Eukaryotic initiation factor 1 (eIF1) is a low molecular weight factor critical for stringent AUG selection in eukaryotic translation. It is recruited to the 43 S complex in the multifactor complex (MFC) with eIF2, eIF3, and eIF5 via multiple interactions with the MFC constituents. Here we show that FLAG epitope tagging of eIF1 at either terminus abolishes its in vitro interactions with eIF5 and eIF2β but not that with eIF5c. Nevertheless, both forms of FLAG-eIF1 fail to bind eIF3 and are incorporated into the 43 S complex inefficiently in vitro. C-terminal FLAG tagging of eIF1 is lethal; overexpression of C-terminal FLAG-eIF1 severely impedes 43 S complex formation and derepresses GCN4 translation due to limiting of eIF2-GTP-Met-tRNA\textsubscript{Met} ternary complex binding to the ribosome. Furthermore, N-terminal FLAG-eIF1 overexpression reduces eIF2 binding to the ribosome and moderately derepresses GCN4 translation. Our results provide the first in vivo evidence that eIF1 plays an important role in promting 43 S complex formation as a core of factor interactions. We propose that the coordinated recruitment of eIF1 to the 40 S ribosome in the MFC is critical for the production of functional 40 S preinitiation complex.

In eukaryotic translation initiation, the small ribosomal subunit (40 S ribosome) binds the eukaryotic initiation factor 2 (eIF2\textsuperscript{1})-GTP-Met-tRNA\textsubscript{Met} ternary complex (TC) to form the 43 S preinitiation complex. Subsequent joining of mRNA carried by eIF4F produces the 48 S preinitiation complex. The eIF3 stimulates recruitment of Met-tRNA\textsubscript{Met} and mRNA to the 40 S ribosome by binding to eIF2 and the eIF4G subunit of eIF4F, either directly or indirectly (for a review, see Ref. 1). The 48 S complex searches for the first AUG codon in the mRNA with the help of low molecular weight factors, eIF1 and eIF1A, and the helicase and its cofactor, eIF4A and eIF4B, respectively. Correct base pairing between the Met-tRNA\textsubscript{Met} anticodon and the AUG codon triggers the hydrolysis of GTP bound to eIF2; this reaction is dependent on the GTPase-activating function of the eIF5 N-terminal domain (NTD). This GTP hydrolysis then leads to dissociation of preassembled eIFs and formation of an initiation complex containing the AUG anticodon base pair in the ribosomal P site. The second GTP-binding protein eIF5B then stimulates joining of the 40 S initiation complex with the 60 S subunit to form the 80 S initiation complex. The elongation of the polypeptide chain starts from the methionine linked to the 80 S initiation complex.

eIFs are highly conserved from yeast to mammals. Mammalian eIFs were purified from a high salt wash fraction (e.g. in a 500 mM KCl buffer) of ribosome-associated proteins and characterized in crude mammalian cell extracts or partially or fully reconstituted initiation systems (1–3). Despite the progress in elucidating the function of individual eIFs, the precise order of and the key element(s) critical for their assembly in vivo remain to be elucidated.

Using yeast Saccharomyces cerevisiae as a model organism, it was proposed that the C-terminal domain (CTD) of eIF5 plays a critical role in the assembly and integrity of the functional 43 and 48 S complexes (4, 5). eIF5-CTD interacts concurrently with the β subunit of eIF2 and the ε subunit of eIF3 via a conserved motif called aromatic/acidic boxes (AA-boxes) 1 and 2 (6). Lysine-rich segments (K-boxes) in eIF2β-NTD are responsible for its binding to the eIF5-CTD (6). These interactions plus interactions between eIF3c and eIF1 (7), between eIF3a and eIF1, and between eIF2β and eIF3a (8) were proposed to mediate formation of the multifactor complex (MFC) containing eIF1, eIF2, eIF3, eIF5, and Met-tRNA\textsubscript{Met} (4). Accumulative evidence supports the model that the constituents of the MFC bind to the 40 S ribosome as a preformed unit to form the 43 S complex (5, 9, 10). Disruption of MFC by an AA-box 2 mutation in eIF5 leads to a defect in Met-tRNA\textsubscript{Met} and mRNA binding to the 40 S ribosome in vitro. Evidence also suggests that this mutation impedes a step subsequent to the 43 S complex formation in vivo (5).

Among the components of the MFC, eIF1 is a small factor (12 kDa) whose importance has just begun to be appreciated. Genetic and biochemical studies indicate that eIF1 is required for discrimination of the 48 S complex against near cognate codon pairing with the tRNA\textsubscript{Met} anticodon, to ensure initiation from AUG only (2, 11, 12). Besides its role in the 48 S complex, in vitro assays using the 40 S ribosome, Met-tRNA\textsubscript{Met}, and a limited set of eIFs indicate that eIF1 stimulates eIF2 TC binding to the 40 S ribosome in yeast (13) and mammals (14).

In this paper, we investigated mutual interactions of eIF1 with other components in the MFC and found that eIF1 binds to the other two components of MFC, eIF2β and eIF5, in addition to eIF3c, as was previously shown (7). These newly identi-
tified eIF1 interactions, but not that with eIF3c, are significantly reduced by tagging of eIF1 with the highly charged FLAG peptide at either terminus. The FLAG-tagged forms of eIF1 reduce binding to eIF3 and are recruited to the 40 S ribosome. C-terminal FLAG-eIF1 produces a recessive lethal phenotype, and its overexpression reduces 43 S complex formation. N-terminal FLAG-eIF1 overexpression also compromises 43 S complex formation and reduces eIF2 binding to the 40 S ribosome. Together these results provide firm evidence that eIF1 plays an important role in promoting 43 S complex formation in vivo.

