mixture of fMet-tRNA_{fMet}, tRNA_{Cys}, and tRNA_{Phe}, again using the pRS170 transcript. IF2 selects the fMet-tRNA_{fMet} toe-

print over the other two (Fig. 10). When added together with IF2, the initiation factor IF1 improves the selection marginally (Fig. 10, lane 6). IF2 does not select uncharged tRNA_{fMet} under otherwise identical conditions (Fig. 10, lane 2). Thus, IF2 selects the initiator tRNA by virtue of at least its formylated methionine; direct tRNA inspection is also possible. Preliminary experiments suggest that the formyl group is essential for the selection (data not shown). Unlike IF3 (Fig. 7, lane 10), IF2 is unable to dissociate elongator tRNAs from preformed complexes to select the initiator tRNA (Fig. 10, lane 8; the increase of the fMet-tRNA_{fMet} toeprint over the control lane is a result of extended complex formation after the preincubation period).

Selection of the initiator tRNA on 70S complexes

70S ribosomes have different tRNA-binding properties than 30S subunits (Nomura and Lowry 1967). We compared factor-free 70S initiation complexes with 30S complexes in toeprinting reactions. We used the pRS170 transcript again as a 'natural' mRNA template. Three elongator tRNAs (tRNA_{fMet}, tRNA_{Cys}, and tRNA_{Phe}) yield toeprints with factor-free 70S ribosomes, just like 30S subunits (Fig. 11). The toeprints occur at the same position (+15) from the cognate codons as in 30S complexes, but they are weaker than the corresponding 30S toeprints. Toeprints probably result from a collision between reverse transcriptase and the 30S subunit,

Figure 6. Sequence and structure of A22_{fMet} is outlined as part of fMet-tRNA_{fMet}. Note the extra pppG on its 5' end and the extra random nucleotide (N) on its 3' end; the cytosine in the anticodon loop is unmodified.

Figure 7. Selection of the initiator tRNA by IF3. In addition to $0.2 \mu\text{M}$ 30S subunits (lane 1), a mixture of the three tRNA species tRNA^{Met}, tRNA^{Cys}, tRNA^{Phe} (tRNAs) at $0.5 \mu\text{M}$ each (lanes 2–7 and 10) or a mixture of fMet–tRNA^{Met}, tRNA^{Cys}, tRNA^{Phe} (tRNAs*) at $0.5 \mu\text{M}$ each (lanes 8 and 9) was added in the toeprinting reactions. Furthermore, IF3 was added in the concentrations (μM) shown above the corresponding lanes. Preincubations were for 10 min at 37°C . (Lane 10) The reaction containing 30S and the tRNA mixture was preincubated for 10 min before IF3 was added and the preincubation continued for another 10 min. Lane 11 contains the sequencing reaction (A lane).

whether or not the 50S particle is present, that is, the 50S particle is positioned away from the elongating reverse transcriptase. Furthermore, the fact that the toeprints are at the same position suggests that the tRNA site used is the same in every complex and is probably equivalent to the P site. The 70S initiation complexes are different from 30S complexes; IF3 excludes the elongator tRNAs from 30S but not from 70S complexes (cf. Fig. 11, lanes 9 and 10), in accordance with previous results (Gottlieb and Davis 1975; Risuleo et al. 1976; Van der Hofstad et al. 1978). Preformed 30S complexes can be chased into 70S complexes by adding 50S subunits (Fig. 11, lanes 11 and 12). In mixed tRNA incubations, tRNA^{Cys} and tRNA^{Phe} compete with the tRNA^{Met} for the same tRNA-binding site on the 70S ribosome, and the resulting toeprints are of about equal strength (Fig. 12, lane 3). If, however, fMet–tRNA^{Met} is used in such a competition experiment, it forms 70S ternary complexes to a lesser extent than the competing elongator tRNAs (Fig. 12, lane 4). Such behavior is not seen in 30S complexes (Fig. 12, lanes 1 and 2). These facts indicate that

70S complexes are formed directly and not via 30S complex intermediates, because 30S intermediates would accept fMet–tRNA^{Met} and the elongator tRNAs equally well. The 70S ribosome is able to find the ribosome-binding site on a message, yet it discriminates against fMet–tRNA^{Met}. This discrimination appears only in competition experiments and not with the single tRNA (Fig. 12, lanes 5 and 6).

