Eukaryotic Initiation Factor (eIF) 1 Carries Two Distinct eIF5-binding Faces Important for Multifactor Assembly and AUG Selection

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Eukaryotic initiation factor (eIF) 1 is a small protein (12 kDa) governing fidelity in translation initiation. It is recruited to the 40 S subunit in a multifactor complex with Met-tRNA\textsuperscript{Met}, eIF2, eIF3, and eIF5 and binds near the P-site. eIF1 release in response to start codon recognition is an important signal to produce an 80 S initiation complex. Although the ribosome-binding face of eIF1 was identified, interfaces to other preinitiation complex components and their relevance to eIF1 function have not been determined. Exploiting the solution structure of yeast eIF1, here we locate the binding site for eIF5 in its N-terminal tail and at a basic/hydrophobic surface area termed KH, distinct from the ribosome-binding face. Genetic and biochemical studies indicate that the eIF1 N-terminal tail plays a stimulatory role in cooperative multifactor assembly. A mutation altering the basic part of eIF1-KH is lethal and shows a dominant phenotype indicating relaxed start codon selection. Cheung \textit{et al.} recently demonstrated that the alteration of hydrophobic residues of eIF1 disrupts a critical link to the preinitiation complex that suppresses eIF1 release before start codon selection (Cheung, Y.-N., Maag, D., Mitchell, S. F., Fekete, C. A., Algire, M. A., Takacs, J. E., Shirokikh, N., Pestova, T., Lorsch, J. R., and Hinnebusch, A. (2007) \textit{Genes Dev.} 21, 1217–1230). Interestingly, eIF1-KH includes the altered hydrophobic residues. Thus, eIF5 is an excellent candidate for the direct partner of eIF1-KH that mediates the critical link. The direct interaction at eIF1-KH also places eIF5 near the decoding site of the 40 S subunit.

In eukaryotic translation, initiation factors (eIFs)\textsuperscript{a} promote dissociation of the 80 S ribosome. They assist binding of Met-tRNA\textsuperscript{Met} and 5’-capped mRNA to the 40 S subunit to form 43 S and 48 S preinitiation complexes, respectively (for a review, see Refs. 1 and 2). The 43 S complex contains eIF1A, eIF1, eIF5, eIF3, and the eIF2-GTP-Met-tRNA\textsuperscript{Met} ternary complex (TC). The eIF4E subunit of eIF4F binds the 5’ cap of mRNA, whereas its eIF4G subunit binds eIF3 in mammals and eIF5 in yeast to recruit the latter factors to the mRNA. The RNA helicase eIF4A and its cofactor eIF4B are required for unwinding the 5’-terminal region of the capped mRNA. The resulting preinitiation complex termed 48 S is believed to undergo the scanning process to position the preinitiation complex onto the first AUG codon of the mRNA.

Prior to AUG recognition, GTP bound to eIF2 appears to be hydrolyzed by the action of the N-terminal residues of eIF5 through a mechanism stimulated by 48 S complex formation. Upon Met-tRNA\textsuperscript{Met} anticodon pairing with the start codon, the G\textsubscript{P} from the GTP hydrolysis is released (3). These events, coupled with a ribosomal conformational change (4, 5), trigger dissociation of eIF1 and eIF2-GDP. The GTPase switch eIF5B promotes joining of the resulting 40 S initiation complex with the 60 S subunit to produce the 80 S initiation complex, an immediate precursor for protein synthesis elongation. The GDP-bound eIF2 is recycled to eIF2-GTP by the action of the pentameric guanine nucleotide exchange factor eIF2B.

eIF1, encoded by \textit{SUI1} in yeast \textit{Saccharomyces cerevisiae}, plays a central role in ensuring the fidelity of translation initiation by destabilizing ribosomal complexes assembled on non-cognate and poorly contexted start codons (6) and by repressing the GTPase activating activity of eIF5 or release of G\textsubscript{P} until precise AUG pairing to the tRNA\textsuperscript{Met} anticodon (3, 7). Yeast eIF1 binds concurrently to eIF5-CTD as well as to the eIF2β and eIF3c subunits, thereby being recruited to the 40 S subunit in the multifactor complex (MFC) formed with eIF2 TC, eIF3, and eIF5 (8, 9). Except for the small eIF1 (12 kDa), minimal binding domains of yeast MFC constituents were identified to be eIF2β-(1–140), eIF3c-(1–156), and eIF5-(241–405) (8, 10). Of these, eIF2β-(1–140) and eIF3c-(1–156) are charged hydrophilic polypeptides. eIF2β-(1–140) regulates the affinity of eIF5-CTD-(241–405) for eIF3c-(1–156), thereby promoting MFC domain; ORF, open reading frame; uORF, upstream ORF; FOA, 5-fluoroorotic acid; sc, single copy; hc, high copy; GST, glutathione S-transferase; aa, amino acids.

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\textsuperscript{b}The atomic coordinates and structure versions (code 2OGH) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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\textsuperscript{4}The abbreviations used are: eIF, eukaryotic initiation factor; TC, ternary complex; MFC, multifactor complex; NTT, N-terminal tail; CTD, C-terminal domain; ORF, open reading frame; uORF, upstream ORF; FOA, 5-fluoroorotic acid; sc, single copy; hc, high copy; GST, glutathione S-transferase; aa, amino acids.
assembly (11). The interaction of eIF3c-(1–156) with eIF1 is important for rapid 43S–48S complex formation, and initiation fidelity is governed by eIF1 (7). The C-terminal minimal MFC binding domain, eIF5–CTD–(241–405), forms a HEAT domain fold with eight α-helices (12, 13). It binds to eIF1 and eIF3c and to eIF2β at two conserved basic and acidic surface sites termed area II and area I, respectively (14). The eIF5-C-terminal tail (aa 396–405) also contributes to eIF2β binding (14).