MATERIALS AND METHODS

Plasmids and Yeast Strains—Plasmids and yeast strains used in this study are listed in Tables I and II, respectively. The 1.5-kb SphI fragment of p1128 (11) containing the chromosomal SUI1 locus was cloned into YCplac111 and YEplac181 (15), yielding YCpL-SUI1 and YEpdL-SUI1. The 1.5-kb SacI-SphI fragment of YCpL-FL-SUI1* ORF, a 5′-half of SUI1 ORF, YCpL-SUI1-FL was cloned into YEplac181 to generate YEpL-FL-SUI1*. The 1.5-kb SacI-SphI fragment of YCpL-FL-SUI1* ORF, YCpL-SUI1-FL was cloned into YEplac181 to generate YEpL-FL-SUI1* and YEpL-SUI1-FL, respectively.

To introduce a NdeI site at the 5′-end of the SUI1 ORF, a 5′-untranslated region DNA was amplified with oligo-1 (5′-GCC GAG CTC GTA AAT CAA TGG AAA TAT GAA ATC-3′) and oligo-2 (5′-GGA TCA ATG TCC ATT GAG TGA AAA CCC ATG AAT TTT AAT GTT C-3′). The resulting plasmid, YCpSUI1Nco, has a unique NdeI site following the 5′-untranslated region and lacks the 5′-half of SUI1 ORF up to the unique BamHII site. The 2.01-kb NcoI-BamHII fragment of p2431 containing the FL-SUI1 ORF 5′-half was subcloned into YCpLSUI1Nco to generate YCpL-FL-SUI1 encoding FL-eIF1 under the GDP promoter.

To introduce an NdeI site at the 5′-end of the SUI1 ORF, the NdeI-SalI fragment of eIF1-TIF5-B6 (covering the unique BamHI site in SUI1 ORF, YCpL-SUI1-FL) was subcloned into YCpL-SUI1Nco altering all 3 K-boxes to alamines to generate YCpL-SUI1Nco plus eIF2β(1-140) cloned in pGEX. The 1.5-kb SacI-SphI fragment of YCpL-FL-SUI1* ORF, a 5′-half of SUI1 ORF, YCpL-SUI1-FL was cloned into YEplac181 to generate YEpL-FL-SUI1*. To introduce an NdeI site at the 5′-end of the SUI1 ORF, the NdeI-SalI fragment of pGEX-TIF5-B6 was cloned into pGEX-FLAG-eIF1 (eIF-FL), the NdeI-SalI fragment of eIF1-TIF5-B6 (covering the unique BamHI site in SUI1 ORF, YCpL-SUI1-FL) was subcloned into YCpL-SUI1Nco. To introduce an NdeI site at the 5′-end of the SUI1 ORF, the NdeI-SalI fragment of pGEX-TIF5-B6 was cloned into YCpL-SUI1Nco. To introduce an NdeI site at the 5′-end of the SUI1 ORF, the NdeI-SalI fragment of pGEX-TIF5-B6 was cloned into YCpL-SUI1Nco. To introduce an NdeI site at the 5′-end of the SUI1 ORF, the NdeI-SalI fragment of pGEX-TIF5-B6 was cloned into YCpL-SUI1Nco.

TABLE I

| Plasmid | Description | Source |
|---------|-------------|--------|
| pGEX vectors | Expression vectors for GST fusions | Amersham Biosciences |
| pGEX-TIF5 | eIF5 cloned in pGEX | Ref. 6 |
| pGEX-TIF5-B6 | eIF5(241-405) cloned in pGEX | Ref. 6 |
| pGEX-TIF5-B6-12A | eIF5(241-405) cloned in pGEX | Ref. 5 |
| pGEX-TIF5-B6-7A | pGEX-TIF5-B6 carrying t5-7A | Ref. 5 |
| pGEX-SUI3 | eIF2β cloned in pGEX | This study |
| pGEX-SUI3-3K | pGEX-SUI3 altering all 3 K-boxes to alamines | This study |
| pGEX-SUI1-FL | eIF2β(1-140) cloned in pGEX | This study |
| pGEX-NIP1-N | eIF5c(1-156) cloned in pGEX | Ref. 4 |
| pHis-NIP1-N | His-tagged eIF5c(1-156) under a T7 promoter | Ref. 4 |
| pHis-SU13AS | His-tagged eIF5β(1-140) under a T7 promoter | Ref. 5 |
| pHis-TIF5-B6 | His-tagged eIF5(241-405) under a T7 promoter | Ref. 4 |
| pET-FL-SU1 | FL-eIF1 cloned under a T7 promoter | This study |
| pET-SU1-FL | eIF1FL cloned under a T7 promoter | This study |
| pT7-7 | Cloning vector with T7 promoter | Ref. 30 |
| pT7-SU11 | eIF1 cloned in pT7-7 | Ref. 7 |
| pT7-SU11-FL | eIF1FL cloned in pT7-7 | This study |
| YCpL-SU11 | Single copy SU11 LEU2 plasmid | This study |
| YEpL-SU11 | High copy SU11 LEU2 plasmid | This study |
| YCpL-SU11-FL | Single copy SU11-FL LEU2 plasmid | This study |
| YCpL-SU11* | Single copy SU11-FL LEU2 plasmid | This study |
| YEpL-SU11* | High copy FL-SU11 LEU2 plasmid | This study |
| pα21 | High copy pα21-FL-SU11-trpC TRP1 plasmid | Ref. 31 |
| pYcplac-αP4p-FL-SU11 | Single copy pYcplac-αP4p-FL-SU11 trpC LEU2 plasmid | This study |
| p170-tMT | High copy pYcplac-αP4p-FL-SU11 IMT UR43 plasmid | Ref. 6 |
| p180 and derivatives | Single copy GCN4 tao2 UR43 plasmids | Ref. 20 |

Yeast strains KAY146, KAY156, KAY178, and KAY142 were constructed by plasmid shuffling (16) using Y217 and the corresponding SUI1 LEU2 plasmids listed in Table II.