To investigate whether the initiation factors IF3 and IF2 select the initiator tRNA on 70S complexes, we included both factors in tRNA competition experiments with 70S ribosomes on the pRS170 transcript. Neither IF3 nor IF2 selects fMet–tRNA^{Met} over tRNA^{Cys} and tRNA^{Phe} on 70S complexes (Fig. 13, lanes 2 and 3). A combination of IF2 and IF3 leads to an increase of the fMet–tRNA^{Met} toeprint (Fig. 13, lane 4). A surprising result is obtained when initiation factor IF1 is added. Even though this factor, itself, does not select fMet–tRNA^{Met} (Fig. 13, lane 5), the toeprint from fMet–tRNA^{Met} is enhanced when IF1 is employed together with IF2 or IF3 (Fig. 13 lanes 6 and 7). Both combinations lead to a stronger selection of the initiator tRNA than the combination of IF2 + IF3. The effect is very striking when all three factors are employed; all three initiation factors yield a clear selection of fMet–tRNA^{Met} (Fig. 13, lane 8). The selection is lost when uncharged tRNA^{Met} is used (Fig. 13, lane 10). Because IF1 does not select the initiator tRNA on 30S or 70S complexes, it is likely that IF1 somehow enables IF3 and IF2 to select fMet–tRNA^{Met} on 70S ribosomes.

Discussion

Previously we used toeprinting to locate a 30S initiation complex on gene 32 mRNA (Hartz et al. 1988), to iden-

Figure 8. Selection of A22^{Met} by IF3. In addition to $0.2 \mu\text{M}$ 30S subunits (left lane), a mixture of A22^{Met}, tRNA^{Cys} and tRNA^{Phe} (tRNAs #) at $0.5 \mu\text{M}$ each was added. Furthermore, IF3 was added at the concentrations (in μM) shown in parentheses. A sequencing lane (A lane) is shown at right.

Figure 9. Influence of IF3 on ternary complex formation with single tRNA species. 0.5 μM of the elongator tRNA_m^{Met}, 0.5 μM of the initiator tRNA fragment A22_f^{Met}, or 0.5 μM of the initiator tRNA_f^{Met} were added together with 0.2 μM 30S subunits and IF3 at the concentrations (in μM) indicated in parentheses above the lanes. Preincubations were for 10 min at 37°C. (A) Actual toeprints; the 5' ends are shown at top. (B) Graph of the relative toeprints, measured by densitometrical scanning; ($\square$) tRNA_f^{Met}, ($\circ$) A22_f^{Met}, ($\triangle$) tRNA_m^{Met}.

tify ribosome-binding sites on the transcript of the *E. coli* *unc* operon (Schaefer et al. 1989) and to study discrimination between dual translation initiation sites in the λ S gene (Bläsi et al. 1989). Toeprint inhibition analysis has been used to monitor the autogenous translational repression of T4 gene 32 by gene 32 protein (McPheeters et al. 1988) and translational repression of T4 *rIIB* by regA protein (Winter et al. 1987). Here we used toeprinting to reinvestigate the tRNA-binding properties of translational initiation complexes. Our data complement a large number of experiments that show the 30S ribosomal subunit to be capable, itself, of locating the initiation domains of mRNAs. Yet the 30S complexes readily accept elongator tRNAs in response to cognate codons within the ribosome-binding site. Thus, a selection mechanism for the initiator tRNA must exist. We have shown that IF2 and IF3 provide initiator tRNA selection in vitro on 30S complexes but not on 70S initiation complexes. For proper tRNA selection with 70S ribosomes, the initiation factor IF1 is needed along with IF2 and IF3.

30S Initiation complexes

30S particles must be competent to bind to the initiation regions on the mRNA because factor-free 30S particles plus initiator tRNA form detectable (by toeprinting) initiation complexes at true initiation codons and not at other AUGs. This is in agreement with the classic experiments of Van Duin and collaborators (Van Duin et al. 1980), which refuted the idea that initiation factor IF3