Human eIF1 exhibits a globular α/β core and an unstructured N-terminal tail (NTT) (15). Hydroxyl-radical footprinting studies identified a surface of its globular core as facing the rRNA helix 24 close to but separated from the P-site (16). N-terminal FLAG tagging of yeast eIF1 impairs its interaction with eIF2β and eIF5 and reduces TC binding to the ribosome in vivo, suggesting that the eIF1-NTT is important for MFC formation. Chueng et al. (17) recently confirmed this idea by finding that the alteration of Phe9 and Phe12 residues of eIF1-NTT to alanines impairs partial 43S complex assembly in vitro. However, the direct physical evidence that eIF1-NTT binds eIF5 or eIF2β has been lacking. Here we report the solution structure of yeast eIF1 and its binding sites for eIF5–241–405 as determined with NMR spectroscopy. These studies identify NTT and a basic/hydrophobic surface of the globular core termed KH as binding sites for eIF5–CTD. Remarkably, the above mentioned report by Cheung et al. (17) further showed that the hydrophobic residues, found here in the eIF1-KH area, also mediate a critical link to the preinitiation complex that suppresses eIF1 release before start codon selection. We propose that eIF5 is an excellent candidate for the direct partner of eIF1-KH that produces such a link.

Materials and Methods

Plasmids and Yeast Strains—Plasmids and yeast strains encoding wild-type, His₅-tagged, or mutant forms of eIF1 were constructed as follows and listed in Table 1. All of the oligonucleotides used in this study are listed in Table S1.

To create pET-His-SUI1, the 0.7-kb Ndel-HindIII fragment of the PCR product, using oligonucleotides eIF1-Nde and eIF1-Hd (Table 1) and p1128 (18) as template, was cloned into pET15b (Novagen). Then the 0.2-kb Ncol-BamHI fragment of pET-His-eIF1 (the Ncol site is located in pET15b upstream of the His-SUI1 ORF) was cloned into YCP-L-SUI1ΔNco (9) to create YCP-L-His-SUI1. To prepare pET-SUI1-His, the 0.33-kb Ndel-Sall-SUI1-His ORF fragment from PCR with oligonucleotides eIF1-Sall and eIF1-Hd was cloned into pET23a (Novagen). Then the 0.7-kb Ndel-HindIII fragment of pET-SUI1-His was cloned into YCP-L-SUI1ΔNde (9) to obtain YCP-L-SUI1-His.

All of the mutants except M4 were created by subcloning the 0.21-kb Ndel-BamHI DNA fragment corresponding to the 5′-half of the mutant eIF1 ORF, generated by PCR as follows, into YCP-L-SUI1ΔNde (9). To prepare the SUI1-M1, -M2, -M3, or -Δ20 segments, PCR was performed with the corresponding mutating primer, the oligonucleotide UFW, and YCP-L-SUI1 DNA as template, creating a 0.7-kb DNA fragment with the mutant eIF1 ORF and 3′-untranslated region, followed by Ndel and BamHI digestion. To create the SUI1-M5 mutant segment, oligonucleotides eIF1-Nde and eIF1-M5-RV were used for PCR.

To create YCP-L-SUI1-M4, we first produced two DNA fragments from separate PCR, one using oligonucleotides eIF1-Nde and eIF1-M4-RV and the other using eIF1-M4-FW and UFW. In both reactions, YCP-L-SUI1 was used as a template. Following DpnI digestion (to remove contamination of bacterial produced and hence methylated YCP-L-SUI1), these two segments (0.3 and 0.4 kb in size, respectively) were gel-purified and combined to perform the second PCR using primers eIF1-Nde and UFW. The 0.4-kb Ndel-HindIII fragment of YCP-L-SUI1ΔNde was replaced with that of the 0.7-kb product of the second PCR.

To create pET- and pGEX-derivatives of the site-directed mutants, listed in Table 1, the 0.7-kb Ndel-HindIII fragments of YCP-L-SUI1 derivatives were cloned into the same sites of plasmid SUI1 LEU2.*

**Table 1.** Plasmids and yeast strains with different eIF1 mutations

| Allele | eIF1 amino acid change(s) | T7 cloning plasmid | GST fusion plasmid | sc SUI1 LEU2 CEN plasmid | gcn2Δ strains |
|--------|---------------------------|--------------------|-------------------|------------------------|-------------|
| Wild type SUI1 | Wild type | pT7-SUI1b | pGEX-SUI1* | YCpL-SUI1c | KAY230 |
| NTT mutations | | | | |
| M1 | aa. 5–12 to DYKDDDDK | pET-SUI1-M1 | NCd | YCpL-SUI1-M1 | KAY333, KAY525 |
| M2 | aa. 11–18 to DYKDDDDK | pET-SUI1-M2 | NC | YCpL-SUI1-M2 | KAY286 |
| M3 | Leu6, Phe6, and Phe7 to Gln | pET-SUI1-M3 | NC | YCpL-SUI1-M3 | KAY539 |
| Δ20 | Deletion of aa. 2–21 | pET-SUI1Δ20 | NC | YCpL-SUI1Δ20 | KAY541 |
| FL-SUI1* | DYSKDDDDL after aa 1 | pET-FL-SUI1* | NC | YCpL-FL-SUI1* | KAY528 |
| Basic surface mutations | | | | |
| M4 | Lys100, Lys101, and His102 to Gln | pET-SUI1-M4 | NC | YCpL-SUI1-M4 | NAa |
| M5 | Lys137, Arg23, Lys28, and Lys30 to Ala | pET-SUI1-M5 | pGEX-SUI1-M5 | YCpL-SUI1-M5 | NA |
| Other constructs | | | | |
| sUI1-1 | Asp9 to Gly | pT7-SUI1-1a | NC | NC | NC |
| His-SUI1 | Six His after aa 1 | pET-His-SUI1 | NC | YCpL-His-SUI1 | KAY250 |
| SUI1-His | Six His after aa 108 | pET-SUI1-His | NC | YCpL-SUI1-His | NA |

