Specific functional interactions of nucleotides at key −3 and +4 positions flanking the initiation codon with components of the mammalian 48S translation initiation complex

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Eukaryotic initiation factor (eIF) 1 maintains the fidelity of initiation codon selection and enables mammalian 43S preinitiation complexes to discriminate against AUG codons with a context that deviates from the optimum sequence GCC[A/G]CCAUGG, in which the purines at −3 and +4 positions are most important. We hypothesize that eIF1 acts by antagonizing conformational changes that occur in ribosomal complexes upon codon-anticodon base-pairing during 48S initiation complex formation, and that the role of −3 and +4 context nucleotides is to stabilize these changes by interacting with components of this complex. Here we report that U and G at +4 both UV-cross-linked to ribosomal protein (rp) S15 in 48S complexes. However, whereas U cross-linked strongly to C1696 and less well to AA1818–1819 in helix 44 of 18S rRNA, G cross-linked exclusively to AA1818–1819. U at −3 cross-linked to rpS5 and eIF2α/H9251, whereas G cross-linked only to eIF2α. Results of UV cross-linking experiments and of assays of 48S complex formation done using α-subunit-deficient eIF2 indicate that eIF2α’s interaction with the +3 purine is responsible for recognition of the +3 context position by 43S complexes and suggest that the +4 purine/AA1818–1819 interaction might be responsible for recognizing the +4 position.

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Eukaryotic ribosomes locate the initiation codon on most mRNAs by a scanning mechanism. A 43S complex comprising a ribosomal 40S subunit, eukaryotic initiation factors [eIFs] 1, 1A, and 3, and an eIF2-GTP/Met-tRNA Met i complex binds to the 5′-cap-proximal region of mRNA with the help of eIF4A, eIF4B, and eIF4F and scans downstream to the initiation codon to form a 48S complex. Initiation codon recognition and base-pairing with the Met-tRNA Met i, anticodon triggers eIF5-mediated hydrolysis of eIF2-bound GTP and, most importantly, subsequent release of phosphate [Algire et al. 2005]. The prevailing model is that this leads to release of eIF2-GDP from the 40S subunit, retaining Met-tRNA Met i in the ribosomal P site, after which eIF5 mediates displacement of other factors and joining of the 60S ribosomal subunit to form an 80S ribosome [Pestova et al. 2000; Unbehaun et al. 2004].

The initiation codon is recognized by base-pairing with the anticodon of Met-tRNA Met i, [Cigan et al. 1988] and is usually the first AUG triplet from the mRNA’s 5′ end. Scanning 40S subunits can bypass the first AUG triplet if it is <10 nucleotides [nt] from the 5′ end of mRNA or if its context deviates from the optimum sequence GCC[A/G]CCAUGG, particularly at −3 and +4 positions [in bold] [Kozak 1986, 1991]. These two context nucleotides are conserved features of mammalian mRNAs and together can enhance translation 20-fold; in yeast, the nucleotide context is less important for initiation codon recognition, and its only common feature is a purine at the −3 position [Kozak 1986, Cavener and Ray 1991]. eIF1 enhances the processivity of scanning and plays the key role in ensuring the fidelity of initiation codon selection by enabling 43S complexes to discriminate against 48S complex formation on non-AUG triplets, on AUG triplets located near the 5′ end.

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of mRNA, and on AUG triplets with suboptimal context (Yoon and Donahue 1992; Pestova et al. 1998, Pestova and Kolupaeva 2002). It also ensures the fidelity of initiation codon selection at the later stage of ribosomal subunit joining by inhibiting premature GTP hydrolysis by eIF2 and by coupling initiation codon recognition with activation of eIF2's GTPase activity (Unbehaun et al. 2004; Valasek et al. 2004; Maag et al. 2005).

eIF1 binds to the interface surface of the 40S subunit between the platform and initiator tRNA, facing the codon–anticodon base pairs but not contacting them directly (Lomakin et al. 2003). This suggests that it promotes scanning and performs its monitoring function indirectly, by influencing the conformation of the platform and the positions of Met-tRNA\(^{\text{Met}}\) and mRNA in ribosomal complexes. To explain eIF1’s mechanism of action, we propose that binding of eIF1–43S complexes induces a scanning-competent conformation that is favorable for rejection of codon–anticodon mismatches and does not permit activation of hydrolysis of eIF2-bound GTP by eIF5. However, on recognizing the initiation codon, a 43S complex would have to undergo conformational changes upon base-pairing to form a 48S complex, which would be antagonized by eIF1. In our model, the conformation of an arrested 48S complex would be stabilized by codon–anticodon base-pairing and by elements within mRNA such as 5′-flanking sequences and context nucleotides. If a 48S complex assembled without eIF1 is insufficiently stable, due to the presence of a noncognate initiation codon, poor context or to the absence of 5′-flanking sequences, it dissociates on delayed addition of eIF1 (Pestova and Kolupaeva 2002). An implication of this model is that the role of context nucleotides (particularly of −3 and +4 positions) is to stabilize an arrested ribosomal complex by interacting specifically with its constituents. Hypotheses that context nucleotides interact with 18S rRNA (e.g., Kozak 1986, Cavener and Ray 1991) in a functionally analogous manner to the Shine-Dalgarno interaction of 16S rRNA and prokaryotic mRNAs have not been substantiated.

In this study we used mRNAs with either 4-thiouridine (“thioU”) or 6-thioguanosine (“thioG”) at −3 and +4 positions [hereafter, −3 and +4] for “zero-length” UV cross-linking of ribosomal proteins, 18S rRNA, and initiation factors in 48S complexes to identify nucleotide-specific interactions of purines and pyrimidines at −3 and +4 that could account for the nucleotide context rule. U and G at +4 both cross-linked to ribosomal protein (rp) S15, but whereas U[+4] specifically cross-linked mostly to C\(_{1696}\) and to some extent to AA\(_{1818-1819}\) in helix 44 of 18S rRNA, G[+4] cross-linked exclusively to AA\(_{1818-1819}\). The base specificity of the interaction of the +4 purine with AA\(_{1818-1819}\) is therefore likely responsible for recognition of this context nucleotide by 43S complexes, so that in addition to monitoring the fidelity of elongator tRNA selection (Ogle et al. 2001), AA\(_{1818-1819}\) might also play a role in initiation codon selection. U[−3] specifically and equally efficiently cross-linked to rpS5 and to eIF2α, whereas G[−3] cross-linked exclusively to eIF2α. The functional involvement of eIF2α in recognizing the [−3] nucleotide was confirmed by assaying 48S complex formation in the presence of α-subunit-deficient eIF2. In the absence of eIF1, the effect of the lack of eIF2α on the efficiency and specificity of 48S complex formation on AUG triplets with different nucleotide contexts was minor. However, in the presence of eIF1, 43S complexes assembled without eIF2α could no longer discriminate the nature of the −3 nucleotide, and 48S complex formation was much less efficient, irrespective of the nucleotide at [−3]. This suggests that interaction of the [−3] nucleotide with eIF2α is generally important for 48S complex formation in the presence of eIF1, but that eIF2α interacts more strongly with a purine than with a pyrimidine residue, increasing the resistance of 48S complexes to dissociation by eIF1, and that this accounts for the −3 nucleotide context rule. The fact that without sucrose density gradient centrifugation 85%–90% of eIF2 remained associated with 48S complexes formed on AUG triplets with G[−3] after eIF5-induced hydrolysis of eIF2-bound GTP could account for the resistance of 48S complexes to eIF1’s dissociating influence after GTP hydrolysis and before the actual ribosomal subunit joining.

