Dissociation of eIF1 from the 40S ribosomal subunit is a key step in start codon selection in vivo

Sarah F. Mitchell
Loyola Marymount University, sarah.mitchell@lmu.edu

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Dissociation of eIF1 from the 40S ribosomal subunit is a key step in start codon selection in vivo

Yuen-Nei Cheung,1 David Maag,2 Sarah F. Mitchell,2 Christie A. Fekete,1 Mikkel A. Algire,2 Julie E. Takacs,2 Nikolay Shirokikh,3 Tatyana Pestova,3 Jon R. Lorsch,2,5 and Alan G. Hinnebusch1,4

1Laboratory of Gene Regulation and Development, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA; 2Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA; 3Department of Microbiology and Immunology, State University of New York Health Science Center at Brooklyn, Brooklyn, New York 11203, USA

Selection of the AUG start codon is a key step in translation initiation requiring hydrolysis of GTP in the eIF2•GTP•Met-tRNA\text{\textsubscript{Met}} ternary complex (TC) and subsequent P\textsubscript{i} release from eIF2•GDP•P\textsubscript{i}. It is thought that eIF1 prevents recognition of non-AUGs by promoting scanning and blocking P\textsubscript{i} release at non-AUG codons. We show that Su\textsuperscript{−} mutations in Saccharomyces cerevisiae eIF1, which increase initiation at UUG codons, reduce interaction of eIF1 with 40S subunits in vitro and in vivo, and both defects are diminished in cells by overexpressing the mutant proteins. Remarkably, Su\textsuperscript{−} mutation ISQLG\textsubscript{93–97}ASQAA (abbreviated 93–97) accelerates eIF1 dissociation and P\textsubscript{i} release from reconstituted preinitiation complexes (PICs), whereas a hyperaccuracy mutation in eIF1A (that suppresses Su\textsuperscript{−} mutations) decreases the eIF1 off-rate. These findings demonstrate that eIF1 dissociation is a critical step in start codon selection, which is modulated by eIF1A. We also describe Gcd\textsuperscript{−} mutations in eIF1 that impair TC loading on 40S subunits or destabilize the multifactor complex containing eIF1, eIF3, eIF5, and TC, showing that eIF1 promotes PIC assembly in vivo beyond its important functions in AUG selection.

[Keywords: AUG selection; Saccharomyces cerevisiae; translation initiation; eIF1]

Supplemental material is available at http://www.genesdev.org.

The eukaryotic translation initiation pathway produces an 80S ribosome bound to mRNA with methionyl initiator tRNA \text{[Met-tRNA\text{\textsubscript{Met}}]} base-paired to the AUG start codon in the ribosomal P-site. The Met-tRNA\text{\textsubscript{Met}} is recruited to the small (40S) ribosomal subunit in a ternary complex (TC) with GTP-bound eIF2, to produce the 43S preinitiation complex (PIC). In budding yeast, this reaction is stimulated by eIF1, eIF1A, eIF3, and eIF5 both in vitro (Danaie et al. 1995; Phan et al. 1998; Asano et al. 2001; Algire et al. 2002) and in vivo (Hinnebusch 2000; Olsen et al. 2003; Fekete et al. 2005; Jivotovskaya et al. 2006). The eIF3, eIF5, eIF1, and TC can be isolated in a multifactor complex (MFC) from yeast (Asano et al. 2000) whose formation promotes binding of all constituent factors to 40S subunits in vivo [Valášek et al. 2002, 2004; Singh et al. 2004; Yamamoto et al. 2005; Jivotovskaya et al. 2006]. The 43S PIC interacts with the 5’ end of mRNA, producing the 48S PIC, and scans the leader until the anticodon of Met-tRNA\text{\textsubscript{Met}} base-pairs with an AUG codon. Scanning is promoted by eIF1, eIF1A, and eIF4F in a reconstituted mammalian system [Pestova et al. 1998; Pestova and Kolupaeva 2002], and genetic data suggest that eIF5, eIF1A, and eIF3 promote scanning in yeast cells [Asano et al. 2001; Nielsen et al. 2004; Fekete et al. 2005]. In the 48S PIC, the GTP bound to eIF2 is partially hydrolyzed to GDP and inorganic phosphate [P\textsubscript{i}] in a manner stimulated by eIF5, but AUG recognition is required for P\textsubscript{i} release from eIF2•GDP•P\textsubscript{i} driving GTP hydrolysis to completion (Algire et al. 2005; Maag et al. 2005). It is thought that eIF2-GDP releases Met-tRNA\text{\textsubscript{Met}} into the P-site, allowing subsequent joining of the 60S subunit [Hershey and Merrick 2000; Pestova et al. 2000]. The eIF2-GDP is recycled to eIF2-GTP by guanine nucleotide exchange factor eIF2B to allow the reassembly of TC.

