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eIF3j Is Located in the Decoding Center of the Human 40S Ribosomal Subunit

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SUMMARY

Protein synthesis in all cells begins with the ordered binding of the small ribosomal subunit to messenger RNA (mRNA) and transfer RNA (tRNA). In eukaryotes, translation initiation factor 3 (eIF3) is thought to play an essential role in this process by influencing mRNA and tRNA binding through indirect interactions on the backside of the 40S subunit. Here we show by directed hydroxyl radical probing that the human eIF3 subunit eIF3j binds to the aminoacyl (A) site and mRNA entry channel of the 40S subunit, placing eIF3j directly in the ribosomal decoding center. eIF3j also interacts with eIF1A and reduces 40S subunit affinity for mRNA. A high affinity for mRNA is restored upon recruitment of initiator tRNA, even though eIF3j remains in the mRNA-binding cleft in the presence of tRNA. These results suggest that eIF3j functions in part by regulating access of the mRNA-binding cleft in response to initiation factor binding.

INTRODUCTION

Protein synthesis in eukaryotes requires initiation factors to recruit initiator tRNA (Met-tRNA$_i$) and messenger RNA (mRNA) to the 40S ribosomal subunit prior to the assembly of active ribosomes. Initiation is thought to occur in ordered steps in which eIF3 associates with free 40S subunits and coordinates ordered binding of a Met-tRNA$_i$-eIF2-GTP complex and two small initiation factors, eIF1 and eIF1A, to form 43S preinitiation complexes (Hinnebusch, 2006). eIF3 prevents premature association of 43S complexes with 60S ribosomal subunits until mRNA has been loaded into the decoding center of the 40S subunit, although the molecular basis for its activity is not yet known. Comprised of 13 nonidentical proteins, the bulk of mammalian eIF3 binds the 40S subunit surface opposite the site of 60S ribosomal subunit binding (Siridechadilok et al., 2005; Srivastava et al., 1992). This position implied a largely indirect role in preventing premature subunit joining (Chaudhuri et al., 1999; Kolupaeva et al., 2005; Majumdar et al., 2003; Trachsel and Staehelin, 1979), although it has been suggested that eIF3 may also disrupt an intersubunit bridge directly (Siridechadilok et al., 2005).

Although relatively little is known about the functions of individual components of eIF3, one of the smallest subunits, eIF3j, is required for high-affinity binding of eIF3 to 40S subunits in vitro and in vivo (Fraser et al., 2004; Nielsen et al., 2006; Unbehaun et al., 2004). eIF3j is a highly conserved subunit (see Figure S1 in the Supplemental Data available with this article online) that can be readily dissociated from the rest of the eIF3 complex during purification (Fraser et al., 2004; Phan et al., 1998; Unbehaun et al., 2004; Valasek et al., 2001b). Deletion of eIF3j in *S. cerevisiae* results in a slow growth phenotype (Valasek et al., 1999), suggesting that while not essential for survival it may play a regulatory role that is required for efficient protein synthesis, or the translation of specific mRNAs. Interestingly, the association of eIF3j with the eIF3 complex is stimulated during the activation of T lymphocytes in an mTOR-dependent fashion (Miyamoto et al., 2005). Its C-terminal end has also been identified as a target of caspase-3 cleavage during apoptosis, implicating this segment of eIF3j in some aspect of translational control (Bushell et al., 2000). Intriguingly, the affinity of eIF3j for the 40S subunit decreases in the presence of mRNA (Benne and Hershey, 1978; Unbehaun et al., 2004), implying a possible role for this subunit in mRNA recruitment. eIF3j has also been linked to 40S subunit biogenesis (Valasek et al., 2001a; Yarunin et al., 2005).

To elucidate the role of eIF3j during the process of initiation, we determined its position on the 40S subunit by site-directed hydroxyl radical probing in the absence of other initiation factors, or in the context of the intact 43S preinitiation complex. Surprisingly, we find that eIF3j binds directly in the mRNA entry channel and aminoacyl (A) site. In addition, using fluorescence-based anisotropy experiments we demonstrate negative cooperativity between eIF3j and eIF1A for 40S binding, indicative of a role for