Biochemical Assays—GST pull-down assays with 35S-labeled proteins, synthesized in a rabbit reticulocyte lysate, were conducted as described previously (7). The amounts of bound 35S-labeled proteins were quantitated with STORM or TYPHOON PhosphorImagers (Amersham Biosciences). Polyhistidine-tagged eIF5-CTD-(241-405), eIF3c (1-156), and eIF2β(1-140) fragments designated His-eIF5-B6, His-NIP1-N, and His-eIF2β-N were expressed and purified from BL21(DE3) carrying pHis-TIF5-B6, pHis-NIP1-N, and pHis-SU13AS, respectively, as described (5). FLAG-eIF1 constructs designated FL-eIF1 and eIF1FL were purified from BL21 (DE3) carrying pET-FL-SU11 and pET-SU11-FL, respectively, as described (17). Peptide sequencing of purified 35S-labeled proteins was performed as described previously (28).

FLAG-tagged eIF2 or HA-tagged eIF3 was affinity-purified from strain KAY42 (gpd-6A-p780FL) (6) or H2557 transformant carrying pLPY-PRT1His-TIF34HA-TIF35FLAG and pLPY-NIP1-TIF32 (10), at-

tached to anti-FLAG (Sigma) or anti-HA (made by preadsorbing protein A-Sepharose beads (Amersham Biosciences) with anti-HA antibodies (Baco) as described (7)) affinity resin, respectively, and used for binding assays with recombinant forms of eIF1.

Co-immunoprecipitation was done essentially as described (6) with the following modifications. Whole cell extracts (WCE) were prepared in buffer A, but immune complex binding and washing were done in buffer

G. Radke, T. Iwamoto, and J. Tomich, unpublished observations.
A supplemented with 0.1% Triton X-100. Polysome analysis was conducted as described previously (4, 5).

To quantitate the amount of factors in the precipitated fractions, we used WCE prepared from a wild type strain (KAY146) as a standard, based on the following information. Using purified FL-eIF1 as a reference, we determined that 40 μg of WCE contains 3.6 pmol of eIF1 (see Fig. 3A). Because the intracellular molar ratio of eIF1, eIF2, eIF3, and eIF5 is 1.97:0.65:0.82 in our WCE, the same amount of WCE was calculated to contain 2.8, 1.9, and 3.0 pmol of eIF2, eIF3, and eIF5, respectively. The amounts of precipitated eIFs were determined in reference to these values. We determined individual eIF levels by anti-FLAG immunoblotting of WCE prepared from strains encoding FLAG-eIF1, -eIF2, -eIF3, or -eIF5 as its sole source.3 The values obtained are in better agreement with data from Ref. 18 than the data from Ref. 19.

GCN4 translational control in KAY142 (crypFL-SU1) was tested by assaying β-galactosidase from its transformant carrying p180 (GCN4::lacZ) or its derivative p226 or p227 (20). The results of these experiments were described in Ref. 21.

All of the biochemical assays were done at least three times, and a typical result is shown.

RESULTS

Yeast eIF1 Interacts with eIF5-CTD via AA-boxes and eIF2β-NTD via K-boxes—It was previously reported that a GST fusion form of eIF5-CTD (165 amino acids, known as GST-eIF5-B6, can interact with35S-labeled eIF1, synthesized in a rabbit reticulocyte lysate (4) (also see Fig. 1A, lanes 2 and 3). Because eIF1 stimulates eIF2 TC binding to the ribosome (13, 14, 22), we also examined whether individual eIF2 subunits can bind eIF1 and found that GST-eIF2β and its C-terminal deletion GST-eIF2β-N (covering amino acids 1–140) specifically bound35S-eIF1 (Fig. 1B, lanes 3 and 4).

To further examine interactions between eIF1 and eIF5 or eIF5β, the bacterial extract containing a recombinant form of eIF1 (r-eIF1) was incubated with GST-eIF5-B6, GST-eIF5β-N, GST-eIF3c-N, or GST alone. GST-eIF3c-N contains the N-terminal 156 amino acids of eIF3c, sufficient for eIF1 binding (4), and was used as a positive control. GST fusion proteins bound to r-eIF1 were one-step purified with glutathione and analyzed by SDS-PAGE. As shown in Fig. 1C, top panel, Coomassie staining of the eluted proteins indicates that GST-eIF3c-N (lane 3) binds to r-eIF1 more strongly than GST-eIF5-B6 (lane 4), and GST alone (lane 5), under these conditions, GST-eIF5-B6 and GST-eIF2β-N bound recombinant eIF1, albeit weakly as determined by Western blotting (Fig. 1C, bottom panel, lanes 9 and 12; note that 5 times less eluted fraction was analyzed in lanes 5–7 than in lanes 8–13). Therefore, r-eIF1 binds GST-eIF5-B6 and GST-eIF2β-N. The treatment of the GST fusion complexes with RNase A prior to the elution step did not reduce these interactions (Fig. 1C, lanes 7, 10, and 13), ruling out the possibility that r-eIF1 was tethered to the proteins via RNA fortuitously bound to r-eIF1 or the GST fusion protein.

As mentioned above, the new interactions of eIF1 with eIF2β and eIF5 are weaker than the previously known interaction between eIF1 and eIF3c (Fig. 1C). However, it is these weaker interactions that contribute to cooperative formation of eIF1-eIF2β-eIF5 subcomplex in the MFC, as shown in Fig. 1, E and F. Consistent with this model, FLAG epitope tagging of eIF1 compromises these interactions, leading to inefficient incorporation of FLAG-eIF1 into the MFC (see Fig. 2C).

Both the novel interactions of eIF1 depend on the AA-boxes of eIF5 and K-boxes of eIF2β, because the interaction between GST-eIF5-B6 and35S-eIF1 was abolished by the AA-box 1 mutation, tff5-12A, or the AA-box 2 mutation, tff5-7A (Fig. 1A, lanes 3–5), and altering all of the lysines in the K-boxes to alanines reduced GST-eIF2β binding to eIF1 by 4-fold (Fig. 1B, lane 5). We also found that these interactions were salt-sensitive, since they were not detected when a buffer containing 150 mM Na+ was used at the washing step (7) instead of a buffer containing 75 mM KCl as employed in Fig. 1 (data not shown).

We confirmed, however, that35S-eIF1 interacts with GST-eIF5-B6 and eIF2β-N in the same binding buffer (7) with 100 mM KCl; this salt concentration has been used in a variety of translation initiation assays (23). The salt sensitivity of these interactions explains our previous failure to identify these interactions.