Genetic studies in yeast showed that eIF1, subunits of eIF2, and eIF5 regulate AUG selection in vivo, by identifying mutations that increase initiation at UUG codons. Such mutations restore translation of the his4-
303 allele lacking the initiation codon, producing the Sui− (Suppressor of initiation codon mutation) phenotype (Donahue 2000). Biochemical analysis of the mammalian system showed that eIF1 antagonizes recognition of non-AUG codons during scanning (Pestova and Kolupaeva 2002). The 40S-binding site of eIF1 was localized near the P-site, and it was proposed that eIF1 promotes an open conformation of the PIC conducive to scanning and restricts base-pairing of Met-tRNA<sub>Met</sub> with non-AUG triplets [Lomakin et al. 2003]. There is biochemical evidence that eIF1 also restrains the GAP [GTPase activating protein] function of eIF5 at non-AUG codons [Unbehaun et al. 2004; Algire et al. 2005]. Consistent with these last results, overexpression of wild-type [WT] eIF1 suppresses the increased initiation at UUG codons conferred by various Sui− mutations in vivo [Valašek et al. 2004].

Our recent experiments with a reconstituted yeast system revealed that AUG recognition stimulates dissociation of eIF1 from the PIC as well as P<sub>i</sub> release from eIF2<sub>P</sub> GDP<sub>P</sub>, [Maag et al. 2005b]. The kinetics of eIF1 dissociation and P<sub>i</sub> release are similar, and a mutation in eIF1 was found to reduce the rates of both reactions in vitro (Algire et al. 2005). This suggested a model wherein eIF1 blocks non-AUG selection by impeding P<sub>i</sub> release, beyond its functions in restraining eIF5 GAP function and promoting scanning. All of these eIF1 functions should be eliminated when AUG base-pairs with Met-tRNA<sub>Met</sub>, as this triggers eIF1 dissociation from the PIC [Maag et al. 2005b]. However, the importance of eIF1 dissociation in controlling the accuracy of AUG selection in vivo was unclear.

If the foregoing model were valid, then it should be possible to obtain a class of Sui− mutations that evoke more rapid eIF1 dissociation when UUG occupies the P-site. Consistent with this, we found previously that the Sui− eIF1 mutation sui1-D83G reduces eIF1 association with native PICs [Valašek et al. 2004]. Here we show that sui1-D83G, sui1-Q84P, and a newly isolated Sui− mutation in eIF1, ISQLG<sub>93–97</sub>ASQAA [abbreviated 93–97 throughout], all decrease eIF1 affinity for 40S subunits in vitro. The 93–97 and Q84P mutations also decrease the stability of eIF1–40S interaction in vivo, and both the Sui− phenotype and impaired 40S binding of eIF1 are partially corrected by overexpressing the mutant proteins. Importantly, the 93–97 mutation elevates the rates of both eIF1 dissociation and P<sub>i</sub> release from eIF2<sub>P</sub> GDP<sub>P</sub>, in reconstituted PICs. The D83G, Q84P, and 93–97 Sui− mutations also increase selection of non-AUGs in the reconstituted mammalian system independent of GTP hydrolysis. In addition, an eIF1A mutation that suppresses UUG initiation decreases [rather than increases] the rate of eIF1 release from initiation complexes. Together, these results provide strong evidence that release of eIF1 from the 40S subunit is a critical step in AUG selection in vivo, and is coupled to P<sub>i</sub> release from eIF2<sub>P</sub> GDP<sub>P</sub>, and the transition to a closed, scanning-incompatible conformation of the initiation complex.