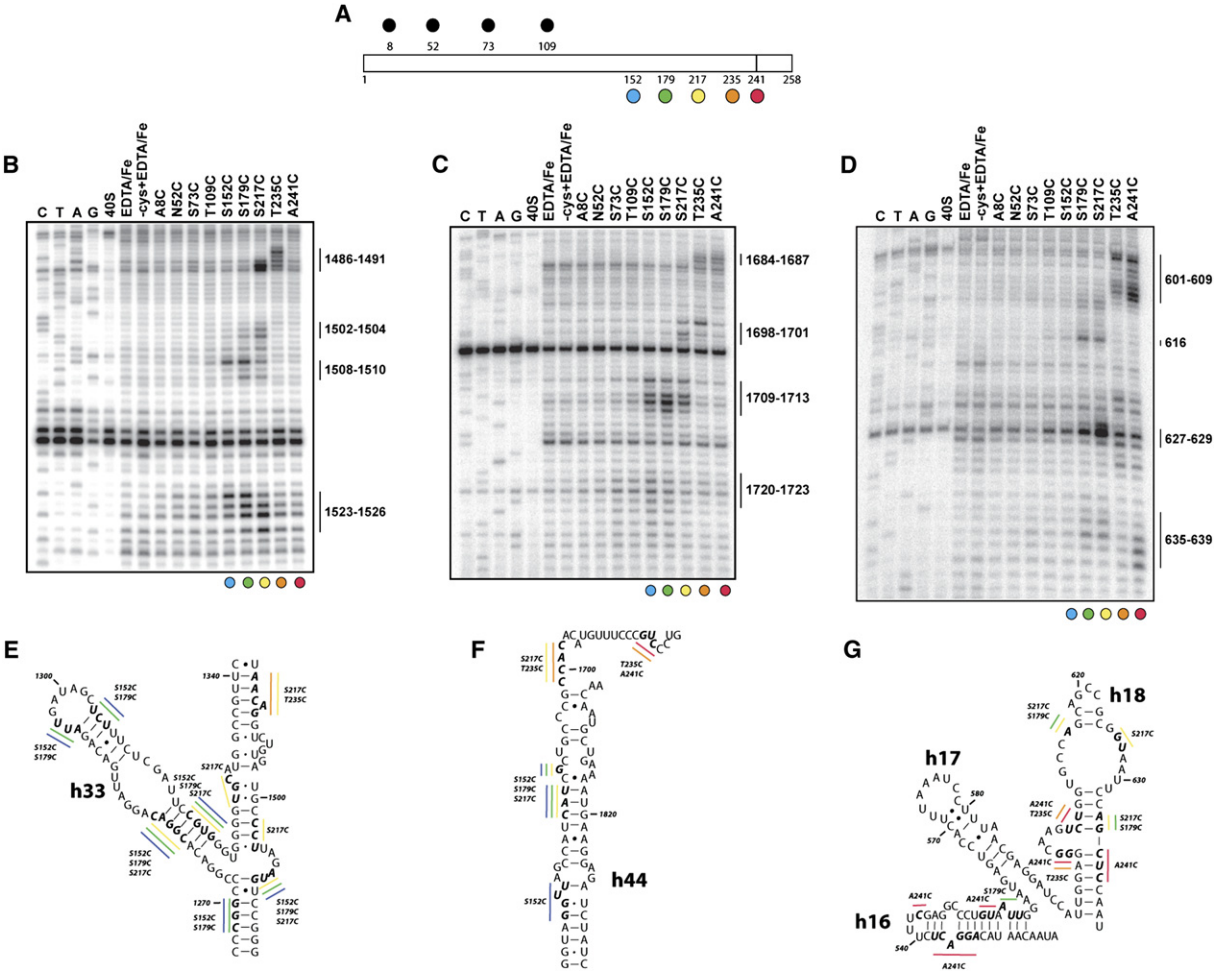

Figure 1. Directed Hydroxyl Radical Probing of 18S rRNA Using BABE-Fe-Modified eIF3j

(A) Schematic of eIF3j indicating positions of single-cysteine mutations introduced to provide BABE-Fe conjugation sites. Position 241 indicates the site of cleavage by caspase-3.

(B–D) Primer extension analysis of 18S rRNA cleaved by BABE-Fe-modified eIF3j. The sequencing lanes are indicated by the letters C, T, A, and G. Other lanes include 40S subunits in the absence or presence of EDTA/Fe, mock-derivatized eIF3j (−cys + EDTA-Fe), or eIF3j derivatized with BABE-Fe at the positions indicated. Nucleotide positions of cleavage sites are indicated next to each denaturing polyacrylamide gel, and colored circles correspond to tethering sites indicated in (A).

(E–G) Detailed regions of 18S rRNA secondary structure indicating cleavage sites.

eIF3j in regulating the assembly of the 43S preinitiation complex. We show that eIF3j and mRNA binding to the 40S subunit is thermodynamically coupled and displays 20-fold negative cooperativity. These results provide a molecular explanation for the previously observed weakening of eIF3j binding to the 40S subunit in the presence of mRNA (Benne and Hershey, 1978; Unbehaun et al., 2004). Our data also indicate that eIF3j maintains its position in the mRNA-binding cleft upon 43S complex formation, suggesting that this subunit of eIF3 regulates 43S-mRNA interaction. These results show that eIF3j associates with the decoding center of the 40S subunit and governs the binding of initiation factors and mRNA to form a scanning-competent initiation complex.

RESULTS AND DISCUSSION

The C Terminus of eIF3j Binds Near Conserved 40S Ribosomal Subunit Nucleotides

We investigated the position of eIF3j on the 40S ribosomal subunit to determine whether its location could explain its reduced affinity upon mRNA binding. To this end, we mapped eIF3j-40S subunit interactions using site-directed hydroxyl radical probing as utilized previously to determine binding sites of proteins on both prokaryotic and eukaryotic ribosomes (Culver and Noller, 2000; Lancaster et al., 2002; Lomakin et al., 2003). Single cysteines were introduced throughout the sequence of eIF3j (Figure 1A) and conjugated to bromoacetamidobenzyl-EDTA-Fe