is necessary for 30S mRNA binding (Suttle and Ravel 1974; see also Maitra et al. 1982; Hershey 1987). The binding of the 30S particle to the mRNA serves to diminish the space to be searched in the hunt for the initiation codon. The Shine and Dalgarno sequence, which interacts with the 3' end of 16S RNA, provides one binding signal and, thus, 'tethers' the 30S particle to the mRNA. The 30S particle probably searches for the initiation codon within some constrained distance from that tether. The fact that toeprints were mostly observed for codons at a certain distance (3–17 nucleotides) from the Shine and Dalgarno sequence (Table 1) supports such a concept. During the hunt, any tRNA can bind to factor-free 30S particles; toeprints (and stable ternary complexes) are formed as long as a cognate codon is within the 'scanning' distance. Many different elongator tRNAs bind to the same tRNA-binding site on factor-free 30S subunits, as evidenced by a corresponding shift (+15 from the cognate codons) in the 3' edge of 30S subunit on the mRNA. The affinity of the elongator tRNAs in initiation complexes varies, as indicated by different toeprint strengths, probably because of the location of cognate codons with respect to the Shine and Dalgarno sequence or other mRNA determinants, or because of an intrinsically higher affinity of some tRNAs for the 30S subunit. With several mRNA templates (T4 gene 32, gene 69, gene 45, gene 1, and regA), the elongator tRNAs compete well with the initiator tRNA for the P site. These data are at odds with the traditional picture that the P site on the 30S subunit excludes the elongator tRNAs on a natural mRNA template (Nomura and

Figure 10. Selection of fMet-tRNA^{Met} by IF2. In addition to 0.2 μ M 30S subunits (lane 1), lane 2 contained a mixture of the three tRNA species, tRNA^{Met}, tRNA^{Cys}, tRNA^{Phe} (tRNAs) at 0.5 μ M each, and lanes 3–8 contained a mixture of fMet-tRNA^{Met}, tRNA^{Cys}, tRNA^{Phe} (tRNAs*) at 0.5 μ M each. Furthermore, IF2 or IF1 plus IF2 was added at the concentrations (in μ M) indicated in brackets. Preincubations of lanes 1–7 were for 10 min at 37°C. In lane 8, the reaction containing 30S and the mixture of tRNAs* was preincubated for 10 min before IF2 was added and the preincubation continued for another 10 min. (Right) Sequencing lane (A lane).

Lowry 1967). The previous experiments were done on phage f2 RNA with one labeled tRNA species in an unlabeled tRNA mixture. It is possible that the initiator tRNA competes well with elongator tRNAs on ribosomes programmed with phage f2 RNA as we found for T4 *rII*B, *orf1*, *orfD* mRNA, and the pRS270 transcript. It should also be noted that contamination of the 30S subunit preparation with initiation factors leads to preferred initiator tRNA binding in 30S initiation complexes [see below]. We encountered such initiator tRNA selection with one 30S subunit preparation and found that it actually contained initiation factors. Because elongator tRNAs bind well to 30S subunits in response to polynucleotides that contain cognate codons (Nomura and Lowry 1967; Risuleo et al. 1976; Canonaco et al. 1986), we conclude that the tRNA-binding characteristics of 30S subunits are similar with natural mRNAs and polynucleotides as templates. 30S subunits bind to *specific* sites on natural mRNA and thus restrict the codons that can be scanned by a tRNA present in the P site.

Another task for the translational machinery might be the discrimination between uncharged and formylmeth-

ionine-charged initiator tRNA. Our titration experiment reveals that tRNA^{Met} with a K_a of $7.4 \times 10^7/\text{M}$ binds almost as well as fMet-tRNA^{Met} with a K_a of $1.0 \times 10^8/\text{M}$. The 30S subunit probably does not discriminate between the two species, in agreement with a previous report (Rudland and Dube 1969). Furthermore, fMet-tRNA^{Met} and tRNA^{Met} behave identically in competition experiments with elongator tRNAs. The binding constants found by toeprinting are in the same binding range as the one reported for *E. coli* Phe-tRNA^{Phe} binding to poly(U)-primed 30S complexes with a K_a of $2.5 \times 10^8/\text{M}$ at 10 mM Mg²⁺ (Kirillov et al. 1980). Yeast tRNA^{Phe} binding in such complexes gives a lower K_a of $1 \times 10^7/\text{M}$ at 30 mM Mg²⁺ (Rose et al. 1983). The lower K_a of $1.8 \times 10^6/\text{M}$ for the unmodified tRNA anticodon stem and loop fragment A22^{Met} (Fig. 5) is surprising because equally strong binding has been reported for *E. coli*