* Isogenic to KAY230 (MATa leu2 lys1 ura3-52 trp1Δ mof2(sui1)-hisG gcn2::hisG p(SUI1 LEU2)) except carrying a sUI1 LEU2 CEN plasmid listed in column 4 instead of p(SUI1 LEU2).*

b Constructed in Ref. 19.
c Constructed in Ref. 9.
d NC, not constructed for this study.
a NA, not applicable because the allele used is unconditionally lethal.

pET-FL-SUI1 has DYSKDDDLK after aa 1, instead of DYSKDDDDLK.

DpsII (19)Δ20, SUI1-His, pGEX-SUI1, pET-SUI1, and pGEX-SUI1 are listed in Table S1.

To create pET- and pGEX-derivatives of the site-directed mutants, listed in Table 1, the 0.7-kb Ndel-HindIII fragments of YCP-L-SUI1 derivatives were cloned into the same sites of plasmid SUI1 LEU2.*
prepared by transferring the SacI-HindIII 1.2-kb SUI1 fragment of the corresponding single copy (sc) mutant plasmid derivatives (Table 1) to YEplac181 (21). YCpUSUI1-M4 and -M5 were likewise constructed by transferring the same segment of sc mutant derivatives to YCplac33 (21). To better express unmodified eIF1 in bacteria, we created pET-SUI1 by subcloning the 0.7-kb Ndel-HindIII wild-type SUI1 fragment from PCR with oligonucleotides eIF1-Nde and eIF1-Hd into pET23a. pET-SUI1-1-His, encoding the sui1-1 (D83G) mutant form of eIF1-His, was constructed by subcloning into pET23a the 0.35-kb Ndel-Sall SUI1-1-His ORF segment from PCR using oligonucleotides eIF1-Nde and eIF1-His-RV and a sui1-1 plasmid as template. The 0.7-kb Ndel-HindIII fragments of YCpL-SUI1-M1 to -M5 were subcloned into pET23a to create pET-His-SUI1-M1 to -M5. The products of these plasmids were used to analyze the folding of the corresponding mutant forms of eIF1 (Fig. 3).

pGB-TIF5-B6 was constructed by subcloning the PCR-amplified BamHI fragment of eIF5 (aa 241–405) ORF into pGBfusions1 (22). pGB1-TIF5-B5.5-BN1 encoding the GB1 fusion form of BNI mutant eIF5-(220–405) will be described elsewhere.5

The GCN2 allele in KAY146 (Table 1) was deleted using pHQ414 (gen2:hisG::URA3::hisG) as described (23) to generate KAY230 (Table 1). The LEU2 SUI1 plasmid in this strain was replaced with the URA3 SUI1 plasmid by naturally segregating the former from a KAY230 transformant carrying the latter to select KAY231. KAY231 was then used for plasmid shuffling using the drug 5-fluoroorotic acid (FOA) (24) to create all of the SUI1 mutant strains listed in column 6 of Table 1. Briefly, the Ura3p enzyme converts FOA into a toxic compound. Thus, the growth of a KAY231 transformant carrying a YCpL-SUI1 (LEU2) derivative (Table 1, column 5) on the FOA medium selects for a sui1A strain lacking p(URA3 SUI1) but still containing the mutant Ycpl-SUI1 derivative (Table 1, column 6) if the mutant SUI1 allele is not lethal.

NMR Spectroscopy—Sample preparation and spectra measurement for NMR spectroscopy were done as described in Ref. 5 B. Lee and K. Asano, manuscript in preparation.

pET23a (Novagen) and pGEX-TIF35 (19), respectively. pGEX-SUI1* was constructed by subcloning the 0.8-kb Ndel-HindIII fragment of pT7-SUI1 into pGEX-TIF35. pGEX-SUI1* is different from pGEX-SUI1 (20) in that the latter has an insertion of a FLAG epitope between the GST and eIF1 moieties. Although we created pGEX-SUI1* to remove concerns from the effect of the FLAG peptide, we did not observe any difference between the results of binding experiments performed with these constructs. Valasek et al. used pGEX-SUI1* as a control to test the effect of sui1-1 on eIF3c binding (7). pT7-SU11-M4 was constructed by subcloning the 0.42-kb AflII-HindIII fragment of YCpL-SUI1-M4 into the same sites of pT7-SUI1 (19).

Besides the plasmids listed in Table 1, the following plasmids were constructed in this study. YEpl-SUI1-M4 and -M5 were

A. superposition of 1H-15N HSQC spectra of 75 μM eIF1-His alone (black) and in the presence of 750 μM GB1-eIF5-(241–405) (red). Residues affected are identified with blue labels. Cross-peaks from the NTT show simple chemical shift changes dependent on the concentration of GB1-eIF5-(241–405). An expansion of the cross-peak for S8 shows this effect for three ligand concentrations (0-, 5-, and 10-fold excess in chemical shift changes dependent on the concentration of GB1-eIF5-(241–405). As an example, the expansion of the signals for Lys104 and His106 shows that the intensity of the arrow