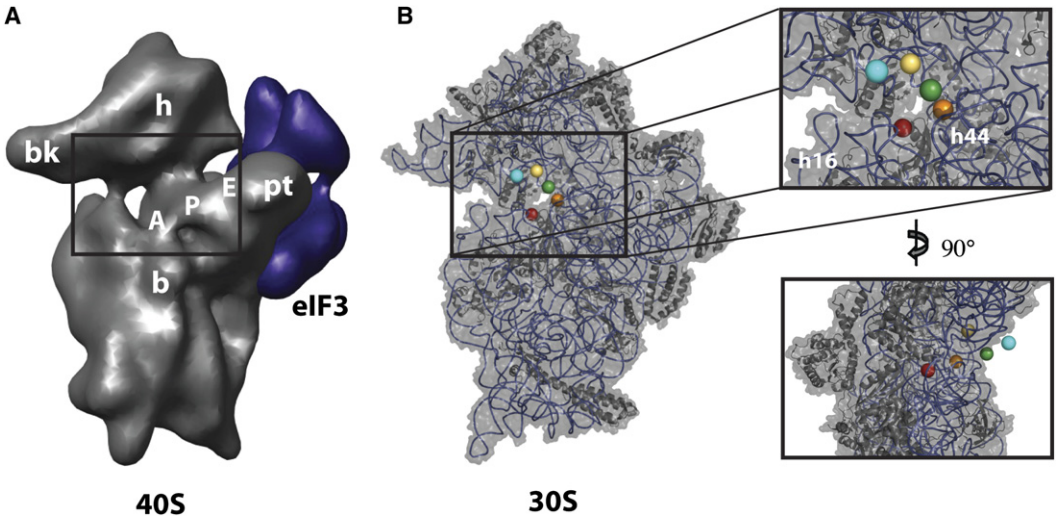

Figure 2. Mapping the Position of eIF3j on the 40S Subunit

(A) Model of eIF3 bound to the 40S subunit based on cryo-EM reconstructions of eIF3-HCV-IRES and HCV-IRES-40S complexes (Siridechadilok et al., 2005). The 40S subunit is shown in dark gray, and eIF3 is shown in purple. Landmarks for the 40S subunit are indicated: A, A site; P, P site; E, E site; bk, beak; b, body; pt, platform; and h, head. The boxed area indicates the corresponding area boxed in the 30S subunit view shown in (B). (B) Modeled positions of eIF3j amino acids in the crystal structure of the *T. thermophilus* 30S (Ogle et al., 2001) with detailed views of the decoding site (right). Helices 16, 18, and 44 of the 30S subunit are indicated as h16, h18, and h44, respectively.

(BABE-Fe). Neither the single-cysteine introductions nor BABE-Fe conjugation affected eIF3j activity, as analyzed by the ability of eIF3j to associate with the 40S ribosomal subunit and stabilize 40S subunit binding to intact eIF3 (Figures S2A–S2D). Each BABE-Fe-modified eIF3j protein was assembled into a 40S-containing complex and incubated with peroxide and ascorbate to produce free radicals; resulting cleavage of the 18S ribosomal RNA was analyzed by primer extension.

Hydroxyl radicals generated from BABE-Fe tethered to all five positions tested in the C terminus of eIF3j resulted in 18S rRNA cleavages in helices 16, 17, 18, 32, 33, 34, and 44 (Figures 1B–1G and Figures S3A–S3C). When hydroxyl radicals were generated from position 241, and to a lesser extent position 235, substantial cleavages occur in nucleotides 601–609 of helix 18, a region constituting the latch of the small ribosomal subunit (Frank et al., 1995). This latch has been proposed to clamp around the incoming mRNA and has been observed to "open" and "close" when the HCV IRES, or eIF1 and eIF1A, bind to the 40S subunit (Passmore et al., 2007; Spahn et al., 2001). Position 241 also cleaves helix 16, which has been identified as a flexible region of the 18S rRNA whose conformation changes when the mRNA-binding cleft opens upon eIF1 and eIF1A binding to the 40S subunit (Passmore et al., 2007). Hydroxyl radicals generated from positions 152, 179, and 217 cleave 18S rRNA at nucleotides 1709–1713 of helix 44, which is located near the P site of the 40S subunit. Regions of the 40S beak and the side of its head are also cleaved by hydroxyl radicals generated from positions 152, 179, and 217 (Figures 1C and 1G). Together, these results support the surprising conclusion that eIF3j is located on the 40S subunit interface, a region highly conserved between prokaryotic and eukaryotic ribosomes.

Modeling Places eIF3j in the mRNA Decoding Site of the 40S Subunit

Cleavage sites identified in the 18S rRNA were mapped onto their positions in the secondary structure of 16S rRNA, revealing that most cleavages corresponded to highly homologous regions of the ribosomal RNA. This enabled modeling of the C-terminal amino acid positions of eIF3j onto the crystal structure of the *T. thermophilus* 30S subunit (Ogle et al., 2001). Although no NMR or crystal structure of eIF3j is available, we were able to successfully triangulate five eIF3j amino acid positions modified with BABE-Fe, each of which produced at least three distinct 18S rRNA cleavages, and thereby determine their approximate position on the crystal structure of the 30S subunit (Figure 2). Unexpectedly, the resulting model places the C terminus of eIF3j directly in the mRNA entry channel and A site of the 40S subunit (Figure 2B). To our knowledge, our finding is the first indication that a subunit of eIF3 binds directly to the ribosomal decoding center and suggests that eIF3 may play a direct role in events that occur in the decoding center of the 40S subunit. In addition, amino acid positions 235 and 241 of eIF3j are located in the mRNA-binding cleft. The only other eukaryotic initiation factor suggested to be similarly positioned is the α subunit of eIF2, which was found to crosslink to the -3 position of an mRNA (Pisarev et al., 2006).