Figure 11. Comparison of 30S and 70S ternary complexes with elongator tRNAs and IF3. In addition to 0.2 μ M 30S or 0.2 μ M 70S (prepared by preincubating 0.2 μ M 30S and 0.24 μ M 50S for 15 min at 37°C) in lanes 1 and 2, the tRNA species indicated above the lanes were added at a concentration of 0.5 μ M (lanes 3–12). In lanes 9–12, the reactions were first incubated with the components in parentheses for 10 min before the next component (IF3 or 50S) was added. Preincubation was continued for 10 minutes more before reverse transcriptase or the next component (IF3 in lane 12) was added. Lane 12 had a total preincubation time of 30 min, whereas the other lanes had a total preincubation time of 20 min at 37°C. The concentration of IF3 added was 0.8 μ M final, and that of 50S was 0.24 μ M final. For better comparison, 5' ends are shown at top of the lanes.

Figure 12. Comparison of initiator tRNA binding in 30S and 70S ternary complexes. Incubations contained 30S or 70S (prepared as in Fig. 11) at $0.2 \mu\text{M}$ and a mixture of the tRNA species $\text{tRNA}_{\text{fMet}}$, tRNA_{Cys} , and tRNA_{Phe} (tRNAs) at $0.5 \mu\text{M}$ each in lanes 1 and 3 or a mixture of the tRNA species $\text{fMet-tRNA}_{\text{fMet}}$, tRNA_{Cys} , and tRNA_{Phe} (tRNAs*) at $0.5 \mu\text{M}$ each in lanes 2 and 4. Lanes 5 and 6 contain $0.2 \mu\text{M}$ 70S ribosomes and $0.5 \mu\text{M}$ $\text{tRNA}_{\text{fMet}}$ or $0.5 \mu\text{M}$ $\text{fMet-tRNA}_{\text{fMet}}$, respectively. Preincubations were for 10 min at 37°C . 5' ends are shown at top of the lanes.

$\text{fMet-tRNA}_{\text{fMet}}$, $\text{tRNA}_{\text{fMet}}$, an anticodon stem and loop fragment of $\text{tRNA}_{\text{fMet}}$ (Rudland and Dube 1969), and also for yeast tRNA_{Phe} and the anticodon stem and loop fragment thereof (Rose et al. 1983). The weaker toeprints with A22_{fMet} could either be the result of a lower binding affinity of A22_{fMet} to 30S subunits or less stable ternary complexes. Both possibilities could well be caused by its unmodified cytidine in the anticodon loop, extraneous nucleotides on the 5' and 3' end, or the triphosphate on the 5' end and do not necessarily mean that some important component for the interaction with the 30S particle is missing.

30S Initiation complexes with initiation factors

If the 30S subunit cannot discriminate $\text{fMet-tRNA}_{\text{fMet}}$ from elongator tRNAs or uncharged $\text{tRNA}_{\text{fMet}}$, what provides the discrimination in the cell? The access of elongator tRNAs to the 30S P site is a serious threat for the proper initiation process and would result either in a shorter protein product or translation of a wrong reading frame.

We show for the first time that IF3 selects the initiator

tRNA on a natural mRNA template. In agreement with Risuleo et al. (1976), we find that IF3 action distinguishes the initiator tRNA from the elongator tRNAs solely because of a unique RNA and not because of its formylmethionine. Furthermore, at least part of the information for initiator tRNA discrimination is located in the fragment A22_{fMet} , which behaves strikingly similar to the whole initiator tRNA with respect to IF3. It has been reported that three GC base pairs adjacent to the anticodon loop are highly conserved in initiator tRNAs (Sprinzl et al. 1987) and that initiator tRNAs have a special anticodon loop conformation (Wrede et al. 1979). The three GC base pairs adjacent to the anticodon loop in the *E. coli* initiator tRNA are important for translational initiation and, when changed, also lead to a different loop conformation (Seong and RajBhandary 1987). These GC base pairs are conserved in the A22_{fMet} fragment and might explain its initiator tRNA-like behavior.