To confirm that eIF1 can bind directly to the trimeric eIF2 complex, we affinity-purified FLAG-tagged eIF2 from yeast and allowed it to bind r-eIF1 expressed from bacteria. The Coomassie-stained gel of the purified eIF2 is shown in Fig. 1D, lane 2. The eIF2r-eIF1 complex was precipitated with anti-FLAG affinity resin and then analyzed by Western blotting. As shown in Fig. 1D, second panel, r-eIF1 bound specifically to FLAG-eIF2 as detected by anti-eIF1 (lanes 4–9). To examine whether this interaction is mediated by eIF3 or eIF5 associated with FLAG-eIF2, we analyzed the complex with antibodies against eIF2α, eIF3g, and eIF5. As shown in Fig. 1D, bottom two panels, we found little eIF3 and eIF5 in the precipitated FLAG-eIF2 fractions (lanes 6 and 9), whereas the amounts of eIF1 and eIF2α in the FLAG-eIF2r-eIF1 fraction was judged to be nearly stoichiometric (lane 6), when compared with their amounts in yeast WCE (lane 3). These results indicate that eIF1 can bind native eIF2 as well as its β subunit.

eIF2β-NTD Interacts Simultaneously with eIF1 and eIF5-CTD—Having observed separate interactions of eIF1 with eIF2β-NTD and eIF5-CTD, we pondered whether these interactions occur simultaneously. If so, the formation of a trimeric eIF1-eIF2β-eIF5 complex as a part of MFC would contribute to stable eIF2 TC binding to the 40 S ribosome. Thus, we tested whether the interaction between GST-eIF5-B6 and eIF1 can be enhanced by the addition of eIF2β-NTD by a bridging mechanism. As a control, we used the eIF3c-NTD segment, since it is known to bridge the same interaction between GST-eIF5-CTD and eIF1 (4). As shown in Fig. 1E, bottom panel, the eIF2β-N segment increases35S-eIF1 binding to GST-eIF5-B6 by forming a bridge, as efficiently as the eIF3c-N segment does (top panel). The addition of equivalent amounts of bovine serum albumin

**TABLE II**

| Strain | Description | Source |
|--------|-------------|--------|
| Y217   | MATα leu2 [sul1 ura3-52 trp1Δ moc2(sui1):hisG(pSU1 URA3)] | Ref. 31 |
| Y218   | Y217 with pSU1 TRP1 replacing pSU1 URA3 | Ref. 31 |
| KAY146 | Y217 with Ycp1-SU11 (SU11 LEU2) replacing pSU11 URA3 | This study |
| KAY156 | Y217 with Ycp1-FL-SU11 (FL-SU11 LEU2) replacing pSU11 URA3 | This study |
| KAY178 | Y217 with Ycp1-FL-SU11 (FL-SU11 LEU2) replacing pSU11 URA3 | This study |
| KAY142 | Y217 with Ycp1-cpr1-FL-SU11 (cpr1-FL-SU11 LEU2) replacing pSU11 URA3 | This study |
| KAY18 | Y217 with Ycp1-FL-SU11 (FL-SU11 LEU2) replacing pSU11 URA3 | This study |
| KAY8   | MATα ura3-52 leu2-3,112 trp1Δ-Δ63 tff3Δ4 gen2Δ Δ1(TIF5 URA3) | Ref. 7 |
| KAY9   | MATα ura3-52 leu2-3,112 trp1Δ-Δ63 tff3Δ4 gen2Δ Δ1(TIF5 URA3) | Ref. 6 |
| KAY37  | MATα ura3-52 leu2-3,112 trp1Δ-Δ63 tff5Δ4 gen2Δ Δ1(TIF5 FL TRP1) | Ref. 6 |

3 H. He, C. R. Singh, and K. Asano, unpublished data.
did not increase GST-eIF5-B6/35S-eIF1 interaction (data not shown), indicating that the increased interaction in Fig. 1E is not due to increasing the efficiency of the pull downs by non-specific mechanisms. Thus, eIF2β-NTD binds simultaneously to eIF1 and eIF5-CTD. Likewise, Fig. 1F shows that the His-eIF5-B6 segment can bridge GST-eIF2β-N and 35S-eIF1, thereby enhancing this interaction (compare lanes 2–4), indicating that eIF5-CTD binds simultaneously to eIF1 and eIF2β-NTD. Together, these results support the idea that a trimeric eIF1-eIF2β-eIF5 complex can form as a part of MFC for stimulation of TC binding to the ribosome.

FLAG Tapping of eIF1 Impairs the Interaction with eIF5-CTD, eIF2β-NTD, and the Native eIF3 Complex in Vitro—To analyze the functional significance of a protein interaction in vivo, it is important to obtain a mutation that specifically reduces it. During the course of our study, we noted that some of the interactions involving eIF1 were compromised by its epitope tagging at either terminus with the bulky highly charged FLAG peptide (DYKDDDDK). As shown in Fig. 2A, middle and bottom panels, C-terminally and N-terminally FLAG-tagged eIF1, designated eIF1-FL and FL-eIF1, respectively, had reduced interactions with GST-eIF2β-N and GST-eIF5-B6 (lanes 3 and 6) but not with GST-eIF3c-N (lane 4). In addition, we found that both forms of FLAG-eIF1 had reduced interactions with the C-terminal HEAT domain of eIF4G2 (lane 5) as well as with the C-terminal domain of eIF3a (GST-TIF32Δ1 (8); data not shown).

To examine the interaction of FLAG-eIF1 derivatives with native eIF3, we affinity-purified native HA epitope-tagged eIF3, attached it to anti-HA affinity resin, and allowed it to
bind recombinant forms of eIF1, FL-eIF1, and eIF1-FL in vitro.

As shown in Fig. 2B, lane 2, the purified HA-eIF3 contains stoichiometric amounts of a, b, and g subunits of eIF3, a substoichiometric amount of eIF5, and no detectable eIF1. This eIF3 complex bound untagged eIF1 very efficiently but bound FLAG-eIF1s less efficiently. Thus, FLAG tagging of eIF1 reduces the interaction with eIF3.