Notably, the C-terminal 16 amino acids of eIF3j are required for its high affinity for the 40S subunit, as cleavage

with caspase-3 reduces the association of eIF3j with the 40S subunit in vitro (Fraser et al., 2004). Our 30S-eIF3j model implies that this region of eIF3j is anchored deep inside the mRNA entry channel (Figure 2B). This anchor point is essential for association of the eIF3 complex with the 40S subunit even though the bulk of eIF3 is located not in the 40S subunit interface but is instead on its backside (Siridechadilok et al., 2005; Srivastava et al., 1992) (Figure 2A).

eIF3j and eIF1A Bind Anticooperatively to the 40S Subunit

Because a segment of eIF3j is located in the 40S subunit A site, we wondered whether it might interact with other initiation factors bound to the 40S subunit interface or block their access to this site. eIF1A, an initiation factor conserved throughout evolution, associates with the A site and is thought to facilitate the recruitment of Met-tRNA$_i$ and direct it to the peptidyl (P) site (Carter et al., 2001; Fekete et al., 2005; Passmore et al., 2007). eIF1A also promotes correct recognition of the initiation codon in association with initiation factor eIF1 (Pestova et al., 1998). To test whether eIF3j interacts with eIF1A on the surface of the 40S subunit, eIF3j was site-specifically labeled with a fluorescent probe and used in anisotropy-based equilibrium binding experiments (Maag and Lorsch, 2003). The anisotropy of labeled eIF3j was determined in the presence of increasing concentrations of 40S subunits and converted into the fraction of eIF3j bound at each 40S concentration (Figure 3A). The 6 nM equilibrium dissociation constant (K_D) for eIF3j-40S complexes increased by a factor of 15 to a K_D value of 90 nM upon the addition of saturating amounts of eIF1A (Figure 3A), demonstrating negative cooperativity between binding of eIF3j and eIF1A on the 40S subunit surface. In addition, binding of fluorescently labeled eIF1A to the 40S subunit was measured in the absence or presence of a saturating amount of unlabeled eIF3j (Figure 3B). The K_D value of 19 nM for eIF1A-40S complexes increased to 230 nM, showing that binding of these proteins is thermodynamically coupled (Figure 3C). In analogous experiments, no coupling was observed between binding of eIF3j and eIF1 on the 40S subunit (data not shown). Together, these results argue that eIF3j interacts directly or indirectly with eIF1A on the 40S subunit surface and present the possibility that eIF3j influences the conformation and activity of eIF1A in recruiting initiator tRNA (Met-tRNA$_i$) and/or decoding the AUG initiation codon.

eIF3j Regulates mRNA Binding to the 40S Subunit

To test whether mRNA influences eIF3j binding and positioning on the 40S subunit, we determined eIF3j-40S subunit complex affinity in the presence of a saturating concentration of an unstructured model mRNA (Maag et al., 2005). The addition of mRNA increased the K_D of eIF3j from 6 nM to 160 nM (Figure 4A), consistent with the previous observation of weakened eIF3j-40S subunit affinity upon mRNA association (Benne and Hershey, 1978;

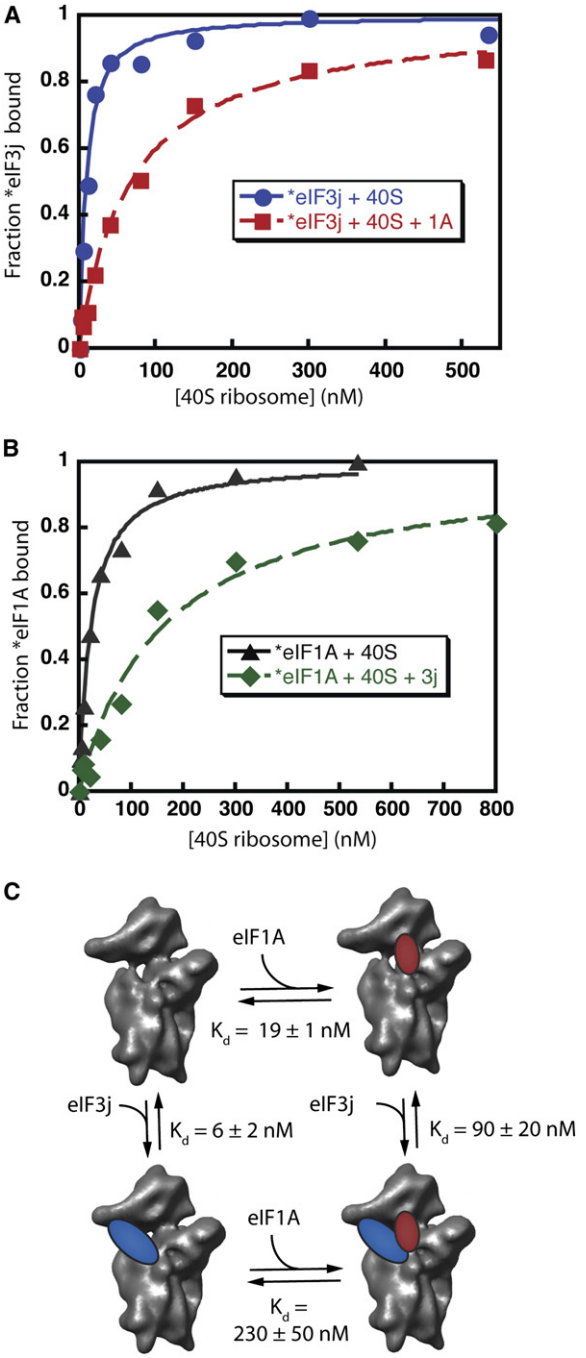

Figure 3. Binding Affinities of eIF3j-40S and eIF1A-40S Subunit Complexes

(A) Equilibrium binding of fluorescently labeled eIF3j to the 40S subunit in the absence or presence of a saturating concentration of eIF1A, as measured by anisotropy.