### Results

**The FDPF<sub>912</sub> and G107R mutations impair TC binding to 40S subunits in vivo and in vitro**

As eIF1 has been implicated in both PIC assembly and AUG selection, we sought to assign these functions to particular residues of the protein through genetic analysis in budding yeast. Whereas the Sui− phenotype signifies reduced stringency of AUG selection, the derepressed translation of GCN4 mRNA [a Gcd− phenotype] is a strong indicator of impaired TC recruitment; that is, 43S PIC assembly. Hence, we constructed alanine substitutions in residues of yeast eIF1 predicted to reside on the surface of the globular domain or unstructured N-terminal tail [NTT] and screened them for Sui− and Gcd− phenotypes. The mutations were made in a plasmid-borne His-SU11 allele [with hexahistidine encoded at the N terminus], and strains with wild-type SU11− replaced by the mutant alleles were produced by plasmid-shuffling. We also constructed His-SU11 alleles containing the previously described mutations G107R [mo2-1] [Cui et al. 1998], D83G [sui1-1], and Q84P [sui1-17] [Yoon and Donahue 1992]. A sui1Δ gcn2Δ strain was used to identify mutations with a Gcd− phenotype, whereas a sui1Δ his4-303 GCN2 strain was used to reveal Sui− phenotypes. The predicted locations in eIF1 of mutations with these phenotypes described in this study are depicted in Figure 1A, and their phenotypes are summarized in Table 1.

We failed to evict SU11+ from the gcn2Δ strain harboring His-SU11-G107R, suggesting that the G107R mutation is lethal in this strain background. Western analysis with anti-His<sub>6</sub> antibodies of whole-cell extracts [WCEs] from the strain harboring both His-SU11-G107R and untagged SU11+ showed that G107R evokes somewhat higher stringency than wild-type expression [Supplementary Fig. S1A], thus implying that the mutation disrupts an essential function of eIF1. G107R on a high-copy [hc] plasmid strongly impairs growth but is not lethal in the his4-303 GCN2 strain, allowing us to characterize its effects on initiation as the only source of eIF1. Western analysis of all other His-SU11 mutants following eviction of SU11+ showed that they contained eIF1 at wild-type or greater levels, except sui1-D83G [Supplementary Fig. S1A; data not shown].

Translation of GCN4 mRNA is repressed in nutrient-replete cells by short upstream ORFs [uORFs 1–4] in its leader. After translating uORF1, ribosomes resume scanning, rebind TC, and reinitiate at uORFs 2–4, after which they dissociate from the mRNA and leave the GCN4 ORF untranslated. In amino acid starvation, the concentration of TC is reduced by phosphorylation of eIF2α by kinase GCN2. As a result, a fraction of 40S subunits scanning downstream after terminating at uORF1 rebind TC only after bypassing uORFs 2–4 and then reinitiate at GCN4 instead. GCN4 translation can be derepressed in gcn2Δ mutants by Gcd− mutations that reduce the rate of TC loading on 40S subunits [Hinnebusch 2005]. gcn2Δ blocks derepression of GCN4 and attendant derepression of histidine biosynthetic enzymes regulated by GCN4,
conferring sensitivity to 3-aminotriazole (3-AT\(^\text{R}\)), an inhibitor of histidine biosynthesis. By restoring derepression of GCN4, Gcd\(^{\text{R}}\) mutations suppress the 3-AT\(^{\text{R}}\) phenotype of gcn2Δ cells.

The His-SUI1 alleles with the mutations FDPF\(_{12}\)/ADPA [abbreviated throughout as 9,12] or ISQLG\(_{93-97}\)/ASQAA [93–97] confer, respectively, strong and weak 3-AT-resistance phenotypes [3AT\(^{\text{R}}\) in the gcn2Δ background, although the slow-growth (Slg\(^{\text{R}}\)) of 93–97 probably diminishes its 3AT\(^{\text{R}}\) phenotype [Table 1, Fig. 1B]. The 3-AT\(^{\text{R}}\) of 9,12 is evident in the presence of SUI1\(^{\text{I}}\) [data not shown], indicating its dominance. The hc G107R allele also confers 3AT\(^{\text{R}}\) despite its strong Slg\(^{\text{R}}\) phenotype in the SUI1\(^{\text{I}}\) strain [Fig. 1B, hc G107R/WT]. These dominant phenotypes suggest that 9,12 and G107R mutations impair eIF1 function rather than merely eliminating the factor from PICs. Among the other mutants we tested, only hc D83G exhibited (weak) 3AT\(^{\text{R}}\).