### Results

**48S complex formation on (CAA)nAUG(CAA)m mRNAs containing thioU and thioG**

We hypothesized that the role of [−3] and [+4] context nucleotides could be to stabilize conformational changes in 48S complexes that occur upon base-pairing, by interacting with elements of these complexes. We investigated their interactions with components of the 48S complex by UV cross-linking using mRNAs that had a single uridine or guanosine (in addition to the AUG codon) at these positions (Fig. 1A). In two mRNAs the context of AUG triplets was good [purines at [−3] and [+4]] and in two it was suboptimal [a pyrimidine at [−3] or [+4]]. Flanking AUG codons with multiple CAA triplets to avoid additional U or G nucleotides also minimized secondary structure and increased initiation efficiency. mRNAs were transcribed in vitro in the presence of thioU or thioG (Fig. 1B), which can be specifically cross-linked to proteins and nucleic acids by low-energy (360-nm) irradiation, yielding “zero-length” cross-links that represent direct contacts with 48S complex constituents. Differences in the specificity/intensity of cross-links between mRNAs containing either thioU or thioG could be indicative of the nucleotide specificity of interactions. ThioG is incorporated less efficiently than thioU into transcripts and may exist in a thio–thione equilibrium that could lead to its misincorporation (Sergiev et al. 1997, Favre et al. 1998). Toe-printing analysis done in the absence of eIF1 [to avoid potential differences in efficiency of 48S complex formation due to context differences of initiation codons] showed that 48S complex assembled equally and efficiently on mRNAs containing...
Contacts of the nucleotide at position \([\pm 4]\) of mRNA with components of the 48S complex

mRNA transcripts used for UV cross-linking contained thioU or thioG and were labeled with \(\textsuperscript{32}P\)-CTP. 48S complexes assembled from 40S subunits, eIF2, eIF3, eIF4A, eIF4B, eIF4F, eIF1A, and Met-tRNA\(_{\text{Met}}\), were purified from unincorporated components by sucrose density gradient centrifugation and cross-linked by irradiation at 360 nm. U\([\pm 2]\) and G\([\pm 3]\) of the initiation codon base-pair with the Met-tRNA\(_{\text{Met}}\) anticodon and thus cannot cross-link to other components of 48S complexes, so radiolabeling of factors, ribosomal proteins, and 18S rRNA can be attributed exclusively to interactions with \([-3]\) and \([\pm 4]\) nucleotides. In control experiments, thioU\([\pm 2]\) or thioG\([\pm 3]\) did not cross-link to components of 48S complexes assembled on mRNA with U and G only in the initiation codon [V.G. Kolupaeva, A.V. Pisarev, C.U.T. Hellen, and T.V. Pestova, in prep.].

Because eIF1 is a functional analog of prokaryotic IF3 (which causes rearrangement of mRNA on 30S subunits) [LaTeana et al. 1995, Shapkina et al. 2000], mRNA cross-linking was assayed in 48S complexes assembled with and without eIF1. RNase-treated samples were analyzed by SDS-PAGE and two-dimensional [2D] gel electrophoresis to identify cross-linked proteins.

A single protein of the same mobility was cross-linked in 48S complexes assembled with or without eIF1 on mRNAs with thioU or thioG at \([\pm 4]\) [Fig. 2A,B, lanes 1,3]. The specificity of UV cross-linking of thioU- and thioG-containing mRNAs did not differ, but consistent with other studies, thioU cross-linked more efficiently than thioG [Nikiforov and Connolly 1992, Fig. 2A,B, lanes 1,3], likely reflecting intrinsic differences in cross-linking efficiencies of these thionucleotides. The low molecular weight of the cross-linked protein indicated that it was a ribosomal protein. Covalently bound mRNA nucleotides cause cross-linked ribosomal proteins to shift “northwest” in 2D gels. Taking this into consideration, cross-linking to \([\pm 4]\) was attributed to rpS15 [Fig. 2C,D]. Its identity was confirmed by mass-spectrometry sequencing of EAPPMEKPEVK and GVDDLQLDM-SYEQLMQLYSAR peptides. rpS15 is a homolog of prokaryotic rpS19, whose position in the crystal structure of the \textit{Thermus thermophilus} 30S subunit is shown in Figure 4 (see below).

To identify the approximate region of cross-linking of \([\pm 3]\) and \([\pm 4]\) nucleotides to 18S rRNA, it was extracted after irradiation of 48S complexes, hybridized with DNA oligonucleotides complementary to different regions, digested with RNase H, and separated by electrophoresis. Attribution of individual \(\textsuperscript{32}P\)-labeled UV-cross-linked fragments of 18S RNA took into account their reduced mobility due to covalently linked mRNA. The exact cross-linked nucleotide was identified by primer extension.

As with cross-linking to ribosomal proteins, cross-linking of 18S rRNA to the \([\pm 4]\) nucleotide was identical in 48S complexes assembled with or without eIF1, and thioG cross-linked less efficiently than thioU [Fig. 3A,B]. In contrast to cross-linking of ribosomal proteins, cross-linking of thioU and thioG to \([\pm 4]\) to 18S rRNA differed significantly. Both nucleotides cross-linked to nucleotides 1652–1863, but further analysis showed that thioG\([\pm 4]\) cross-linked exclusively to nucleotides 1815–1863 [Fig. 3A,B, lanes 3], whereas thioU\([\pm 4]\) cross-linked to this region weakly but cross-linked strongly to nucleotides 1652–1796 [Fig. 3A,B, lanes 6]. Cross-linking sites were then determined precisely: ThioU\([\pm 4]\) cross-linked mostly to C\(_{1696}\) and to some extent to AA\(_{1818}\) [Fig. 3D], whereas thioG\([\pm 4]\) cross-linked to C\(_{1696}\) and to some extent to AA\(_{1818}\) [Fig. 3C]. In control experiments [Fig. 3C–E, lanes 2], primer extension was done on 48S complexes assembled on mRNAs containing thioU or thioG at \([\pm 3]\). The positions of C\(_{1696}\) and AA\(_{1818}\) in h44 of 18S rRNA are shown on the secondary structure of 18S rRNA [Fig. 3F,G] and are mapped onto the corresponding nucleotides of 16S rRNA in the crystal structure of the \textit{T. thermophilus} 30S subunit [Fig. 4]. In conclusion, in 48S complexes, U\([\pm 4]\) in mRNA specifically cross-linked to C\(_{1696}\) and to some extent to AA\(_{1818}\) whereas G\([\pm 4]\) cross-linked exclusively to AA\(_{1818}\). U and G both also cross-linked to rpS15.
Recognition of initiation codon context

Unlike the [4] nucleotide, thioU or thioG at [3] did not cross-link to 18S rRNA, but thioU at [3] cross-linked specifically with equal efficiency to two proteins whereas thioG cross-linked only to the larger one [Fig. 2A,B, lanes 2, 4]. As for the [4] nucleotide, cross-linking was identical in 48S complexes formed with or without elf1 and was less efficient with thioG than thioU. The size of the smaller protein [21 kDa] indicated that it was a ribosomal protein. Taking into consideration the “northwest” shift of cross-linked proteins in 2D gels, we attributed cross-linking of U[3] to rpS5 [Fig. 2E,F]. Its identity was confirmed by mass-spectrometry sequencing of QAVDVFPLR and TIAEC*LADELINAAK peptides. It is a homolog of prokaryotic rp57, shown on the crystal structure of the T. thermophilus 30S subunit [Fig. 4]. The ~38-kDa molecular weight of the larger protein indicated that it could be elf2α or a subunit of elf3. To identify it, we assembled 48S complexes using elf2 with a truncated α-subunit (“Δelf2α”) from HeLa cells [Fig. 2C] and then exploited the observation that elf5-induced hydrolysis of elf2-bound GTP in 48S complexes releases elf2 but not elf3 [Unbehaun et al. 2004]. The N-terminal sequence of Δelf2α [PGLS, identical to that of intact elf2α] and its mobility in SDS-PAGE indicated that it was C-terminally truncated by 1.5–2 kDa, such cleavage is mediated by caspases [Satoh et al. 1999]. elf2 containing Δelf2α was ~30% as active in 48S complex formation as intact elf2 [data not shown]. The lower activity may be due to the substoichiometric amount of Δelf2α in elf2 compared with intact elf2 [Fig. 2G]. The ~38-kDa protein cross-linked to thioU and thioG at [3] was identified as elf2α by cross-linking 48S complexes assembled with elf2 containing Δelf2α on mRNA with thioU[3] [which yielded a cross-linked protein with altered mobility] and by cross-linking 48S complexes after incubation with elf5 [which led to specific loss of this band] [Fig. 2H, lanes 2, 3]. Cross-linking of elf2α to [3] was specific: No cross-linking was observed to [4] and only very little to [2] [V.G. Kolupaeva, A.V. Pisarev, C.U.T. Hellen, and T.V. Pestova, in prep.]. In conclusion, in 48S complexes, thioU[3] in mRNA cross-links specifically to rpS5 and elf2α, whereas thioG[3] cross-links exclusively to elf2α.