(B) Equilibrium binding of fluorescently labeled eIF1A to the 40S subunit in the absence or presence of a saturating amount of eIF3j, as measured by anisotropy.

(C) Diagram of the thermodynamic framework for the binding of eIF3j and eIF1A to the 40S subunit.

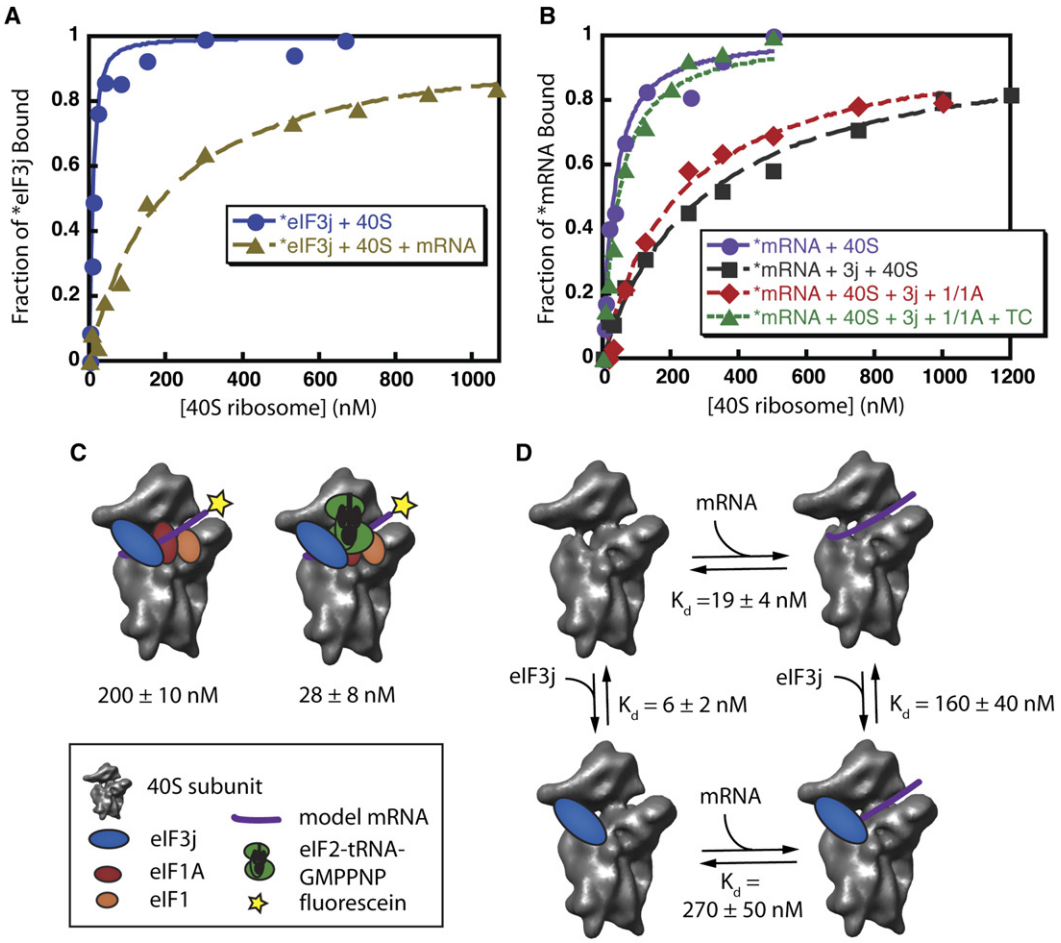

Figure 4. Effects of eIF3j and Other Factors on mRNA-40S Subunit Affinity

(A) Equilibrium binding of fluorescently labeled eIF3j to the 40S subunit in the absence or presence of a saturating amount of a short unstructured mRNA.

(B) Equilibrium binding of fluorescently labeled mRNA to the 40S subunit in the absence or presence of saturating concentrations of initiation factors, as measured by anisotropy.

(C) Summary of the K_D values for each experiment; the cartoons indicate components added.

(D) Thermodynamic framework for binding of eIF3j and mRNA to the 40S subunit.

Unbehaun et al., 2004). Together, these results raise the possibility that eIF3j is directly involved in the process of mRNA recruitment.