B. ensemble of yeast eIF1 models (residues 20–108) calculated from the NMR data. C, ribbon diagram of the globular core of yeast eIF1. D, surface representation of eIF1. The residues of eIF1 affected by the addition of eIF5-CTD are colored in yellow. Right, same orientation as in B and C; left, orientation turned by 180° around the x axis. All affected residues are located on one face of the globular domain and in the NTT.
25. NMR experiments for eIF1 backbone assignment and structure determination have been carried out with the sample of 0.9 mM $^{15}$N-, $^{15}$C-labeled eIF1 in a buffer containing 20 mM Tris, 300 mM NaCl, 5 mM $\beta$-mercaptoethanol, pH 7.2. Binding NMR experiments have been carried out in 20 mM Tris, 1 mM NaCl, 5 mM $\beta$-mercaptoethanol, pH 7.2, buffer with 0.075 mM $^{15}$N eIF1 and 0.075–1.5 mM unlabeled GB1-eIF5-(241–405). Structural figures were produced with MolMol and PyMOL (available on the World Wide Web). To investigate whether the eIF1 mutants described here are folded, we expressed $^{15}$N-labeled M1–M5 mutant forms of His-eIF1 and the D83G form of eIF1-His and recorded $^{1}H$-$^{15}N$ correlated HSQC spectra.

**Biochemical Techniques—**Protein interactions in vitro (11) and the preinitiation complex formation in vivo (26, 27) were assayed as described previously.

**RESULTS**

**Solution Structure of Yeast eIF1 and Identification of the eIF5-binding Face**—To study the effect of eIF5-(241–405) on the structure of yeast eIF1, we first studied NMR spectra of its C-terminally or N-terminally hexahistidine-tagged forms termed eIF1-His and His-eIF1 alone. In vivo assessment of the function of eIF1-His and His-eIF1 is described in the supplemental materials. Both eIF1 constructs exhibited excellent two-dimensional $^{1}H$-$^{15}N$ HSQC spectra, indicating that the protein is well behaved and folded with His6 tags on either termini. Fig. 1A displays the $^{1}H$-$^{15}N$ HSQC spectrum of eIF1-His in black. The resonances were assigned, using standard multiple-resonance experiments, and the structure was determined as described previously for human eIF1 (15), except that CYANA was used for structure calculation instead of XPLOR. The resulting structure of the globular core (residues 24–108) is shown in Fig. 1, B and C (Table 2). As expected from the high sequence homology (87% similarity and 63% identity), the structure is very similar to that of human eIF1 (15). However, in contrast to the human protein, yeast eIF1 contains a segment (residues 87–106) that adopts two distinct but similar conformations as manifested with two sets of backbone resonances for 13 of the 20 residues, and the resonances of both forms could be assigned unambiguously (Fig. 1A). The two conformations are in slow exchange, since two sets of signals are observed. The relative populations are salt-dependent, and the minor conformation at high salt becomes the major conformation at low salt. The dependence of relative signal intensities on the salt concentration identifies this effect as two interconverting conformations rather than a heterogeneity of the covalent structure. The small differences of chemical shifts show that the two structures must be very similar and would be difficult to individually determine with NMR spectroscopy.

To identify the binding site for eIF5, we pursued an NMR mapping approach. Since previous evidence suggested that the eIF1-binding activity of eIF5 resides in its C-terminal domain (9), we expressed C-terminal fragments with different sizes. Among these, an eIF5-(241–405) fragment behaved best in terms of solubility and NMR spectral appearance. However, even this segment aggregated and exhibited poor NMR spectra. Thus, we used a N-terminal fusion with the B1 domain of streptococcal protein G (GB1) as a solubility enhancement tag (22).

This fusion protein behaved well and exhibited good NMR spectra but required high salt concentrations to remain monomeric. At high salt, the eIF1/eIF5 interaction is rather weak but clearly identifiable in the NMR experiments (see below). Lower salt increases the affinity but causes broadening of both the eIF1 and eIF5 spectra so that spectral changes cannot be monitored.

Thus, all NMR titrations had to be carried out at 1 M NaCl. We added unlabeled GB1-eIF5-(241–405)-His to the two different tagged forms of eIF1. Only eIF1-His was affected by the addition of GB1-eIF5-(241–405)-His, consistent with in vitro binding studies showing that an N-terminal His tag prevents binding, as mentioned above. Of 108 yeast eIF1 amino acids, 17 were affected by eIF5-(241–405) (Fig. 1A). Among these, residues Ser8, Asp10, Phe12, and Ala13 are located in the unstructured NTT and experience ligand-dependent chemical shift changes (fast exchange limit). The second group consists of Arg85, Lys87, Val88, Cys89, Phe91, Ile93, Ser94, Gln95, Gly97, Leu98, Lys100, Lys101, Lys104, Ile105, and His106. These residues adopted two conformations in free eIF1 (see above); the addition of GB1-eIF5-(241–405)-His caused the minor conformation to disappear in a manner proportional to the ligand concentration. This indicates that eIF5 binding competes with the intramolecular interactions that stabilize the minor conformation. All affected residues cluster in two separate regions of eIF1 (Fig. 1D) defining potential binding sites for eIF5-(241–405), one at the NTT (aa 1–23) and the other at a basic surface area of the globular core (Fig. 1D). We call this site the KH area after the characteristic lysine (K) and hydrophobic residues.

To confirm that the basic surface of eIF5-(241–405) is the primary eIF1-binding face (14), we mixed $^{15}$N-labeled eIF1-His with the BN1 mutant form of GB1-eIF5-(220–405)-His, altering six basic amino acids (Lys$^{367}$, Lys$^{369}$, Lys$^{372}$, Lys$^{375}$, Lys$^{377}$, Lys$^{379}$, Lys$^{380}$, and Lys$^{382}$) in the B1 domain. The eIF5-bound eIF1-His exhibited enhanced signals at the sites of these residues, indicating that eIF5 is bound to both the eIF1-His and the eIF1-His fragment, suggesting that this is the site of eIF5 binding. To confirm that eIF5 binding is specific, we expressed a series of mutant forms of eIF1-(241–405) and tested their binding to eIF5 in vitro. Among these, eIF1-D83G and eIF1-D83N were found to bind eIF5, while other mutants did not.