48S complex formation on CAA-GUS mRNAs with upstream AUGs in different nucleotide contexts in the presence of α-subunit- and β-subunit-deficient mammalian elf2

UV cross-linking data showed that G residues at [4] and [3] in mRNA specifically bound AA\textsubscript{1815-1819} of 18S rRNA and elf2α, respectively. To prove elf2α’s functional role in recognizing initiation codon context, we compared 48S complex assembly using complete elf2, elf2 lacking either elf2α (elf2β) or elf2β (elf2γ), or elf2βγ with recombinant elf2α on (CAA)n-AUGbad/bad-GUS, (CAA)n-AUGbad/good-GUS, and (CAA)n-AUGgood/bad-GUS mRNAs. These mRNAs have an un-

Figure 2. Contacts of nucleotides at [3] and [4] positions of mRNA with ribosomal proteins and factors in 48S complexes. (A,B) UV cross-linking of [32P]-labeled (CAA)n-AUG-[CAA]m RNAs containing 4-thioU or 6-thioG at [3] and [4] as indicated with components of 48S complexes assembled with [A] or without [B] elf1, assayed by SDS-PAGE and autoradiography. The positions of molecular weight markers [MW] are shown on the left. (C–F) Analysis by 2D electrophoresis of ribosomal proteins UV-cross-linked to [32P]-labeled (CAA)n-AUG-[CAA]m RNAs containing 4-thioU at [4] (C,D) and at [3] (E,F), (C,E) Gels of 40S subunit proteins stained with Simply Blue Safe Stain. (D,F) Autoradiographs of gels from C and E. Positions corresponding to radioactive spots [D,F] on stained gels [C,E] are shown in red. The positions of some ribosomal proteins based on sequencing data or according to Madjar et al. [1979] are indicated. (C) elf2 with full-length [lanes 2, 4] and truncated [lanes 1, 3] elf2α assayed by SDS-PAGE and Coomassie staining [lanes 1, 2] or immunoblotting [lanes 3, 4, elf2α subunits are indicated on the left. (H) UV cross-linking of [32P]-labeled (CAA)n-AUG-[CAA]m mRNA derivative containing 4-thioU at [3] with components of 48S complexes assembled using elf2 with intact elf2α [lane 1], elf2 with truncated elf2α [lane 2], and elf2 with intact elf2α and elf5 [lane 3], assayed by SDS-PAGE and autoradiography. Positions of elf2α and rpS5 are indicated on the left.

structured 5′-UTR lacking potential near-cognate initiation codons and contain additional AUG triplets in different contexts upstream of the GUS initiation codon (Fig. 5A; Pestova and Kolupaeva 2002). The AUGbad/bad triplet contained [−3] and [+4] pyrimidines, AUGbad/good had a [−3] pyrimidine and a [+4] purine, and AUGgood/bad had a [−3] purine and a [+4] pyrimidine. Small quantities of eIF2βγ and eIF2αγ (Fig. 5B, lanes 2,3) were obtained using standard eIF2 purification procedures [Anthony et al. 1990; Materials and Methods]. eIF1 is the principal factor that allows recognition of initiation codon context by scanning 43S complexes, so 48S complexes were assembled with and without eIF1. 48S complex formation did not depend on when eIF1 was added, and in all experiments described in this section identical data were obtained if eIF1 was added simultaneously with other translation components or if 48S complexes were first assembled without eIF1 and were then incubated with eIF1 for 15 min more. Consistent with our previous report [Pestova and Kolupaeva 2002], ∼90% of 43S complexes assembled with eIF1 and complete eIF2 were first assembled without eIF1 and were then incubated with eIF1 for 15 min more. 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eIF2 (Fig. 5C, lanes 4,5). No differences in 48S complex formation were detected on [CAA]n-AUGbad/good-GUS and [CAA]n-AUGood/bad-GUS mRNAs in the presence of eIF2αe or complete eIF2 (data not shown). Consistently, there was no difference in cross-linking of eIF2α to thioU or thioG at [−3] or [−4] of mRNA in 48S complexes with complete eIF2 or eIF2αe (Fig. 5D, lanes 1,2; data not shown).

The role of eIF2α in 48S complex formation was investigated using [CAA]n-AUGbad/good-GUS and [CAA]n-AUGood/bad-GUS mRNAs. This combination of mRNAs containing upstream AUG triplets with only one purine at either [−3] or [−4] was optimal for these studies because 48S complex formation on the first AUG triplet of [CAA]n-AUGbad/bad-GUS mRNA in the presence of eIF1 is inefficient even with complete eIF2, and quantitating possible reductions in 48S complex formation with eIF2βγ reliably would be difficult. On the other hand, 48S complex formation on the first AUG of [CAA]n-AUGgood/good-GUS mRNA would be too efficient to permit detection of potential leaky scanning.

In addition, [CAA]n-AUGbad/good-GUS and [CAA]n-AUGgood/bad-GUS mRNAs allow the relative effects of purines at different positions on the efficiency of 48S complex formation to be compared. With complete eIF2 and without eIF1, 48S complexes formed almost exclusively on the first AUG triplet on both mRNAs (Fig. 5F,G, lanes 5). In the presence of eIF1, complex formation on the first AUG triplet with a [−3] purine was more efficient, constituting ∼80% of 48S complexes whereas 48S complex formation on the first AUG with a [−4] purine constituted ∼50% of the total (Fig. 5F,G, lanes 6). The [−3] purine was therefore relatively more important for these mRNAs than that at [−4]. In the absence of eIF1, total 48S complex formation on the two AUGs for both mRNAs was only 10% lower with eIF2βγ than with complete eIF2. Initiation on both mRNAs was slightly leakier: 10−15% of 48S complexes formed on the GUS AUG with eIF2βγ, whereas only ∼4% of 48S complexes formed there with complete eIF2 (Fig. 5F,G, lanes 1,5).

Although it had only a minor effect in the absence of eIF1, the lack of eIF2α strongly affected 48S complex formation in elF1’s presence. Total 48S complex formation with eIF2βγ and eIF1 on the two AUG triplets of both mRNAs was reduced threefold (Fig. 5F,G, cf. lanes 2,6). The relative reduction in 48S complex formation on the first AUG triplet was higher with [CAA]n-AUGgood/bad-GUS mRNA, in which case the ratio between 48S complex formation on the first and second AUG triplets fell to 1:1 from 4:1 in the presence of complete eIF2 (Fig. 5G, lanes 2,6) and became similar to the ratio of 48S complex formation on the first [with an unfavorable [−3] pyrimidine] and second AUGs of [CAA]n-AUGbad/good-GUS mRNA (Fig. 5F, lanes 2,6). This result suggests that in the absence of eIF2α, 43S complexes cannot sense the nature of the [−3] nucleotide. The similar relative efficiencies of 48S complex formation on the two AUG triplets on both mRNAs despite the first AUG triplet of [CAA]n-AUGbad/good-GUS mRNA having a favorable [−4] purine suggest that the “−4 nucleotide rule” might be secondary to the “−3 nucleotide rule” and may not function efficiently in the absence of the eIF2α/[−3] nucleotide interaction. The fact that 48S complex formation with eIF2α-deficient eIF2 was strongly reduced on both AUGbad/good and AUGgood/bad suggests that interaction of eIF2α with the [−3] nucleotide is, irrespective of its nature, generally important for resistance of 48S complexes to dissociation by eIF1. Addition of recombinant eIF2α to reaction mixtures containing eIF2βγ restored the efficiency of 48S complex formation on both AUG codons to the level observed with complete eIF2 (Fig. 5F,G, lanes 2,4,6). Consistently, recombinant eIF2α added to reaction mixtures with eIF2βγ was cross-linked to thioU or thioG at [−3] in 48S complexes (Fig. 5D, lanes 3,4), E). 43S complexes became slightly less leaky and fewer 48S complexes assembled on the GUS AUG codon with eIF2βγ and recombinant eIF2α than with native complete eIF2 (Fig. 5F,G, lanes 4,6). The eIF2α N-terminal domain may interact with the [−3] nucleotide, in which case the N-terminal tag may influence this interaction, but we were reluctant to tag the C terminus of...
eIF2α because its C-terminal domain interacts with eIF2γ. In conclusion, these data suggest that interaction of eIF2α with the [−3] nucleotide is generally important for 48S complex formation in the presence of eIF1, and that eIF2α likely interacts more strongly with a purine at [−3], protecting 48S complexes more from dissociation by eIF1.