To determine whether eIF3j influences the association of mRNA with the 40S subunit, anisotropy-based binding experiments were performed using fluorescently labeled AUG-containing mRNA in a system previously shown to require only a subset of initiation factors for mRNA recruitment and correct AUG codon recognition (Maag et al., 2005). This system allows eIF3j function to be analyzed in the absence of the intact eIF3 complex, although it should be noted that the eIF3 complex may play additional roles in mRNA recruitment (Hinnebusch, 2006). This short mRNA of low predicted secondary structure has been shown to correctly associate with the mRNA-binding cleft using an environmentally sensitive pyrene dye attached to the 3′ end (Studer et al., 2003; data not shown) and was found by anisotropy to have a K_D of 19 nM for the 40S subunit in the absence of initiation factors (Figure 4B). This value, similar to that obtained for mRNA binding to the prokaryotic 30S subunit in the absence of a Shine-Dalgarno sequence and initiation factors (Studer and Joseph, 2006), is consistent with the mRNA-binding cleft being highly conserved between the prokaryotic and eukaryotic small ribosomal subunits. However, the binding of this mRNA to human 40S subunits is ~100-fold tighter than values previously reported for a model mRNA sequence using purified yeast 40S subunits (Maag et al., 2005). This disparity could reflect inherent species differences, or this difference in affinity could result from the fact that our assay uses direct anisotropy measurements whereas the previous work utilized a competition assay with two

different mRNAs. In addition, this difference could reflect the sensitivity of mRNA sequence and structure on the affinity for 40S subunits in the absence of initiation factors, and it is also possible that the fluorophore may influence the affinity of the mRNA for the 40S subunit in our system.

In keeping with our data showing negative cooperativity of eIF3j and mRNA binding to the 40S subunit, the addition of a saturating concentration of eIF3j increased the K_D of mRNA for the 40S from 19 nM to 270 nM (Figures 4B and 4D). This shows that binding of mRNA and eIF3j to the 40S subunit is thermodynamically coupled (Figure 4D). The addition of eIF1 and eIF1A slightly decreased the K_D to 200 nM, suggesting that they may play a direct role in mRNA recruitment in the presence of eIF3j. However, a substantial recovery of the affinity of mRNA for the 40S subunit in the presence of eIF3j was only achieved upon addition of eIF2/Met-tRNA$_i$ (Figure 4B). Previously published data using yeast components showed that upon recruitment of eIF2/Met-tRNA$_i$, the K_D of an unstructured mRNA for the 40S subunit in a competition assay was decreased to <2 nM (Maag et al., 2005). Since that assay did not include eIF3j, we tested the effect of eIF2/Met-tRNA$_i$ recruitment in the absence of eIF3j to determine whether the presence of eIF3j antagonizes mRNA binding even in the presence of eIF2/Met-tRNA$_i$. Using saturating amounts of eIF2/Met-tRNA$_i$ in the absence of eIF3j, the K_D of mRNA for the 40S was found to be 12 ± 1 nM compared to 28 ± 8 nM in the presence of eIF3j (Figures 4B and 4C and Figures 5A and 5B). The observation that eIF3j antagonizes mRNA binding to a small degree even in the presence of eIF2/Met-tRNA$_i$ suggests that eIF3j may remain bound to the 40S subunit even after mRNA recruitment. It is interesting to note that the short mRNA used in our study binds to human 40S subunits less tightly in the presence of saturating eIF2/Met-tRNA$_i$ than is observed for a similar unstructured mRNA binding to yeast 40S subunits (Maag et al., 2005 and Figure 5). This discrepancy may simply reflect differences between the assays and mRNAs used to measure mRNA affinity in each study. Alternatively, it is also possible that these data reflect the fact that AUG recognition in yeast does not have the same context requirements as mammalian AUG recognition (Hamilton et al., 1987; Kozak, 1987).

The C Terminus of eIF3j Remains Bound to the mRNA Entry Channel in the 43S Complex

To better understand the role of eIF3j during translation initiation, we tested whether the 18S rRNA cleavage pattern induced by BABE-Fe-modified eIF3j changes when assayed in the context of the intact 43S complex. In particular, we wondered whether eIF3j remains in the 40S subunit mRNA-binding cleft in the presence of eIF1, eIF1A, eIF2/Met-tRNA$_i$, and eIF3, since this complex is competent for mRNA recruitment and AUG recognition on unstructured mRNA (Pestova and Kolupaeva, 2002). To this end, each BABE-Fe-modified eIF3j protein was assembled into an eIF3j-43S complex and cleavages in two regions of the 18S rRNA were analyzed and compared to

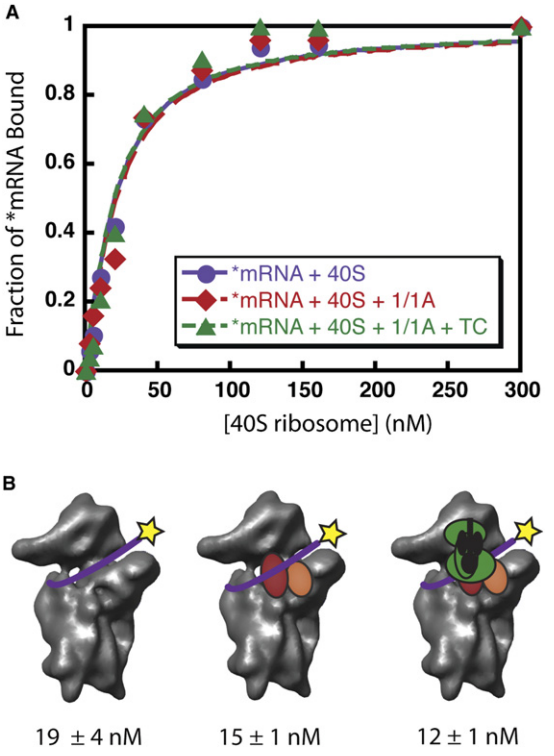

Figure 5. Analysis of mRNA Binding to the 40S Subunit in the Absence of eIF3j

(A) Equilibrium binding of fluorescently labeled mRNA to the 40S subunit in the absence or presence of saturating concentrations of initiation factors, as measured by anisotropy.