As shown previously, IF3 enhances the exchange rate of elongator tRNAs that are bound in polynucleotide-primed 30S complexes (Pon and Gualerzi 1974). The cor-

Figure 13. Selection of initiator tRNA on 70S complexes. In addition to $0.2 \mu\text{M}$ 70S ribosomes (prepared as in Fig. 11), a mixture of the three tRNA species $\text{fMet-tRNA}_{\text{fMet}}$, tRNA_{Cys} , and tRNA_{Phe} (tRNAs*) at $0.5 \mu\text{M}$ each was added in lanes 1–8 or a mixture of the three tRNA species $\text{fMet-tRNA}_{\text{fMet}}$, tRNA_{Cys} , and tRNA_{Phe} (tRNAs) at $0.5 \mu\text{M}$ each was added in lanes 9 and 10. Initiation factors were added at the concentrations (in μM) indicated in parentheses above the lanes. Preincubations were for 20 min at 37°C . (Right) Sequencing lane (A lane). 5' ends are shown at the top of the lanes.

responding initiator tRNA-containing complexes are more resistant to IF3-induced destabilization than are complexes with elongator tRNAs (Risuleo et al. 1976). The results from our tRNA competition experiments on a natural mRNA template are consistent with these earlier data. In agreement with earlier reports (Risuleo et al. 1976; Van der Hofstad et al. 1978), we also found that IF3 acts in a concentration-dependent manner on initiator tRNA-containing complexes. IF3 enhances complex formation at an IF3/30S ratio of 1 : 1, but this effect is reversed at higher IF3/30S ratios. A second binding site for IF3 could exist which, when occupied, inhibits complex formation. More complicated kinetic models are also possible, which require only one IF3-binding site on the 30S particle. Unfortunately, concentration-dependent IF3 action led to confusion about the role of IF3. Van der Hofstad et al. (1978) reported that the binding of IF3 and the binding of the initiator tRNA and IF2 were exclusive on the 30S subunit and thus proposed a different pathway for initiation complex formation. Their measurements were done at a high IF3/30S ratio. Using an IF3/30S ratio of 1 : 1, Pon and Gualerzi (1986) later showed that the proposed pathway was incorrect for physiologically appropriate initiation factor quantities.

The role of IF3 in initiator tRNA selection has so far been underestimated because of the reports that initiator tRNA binds most strongly to the 30S P site (Bretscher and Marcker 1966; Nomura and Lowry 1967), that IF2 is involved in the binding of the initiator tRNA (Jay and Kaempfer 1974; see below), and that elongator tRNAs are complexed with elongation factor Tu (EFTu) [thus preventing them from binding during initiation (but see Gnirke and Nierhaus 1986)]. Additionally, IF3 was associated with binding of the 30S subunit to natural mRNA but not to synthetic polynucleotides (Suttle and Ravel 1974). However, these data for mRNA binding to ribosomes were gained mainly by measuring the initiator tRNA (!) binding in 30S initiation complexes (Wahba et al. 1969; Jay and Kaempfer 1974; Suttle and Ravel 1974). The finding that IF3 did not stimulate the binding of fMet-tRNA^{Met} or elongator tRNAs to polynucleotide primed 30S but did stimulate the binding of fMet-tRNA^{Met} to natural mRNAs led to an artificial distinction between polynucleotide templates and natural mRNA templates. Our explanations of these data are twofold: (1) IF3 does not stimulate the binding of elongator tRNAs but does stimulate the binding of initiator tRNA as shown in this study; and (2) IF3 probably dissociates fMet-tRNA^{Met} from 30S complexes on non-AUG initiation codons of a random poly(A,U,G) template and therefore might not have an overall stimulating effect (Berkhout et al. 1986). Recent measurements of 30S mRNA binary complex formation did not indicate any effect of IF3 [nor any effect of the other initiation factors (Calogero et al. 1988)].

IF2 also selects the initiator tRNA in direct competition with elongator tRNAs. This selection is absolutely dependent on the formylmethionine charging of the initiator tRNA. Even though the stimulation of fMet-tRNA^{Met} binding into 30S initiation complexes by IF2 is

well-documented (Wahba et al. 1969; Dubnoff et al. 1972), a selective function had not been proved. IF2 is able to form binary complexes with initiator tRNA, which is dependent on its formylation (Sundari et al. 1976; Van der Hofstad et al. 1977; Petersen et al. 1983). Nevertheless, IF2 probably acts on the 30S level (Canonaco et al. 1986; Gualerzi and Wintermeyer 1986) and mainly influences the binding of the initiator tRNA to the 30S subunit (Vermeer et al. 1973), in striking similarity to IF3. Yet the selective mechanism must be different from that of IF3. IF2 does not dissociate the elongator tRNA complexes (as IF3 does) but even stabilizes them (Canonaco et al. 1986). Our data agree with the notion that IF2 selects fMet-tRNA^{Met} by kinetically favoring the steps leading to 30S initiation complexes containing fMet-tRNA^{Met} (Canonaco et al. 1986). In essence, IF2 action not only discriminates the initiator tRNA from elongator tRNAs but also provides discrimination against uncharged and probably unformylated initiator tRNA. It thus connects the fidelity of translation initiation to the intracellular redox state and the one carbon metabolism via tetrahydrofolate (the formyl group donor); of course, methionine, itself, is the other important component in the one carbon metabolism (Danchin 1973).