**FLAG Tagging of eIF1 Disrupts the Interaction with the eIF3 Complex in Vivo**

To test the in vivo effect of FLAG tagging of eIF1, we constructed single copy plasmids YcPL-FL-SU11 (FL-SU11 LEU2) or YcPL-SU11-FL (SU11-FL LEU2) encoding FL-eIF1 or eIF1-FL, respectively (see Tables I and III). The FL-eIF1 construct contains a Met residue inserted between the FLAG peptide and the second codon of eIF1. To avoid possible leaky scanning that might allow expression of an untagged eIF1 from the FL-eIF1 plasmid, we also constructed YcPL-FL-SU11*, altering the second Met residue of FL-eIF1 to Leu. This eIF1 derivative was designated FL-eIF1*.

To verify the expression of FLAG-eIF1 derivatives in vivo, we introduced the above mentioned plasmids to a wild-type yeast strain, Y217 (Table II) and analyzed the resulting transformants for immunoblotting. As shown in Fig. 3A, top panel, immunoblotting with anti-FLAG antibodies showed that YcPL-FL-SU11 (lanes 1 and 2) and YcPL-SU11-FL produced FLAG-eIF1 species migrating at 16 and 18 kDa, whereas YcPL-SU11-FL produced eIF1-FL migrating at 14 kDa (lanes 8 and 9).
confirmed that these species indeed reacted with anti-eIF1 antibodies (Fig. 3A, bottom panel). The 16-kDa species of FL-eIF1 and the 14-kDa species of eIF1-FL comigrated with recombinant forms of FL-eIF1 and eIF1-FL (with predicted masses of 13.4 and 13.3 kDa, see Table III), respectively, indicating their analogy in electrophoretic mobility. We do not know how the larger, 18-kDa FL-eIF1* or FL-eIF1 was produced. Finally, comparison with the amount of endogenous eIF1 expressed solely from the plasmids under column 3. For lethal SUI1-FL expressing FL-eIF1* or eIF1-FL, respectively, or an empty vector to strain Y217 allowed us to judge the expression level of the three FLAG-eIF1 derivatives to be equivalent to that of endogenous eIF1 co-precipitated specifically with HA-eIF3, together with the eIF3b subunit, eIF2 and eIF1-FL (with FL-eIF1* or FL-eIF1 was produced). These data are consistent with the idea that the FLAG tagging of eIF1 impairs its recruitment to the 40 S ribosome.

As summarized in Table IV, we consistently find 20–30% of total MFC components (eIF1, -2, -3, and -5) in the 40 S ribosomal fractions isolated from wild type yeast, and the molar levels of these proteins are roughly equivalent. In agreement with in vitro binding assays between native eIF3 and FLAG-eIF1 derivatives (Fig. 2B), little of the FLAG-eIF1 construct interacts with HA-eIF3 (Fig. 2C, bottom panel, lanes 8, 11, and 17). Thus, all three forms of FLAG-eIF1 cannot bind eIF3 efficiently in vivo.

Interestingly, we found that endogenous eIF1 was not associated with HA-eIF3 in the presence of eIF1-FL (Fig. 2C, fifth panel, lane 17), although it was associated with HA-eIF3 in the presence of FL-eIF1* (lane 8) to the same degree as found in the vector control transformant (lane 5). Thus, eIF1-FL has a dominant negative effect on native eIF1 binding to eIF3, whereas FL-eIF1* does not. Because the in vitro binding assay in Fig. 2B indicates that eIF1-FL can bind HA-eIF3, albeit less efficiently than wild type eIF1, we presume that the weak association of eIF1-FL with eIF3-HA interfered with the latter’s binding to endogenous eIF1. Perhaps, the washing step in the coimmunoprecipitation experiment was harsh enough to disrupt this weak interaction. These results are consistent with the more severe dominant negative effect of eIF1-FL overexpression (see Figs. 4 and 5).

| Allele | Product | Plasmid | Expression | Growth |
|--------|---------|---------|------------|--------|
| SU1    | eIF1    | YCpL-SU1 | (1)        | WT     |
| FL-SU1 | FL-eIF1 | YCpL-FL-SU1 | 1–2       | WT     |
| FL-eIF1* | YCpL-FL-SU1* | 1–2       | WT     |
| SUI1   | eIF1-FL | YCpL-SUI1-FL | 1        | WT     |
| SUI1-FL | eIF1-FL | YCpL-SUI1-FL | 1–2     | WT     |

*FL-eIF1 contains MDYKDDDKM, followed by the natural eIF1 peptide from the second amino acid. FL-eIF1 replaces the second Met of eIF1 with Leu. eIF1-FL contains DYKDDDDK, following the entire eIF1 peptide. Predicted molecular masses are 13,429, 13,411, and 13,298 Da, respectively, compared with 12,304 Da for wild type. Transcription of all these alleles starts from the natural SU1 promoter, except for FLAG-eIF1 starting from a stronger GPD promoter.

**Table II.** Phenotypes of differently FLAG-tagged SUI1 alleles analyzed in this study

| Allele | Product | Plasmid | Expression | Growth |
|--------|---------|---------|------------|--------|
| SU1    | eIF1    | YCpL-SU1 | (1)        | WT     |
| FL-SU1 | FL-eIF1 | YCpL-FL-SU1 | 1–2       | WT     |
| SUI1   | eIF1-FL | YCpL-SUI1-FL | 1        | WT     |
| SUI1-FL | eIF1-FL | YCpL-SUI1-FL | 1–2     | WT     |

**Table III.** Expression levels were tested after evicting the URA3 SUI1 plasmid on FOA medium except for lethal alleles. Thus, the values under columns 4 and 5 are the relative amounts of eIF1 expressed solely from the plasmids under column 3. For lethal SUI1-FL alleles, expression level was examined in the presence of p[URA3 SUI1] in Y217, since the level of untagged 12-kDa eIF1 species from these alleles was indistinguishable from that of native eIF1 from the control SU1 deletion strain Y217 harboring a URA3 SUI1 plasmid (Table II), and the latter plasmid was removed by growth on medium supplemented with 5-fluoroorotic acid (FOA) that is toxic to cells expressing the Ura3p enzyme. As shown in Fig. 3B, lane c, we found that the cells expressing eIF1-FL did not grow on the FOA medium, indicating that C-terminal FLAG tagging of eIF1 is lethal. Because very little eIF1-FL was associated with the 40 S ribosome (Fig. 4C), we suggest that the inability...
of at least this protein to bind the ribosome produced the rate-limiting defect.