(B) Summary of the K_D values for each experiment; the cartoons indicate components added.

those observed using an eIF3j-40S complex. One of the regions chosen was that surrounding the ribosomal peptidyl (P) site, which is expected to change significantly upon 43S complex formation. As might be expected, many of the cleavage positions observed in eIF3j-40S complexes disappear upon formation of an eIF3j-43S complex (Figure 6A). This likely results from rRNA protection by the other initiation factors that associate with this region of the 40S subunit, but may also indicate 40S conformational changes or a change in the position of eIF3j in the 43S complex.

Notably, however, one cleavage position that remains unchanged in location and intensity in the 43S versus 40S complex is that induced at nucleotide 1698 by eIF3j modified with BABE-Fe at position 235 (Figure 6A). This cleavage site is located in the ribosomal A site, suggesting that, within the detection limits of this method, amino acid 235 of eIF3j remains unchanged in position within the mRNA-binding cleft in the 43S complex compared to the 40S subunit alone. To further examine the position of the C terminus of eIF3j in the 43S complex, we also determined the positions and intensities of cleavages in helix 18 (Figures 6B and 2). Surprisingly, the cleavage intensities obtained from hydroxyl radicals produced from

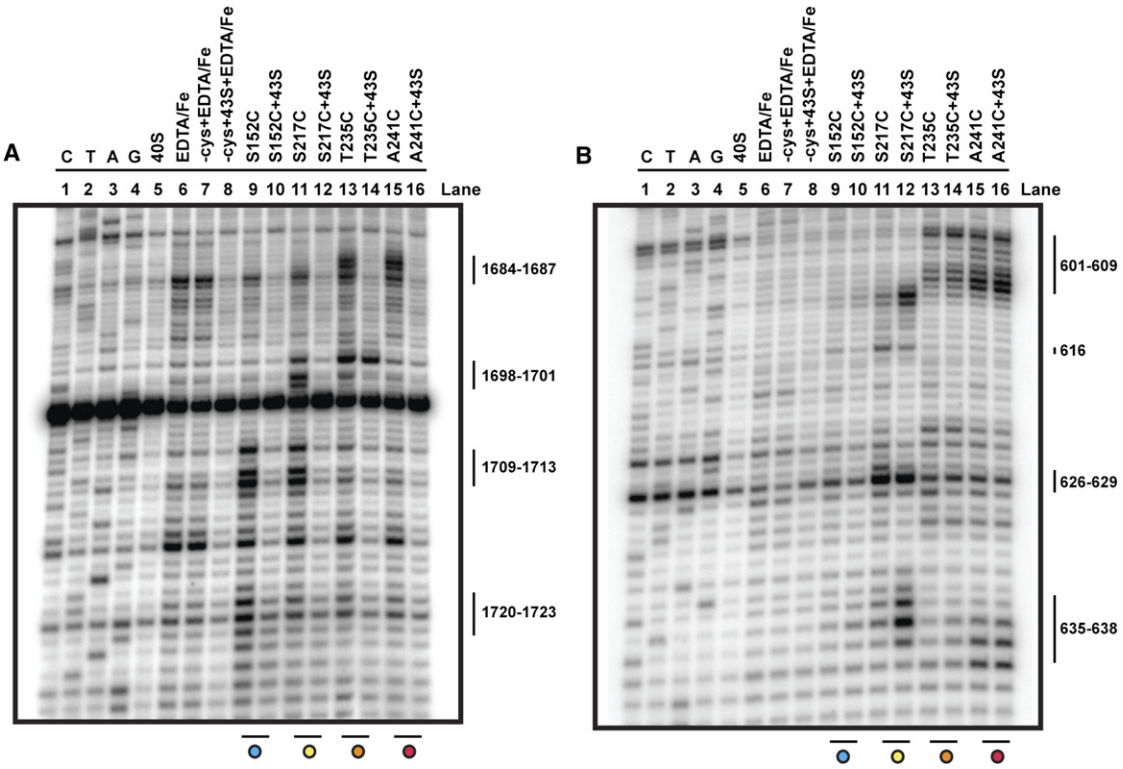

Figure 6. Directed Hydroxyl Radical Probing of 18S rRNA from BABE-Fe-eIF3j-43S Complexes

Primer extension analysis of 18S rRNA in the A site (A) and helix 18 (B) cleaved by BABE-Fe-modified eIF3j incorporated into 43S complexes. The sequencing lanes are indicated by the letters C, T, A, and G. Other lanes include 40S subunits in the absence or presence of EDTA/Fe, mock-derivatized eIF3j (−cys + EDTA-Fe) in the absence (lane 7) or presence (lane 8) of the 43S complex, or eIF3j derivatized with BABE-Fe at the positions indicated (lanes 9–16). The lanes corresponding to eIF3j in the absence (lanes 9, 11, 13, and 15) or presence (lanes 10, 12, 14, and 16) of the 43S complex are indicated, and colored circles correspond to tethering sites indicated in Figure 1A.

positions 235 and 241 in eIF3j do not change in position or intensity, which supports the conclusion that this region of eIF3j remains associated with the mRNA entry channel in the 43S complex (Figure 6B).