70S Initiation complexes

We obtained toeprints with initiator tRNA and elongator tRNAs on factor-free 70S initiation complexes at the same positions (+15 from the codons) as in 30S complexes. The most likely explanation is that these tRNAs bind to the same site as in 30S complexes and that the edge of the ribosome into which reverse transcriptase collides is composed of 30S material. This finding has implications for the positioning of the 50S subunit with respect to the 30S subunit and the mRNA. A current model proposes the 50S subunit to be bound to the 30S subunit away from the mRNA, which is in accordance with our finding (Gold 1988).

Initiation complexes with 70S ribosomes are weaker at toeprinting. The 70S complexes are formed more slowly than those with 30S subunits. After 30S complexes have formed, addition of 50S subunits yields stronger toeprints from 70S complexes than those obtained when starting the incubation with 70S particles and incubating for the same amount of time. The 70S complexes probably do not form via a 30S intermediate because fMet-tRNA^{Met} is bound less well than tRNA^{Cys} and tRNA^{Phe} in competition experiments on 70S complexes but is bound equally well on 30S complexes (the hypothetical intermediates). These results on a natural mRNA contradict previous data showing that neither initiator nor elongator tRNAs can form 70S complexes directly from 70S on natural mRNA (Nomura and Lowry 1967; Nomura et al. 1967). Most likely, the slower formation of 70S complexes without initiation factors prevented their detection. Our data again indicate a similarity in the behavior of natural mRNA and synthetic polynucleotides, which readily form 70S complexes

with elongator tRNAs, but not with fMet-tRNA^{Met} if elongator tRNAs are present (Nomura and Lowry 1967; Guthrie and Nomura 1968).

Another way to distinguish 70S from 30S complexes is their behavior with IF3. IF3 does not provide a selection mechanism for the initiator tRNA on 70S initiation complexes, as it does on 30S initiation complexes. The reason is that IF3 cannot dissociate elongator tRNAs from 70S complexes, probably because it does not bind to 70S (Gottlieb and Davis 1975; Risuleo et al. 1976; Van der Hofstad et al. 1978). The other selector of the initiator tRNA on 30S initiation complexes, IF2, also does not work on 70S initiation complexes. How is initiator tRNA selection achieved in vivo? The key component seems to be IF1, which does not select the initiator tRNA. If added together with IF3 or IF2, or both, selection is achieved gradually. We interpret this IF1 action as allowing IF3 and IF2 to resume their function on 70S complexes. IF1 could either facilitate IF3 and IF2 action on the 70S subunit or support the dissociation of 70S so that IF3 and IF2 can resume their function on the 30S subunit. We cannot distinguish those two possibilities in our experimental system. We favor the latter possibility; IF1 alters the dissociation of 70S by increasing both the forward and backward rates of the association reaction (Godefroy-Colburn et al. 1975). Furthermore, there is good evidence that 30S subunits are formed transiently in vivo (Kaempfer et al. 1968; Martin and Webster, 1975), and in vitro experiments indicate that 70S initiation complexes form via a 30S complex intermediate if initiation factors are present (Nomura et al. 1967; Blumberg et al. 1979). All current schemes of translational initiation use a 30S complex intermediate (Gualerzi and Pon 1981; Hershey 1987). The dissociation of 70S ribosomes in vivo is supported by the low intracellular Mg²⁺ concentration (Chaires et al. 1981). So far, IF3 has been implicated as the major factor in 70S dissociation (Kaempfer 1972). If, in the presence of IF1, 70S ribosomes initiate via 30S intermediates, IF1 might be very important for 70S dissociation.