To test whether this lethal effect of eIF1-FL can be overcome by mass action, we constructed YEpL-SUI1-FL encoding eIF1-FL on a high copy vector. As shown in Fig. 2C, lanes 19–21, co-immnoprecipitation of HA-eIF3 in KAY107 (HA-TIF34) transformant carrying this plasmid confirms that eIF1-FL associates with HA-eIF3 when overproduced. However, the SU11-FL allele on this plasmid did not complement the SU11 deletion (Fig. 3B, lane g), although it expressed eIF1-FL at a 6–8 times higher level than Ycp-SU11-FL (Table III). These results indicate that increased gene dosage cannot complement the rate-limiting defect of eIF1-FL. Consistent with this idea, we found that the 43 S complex formation in the presence of excess eIF1-FL was severely inhibited, with fewer MFC components (eIF1, eIF2, eIF3, and eIF5) associated with the 40 S ribosome (Fig. 4F, lanes 7 and 8). Thus, excess eIF1-FL impedes 43 S complex formation rather than restoring it.

Together with the data in Fig. 2C, lanes 19–21, these results suggest that excess eIF1-FL recovers complex formation with eIF3 by mass action, but this complex cannot be recruited to the 40 S ribosome. Therefore, C-terminal FLAG tagging might impair efficient binding of MFC to the 40 S ribosome in addition to impairing incorporation into the MFC (see “Discussion”). The severe inhibition of 43 S complex formation caused by eIF1-FL overexpression obviously impaired general translation initiation, because the Y217 transformant carrying YEplac111 grew slowly at all of the temperatures tested (hence a dominant slow growth phenotype), concomitant with a reduced polysome content, as shown in Fig. 3D.

In contrast to the effect of eIF1-FL, the cells expressing FL-eIF1 or FL-eIF1* grew on the FOA medium (Fig. 3B, lanes d and e), indicating that these alleles are not lethal. Immunoblot analyses showed that the resultant FOA resistant strains KAY156 (FL-SU11) and KAY178 (FL-SU11*) expressed untagged eIF1 (12 kDa) besides FL-eIF1 (16 and 18 kDa) (Fig. 3C, lanes 1–9), although the strains were Ura– and therefore lacked the URA3 plasmid carrying the native eIF1 allele. Notably, untagged eIF1 was diminished but not completely eliminated in KAY178 encoding FL-eIF1* compared with that in KAY156 encoding FL-eIF1 (Fig. 3C, lanes d and e). Having observed severe inhibition of 43 S complex formation with this idea, we found that the 43 S complex formation in the second Met between FLAG and eIF1 peptides, the generation of untagged eIF1 in KAY178 plasmid carrying the native eIF1 allele. Notably, untagged eIF1 was diminished but not completely eliminated in KAY178 encoding FL-eIF1* compared with that in KAY156 encoding FL-eIF1 (Fig. 3C, lanes d and e).
S ribosomes that are bound to the GCN4 leader following uORF1 translation dissociate after they translate uORF4 efficiently. When stars are carved for amino acids, uncharged tRNAs accumulate and activates eIF2 kinase Gen2p. Phosphorylated eIF2 competitively inhibits the action of the guanine nucleotide exchange factor eIF2B, thereby blocking Met-tRNA\textsubscript{iMet} delivery to the 40 S ribosomes. Under these conditions, 40 S ribosomes migrating down the GCN4 leader rebind TC only after they pass uORF4, resulting in efficient GCN4 translation. Gcd\textsuperscript{−} mutations derepress GCN4 translation independent of eIF2 phosphorylation (hence action of Gen2p), due to reduced Met-tRNA\textsubscript{iMet} binding to the ribosome. Therefore, excess eIF1-FL would be expected to induce GCN4 translation, since it severely impedes 43 S complex formation, as shown in Fig. 4F.

To test this possibility, we introduced a high copy eIF1-FL plasmid and control plasmids to a gen2Δ strain. As shown with the vector control transformant (Fig. 5A, line 1), the gen2Δ strain is sensitive to 3-AT, since it inhibits a histidine biosynthesis enzyme, and this inhibition cannot be rescued by activating the general control pathway due to gen2Δ. As shown in Fig. 5A, line 7, the gen2Δ transformant carrying high copy eIF1-FL plasmid is now 3-AT-resistant, just as observed with the control transformant carrying high copy eIF5 plasmid (line 8). Overexpression of eIF5 sequesters eIF2 TC in a partially formed MFC by its direct interaction with the latter, thereby impeding 43 S complex formation (6). Note, however, that the 3-AT resistance observed with eIF5 and eIF1-FL overexpression is not as strong as the normal 3-AT resistance conferred by Gen2p kinase (Fig. 5B). The Gcd\textsuperscript{−} phenotype of the eIF1-FL transformant is due to C-terminal FLAG tagging of eIF1 but not due to eIF1 overexpression per se, because overexpression of native eIF1 did not suppress the 3-AT sensitivity of the gen2Δ strain (Fig. 5A, line 3). Importantly, the Gcd\textsuperscript{−} phenotype of eIF1-FL transformant is due to limiting eIF2 TC binding to the ribosome, because co-overexpression of all three eIF2 subunits and tRNA\textsubscript{iMet} suppressed 3-AT resistance conferred by eIF1-FL overproduction as well as that caused by eIF5 overproduction (Fig. 5C, compare lanes 14 and 15 with lanes 17 and 18). Immuno blot analyses in Fig. 5D indicate that the reduction in 3-AT resistance by eIF2 TC overexpression occurred without altering the level of eIF1-FL. These results strongly support the physiological relevance of the inhibition of 43 S complex formation by excess eIF1-FL, as observed in Fig. 4F.