**Interaction of eIF2α with the [−3] nucleotide of mRNA in 48S complexes after eIF5-induced hydrolysis of eIF2-bound GTP**

Our data suggest that eIF2α’s interaction with the [−3] nucleotide stabilizes 48S complexes against dissociation by eIF1. However, it is generally accepted that eIF5-induced hydrolysis of eIF2-bound GTP leads eIF2 to dissociate from 48S complexes. If eIF1 can dissociate aberrant initiation complexes after GTP hydrolysis, then one would expect that if ribosomal subunit joining does not occur immediately after this event, then even 48S complexes assembled on AUG triplets with a [−3] purine would be dissociated at this stage. However, 48S complexes formed with eIF1 on the good context AUG codon of the GUS ORF of (CAA)n-AUGbad/bad-GUS mRNA remained intact after 15 min incubation with eIF5 (Fig. 6A, lanes 1, 2). This result was not due to a hypothetical inability of eIF1 to discriminate the context of the initiation codon after hydrolysis of eIF2-bound GTP, because ~95% of 48S complexes formed on the first bad context AUG of the same mRNA without eIF1 could still be dissociated by eIF1 after incubation with eIF5 [Fig. 6B, lanes 2, 4]. The upstream AUG triplet had poor context at [−3] and [+4] whereas the downstream AUG triplet had good context at both positions. The [+4] purine could conceivably be sufficient to stabilize 48S complexes after hydrolysis of eIF2-bound GTP. However, re-
taining a purine only at \([3]\) of the first AUG codon in \([\text{CAA}]_n\cdot\text{AUGgood/bad-GUS}\) mRNA yielded 48S complexes that were as resistant to eIF1-mediated dissociation after GTP hydrolysis as eIF5-untreated 48S complexes (data not shown). To account for this result, we tested if interaction of the \([-3]\) purine switches from eIF2 to another component of the 48S complex after GTP hydrolysis, which could render 48S complexes resistant to eIF1-mediated dissociation in the absence of eIF2. Just as for eIF5-untreated 48S complexes, no specific interaction was detected between thioG\([3]\) and 18S rRNA or any ribosomal protein after eIF5-induced hydrolysis of eIF2-bound GTP (Fig. 6D; data not shown). These results suggest that eIF5B promotes dissociation of eIF2 from the 48S complexes after GTP hydrolysis of eIF2-bound GTP, which is nevertheless not complete if the mRNA has a \([-3]\) purine. The absence of UV cross-linking of eIF2 to thioU\([3]\) by 70\% (Fig. 6D, lane 4; data not shown). These results suggest that eIF5B promotes dissociation of eIF2 from the 48S complexes after hydrolysis of eIF2-bound GTP, which is nevertheless not complete if the mRNA has a \([-3]\) purine. The absence of UV cross-linking of eIF2 to either thioU or thioG at \([-3]\) after treatment of 48S complexes with eIF5, eIF5B, and 60S subunits (Fig. 6D, lane 5; data not shown) indicated complete conversion of 48S complexes into 80S ribosomes and confirmed that incubation with eIF5 alone or together with eIF5B in identical conditions (Fig. 6D, lanes 3, 4) led to complete hydrolysis of eIF2-bound GTP. In case some eIF1 was lost from 48S complexes during their initial purification by sucrose density gradients, we compared the effect of adding eIF5 alone or together with eIF1–48S complexes on UV cross-linking of eIF2 to thioG\([3]\): No difference was detected, which means that eIF2 release was not affected (data not shown).

eIF5-induced hydrolysis of eIF2-bound GTP, therefore, does not completely dissociate eIF2 from 48S complexes, and the fact that the nature of the \([-3]\) nucleotide influences eIF2 release suggests that mRNA stabilizes binding of eIF2–48S complexes after GTP hydrolysis through interaction of eIF2 with the \([-3]\) nucleotide. The fact that only a small fraction of eIF2 was released from 48S complexes assembled on AUG codons with a \([-3]\) purine upon hydrolysis of eIF2-bound GTP could account for resistance of these complexes to dissociation by eIF1.

**Figure 6.** Influence of eIF5-induced hydrolysis of eIF2-bound GTP on 48S complex formation on AUG triplets in good and bad context \(A,B\) and on UV cross-linking of eIF2\(\alpha\) to the \([-3]\) nucleotide of mRNA in 48S complexes \(C,D\). \(A,B\) Toe-print analysis of 48S complexes assembled on \((\text{CAA})_n\cdot\text{AUGbad/bad-GUS}\) mRNA from 40S subunits, Met-tRNA\(^{\text{Met}}\), and eIFs as indicated. Toesprints due to 48S complexes are shown on the left. \((C,D)\) UV cross-linking of \(^{32}\text{P}-\text{labeled (CAA)}_n\cdot\text{AUG}-(\text{CAA})_m\) mRNA derivatives containing 4-thioU or 6-thioG at \([-3]\) with components of 48S complexes before and after incubation with eIF5, eIF5B, and 60S subunits, as indicated. In lanes 2 48S complexes incubated with eIF5 were subjected to sucrose density gradient centrifugation before UV cross-linking. Cross-linked proteins were assayed by SDS-PAGE and autoradiography. eIF2\(\alpha\) and rpS5 are indicated on the right.
et al. 2000), so we assayed interactions of the mRNA on 30S subunits (La Teana et al. 1995; Shapkina IF3, a functional analog of eIF1, alters the position of
with and without eIF1

Discussion
eIF1’s position on the 40S subunit between the platform and initiator tRNA suggests that it acts indirectly to ensure the fidelity of initiation codon selection and, specifically, to enable 43S complexes to discriminate against AUG triplets in suboptimal context (Pestova and Kolupaeva 2002, Lomakin et al. 2003). The finding that the C-terminal domain of prokaryotic IF3 (which is not homologous to eIF1) can bind the same region of the 40S subunit and perform many of eIF1’s functions in initiation codon selection, including enabling 43S complexes to recognize initiation codon context, also favors an indirect mode of action for eIF1 (Lomakin et al. 2006). Our hypothesis that eIF1 acts by antagonizing conformational changes in the 48S complex that occur as a result of initiation codon recognition and base-pairing with the anticodon suggests that the role of the key -3 and +4 context nucleotides is to stabilize such changes by interacting with components of the 48S complex. Here, we used UV cross-linking to characterize and compare the specificity of interactions of thioU and thioG at these positions with constituents of this complex. In a separate study, we used mRNAs containing single thioU residues at positions -26 to +11 to map the mRNA path on the 40S subunit in 48S complexes (Ogle et al. 2001). The equivalent prokaryotic nucleotides [AA1492–1493 in T. Thermophilus] flip out upon binding of cognate aminoacyl tRNA to the A-site during elongation and interact with the minor groove of the first two base pairs of the base-paired codon–anticodon helix, thereby monitoring the fidelity of elongator tRNA selection (Ogle et al. 2001). Flipping out of AA1492–1493 also occurs during prokaryotic initiation when IF1 binds to the A-site area of the 30S subunit; these bases splay apart whereas they stack together when cognate tRNA binds to the A-site (Carter et al. 2001). Binding of eIF1A, the eukaryotic IF1 homolog (Battiste et al. 2000) or other factors to the 40S subunit might also alter the conformation of the upper part of h44 and flip out AA1818–1819. Such conformational changes could account for “zero-length” cross-linking of thioU and thioG at [+4] to AA1818–1819 in 48S but not 80S complexes. Cross-linking of thioU[+4] to C1696 in 48S complexes was not consistent with cross-linking of thioU[+4] to the equivalent of rabbit C1691 in H28 of 18S rRNA in human 80S complexes (Bulygin et al. 2005). This discrepancy cannot be explained by the difference in positions of mRNA in 48S and 80S complexes: In our recent experiments C1691, cross-linked specifically to thioU[8] in 48S and 80S complexes (V.G. Kolupaeva, A.V. Pisarev, C.U.T. Hellen, and T.V. Pestova, in prep.), consistent with cross-linking of thioU[+4] to the equivalent nucleotide [C1393] in prokaryotic 70S complexes [Rinke-Appel et al. 1993]. In prokaryotes, C1400 and AA1492–1493 [equivalents of rabbit C1696 and AA1818–1819] are opposite each other, flanking the mRNA [Fig. 4]. Cross-linking of thioU to