Concluding remarks

Translational initiation can be thought of in the following way. Termination of translation of any message releases free ribosomal subunits (Martin and Webster 1975). The free 30S particle, with or without initiation factors, is able to recognize the initiation domain of most mRNAs so that a new round of translation can occur. In the work reported here, a large number of mRNAs were shown to be recognized in the initiation domain by factor-free 30S particles. Recognition of the mRNA effectively tethers the initiation codon and several other codons close to the anticodon of a tRNA bound in the ribosomal P site. It is these several other codons that present a problem for the initiation process, because at first approximation, factor-free 30S subunits do not select one tRNA over another. Recognition of the initiation codon over nearby codons in each message requires the joint action of IF2, IF3, and the initiator

tRNA. The two initiation factors appear to use opposite ends of the tRNA to accomplish selectivity. Finally, IF1 may largely facilitate subunit dissociation, allowing IF2 and IF3 to select the initiator tRNA on the 30S particles to which they bind.

Materials and methods

tRNA

Total *E. coli* tRNA and the uncharged *E. coli* tRNA species tRNA^{Met} and tRNA^{Phe} were purchased from Boehringer-Mannheim. All other tRNAs were purchased from Subriden RNA. fMet-tRNA^{Met} was prepared by D. Dix and J. Binkly, according to Dubnoff and Maitra (1971). The tRNA preparation used in experiments of Figures 2, 4, and 7 was ~60% charged and 39% charged and formylated, and the tRNA preparation used in experiments of Figures 10, 12, and 13 was ~40% charged and 12% charged and formylated. The synthetic tRNA fragment A22^{Met} was generated by T7 transcription from the fragments pts (top strand) and floop (bottom strand), both shown in Table 2, according to Milligan et al. (1987). The 3' nucleotide of purified (20% polyacrylamide gel electrophoresis) A22^{Met} was determined by 3'-end-labeling with ³²pCp, according to Beckett and Uhlenbeck (1984), followed by RNase digestion and two-dimensional thin layer chromatography (Nishimura 1972).

Enzymes

AMV-reverse transcriptase and MMLV-reverse transcriptase were obtained from Life Sciences, and Bethesda Research Laboratories, respectively. T7 RNA polymerase was provided by O. Uhlenbeck. The Klenow fragment of *E. coli* DNA polymerase I, T4 polynucleotide kinase, and T4 RNA ligase were purchased from New England Biolabs.

Initiation factors

E. coli initiation factors were a kind gift from C. Gualerzi. Initiation factor IF3 was also provided by D. Dix.

Ribosomes

30S and 50S subunits, prepared according to Kenney et al. (1979), were a gift from R. Traut. 70S ribosomes were generated by incubating 30S (0.2 μM) + 50S (0.24 μM) in our standard buffer (see below) for 15 min at 37°C prior to use.

mRNAs

Crude bacteriophage T4 mRNAs were prepared from *E. coli* NapIV infected with T4-33amN134-55amBL292 and were harvested 12 min after infection. The procedure for cell growth, infection, and extraction of the RNA is outlined in McPheeters et al. (1986). In vitro RNAs from the PvuII-cut plasmids pRS170 and pRS270 were synthesized with T7 polymerase, according to Lowary et al. (1986). The RNAs were purified on a 6% polyacrylamide gel.

Plasmids

The plasmid pRS170 contains the sequence from -92 to 107 of gene 32 (Krisch and Allet 1982), and the plasmid pRS270 contains a synthetic ribosome-binding site as shown in McPheeters et al. (1988). Both sequences are downstream from a T7 promoter. They were constructed by R. Saunders, who cloned the

Table 2. Description of oligonucleotides

Oligonucleotide	Sequence	mRNA target
rIIIP3	cctgttgggtgtaattaccacttg	<i>rIIB</i> , from 58 to 81
32loopD	atcttcagaagaaaaccttt	gene 32, from 60 to 80
32P2	gcttgaccgttaccgcat	gene 32, from 109 to 117
nama69	caccggcagtttctaatttatctg	gene 69, from 166 to 189
nama45	ccgagaaaaccgttcaaatcg	gene 45, from 164 to 184
pdex1	gaagctttttcgcgaatagta	<i>orf1</i> , from 85 to 106
namat1	gtagtatcttttcactac	gene 1, from 31 to 49
rega p1	ctttattattagcaattcccatc	<i>regA</i> , from 61 to 84
magnag 47	cattgcctctgttttagtct	<i>orfD</i> , from 38 to 57
LP134	gttgggtaacgccagg	<i>lacZ</i> , gene from 57 to 73
pts	taatacgactcactatag	—
floop	attatgctgagtgatccagccgagattgggcttc	—