**TABLE 2**

| Parameters                      | Values  |
|---------------------------------|---------|
| CYANA target function ($\AA^2$) | 0.8 $\pm$ 0.1 |
| No. of "acting" distance constraints (upper/lower) | 570/0 |
| Nuclear Overhauser effect       | 72/72   |
| Hydrogen bond                   | 0.02 $\pm$ 0.002 |
| Maximum (Å)                     | 0.18    |
| No. of torsion violations       | 0.01 $\pm$ 0.002 |
| Root mean square                | 0.02, 0.06 |
| Maximum (Å)                     | 2.6     |
| Angle constraint violations     | 0.25 $\pm$ 0.05 |
| Root mean square                | 0.27    |
| Maximum (degrees)               | 4.0     |
| van der Waals distance violations | 2.4 $\pm$ 0.3 |
| Sum                             | 0.25    |
| Maximum (Å)                     | 0.27    |
| Root mean square deviations to mean structure (Å) | 0.6 $\pm$ 0.2 |
| Full protein without NTT (residues 24–108) | 0.4 $\pm$ 0.1 |
| Backbone                        | 1.3 $\pm$ 0.3 |
| All heavy atoms                 | 1.0 $\pm$ 0.2 |
| Protein without all flexible regions (residues 24–30, 39–69, 77–108) | 0.4 $\pm$ 0.1 |
| Backbone                        | 1.3 $\pm$ 0.3 |
| All heavy atoms                 | 1.0 $\pm$ 0.2 |
and Arg$^{382}$) to Gln (see Fig. 7a for the basic area II) involved in eIF1 binding. As expected, the BNI mutant form of the GB1-eIF5 segment did not change the HSQC spectra of $^{15}$N-labeled eIF1-His (data not shown). The NMR spectrum of GB1-eIF5-(220–405)-His/RN1 sample displayed well dispersed chemical shifts, confirming that the BNI mutant eIF5-CTD is folded (data not shown). Thus, the BNI mutation impaired the eIF1/eIF5-(220–405) interaction by altering the surface residues of eIF5 without largely unfolding the structure.

**Effect of eIF1-NTT Mutations on Binding to eIF Partners in Vitro**—Next, we performed site-directed mutagenesis to test the significance of the eIF1-NTT for binding GST-fusion forms of MFC partner fragments, eIF5-(241–405), eIF2β-(1–140), and eIF3c-(1–156). As shown in Fig. 2A, the M1 and M2 mutations change residues 5–12 and 11–18 to the FLAG epitope (DYKDDDDK). We expressed $^{35}$S-labeled wild-type and mutant eIF1 in reticulocyte lysate and tested their interactions. As shown in Fig. 2B, both M1 and M2 significantly reduced $^{35}$S-labeled eIF1 binding to GST-eIF5-(241–405) as well as to GST-eIF2β-(1–140). However, they did not alter its binding to GST-eIF3c-(1–156). Thus, the NTT is an important binding element for eIF5 and eIF2β. Given that the FLAG epitope sequence is rich in acidic residues, we reasoned that hydrophobic residues in the N-terminal half of the eIF1-NTT are important. To test this, we created SUII-M3, altering conserved Leu$^{9}$, Phe$^{9}$, and Phe$^{12}$ to polar glutamine residues (Fig. 2A). As expected, M3 impaired eIF1 binding to eIF2β and eIF5 but not to eIF3c (Fig. 2B).

**Effect of eIF1 Basic Surface Mutations on Binding to eIF Partners in Vitro**—To examine the basic eIF5-binding face of eIF1, we simultaneously altered Lys$^{105}$, Lys$^{101}$, Lys$^{104}$, and His$^{100}$ to glutamines, creating mutant M4. M4 also reduced binding to eIF2β and eIF5 but not to eIF3c (Fig. 2B). Thus, the interactions of eIF1 with eIF2β and eIF5 depended on the same NTT and basic areas when eIF2β and eIF5 each was the sole binding partner.

Because eIF3c-(1–156) can stabilize the eIF1-eIF5-(241–405) complex by mutual cooperativity (8), we searched for a potential eIF3c-binding face on the surface separate from the eIF5 binding areas. We created mutant M5 altering Lys$^{52}$, Arg$^{53}$, Lys$^{59}$, and Lys$^{60}$ to alanines (Fig. 2A), because (i) these...
the $^{1}H$-$^{15}N$ HSQC spectra of $^{15}N$-labeled sample of the mutant show significant resonance line broadening and peak disappearance, suggesting at least partial unfolding of the protein (Fig. 3). By contrast, $^{15}N$-labeled elf1 mutants $M1$–$M5$ showed $^{1}H$-$^{15}N$ HSQC spectra of the same quality as wild type, indicating that these new mutants are folded (Fig. 3).