UV cross-linking in 48S complexes formed with and without eIF1

IF3, a functional analog of eIF1, alters the position of mRNA on 30S subunits [La Teana et al. 1995; Shapkina et al. 2000], so we assayed interactions of the -3 and +4 nucleotides in 48S complexes assembled with and without eIF1. The interactions of thioU or thioG at both positions were unaffected by eIF1. Even if eIF1 influences the positions of mRNA or Met- tRNA\textsuperscript{Met} in scanning ribosomal complexes, the final conformation of 48S complexes with established codon–anticodon base-pairing appears not to depend on eIF1’s involvement in their assembly. We detected eIF1 in 48S complexes after eIF5-induced hydrolysis of eIF2-bound GTP [Unbehaun et al. 2004], but the observation that eIF1 was released from minimal yeast initiation complexes following codon–anticodon base-pairing [Maag et al. 2005] suggests that in mammalian 48S complexes, eIF1 might be displaced from its original location on the 40S subunit but be retained in these complexes by interaction with eIF3. If this is so, the apparently identical position of mRNA in 48S complexes assembled with and without eIF1 is not surprising.

UV cross-linking to the [+4] position

Both thioU[+4] and thioG[+4] cross-linked to rpS15. However, whereas thioU cross-linked weakly to AA1818–1819 and strongly to C1696 in h44 of 18S rRNA, thioG cross-linked exclusively to AA1818–1819. Specific mRNA cross-linking to components of the 48S complex has not previously been analyzed, so we compared our data with mRNA cross-linking in eukaryotic 80S complexes phased by cognate tRNA and in prokaryotic 70S complexes. Cross-linking of thioU[+4] to rp15 in 48S complexes was consistent with the same interaction in 80S complexes [Bulygin et al. 2005], rpS19, the prokaryotic homolog of rpS15, is located in the head of the 30S subunit [Fig. 4, Wimberly et al. 2000]. Its C-terminal tail points toward the interface side but does not reach the A-site codon, so cross-linking of rpS15 is likely due to N- or C-terminal extensions relative to prokaryotic rpS19.

Cross-linking of mRNA to AA1818–1819 has been detected with midranger nucleotide derivatives but not with “zero-length” cross-linkers: No cross-linking of AA1818–1819 to thioU[+4] was observed in phased or unphased 80S complexes [Demeshkina et al. 2000, Bulygin et al. 2005]. The equivalent prokaryotic nucleotides [AA1492–1493 in T. Thermophilus] flip out upon binding of cognate aminoacyl tRNA to the A-site during elongation and interact with the minor groove of the first two base pairs of the base-paired codon–anticodon helix, thereby monitoring the fidelity of elongator tRNA selection (Ogle et al. 2001). Flipping out of AA1492–1493 also occurs during prokaryotic initiation when IF1 binds to the A-site area of the 30S subunit; these bases splay apart whereas they stack together when cognate tRNA binds to the A-site [Carter et al. 2001]. Binding of eIF1A, the eukaryotic IF1 homolog (Battiste et al. 2000) or other factors to the 40S subunit might also alter the conformation of the upper part of h44 and flip out AA1818–1819. Such conformational changes could account for “zero-length” cross-linking of thioU and thioG at [+4] to AA1818–1819 in 48S but not 80S complexes. Cross-linking of thioU[+4] to C1696 in 48S complexes was not consistent with cross-linking of thioU[+4] to the equivalent of rabbit C1691 in H28 of 18S rRNA in human 80S complexes [Bulygin et al. 2005]. This discrepancy cannot be explained by the difference in positions of mRNA in 48S and 80S complexes: In our recent experiments C1691, cross-linked specifically to thioU[8] in 48S and 80S complexes (V.G. Kolupaeva, A.V. Pisarev, C.U.T. Hellen, and T.V. Pestova, in prep.), consistent with cross-linking of thioU[+4] to the equivalent nucleotide [C1393] in prokaryotic 70S complexes [Rinke-Appel et al. 1993]. In prokaryotes, C1400 and AA1492–1493 [equivalents of rabbit C1696 and AA1818–1819] are opposite each other, flanking the mRNA [Fig. 4]. Cross-linking of thioU to
both sites suggests that structural rearrangements in 48S complexes cause them to be closer to each other than their equivalents in prokaryotic 30S subunit/70S ribosome crystal structures. The inability of thioG to cross-link to C\textsubscript{1604}, might be due to its specific interaction with A\textsubscript{1818} and/or A\textsubscript{1819}, which could cause further structural adjustments that preclude this cross-link.

**UV cross-linking to the [-3] position**

In 48S complexes, neither thioU nor thioG at [-3] cross-linked efficiently to 18S tRNA, but thioU[-3] cross-linked to rpS\textsubscript{2} and eIF\textsubscript{2}α and thioG[-3] cross-linked only to eIF\textsubscript{2}α. Cross-linking of rpS\textsubscript{2} to the [-3] position is consistent with the similar paths of mRNA on eukaryotic 40S and prokaryotic 30S subunits: In prokaryotic ribosomal complexes, thioU[-3] cross-links specifically to rpS\textsubscript{7}, a homolog of eukaryotic rpS\textsubscript{5} (Fig. 4; La Teana et al. 1995). Cross-linking of the [-3] nucleotide to rpS\textsubscript{5} was also observed in eukaryotic 80S complexes (Demeshkina et al. 2003); this study also reported its cross-linking to rpS\textsubscript{26} (and to rpS\textsubscript{2} and rpS\textsubscript{3}, which we suggest was non-specific, taking into account the positions of prokaryotic analogs of eukaryotic rpS\textsubscript{2} and rpS\textsubscript{3} in the 30S subunit, Wimberly et al. 2000). In contrast to the study by Demeshkina et al. (2003), we saw specific cross-linking of rpS\textsubscript{26} to mRNA in 48S complexes only to thioU at [-8] to [-11] but not at all at [-3] (V.G. Kolupaeva, A.V. Pisarev, C.U.T. Hellen, and T.V. Pestova, in prep.).

The mRNA path in 48S complexes has not been studied, so specific cross-linking of eIF\textsubscript{2}α to [-3] in mRNA has not been reported. eIF\textsubscript{2}α consists of structured N-terminal and C-terminal domains that are mobile relative to each other; the latter binds eIF\textsubscript{2}γ (Yatime et al. 2004). eIF\textsubscript{2}α might thus bind the [-3] nucleotide either through the N-terminal domain or through its unstructured C-terminal tail. The absence of the ~10 C-terminal amino acids of eIF\textsubscript{2}α and, interestingly, of eIF\textsubscript{2}β did not influence this interaction. Consistent with the affinities to Met-tRNA\textsuperscript{Met}\textsubscript{2} of eIF\textsubscript{2}α-GTP and eIF\textsubscript{2}α-GDP differing by only one order of magnitude (Kapp and Lorsch 2004), in the absence of sucrose density gradient centrifugation, eIF\textsubscript{5}-induced hydrolysis of eIF\textsubscript{2}β-bound GTP did not lead to complete dissociation of eIF\textsubscript{2}β from 48S complexes so that 30%–35% and 85%–90% of eIF\textsubscript{2}α could still cross-link to thioU[-3] and thioG[-3], respectively. The fact that the nature of the [-3] nucleotide influenced its cross-linking to eIF\textsubscript{2}α after eIF\textsubscript{5}-induced GTP hydrolysis suggests that the eIF\textsubscript{2}α-mRNA interaction influences release of eIF\textsubscript{2}α during subunit joining. It is possible that without this interaction, GTP hydrolysis would result in greater and even complete eIF\textsubscript{2}α dissociation.