Notably, cleavage of helix 18 by hydroxyl radicals generated from position 217 in eIF3j appears to significantly enhance upon formation of the 43S complex (Figure 6B, compare lanes 11 and 12). This may suggest an interesting conformational reorganization in the complex upon 43S complex formation.

eIF3j Influences Multiple Steps of the Initiation Pathway

Previously, eIF3j was found to regulate eIF3 association with the 40S subunit (Fraser et al., 2004; Nielsen et al., 2006; Unbehaun et al., 2004) through an interaction between the N terminus of eIF3j and the RNA recognition motif (RRM) of the eIF3 subunit eIF3b (ElAntak et al., 2007; Nielsen et al., 2006). The data presented above indicate that eIF3j interacts with eIF1A in the ribosomal A site, an activity that would likely influence assembly of the 43S complex. Our findings also show that eIF3j regulates access to the mRNA-binding cleft, reducing the affinity of mRNA until Met-tRNA$_i$ is present in the P site. However, even after Met-tRNA$_i$ association, the C terminus of eIF3j remains in the mRNA-binding cleft, implying that eIF3j may influence mRNA-40S subunit association during scanning and AUG recognition. Our results also help explain the consequences of caspase cleavage of eIF3j during apoptosis, whereby in addition to destabilizing eIF3-40S complexes, internal cleavage of eIF3j could prevent regulated mRNA access to 40S subunits (Fraser et al., 2004). Taken together, our results now firmly establish that in addition to its function as a scaffolding complex, a portion of eIF3 is present in the decoding center of the 40S subunit and facilitates the recruitment of mRNA to the 40S subunit.

EXPERIMENTAL PROCEDURES

Sample Purification

Detailed sample purification protocols are described in the Supplemental Experimental Procedures. Briefly, recombinant human eIF1 and eIF1A proteins were expressed in *E. coli* as maltose-binding protein fusion constructs, cleaved using recombinant TEV protease, and purified by ion-exchange chromatography to yield untagged proteins. Recombinant wild-type and single-cysteine eIF3j proteins were

expressed and purified using a baculovirus expression system similar to that reported previously (Fraser et al., 2004) with modifications to obtain untagged proteins. Human eIF3 was purified according to a published protocol (Damoc et al., 2007) with minor modifications in order to remove endogenous eIF3j. Human eIF2 was purified from HeLa cell lysate as described previously (Benne et al., 1976) with modifications using hydroxyapatite and gel filtration columns. Human 40S ribosomal subunits were purified according to published protocols (Falvey and Staehelin, 1970; Fraser et al., 2004) with extensive modifications in order to obtain pure undamaged subunits that maintain high binding affinity for eIFs and mRNA.

All 40S subunit complexes were analyzed by sucrose density gradients as described previously (Fraser et al., 2004) with modifications (see the Supplemental Experimental Procedures).

Derivatization of eIF3j Proteins with BABE-Fe and Fluorescein

Conjugation of BABE-Fe (Dojindo Molecular Technologies) to single-cysteine eIF3j proteins was carried out according to a published protocol (Spanggord et al., 2005 and Supplemental Experimental Procedures). Conjugation of a single-cysteine eIF3j protein (S152C) and a single-cysteine eIF1A protein (D142C) with fluorescein-5-maleimide (Pierce) was carried out using a similar protocol to that described for BABE-Fe conjugation (Spanggord et al., 2005) with minor modifications (Supplemental Experimental Procedures). All BABE-Fe- and fluorescein-5-maleimide-conjugated proteins were verified by electrospray mass spectrometry (data not shown).

Directed Hydroxyl Radical Probing

Complexes containing either mock-derivatized eIF3j (−Cys) or BABE-Fe-eIF3j bound to salt-washed 40S subunits were formed and radical probing was carried out as described (Culver and Noller, 2000; Lomakin et al., 2003) with minor modifications (Supplemental Experimental Procedures). Detection of 18S rRNA cleavage by BABE-Fe-eIF3j was carried out by reverse transcription as described (Culver and Noller, 2000; Lomakin et al., 2003) with modifications (Supplemental Experimental Procedures).

Modeling

The eIF3j-40S complex was modeled using the structure of the *T. thermophilus* 30S subunit (Ogle et al., 2001). PyMOL (DeLano, 2002) was used for analysis of the hydroxyl radical cleavage sites in the context of the 30S ribosomal subunit structure. The cleavage radius of hydroxyl radicals was defined according to strong, medium, and weak cleavages, as described (Joseph and Noller, 2000). The corresponding cleavage sites on the 16S rRNA were determined, and spheres corresponding to the positions of individual amino acids in eIF3j were positioned in the structure of the 30S subunit.

Fluorescent Labeling of Model mRNA

Chemically synthesized 20-mer RNA (5′-AAGGAGGUAAAAU GUUUGCU-3′; from IDT) was modified at the 5′ end using a two-step procedure. A phosphorothioate moiety was first introduced at the 5′ hydroxyl using ATPγS and T4 polynucleotide kinase (T4PNK), and subsequently conjugated to fluorescein-5-maleimide (as described in detail in the Supplemental Experimental Procedures).

Anisotropy-Based Binding Experiments

Fluorescence anisotropy was used to measure binding affinities of fluorescein-labeled RNA, eIF3j, and eIF1A to purified 40S subunits. These experiments were performed according to a published protocol (Maag et al., 2006; Maag and Lorsch, 2003) with minor modifications (as described in detail in the Supplemental Experimental Procedures).

Supplemental Data

Supplemental Data include four figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.molecule.org/cgi/content/full/26/6/811/DC1/.

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