The elf1-NTT Is Required for Its Cooperative Incorporation into MFC—Having observed that the binary interactions of elf1 with elf2β and elf5 depend on the same interfaces, NTT and 4KH, we wished to determine which surface is more critical for elf1 incorporation into the MFC. Thus, we tested the effect of elf1 mutations on its binding to a binary complex of elf5-(241–405) and GST-elf2β-(1–140), a reaction mimicking cooperative MFC formation (9). We also used, as elf1 mutants, previously characterized constructs, FL-elf1 and elf1-FL, with the FLAG epitope introduced to the N and C terminus of elf1, respectively (9); FL-elf1 becomes important in subsequent in vivo studies (see Figs. 5 and 6). In this experiment, $^{35}S$-labeled elf1 was mixed with His-elf5-(241–405) and GST-elf2β-(1–140) in a trace amount compared with the latter two. Subsequently, the complex was pulled down with glutathione resin and analyzed by SDS-PAGE. The data in Fig. 4A, panel 1, and its quantification in Fig. 4B confirmed that elf1 binding to GST-elf2β-(1–140) was $>3$-fold increased due to bridging by elf5-(241–405), indicating mutual cooperativity between elf1 interactions with elf5 and elf2β (9). Likewise, the binding of elf1-FL greatly increased by the presence of elf5-(241–405), and the binding of elf1-M4 was also slightly but significantly increased (Fig. 4, A, panels 4 and 5, and B, columns 3–6). Note that both the mutants would interfere with interaction at the KH interface (also see “Discussion”). In contrast, the NTT mutants $M1$ and $M2$ and N-terminal FLAG-elf1 (FL-elf1) did not bind at all to the GST-elf2β-(1–140)elf5-(241–405) complex (Fig. 4A, panels 2, 3, and 6). These results indicate a more critical role for elf1-NTT in MFC incorporation or formation.

To test if elf1-NTT mediates elf1 incorporation into MFC in vivo, we expressed mutant elf1 in yeast encoding HA-elf3i. As shown in Fig. 4C, HA-tagged elf3 immunoprecipitates endogenous elf1 (lanes 5, 8, and 11), but not elf1-M1 (lane 8) or elf1-M2 (lane 11), which migrated more slowly than wild-type elf1 due to amino acid composition changes (upper bands in third gel). We previously showed that FLAG-elf1 is likewise defective in binding to HA-elf3 in this assay (9). Because the mutant versions of elf1 under the study were expressed in the presence of wild-type elf1, the effect of these mutations on elf1 binding to elf3 in vivo might be minor if it was examined in the absence of wild-type elf1. Nevertheless, the co-immunoprecipitation studies indicate that the elf1-NTT plays at least a stimulatory role in its incorporation into the MFC in vivo.

The Effect of elf1 Surface Mutations on Yeast Growth—To study the effect of elf1 mutations on yeast growth, we introduced sc YCpL-SU1 plasmid derivatives encoding elf1 mutants under the natural promoter (Table 1, column 5) to
yeast carrying chromosomal sui1 deletion and a centromeric URA3 elf1 plasmid. All of the sc elf1-NTT mutant plasmids (M1, M2, and M3) replaced the URA3 elf1 plasmid in the sui1Δ background, indicating that the NTT mutations are not lethal (data not shown). Interestingly, the resulting M1 and M3 strains carrying the mutant protein as the sole elf1 source grew slowly in the complex-rich medium at the permissive temperature of 30 °C and more slowly at a higher temperature (Fig. 5A, rows 2 and 4). Yeast mutant with SLIIIn1-A20 that was deleted in the NTT region from residues 2–21 was also viable, since the mutant strain grew just like wild-type (Fig. 5A, row 6). These results suggest that the NTT mutations M1 and M3 negatively affect yeast growth and translation. Immunoblot analysis shows that all of the employed elf1 mutants were expressed at a level nearly identical to or higher than that of the wild-type elf1 (Fig. 5B). Thus, the elf1-NTT is not essential for yeast growth, and hence, its role in MFC assembly is only stimulatory (see “Discussion”).

In contrast, M4 and M5, altering the basic binding faces, were unconditionally lethal when examined by plasmid shuffling with the corresponding YCpl-SU11 derivatives (data not shown) or with the high copy (hc) YEpL-SU11 derivatives (Fig. 6A). Since the mutant protein was overexpressed from the hc plasmids beyond the level of endogenous elf1 (Fig. 6B), the lethality of the mutants is due to their functional defect. Thus, both of the basic interfaces of elf1 mediate essential functions in translation initiation.

Evidence That elf1-NTT Promotes TC Binding to the Ribosome in Vivo—To further study elf1-NTT in vivo, we used the GCN4-dependent general (amino acid) control response as a sensitive indicator of elf1 activities (2). Gcn4p encodes a transcription factor that activates transcription of genes controlled under the general amino acid control response. Translation of GCN4 mRNA is regulated by a mechanism involving a series of short upstream ORFs (uORFs) in its 5′ leader. Under nonstarvation conditions, translation reinitiation between uORF1 and one of the uORFs from uORF2 to -4 occurs rapidly, dissociating ribosomes from GCN4 mRNA and repressing GCN4 translation. However, upon amino acid starvation, the Gcn2p elf2α kinase is activated to lower the eIF2-GTPMet-tRNA3Met TC level. This slows ribosomes’ acquisition of TC following uORF1 translation, allowing them to bypass inhibitory uORFs. Thus, GCN4 is induced (derepressed) during amino acid starvation, conferring a 3-aminotriazole-resistant growth. When elf1 mutations decrease the cellular TC level or TC binding to the ribosome, the ribosomes migrating along GCN4 mRNA after uORF1 translation bypass uORF2 to -4, hence inducing GCN4 translation in the absence of amino acid starvation signal; GCN4 translation is constitutive, occurring even in the
absence of the activator Gcn2p (general control derepressed, or Gcd⁻ phenotype).