The finding that eIF\textsubscript{5}A enhances release of eIF\textsubscript{2}α from 48S complexes after GTP hydrolysis merits special attention. Although unlike its prokaryotic homolog IF\textsubscript{2}, binding of eIF\textsubscript{5}A to Met-tRNA\textsuperscript{Met}\textsubscript{2} has not been shown directly, this interaction might occur on the 40S subunit and after binding to 48S complexes, eIF\textsubscript{5}A might compete with eIF\textsubscript{2} for interaction with Met-tRNA\textsuperscript{Met}\textsubscript{2}. Weakening of eIF\textsubscript{2}/Met-tRNA\textsuperscript{Met}\textsubscript{2} binding after hydrolysis of bound GTP could permit an interaction between Met-tRNA\textsuperscript{Met}\textsubscript{1} and the C-terminal domain IV of eIF\textsubscript{5}B to be established, and consequently promote release of eIF\textsubscript{2}α. However, complete release of eIF\textsubscript{2}α from 48S complexes assembled on mRNA containing thioG[-3] occurred only after ribosomal subunit joining, which suggests that eIF\textsubscript{2}α is completely released only during the actual ribosomal subunit joining event promoted by eIF\textsubscript{5}B. mRNA, therefore, influences release of eIF\textsubscript{2}α as well as of eIF\textsubscript{3} from initiation complexes (Unbehaun et al. 2004).

**Activities of α-subunit- and β-subunit-deficient eIF2 in 48S complex formation**

Specific UV cross-linking to thioG[-3] in mRNA in 48S complexes suggests that eIF\textsubscript{2}α is involved in recognition of initiation codon context and thus in initiation codon selection. The functionality of this interaction was confirmed in experiments on 48S complex formation in the presence of eIF\textsubscript{2}α-deficient eIF\textsubscript{2}βγ on two mRNAs, both containing two AUG triplets, of which the first had a purine residue either at [-3] or at [-4]. With complete eIF\textsubscript{2}β but without eIF\textsubscript{1}, 48S complexes formed almost exclusively on the first AUG triplets of both mRNAs, but in the presence of eIF\textsubscript{1}, 48S complex formation was more efficient on the AUG triplet with the [-3] purine (80% of total 48S complexes) than with the [-4] purine (50% of total 48S complexes). In the absence of eIF\textsubscript{1}, the lack of eIF\textsubscript{2}α had little effect on the efficiency or specificity of 48S complex formation so that 43S complexes stopped efficiently on the first AUG triplet irrespective of its context. In eIF\textsubscript{1}’s presence, the lack of eIF\textsubscript{2}α strongly influenced 48S complex formation. First, the combined efficiency of 48S complex formation on two AUG triplets on both mRNAs was threefold lower than with complete eIF\textsubscript{2}α. Second, whereas the ratio of 48S complexes formed on the first AUG triplet with a [-3] purine and on the second AUG triplet was 4:1 in the presence of complete eIF\textsubscript{2}α, it fell to 1:1 in the absence of eIF\textsubscript{2}α and became similar to the ratio of 48S complex formation on mRNA with two AUG triplets in which the first was flanked by a [-3] pyrimidine. In the presence of eIF\textsubscript{1}, 43S complexes assembled without eIF\textsubscript{2}α therefore could not sense the nature of the [-3] nucleotide and 48S complexes formed with equal efficiency on AUG triplets whether there was a purine or a pyrimidine at [-3]. This result confirmed the suggested role for eIF\textsubscript{2}α in discriminating the [-3] context nucleotide. The reduced efficiency of 48S complex formation on the AUG triplet with a [-3] pyrimidine in the absence of eIF\textsubscript{2}α also suggests that eIF\textsubscript{2}α’s interaction with the [-3] nucleotide, irrespective of its nature, is generally important for 48S complex formation in the presence of eIF\textsubscript{1} but that it is the strength of interaction (which is higher for purines) that is responsible for the [-3] context rule. Our finding that eIF\textsubscript{2}α is not fully released from 48S complexes upon eIF\textsubscript{5}A-induced GTP hydrolysis and that the extent of its release depends on the nature of the [-3] nucleotide (being only 10%–15% with G at this position) could ac-
count for the resistance of 48S complexes to eIF1-mediated dissociation after hydrolysis of eIF2-bound GTP and before the ribosomal subunit joining step.

Although interaction of the [+4] nucleotide with rpS15 was not base-specific and rpS15 is also cross-linked to thioU[A] [5] [V.G. Kolupaeva, A.V. Pisarev, C.U.T. Hellen, and T.V. Pestova, in prep.], we cannot exclude the possibility that the rpS15–[+4] nucleotide interaction is important for initiation codon selection. We cannot directly test the functional importance of interaction of the [+4] nucleotide with AA1818–1819, but the base specificity of this interaction points to the fact that AA1818–1819 are involved not only in monitoring the fidelity of elongator tRNA selection, but also in selection of the initiation codon during initiation. By analogy with eIF2α, the interaction of the [+4] nucleotide with components of the 48S complex AA1818–1819 and/or rp S15 might also be generally important to stabilize 48S complexes assembled on AUG triplets whether they have a purine or a pyrimidine at [+4].

Materials and methods

Plasmids

Vectors for expression of His₆-tagged eIF1, eIF1A, eIF4A, eIF4B, eIF5, and *Escherichia coli* methionyl-tRNA synthetase, and for [CAA]ₙ–AUG-[CAA]ₙ mRNA transcription have been described [Pestova et al. 1996, 1998, 2000; Pestova and Kolupaeva 2002; Lomakin et al. 2006]. The bovine eIF2α coding region was amplified by PCR from pUKC50 [Green et al. 1991] and cloned between BamH1 and HindIII restriction sites of pET28b [Novagen] yielding pET[His₆]-eIF2α. Translation vectors for [CAA]ₙ–AUG-[CAA]ₙ, mRNA derivatives containing U or G at [3] or [+4] were made by inserting complementary oligonucleotides corresponding to a T7 promoter, 5'–UTR, coding region, and Smal restriction site between the BamH1 and HindIII sites of pBR322. [CAA]ₙ–AUGbad/good-GUS and [CAA]ₙ–AUGgood/bad-GUS transcription vectors were made using the same strategy as for the [CAA]ₙ–AUGbad/bad-GUS vector [Pestova and Kolupaeva 2002].

*In vitro transcription*

All mRNAs were transcribed using T7 RNA polymerase. [CAA]ₙ–AUGbad/bad-GUS, [CAA]ₙ–AUGgood/bad-GUS, and [CAA]ₙ–AUGbad/good-GUS mRNAs were transcribed as described [Pestova and Kolupaeva 2002]. For UV cross-linking experiments, [³²P]-labeled derivatives of [CAA]ₙ–AUG-[CAA]ₙ mRNA containing 4-thioU or 6-thioG [8 × 10⁶ c.p.m./µg] were transcribed from Smal-digested plasmids in the presence of 4-thioUTP [Ambion] or 6-thioGTP [Iena Bioscience, [α²⁵P]-CTP [222 Tbq/mmol], and a RAC transcription primer [Drachman]. For toe-printing experiments, derivatives of [CAA]ₙ–AUG-[CAA]ₙ mRNA were transcribed from Ealg-digested plasmids.

*Purification of factors and ribosomal subunits, and aminoaaclylation of initiator tRNA*

40S and 60S subunits, eIF2, eIF3, eIF4F, and eIF5B were purified from rabbit reticulocyte lysate [RRL] and recombinant eIF2α, eIF1A, eIF4A, eIF4B, eIF5, and *E. coli* methionyl-tRNA synthetase were expressed in *E. coli* BL21(DE3) and purified as described [Pestova et al. 1996, 1998, 2000; Lomakin et al. 2006]. α-Subunit-deficient eIF2α was purified as described [Anthony et al. 1990]. β-Subunit-deficient eIF2α is always obtained in small amounts during eIF2 purification from RRL [Pestova et al. 2000] as a peak eluted from MonoQ two fractions earlier than complete eIF2α. eIF2α with a truncated α-subunit was purified in small quantities from HeLa cells using the purification procedure previously described for eIF2 from RRL [Pestova et al. 2000] as a peak eluted from MonoQ slightly earlier than complete eIF2α. Recombinant eIF2α was expressed in *E. coli* BL21(DE3) and purified from Ni²⁺–NTA [Qiagen] and MonoQ. Total native rabbit tRNA [Novagen] was aminoaacylated by recombinant methionyl-tRNA synthetase as described [Pestova et al. 1996].