In contrast to the effect of eIF1-FL, we did not observe a dominant Gcd\textsuperscript{−} phenotype with any FL-eIF1 or FL-eIF1\textsuperscript{*} construct either on a single copy or high copy vector (Fig. 5A, lines 4 and 5; data not shown for FL-eIF1). Immuno blot analyses indicate that FL-eIF1\textsuperscript{*} level from the high copy vector is ~3-fold higher than eIF1-FL level from the same vector and that the level of untagged eIF1 expression from the high copy FL-eIF1\textsuperscript{*} plasmid is low (Table III). Thus, we believe that the effect of FL-eIF1\textsuperscript{*} on GCN4 expression is smaller than that of eIF1-FL. Alternatively, the residual expression of untagged eIF1 from the high copy FL-eIF1\textsuperscript{*} plasmid may be sufficient to competitively diminish its effect on GCN4 expression.

**FL-eIF1 Reduces eIF2 Binding to the 40 S Ribosome**—In an effort to uncover any detectable effect of N-terminal FLAG tagging of eIF1, we constructed strain KAY142 (\textsuperscript{cryp}FL-SU11) that encodes FL-eIF1 under the GPD promoter as the sole source of eIF1. Due to the change in promoter, this construct

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**Fig. 4.** Effect of FLAG-eIF1 expression on eIF1 binding to 40 S ribosome and overall 43 S complex formation in vivo. Wild type strain Y217 transformants carrying YCpLacl 11 (A), YCpL-FL-SU11\textsuperscript{*} (B, YCpL-SU1-FL (C), and YEpL-SU1-FL (F) were grown in SC-ura-leu medium and subjected to polysome analysis on a 15–40% sucrose gradient as described (4). One-third of the top to middle fractions encompassing free eIFs and 40 and 60 S ribosomes (lanes 2–11) are analyzed by SDSPAGE and immunoblotting with anti-FLAG antibodies indicated to the side of each panel, together with 2% of input amount used for loading on the sucrose gradient (lane 1). Anti-FLAG antibodies are used to produce the fifth panels (B, C, and F) to detect FLAG-eIF1 specifically. D and E, amounts of endogenous eIF1 (columns 1 and 2, detected by anti-eIF1) or FLAG-eIF1 (columns 3 and 4, detected by anti-FLAG) in combined fractions encompassing free eIFs and 40 S ribosomes (lanes 2–6 (free) or 7–8 (40 S) were quantitated by the NIHImage software and compared with the input amount in lane 1.
eIF1 was also highly expressed in this strain (Fig. 3B, lanes 10–13). Strain KAY142 grows normally at all of the temperatures tested (Table III) and displayed a normal polysome profile (Fig. 3E), indicating that general translation initiation is not inhibited by the FL-eIF1 overexpression.

As shown in Fig. 6B, sucrose gradient analyses of cell extracts prepared from KAY142 indicate that the level of eIF2 on the 40 S ribosome was modestly reduced compared with wild type KAY146 (Fig. 6A), without altering the ribosomal association of eIF3 (as detected by anti eIF3a and eIF3b antibodies) and eIF5. Densitometric measurements from three independent experiments demonstrated a 2-fold reduction in eIF2 association with 40 S ribosome (Fig. 6C, bottom panel) and no alteration of eIF3 and eIF5 association with 40 S ribosome (top to third panels). Consistent with the results shown in Fig. 4B, N-terminal FLAG tagging of eIF1 reduces the level of FL-eIF1 binding to the 40 S ribosome, compared with the native form expressed from the same allele, eIF2-GPDFL-SUI1 (Fig. 6D). Together, these results indicate that FL-eIF1 overexpression partially impedes 43 S complex formation in vivo.

We believe that the 2-fold decrease in eIF2 binding to the ribosome is significant, because we found that GCN4 translation from a GCN4::lacZ reporter plasmid was partially derepressed in strain KAY142, consistent with this observation (data not shown).

**DISCUSSION**

eIF1 plays a critical role in accurate selection of AUG as a start codon as shown both by yeast genetics and mammalian biochemistry (see Introduction). In this report, our analyses of FLAG-tagged forms of eIF1 as mutants provided in vivo evidence for a second function of eIF1 in promoting the 43 S complex formation. Specifically, we found that FLAG-tagged forms of eIF1 reduce their binding to eIF3 in vitro (Fig. 2B), thereby reducing incorporation into the 43 S complex in vivo (Fig. 4, A–E). Importantly, C-terminal FLAG-eIF1 overexpression severely impedes the 43 S complex formation in vivo (Fig. 4F) and confers a dominant Gcd phenotype that is suppressible by overexpression of eIF2 TC (Fig. 5). N-terminal FLAG-eIF1 overexpression also reduces binding of eIF2 to the 40 S ribosome (Fig. 6) and moderately derepresses GCN4 translation (data not shown). These results indicate that the incorporation of eIF1 into the MFC is critical for formation of 43 S complex in vivo. eIF1 was recently shown to stimulate eIF2 TC binding to the 40 S ribosome in vitro both in yeast (13) and mammals (14). Together with our in vivo data, these data strongly support the second role of eIF1 in promoting 43 S complex assembly. The dual role for a single factor in 43 S complex formation and accurate AUG selection was first proposed for eIF5, the GAP for eIF2 (4, 5).

How does eIF1 promote the 43 S complex formation? eIF1 appears to be a part of nucleation site for the MFC formation, composed of eIF2β-NTD, eIF3c-NTD, eIF5-CTD, in addition to eIF1 itself (see Fig. 1G). Consistent with this idea, we provided biochemical evidence indicating that a trimeric complex can be formed between eIF1, eIF2β-NTD, and eIF5-B6 (Fig. 1, E and F). Because eIF3 and eIF2 can bind directly (8) and have independent ribosome-binding sites (26), eIF1 should be able to facilitate 43 S complex formation in a cooperative manner, in concert with the action of eIF5-CTD. We believe that the ribosome-binding defect observed with C-terminal and N-terminal FLAG-eIF1 (Fig. 4, B, C, E, and F) is due to severing eIF1 from eIF2, eIF5, and eIF3 (Fig. 2), thereby impairing this mechanism of 43 S complex promotion by eIF1.