Yeast gcn2Δ strains are sensitive to 3-aminotriazole due to an inability to express the general control response (Fig. 5C, row 1). All of the gcn2Δ strains carrying the eIF1-NTT mutations were clearly 3-aminotriazole-resistant (Fig. 5C), with an attendant increase in GCN4-lacZ expression from p180 (Fig. 5D), as examined for M1–M3. We previously showed that the FLAG-
eIF1-encoding yeast strain also shows an elevated GCN4 expression (9). Thus, these mutations derepress the Gcn4p-dependent general control even in the absence of Gcn2p (Gcd⁻). hc TC suppressed the Gcd⁻ phenotype of the FLAG-eIF1 construct, indicating that this phenotype results from a defect in TC recruitment to the 40 S subunit (Fig. 5C, row 6), like Gcd⁻ phenotypes of eIF3c and eIF5-CTD mutants (7, 28) or the eIF1 9–12 mutant also altering the NTT (17). These results support our previous proposal that eIF1-NTT is involved in TC binding, to the ribosome through contributing to MFC formation.

Interestingly, however, hc TC had little or no effect on Gcd⁻ phenotypes of other eIF1-NTT mutants (Fig. 5C, d), suggesting that these latter phenotypes are not due to a tRNAᵣᵣ recruitment defect. The observation made with M1 and M3 may be complicated by their rather toxic effect on yeast growth in the rich medium (Fig. 5A). However, the lack of Gcd⁻ phenotype suppression for M2 and Δ20 mutations, which did not produce any growth defect, contrasts with the Gcd⁻ phenotypes by N-terminal FLAG tagging (this study) and 9–12 mutation (17). Thus, Gcd⁻ phenotypes of eIF1-NTT mutants appear to be confounded by the effect on processes in initiation other than TC binding to the ribosome (see “Discussion”).

The KH Area Mediates the Essential Function of eIF1 in AUG Selection—The eIF1 mutations increasing the eIF2 GTPase activation (29) or spontaneous eIF1 release (4) relax stringency of start codon selection and allow translation from UUG codons (Sui⁻), or suppressor of initiation codon mutation phenotype). We tested the Sui⁻ phenotypes of our eIF1 mutants by reporter assays using HIS4-lacZ alleles with altered start codons. The efficiency of translation of the mutant his4-lacZ allele from the UUG codon is between 2 and 10% of that of wild-type HIS4-lacZ from normal AUG codon in wild-type yeast, depending on the strain background. Sui⁻ mutations would significantly increase this ratio. We found that none of the viable NTT mutations tested, M1, M2, or M3, showed the Sui⁻ phenotype (data not shown). However, the lethal M4 mutation altering the KH area, but not the M5 mutation altering the KR area, significantly increased translation from UUG codons in a dominant fashion when the mutant protein was expressed from a hc plasmid (Fig. 6C). This suggests that the KH area of eIF1 is important for stringent AUG selection by the ribosome.

To test if the lethal mutant eIF1 proteins are firmly anchored to MFC or the preinitiation complex, we expressed them from sc plasmids in a strain encoding FLAG-eIF1 (used in Fig. 5). The purpose of expression in this strain is to differentiate the plasmid-borne eIF1 mutant from the host-encoded FLAG-eIF1 by size (9). As shown in Fig. 6D, panel 1, sucrose gradient analyses indicated that, in the absence of other eIF1 species, FLAG-eIF1 bound to the 40 S subunit (lanes 6 and 7) without firmly associating with the free MFC (lanes 4 and 5), in agreement with the defect of FLAG-eIF1 observed in vitro (9) and in vivo (Fig. 5C, row 6). When wild-type eIF1 was expressed, the majority of eIF1 species associated with the 40 S subunit was the wild-type eIF1 (lanes 7 and 8) due to lower abundance of FL-eIF1 in the presence of wild-type eIF1 (Fig. 6E, lanes 1–6). Immunoblotting with anti-FLAG (Fig. 6D, panel 2, bottom gel) indicated that a much smaller fraction of FL-eIF1 bound to the 40 S subunit in lanes 7 and 8 than in the absence of wild-type eIF1 (panel 1, lanes 1–6).
Functional Interfaces of eIF1

**FIGURE 7. Model of MFC assembly with eIF5-CTD as an essential assembly core.** eIF1 and eIF5-CTD are depicted with the outline of a space-filled model, together with the partners in each step of preinitiation complex assembly. On eIF1, blue circles represent the basic surfaces; the area KR or KH is located on the side toward or away from the viewer, respectively. In all of the panels (a–d), eIF5-CTD is outlined in the same orientation as in a, with E396 (indicated by x) at the start of the C-terminal tail (aa 396–405) closest to the viewer. Acidic area I and basic area II of eIF5-CTD are drawn in red and blue, respectively. The 40 S ribosome surface near the P-site is depicted in gray. NTTs of eIF2β (light blue) and eIF3c (light green) are depicted as hypothetical tubes that wrap around the eIF1/eIF5-CTD core complex. In a, thick arrows indicate essential interactions mediating the MFC. Dashed thick arrows also indicate essential interactions, but they are likely to be essential at later steps in the preinitiation complex function. The interacting surface of eIF5-CTD for eIF1-KH is approximate.

Bottom two gels, lanes 6 and 7). These results suggest that the ectopically expressed wild-type eIF1 outcompeted the host-encoded FLAG-eIF1 from the 40 S subunit. (As shown in Fig. 6E, lanes 1–6, however, the cellular abundance of FLAG-eIF1 was severely reduced in the presence of wild-type eIF1 for an unknown reason. Thus, we could not rule out the possibility that the reduced binding of FL-eIF1 to the 40 S subunit was due to its reduced abundance.)