*UV cross-linking experiments*

48S complexes were assembled on [³²P]-labeled derivatives of [CAA]ₙ–AUG-[CAA]ₙ mRNA containing 4-thioU or 6-thioG at [3] or [+4] by incubating 100 ng of mRNA, 10 pmol Met-tRNA⁹Met, 8 pmol 40S subunits, 5 µg of different forms of eIF2, 15 µg of eIF3, 2.5 µg of eF4A, 0.5 µg of eIF4B, 2.5 µg of eIF4F, 0.2 µg of eIF1A, 0.2 µg of eIF1, and 3 µg of recombinant eIF2α (as indicated) in 100 µL of buffer A [20 mM Tris at pH 7.5, 100 mM KAc, 2 mM DTT, 2.5 mM MgAc₂, 0.25 mM spermidine] containing 1 mM ATP, 0.4 mM GTP, or 0.4 mM GTP when eIF5 was included for 10 min at 37°C. In reaction mixtures shown in Figures 2H [lane 3] and 6C, D [lanes 2] hydrolysis of eIF2-bound GTP was induced by incubating with 1 µg of eIF5 for 15 min. 48S complexes were purified by centrifugation in a Beckman SW55 rotor for 1 h and 40 min at 4°C and 50,000 rpm in 10%–30% sucrose density gradients prepared in buffer A. [³²P]-labeled mRNA in ribosomal fractions was monitored by Cherenkov counting. Equal amounts of counts (∼200,000 c.p.m.) of peak fractions were irradiated at 360 nm for 30 min on ice using a UV-Stratalinker [Stratagene] and used to identify cross-linked proteins and nucleotides in 18S rRNA. For experiments shown in Figure 6C [lane 3] and D [lanes 3, 5], 48S complexes were purified by sucrose density gradient centrifugation, diluted threefold with buffer A + 0.4 mM GTP, incubated for 15 min at 37°C with 1 µg of eIF5, 1 µg of eIF5B, or 8 pmol 60S subunits, as indicated, and then subjected to UV irradiation.

*Identification of cross-linked proteins*

To identify UV-cross-linked eIFs, −20 µL of cross-linked ribosomal fractions containing equal amounts of counts were treated with RNase A and subjected to electrophoresis in NuPAGE 4%–12% Bis-Tris-Gel [Invitrogen] followed by autoradiography. UV-cross-linked ribosomal proteins were identified by acidic-SDS 2D gel electrophoresis. Complete cross-linked peak fractions (∼200,000 c.p.m.) were combined, transferred to buffer B [20 mM Tris-HCl at pH 7.5, 50 mM KCl, 2 mM MgCl₂, 2 mM DTT, 0.1 mM EDTA], concentrated on microcon YM10 centrifugal filter units [Millipore] to 100 µL of final volume, and treated with RNase A for 30 min at 37°C. These samples were mixed with 100 µL of 40S subunits [OD₂₆₀ = 100 o.u./mL] in buffer B. Proteins were extracted from these mixtures with 100 mM MgCl₂ in 67% acetic acid and precipitated with acetone [Hardy et al. 1969]. Samples were then resuspended in 8 M urea, 1% 2-mercaptoethanol, 10 mM bis-tris acetate [pH 4.2], incubated for 15 min at 37°C, and subjected to first-dimension electrophoresis [Yusupov and Spirin 1988] in 120-mm-long glass tubes with a 2.4-mm inner diameter. First-dimension gels were incubated for 10 min in cathode buffer and combined with second-dimension gels, which had been prepared as described [Schagger and von Jägow 1987]. The separating gel [16.5% T and 3% C] contained 13.3% w/v glycerol. Gels were run for 12 h at 40 mA, stained with Simply Blue Safe Stain [Invitrogen], and destained with water for LC-nanospray tandem...
mass spectrometry of peptides derived by in-gel tryptic digestion at an in-house facility, or fixed with 10% methanol/5% glycerol for drying and autoradiography.

Identification of cross-linked nucleotides in 18S rRNA

After irradiating 48S complexes, rRNA, mRNA, and tRNA were phenol-chloroform extracted and ethanol precipitated. Regions of 18S rRNA cross-linked to [32P]-labeled mRNAs were first identified by RNase H digestion of 18S rRNA hybridized with a panel of -20-mer DNA oligonucleotides complementary to different regions of 18S rRNA essentially as described (Dontsova et al. 1992). 18S rRNA fragments were separated by electrophoresis in 12% denaturing PAGE. Cross-linked and uncross-linked 18S rRNA fragments were visualized by autoradiography and methylene blue staining, respectively. Cross-linked regions were identified and attributed to corresponding uncross-linked fragments of 18S rRNA on stained gels taking into account the reduced mobility of cross-linked RNA fragments due to covalently bound 64-nt mRNA. Precise identification of cross-linked nucleotides in 18S rRNA was done by primer extension inhibition using primers 5'-CAAGTTCGACCCGTTCTC-3' and 5'-CC TTTCGAGGTCCACC-3' complementary to nucleotides 1783-1799 and 1840-1856 of 18S rRNA respectively, chosen on the basis of RNase H digestion.

Toe-printing analysis of 48S initiation complexes

Ribosomal 48S complexes assembled on [CAA]n-AUGbad/bad-GUS, (CAA)n-AUGgood/bad-GUS, and (CAA)n-AUGbad/good-GUS mRNAs, and derivatives of (CAA)n-AUG-(CAA)m mRNAs were analyzed by primer extension using AMV RT essentially as described (Pestova and Kolupaeva 2002). Reaction mixtures (40 µL) containing 2 pmol mRNAs, 5 pmol Met-tRNAMet was first incubated in buffer A (+1 mM ATP + 0.4 mM GTP) at 37°C for 10 min, and in some cases for 15 min more with 1 µg of elf5S or 1 µg of elf5F and 0.3 µg of elf1A (Fig. 6A,B). Toe-printing analysis was done using [32P]pDATP and primers 5'-CATGACATTAACC-3' and 5'-CGCGCTTTCCCACCAA CG-3's complementary to prPB322 nucleotides 4307-4319 and GUS nucleotides 97-114, respectively. cDNA products were analyzed by electrophoresis through 6% polyacrylamide sequencing gel. Phosphorimage analysis was used to quantify the efficiency of initiation complex formation. All values presented in Results are the average of at least three independent experiments.

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References

Algire, M.A., Maag, D., and Lorsch, J.R. 2005. Pi release from elf2, not GTP hydrolysis, is the step controlled by start-site selection during eukaryotic translation initiation. Mol. Cell 20: 251-262.

Anthony Jr., D.D., Kinzy, T.G., and Merrick, W.C. 1990. Affinity labeling of eukaryotic initiation factor 2 and elongation factor 1 α β γ with GTP analogs. Arch. Biochem. Biophys. 281: 157-162.

Battiste, J.L., Pestova, T.V., Hellen, C.U., and Wagner, G. 2000. The elf1A solution structure reveals a large RNA-binding surface important for scanning function. Mol. Cell 5: 109-119.

Bulygin, K., Chavatte, L., Frolova, L., Karpova, G., and Favre, A. 2005. The first position of a codon placed in the A site of the human 80S ribosome contacts nucleotide C1696 of the 18S rRNA as well as proteins S2, S3, S3a, S30, and S15. Biochemistry 44: 2153-2162.

Carter, A.P., Clemons, W.M., Brodersen, D.E., Morgan-Warre, R.J., Hartsch, T., Wimberley, B.T., and Ramakrishnan, V. 2001. Crystal structure of an initiation factor bound to the 30S ribosomal subunit. Science 291: 498-501.