gene 32 DNA into the *Bgl*II-cut (and blunted) site of pBC26 [Childs et al. 1985]. The small *Hind*III–*Sac*I fragment was subsequently ligated to the large *Hind*III–*Sac*I fragment of pT7-2 (U.S. Biochemical Corporation) to yield the pRS170 plasmid. To construct the pRS270 plasmid, the oligodeoxynucleotide corresponding to the synthetic operator region of pTS26 RNA [shown in Fig. 9 of McPheeters et al. (1988)] was ligated to the large *Hind*III–*Pst*I fragment of pBC26. The small *Hind*III–*Sac*I fragment of that construct was ligated to the large *Hind*III–*Sac*I fragment of pT7-2.

Oligodeoxynucleotides

Oligodeoxynucleotides were synthesized on an Applied Biosystems Model 380A DNA Synthesizer and purified by preparative polyacrylamide gel electrophoresis. The sequences and annealing places in the mRNAs are shown in Table 2.

Extension inhibition

Extension inhibition (or toeprinting) was performed essentially as described in Hartz et al. (1988) with the following modifications: AMV reverse transcriptase (~1 unit per reaction) was used exclusively for primer extension on crude *in vivo* RNA and the sequencing reactions. MMLV reverse transcriptase (~200 units per reaction) was employed with the *in vitro* transcripts. In the experiment of Figure 11, only 25 units of MMLV reverse transcriptase was used per reaction. Primers labeled with ³²P, complementary to the mRNAs of interest (see Table 2), were annealed as described in Hartz et al. (1988). Ten-microliter toeprinting reactions were prepared in standard buffer [10 mM Tris-acetate (pH 7.4), 60 mM NH₄Cl, 6 mM β-mercaptoethanol, 10 mM Mg acetate] and contained either 3 μg total RNA or 6.7 nM of the *in vitro* transcripts, along with ribosomes and tRNAs, as specified in Results. Reactions with all ingredients were prepared on ice and preincubated at 37°C, as specified in the figure legends, before reverse transcriptase was added while the reactions were incubating.

Binding constants

The strength of the toeprint stop compared with the extent to which reverse transcriptase reads through to the 5' end of the mRNA is a measure of how many initiation complexes have formed and 'survived' reverse transcription. The relative toeprint is defined as toeprint stop/5' end plus toeprint stop, and it reflects the fraction of mRNAs that formed initiation com-

plexes. By altering the concentration of tRNA under a saturating concentration of 30S subunits and limiting mRNA concentration, the relative toeprint should reflect the binding of the tRNA into the 30S initiation complex. To quantify the toeprint stop and the 5'-end stop, the autoradiographs were scanned on a Hoffer GS300 densitometer, and the peaks electronically integrated. The amount of complexes formed was subtracted from the original tRNA concentration to calculate the free tRNA concentration. A bimolecular binding function using an algorithm of Caceci and Cacheris (1984) was fitted to the binding data. The best fit was obtained when a relative toeprint of 40% for fMet–tRNA^{fMet}, 42% for tRNA^{fMet}, and 53% for A22^{fMet} was assumed as maximal binding (100%).

Acknowledgments

We thank J. Binkley for the preparation of fMet–tRNA^{fMet} and D. Dix for the provision of initiation factor IF3 and fMet–tRNA^{fMet}. We also thank R. Traut for the provision of 30S and 50S ribosomal subunits and R. Saunders for constructing the pRS170 and pRS270 plasmids. We could have done little of this work if C. Gualerzi had not given us a large supply of purified *E. coli* initiation factors. We are also grateful to E. Fish, T. Hollingsworth, D. Irvine, and C. Tuerk for their helpful discussions. This work was supported by a research grant from the National Institutes of Health (GM-28685).

Note added in proof

The competition of tRNA^{Cys} and tRNA^{Phe} with initiator tRNA and the selection of initiator tRNA by IF3 seen in Figure 7 (at 10 mM Mg²⁺) are also observed at the more physiological Mg²⁺ concentration of 6 mM Mg²⁺. This corresponds to an even lower free Mg²⁺ concentration as the reactions contain 1.5 mM nucleoside triphosphates.

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Genes Dev. 1989, **3**:

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