Importantly, sc eIF1-M4 and eIF1-M5 did not bind to the 40 S subunit (Fig. 6D, panels 3 and 4), demonstrating a defect in the 40 S subunit binding. Compared with the vector control (panel 1), smaller fractions of FL-eIF1 bound to the 40 S subunit in the presence of these eIF1 mutants (panel 3, lanes 6 and 7, and panel 4, lane 7), suggesting that eIF1-M4 and eIF1-M5 can inhibit FL-eIF1 binding to the 40 S subunit, possibly by competing with FL-eIF1 for MFC partners albeit weakly (see below). Furthermore, less eIF1-M4 or eIF1-M5 was found in the MFC peak (panels 3 and 4), consistent with their defective binding to MFC partners (Figs. 2 and 3). These results together confirm that the KR area altered by M5 is both a part of the 40 S subunit binding face (16) and an MFC incorporation site via eIF3c (Fig. 2) and further indicate that the KH area altered by M4 is an indirect but critical 48 S complex binding site. Together with a strong Sui− phenotype with the eIF1 93–97 mutant altering Ile93, Leu96, and Gly97, found in the KH area (also see Fig. 2A) and the demonstration of its spontaneous release from the simplified 48 S complex in vitro (17), our results suggest that the direct interaction of eIF5-CTD with eIF1-KH mediates the critical link of eIF1 to the 48 S complex that prevents its release before start codon selection.

**DISCUSSION**

The structure of yeast eIF1 presented here was used as a basis for identifying binding faces for MFC components. The NMR titration experiments identified two well defined eIF1 regions important for eIF5-(241–405) binding, although the spectroscopic changes were small, in part due to the experimental conditions of high salt we had to use. The interaction between these proteins is known to be salt-dependent (9), with higher salt concentrations resulting in weaker binding. All NMR binding experiments had to be conducted at 1 M NaCl due to inherent aggregation of eIF5-(241–405) at low salt (data not shown), thus sacrificing the strength of the interaction to maintain readability of the spectra. Despite being weak, the interactions identified are very specific even at high salt concentration; lowering the salt concentration gradually increases the affinity but is not expected to change the geometry of the complex.

Besides the experimental conditions, the apparently weak effect of eIF5-(241–405) on 15N-labeled eIF1-His (Fig. 1) may also be due to the C-terminal His tag introduced, which would perturb the interaction at the KH area made of eIF1 C-terminal residues. Consistent with this, the eIF1-His allele is lethal when expressed from an sc plasmid, as observed with the eIF1-FL allele (9). In contrast, the N-terminal His tagging shows no growth or Gcd phenotype (see supplemental materials), confirming that although His-eIF1 may be partially defective in eIF5 binding, this is not sufficient to severely impact MFC assembly due to the mutual cooperativity of the complex formation. Understanding why the sc eIF1-His or eIF1-FL allele is lethal would require better understanding of the preinitiation complex components that bind the KH area during the postassembly steps, as discussed below.

Of the two eIF5 interfaces, we provided substantial evidence that eIF1-NTT plays a stimulatory role in MFC assembly both in vitro (Figs. 3 and 4A) and in vivo (Fig. 4C). That this function of eIF1-NTT is not essential (Fig. 5A) is consistent with the previous finding that human eIF1 deleting the NTT can promote formation of the reconstituted 48 S complex on the start codon in vitro (16). Together with the recent findings of Cheung et al. using the eIF1 9–12 mutant (17), these results establish that the interaction of eIF1-NTT with eIF5-CTD, as observed in Fig. 1, is an important part of MFC-linking interactions. However, the stimulatory, but not essential, function for eIF1-NTT in MFC assembly should not be taken as evidence against the crucial role played by the MFC formation. We previously showed that eIF5-CTD bridges the interaction between eIF2β and eIF3c (11) and that point mutations in eIF5-CTD produce temperature-sensitive (Ts−) and Gcd− phenotypes that are suppressible by hc TC (28) (thick arrows in Fig. 7a). These results provide strong evidence that the eIF5-CTD plays an essential role in Met-tRNA7 Met recruitment to the ribosome and hence in 43 S complex assembly. We propose that eIF1-NTT assists the eIF5-CTD-driven MFC assembly by providing mutual cooperative
interaction faces, as illustrated in Fig. 7a (thin arrow) and Fig. 7b (red arrow) (also see below).

Our finding that Gcd– phenotypes caused by eIF1-M2 and -Δ20 mutants were not suppressed by hc TC (Fig. 5C) suggests that eIF1-NTT is also involved in other steps of translation initiation. For example, if these mutants delay response to AUG recognition, the ribosomes that have acquired TC before GCN4 uORF start codons may skip these codons, such that they instead translate GCN4. This mechanism can lead to a Gcd– phenotype that is not suppressible by hc TC. Since the NTT and CTT of eIF1A are implicated in regulating ribosome conformation during the scanning and AUG selection processes (30), it would be intriguing to investigate the role of eIF1-NTT in these processes.

We also presented evidence that the basic part of the second eIF5 interface termed KH plays an essential role and probably would be intriguing to investigate the role of eIF1-NTT in these processes.

Finally, are the eIF1-eIF5-CTD interactions as observed in Fig. 1 retained throughout the initiation process? As illustrated in Fig. 7a, we believe that this is the case at least at the initial MFC assembly step, since eIF1-M4 altering the KH area appears to reduce the interaction with the eIF2β-(1–140)-eIF5-(241–405) complex (Fig. 4, A and B) as well as its incorporation into MFC in vivo (Fig. 6D). Since the second basic surface of eIF1 termed KR binds the eIF3c peptide (Fig. 2), it is possible that the eIF3c peptide wraps around the eIF1-eIF5 complex in MFC by binding to eIF1-KR and the eIF3c-binding basic surface of eIF5-CTD termed area II (14) (Fig. 7, b–d). More work combining genetic, biochemical, and structural approaches is needed to study factor communication during translation initiation.

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