Cavener, D.R. and Ray, S.C. 1991. Eukaryotic start and stop translation sites. Nucleic Acids Res. 19: 3185-3189.

Cigan, A.M., Feng, L., and Donahue, T.F. 1988. Met-tRNAMet functions in directing the scanning ribosome to the start site of translation. Science 242: 93-97.

Demeshkina, N., Repkova, M., Ven'yaminova, A., Graifer, D., and Karpova, G. 2000. Nucleotides of 18S rRNA surrounding mRNA codons at the human ribosomal A, P, and E sites: A crosslinking study with mRNA analogs carrying an aryl azide group at either the uracil or the guanine residue. RNA 6: 1727-1736.

Demeshkina, N.A., Laletina, E.S., Meshchaninova, M.I., Repkova, M.N., Ven'ya mimova, A.G., Graifer, D.M., and Karpova, G.G. 2003. The mRNA codon environment at the P and E sites of human ribosomes deduced from photo crosslinking with puUUGUU. Mol. Biol. 37: 147-155.

Dontsova, O., Dokudovskaya, S., Kopylov, A., Rinke-Appel, J., Jünke, N., and Brimacombe, R. 1992. Three widely separated positions in the 16S RNA lie in or close to the ribosomal decoding region, a site-directed cross-linking study with mRNA analogues. EMBO J. 11: 3105-3116.

Favre, A., Saintomé, C., Fourrey, J.L., Clivio, P., and Laugäa, P. 1998. Thionucleobases as intrinsic photoaffinity probes of nucleic acid structure and nucleic acid–protein interactions. J. Photochem. Photobiol. B 42: 109-124.

Green, S.R., Fullekrug, J., Sauer, K., and Tuite, M.F. 1991. Isolation and characterisation of a bovine cDNA encoding eukaryotic initiation factor 2 α. Biochim. Biophys. Acta 1090: 277-280.

Hardy, S.J., Kurland, C.G., Voynow, P., and Mora, G. 1969. The 30S ribosomal subunit under the influence of the initiation factor eIF2. J. Biol. Chem. 244: 2897-2905.

Kapp, L.D. and Lorsch, J.R. 2004. GTP-dependent recognition of the methionine moiety on initiator tRNA by translation factor elf2. J. Mol. Biol. 335: 923-936.

Kozak, M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44: 283-292.

——. 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. 266: 19867-19870.

La Teana, A., Gualerzi, C.O., and Brimacombe, R. 1995. From stand-by to decoding site. Adjustment of the mRNA on the 30S ribosomal subunit under the influence of the initiation factors. RNA 1: 772-782.

Lomakin, I.B., Kolupaeva, V.G., Marintchev, A., Wagner, G., and Pestova, T.V. 2003. Position of eukaryotic initiation factor elf1 on the 40S ribosomal subunit determined by directed hydroxyl radical probing. Genes & Dev. 17: 2786-2797.
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Lomakin, I.B., Shirokikh, N.E., Yusupov, M.M., Hellen, C.U.T., and Pestova, T.V. 2006. The fidelity of translation initiation: Reciprocal activities of eIF1, IF3 and YcH. *EMBO J.* **25**: 196–210.

Maag, D., Fekete, C.A., Grzynski, Z., and Lorsch, J.R. 2005. A conformational change in the eukaryotic translation preinitiation complex and release of eIF1 signal recognition of the start codon. *Mol. Cell* **17**: 265–275.

Madjar, J.J., Arpin, M., Buisson, M., and Reboud, J.P. 1979. Spot position of rat liver ribosomal proteins by four different two-dimensional electrophoreses in polyacrylamide gel. *Mol. Gen. Genet.* **171**: 121–134.

Nikiforov, T.T. and Connolly, B.A. 1992. Oligodeoxynucleotides containing 4-thiouridine and 6-thiodeoxyguanosine as affinity labels for the Eco RV restriction endonuclease and modification methylase. *Nucleic Acids Res.* **20**: 1209–1214.

Ogle, J.M., Brodersen, D.E., Clemons Jr., W.M., Tarry, M.J., Carter, A.P., and Ramakrishnan, V. 2001. Recognition of cognate transfer RNA by the 30S ribosomal subunit. *Science* **292**: 897–902.

Pestova, T.V. and Kolupaeva, V.G. 2002. The roles of individual eukaryotic translation initiation factors in ribosomal scanning and initiation codon selection. *Genes & Dev.* **16**: 2906–2922.

Pestova, T.V., Hellen, C.U.T., and Shatsky, I.N. 1996. Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. *Mol. Cell. Biol.* **16**: 6859–6869.

Pestova, T.V., Borukhov, S.I., and Hellen, C.U.T. 1998. Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. *Nature* **394**: 854–859.

Pestova, T.V., Lomakin, I.B., Lee, J.H., Choi, S.K., Dever, T.E., and Hellen, C.U.T. 2000. The joining of ribosomal subunits in eukaryotes requires eIF5B. *Nature* **403**: 332–335.

Rinke-Appel, J., Junke, N., Brimacombe, R., Dukudovskaya, S., Dontsova, O., and Bogdanov, A. 1993. Site-directed cross-linking of mRNA analogues to 16S ribosomal RNA; A complete scan of cross-links from all positions between ‘+1’ and ‘+16’ on the mRNA, downstream from the decoding site. *Nucleic Acids Res.* **21**: 2853–2859.

Satoh, S., Hiijikata, M., Handa, H., and Shimotohno, K. 1999. Caspase-mediated cleavage of eukaryotic translation initiation factor subunit 2a. *Biochem. J.* **342**: 65–70.

Schagger, H. and von Jagow, G. 1987. Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**: 368–379.

Sergey, P.V., Lavrik, I.N., Wlasoff, V.A., Dokudovskaya, S.S., Dontsova, O.A., Bogdanov, A.A., and Brimacombe, R. 1997. The path of mRNA through the bacterial ribosome: A site-directed crosslinking study using new photoreactive derivatives of guanosine and uridine. *RNA* **3**: 464–475.

Shapkina, T.G., Dolan, M.A., Babin, P., and Wollenzien, P. 2000. Initiation factor 3-induced structural changes in the 30S ribosomal subunit and in complexes containing tRNA^{Met} and mRNA. *J. Mol. Biol.* **299**: 615–628.

Unbehaun, A., Borukhov, S.I., Hellen, C.U.T., and Pestova, T.V. 2004. Release of initiation factors from 40S complexes during ribosomal subunit joining and the link between establishment of codon–anticodon base-pairing and hydrolysis of eIF2-bound GTP. *Genes & Dev.* **18**: 3078–3093.

Valasek, L., Nielsen, K.H., Zhang, F., Fekete, C.A., and Hinniush, A.G. 2004. Interactions of eukaryotic translation initiation factor 3 (eIF3) subunit NIP1/c with eIF1 and eIF5 promote preinitiation complex assembly and regulate start codon selection. *Mol. Cell. Biol.* **24**: 9437–9455.

Wimberly, B.T., Brodersen, D.E., Clemons Jr., W.M., Morgan-Warren, R.I., Carter, A.P., vonrhein, C., Hartsch, T., and Ramakrishnan, V. 2000. Structure of the 30S ribosomal subunit. *Nature* **407**: 327–339.

Yatime, L., Schmitt, E., Blanquet, S., and Mechulam, Y. 2004. Functional molecular mapping of archaean translation initiation factor 2. *J. Biol. Chem.* **279**: 15984–15993.

Yoon, H. and Donahue, T.F. 1992. The *suil* suppressor locus in *Saccharomyces cerevisiae* encodes a translation factor that functions during tRNA^{Met} recognition of the start codon. *Mol. Cell. Biol.* **12**: 248–260.

Yusupov, M.M. and Spirin, A.S. 1988. Hot tritium bombardment technique for ribosome surface topography. *Methods Enzymol.* **164**: 426–439.

Yusupova, G.Z., Yusupov, M.M., Cate, J.H., and Noller, H.F. 2001. The path of messenger RNA through the ribosome. *Cell* **106**: 233–241.
Specific functional interactions of nucleotides at key −3 and +4 positions flanking the initiation codon with components of the mammalian 48S translation initiation complex

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