A second, less established mechanism for eIF1 to promote 43 S complex formation would be to assume that eIF1 allosterically activates either MFC or the 40 S ribosome for 43 S
**TABLE IV**

Relative eIF levels in free and 40 S ribosome fractions in yeast

| eIF or its subunit | Protein expression (percentage of eIF5 level) | Percentage found in 40 S fraction | Percentage found in free fraction |
|--------------------|---------------------------------------------|-----------------------------------|----------------------------------|
| eIF3a              | 65 ± 20\%                                   | 22 ± 3                            | 36 ± 8                           |
| eIF3b              | 65 ± 20\%                                   | 21 ± 3                            | 37 ± 8                           |
| eIF2α              | 93 ± 13\%                                   | 25 ± 9                            | 55 ± 20                          |
| eIF5               | 100 ± 23                                    | 28 ± 7                            | 72 ± 15                          |
| eIF1               | 122 ± 44                                    | 20 ± 4                            | 61 ± 13                          |

\(^1\) Based on the expression level of the eIF3c subunit.

\(^2\) Based on the expression level of the eIF2β subunit.

Fig. 6. FL-eIF1 overexpression partially inhibits 43 S complex formation in vivo. Strains KAY142 (qpr,FL-SU11) (B) and its isogenic wild type KAY146 (A) were grown in YPD and subjected to polysome analysis exactly as in Fig. 4. Lane 1, 5% of input amount of cell extracts. Lanes 2–11, 50% of sucrose gradient fractions. A, eIF distribution between free and 40 S fractions. Amounts of eIF3a, eIF3b, eIF5, and eIF2α in combined fractions 2–7 (free) or 8 and 9 (40 S) were compared with the total amount of each factor in lanes 2–11. We compared with this amount rather than the input amount in lane 1 to avoid larger S.D. values (see Table IV). B, amounts of untagged eIF1 and FL-eIF1 in free and 40 S fractions from KAY142 were quantitated exactly as in Fig. 4, D and E.

complex formation. The idea that eIF1 might allosterically control eIF2 TC binding to the 40 S ribosome is not new but has yet to be proven (23). In this study, we observed that overexpression of C-terminal FLAG-eIF1 can restore binding of the mutant eIF1 to eIF3 (Fig. 2C, lanes 19–21), but the MFC formed with the mutant eIF1 appears to be defective in binding to the 40 S ribosome (Fig. 4F). Because it was shown recently that eIF1 can directly bind to the 40 S ribosome in vitro at a high affinity (27), one might suspect that this direct contact is the major driving force for MFC binding to the ribosome and is inhibited by eIF1-FL. However, we believe that this is not the case, because the prt-t mutation altering the eIF3b subunit impairs eIF1 as well as eIF3 and TC binding to the 40 S ribosome in cell extracts, indicating that the recruitment of MFC constituents, including eIF1, depends on eIF3 (10). If so, it would be attractive to propose that eIF1 allosterically stimulates the binding of other factors (likely eIF3) to bind the 40 S ribosome and that C-terminal FLAG tagging impairs this function, in addition to impairing incorporation into the MFC. Indeed, a similar role in MFC binding to the ribosome could be proposed for eIF5, since it enhances the ability of the eIF3a-eIF3c subcomplex to bind the 40 S ribosome (26).

The effect of FLAG tagging of eIF1 on the 43 S complex formation appears to be different between N-terminal and C-terminal FLAG-eIF1 (Figs. 2–6), although both forms lower the affinity with individual MFC partners in a similar manner (Fig. 2A). This difference may arise from different locations of the tag introduced, since the C terminus of eIF1 ends in an antiparallel β-sheet, whereas its N-terminal 20-amino acid–long segment is unfolded and contains conserved hydrophobic/acidic residues, suggestive of interaction with other partners (28). The antiparallel β-sheet domain of eIF1 may be important for MFC binding to the ribosome, either through direct contact with the ribosome as proposed previously (27) or through somehow activating eIF3, as suggested above.

In the case of N-terminal FLAG-eIF1 (FL-eIF1 or FL-eIF1*), the in vitro binding defect with eIF3 appears to be more severe than the defect caused by eIF1-FL (Fig. 2B, compare lanes 6...
We consistently observed that FL-eIF1 was not incorporated into the MFC in vivo (Fig. 2C). Thus, the N-terminal portion of eIF1 may be more critical for incorporation into the MFC. Despite the more severe defect in eIF3 binding, FL-eIF1 overexpression from the GPD promoter, resulted in a minor decrease in the level of eIF2 on the 40 S ribosome (Fig. 6) and derepressed GCN4 translation (data not shown; also see Ref. 21). A possible explanation for these findings might be to propose that FL-eIF1 separately interacted with eIF2 by mass action, thereby sequestering a part of eIF2 from binding to the ribosome. It is possible that the inhibition of TC binding in this manner was very moderate and resulted in only a minor increase in GCN4 expression.

Consistent with a defect in eIF3 binding (Fig. 2C) and possibly an additional defect in MFC binding to the ribosome (Fig. 4F), the SU11-FL allele encoding eIF1-FL was recessive lethal, regardless of the copy number of the vector (Fig. 3B and Table III). We could not judge the recessive phenotypes of N-terminal FLAG-eIF1-encoding alleles, because untagged eIF1 was produced from the same expression plasmids, possibly due to proteolytic cleavage of the tag (Fig. 3C). Therefore, it would be important to generate and characterize eIF1 mutant derivatives that cannot bind MFC (like FL-eIF1 or FL-eIF1*) but do not produce proteolytic derivatives that might complement the phenotypes produced by the mutations themselves.

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Efficient Incorporation of Eukaryotic Initiation Factor 1 into the Multifactor Complex Is Critical for Formation of Functional Ribosomal Preinitiation Complexes in Vivo

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