Consistent with their 3AT\(^{\text{R}}\)/Gcd\(^{\text{R}}\) phenotypes, the 9,12, hc G107R/WT and 93–97 mutations produced 2.5-fold to fourfold derepression of a GCN4-lacZ reporter in nonstarved gcn2Δ cells compared with the single-copy (sc) or hc SUI1\(^{+}\) alleles [Fig. 1C]. Similarly, hc G107R and 93–97 evoked approximately sixfold derepression of GCN4-lacZ in nonstarved GCN2\(^{+}\) cells [Fig. 1D]. Starva-
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Table 1. Growth of His-SUI1 mutants on media indicating Gcd+ or Sui+ phenotypes

<table>
<thead>
<tr>
<th>His-SUI1 alleles</th>
<th>gcn2Δ</th>
<th>GGCN2 his4-303b</th>
</tr>
</thead>
<tbody>
<tr>
<td>sc wild type</td>
<td>++++</td>
<td>+/+</td>
</tr>
<tr>
<td>hc wild type</td>
<td>++++</td>
<td>+/+</td>
</tr>
<tr>
<td>sc FDPP9ADPA</td>
<td>++++</td>
<td>+/+</td>
</tr>
<tr>
<td>is SQLG95G95ASQAA</td>
<td>+++</td>
<td>+/+</td>
</tr>
<tr>
<td>hc SQLG95G95ASQAA</td>
<td>+++</td>
<td>+/+</td>
</tr>
<tr>
<td>hc G107R/WT</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>hc G107R</td>
<td>NA²</td>
<td>NA</td>
</tr>
<tr>
<td>sc D83G</td>
<td>++</td>
<td>+/+</td>
</tr>
<tr>
<td>hc D83G</td>
<td>++</td>
<td>+/+</td>
</tr>
<tr>
<td>sc Q84P</td>
<td>++</td>
<td>+/+</td>
</tr>
<tr>
<td>hc Q84P</td>
<td>++++</td>
<td>+/+</td>
</tr>
</tbody>
</table>

¹Strains containing the indicated His-SUI1 alleles were derived by plasmid shuffling from CHY01 [MATa ura3-52 leu2-3 leu2-112 trplΔ63 gcn2Δ sui1Δ::hisg [TRP1 GCN4-lacZ] p1200 [sc URA3 SUI1].
²Strains containing the indicated His-SUI1 alleles were derived by plasmid shuffling from CY03 [MATa ura3-52 leu2-3 leu2-112 trplΔ63 his4-303(AUU)] sui1Δ::hisg p1200 [sc URA3 SUI1].
³[SC-L] Synthetic complete medium lacking leucine, [3-AT] SC-L supplemented with 20 mM 3-AT, [-His] SC lacking leucine and histidine, [37°C] SC with incubation at 37°C. Growth was scored after incubating at 30°C unless specified otherwise.
⁴[NA] Not applicable, as we did not obtain the hc G107R mutant in this background lacking SUI1⁺.

The strong 3-AT²/Gcd⁻ phenotype of the 9.12 mutation was suppressed by overexpressing all three eIF2 subunits and tRNÁMet from a hc plasmid [hc TC] [Fig. 1B]. This suggests that 9.12 derepresses GCN4 translation by reducing the rate of TC binding to 40S subunits scanning the GCN4 leader after translating uORF1, allowing them to bypass uORFs 2-4 and reinitiate at GCN4 instead. Increasing the TC concentration with hc TC would restore efficient TC loading by mass action and prevent bypass of uORFs 2-4. [We did not obtain consistent results on suppression of the 3-AT² phenotype of the hc G107R/WT strain by hc TC, possibly because of copy number fluctuations resulting from the presence of two different hc plasmids in the same cells.]

To test our hypothesis that Gcd⁻ mutations in eIF1 impair TC loading on 40S subunits, we measured the kinetics of this reaction in a reconstituted system consisting of eIF1, eIF1A, preassembled TC, 40S subunits, and a model mRNA. As shown in Figure 1E and Table 2 [columns vi-vii], the 9.12 and G107R mutations greatly reduced the rate of TC binding to 40S subunits, whereas saturating 93-97 conferred nearly wild-type loading kinetics. These findings suggest that the Gcd⁻ phenotypes of 9.12 and G107R result directly from impairing eIF1 activity in TC recruitment. As discussed below, we believe that 93-97 affects TC recruitment in vivo by disrupting the MFC or impairing the 40S binding of eIF1 itself.

To determine if the defects in TC loading conferred by 9.12 and G107R reflect impaired 40S binding of eIF1, we measured the apparent dissociation constants (K₉) in experiments using fluorescent tetramethylrhodamine-tagged eIF1 [eIF1-TAMRA] and [1] 40S subunits alone; [2] 40S and eIF1A [40S•A complex]; [3] 40S, eIF1A, and TC (43S PIC); or [4] 40S, eIF1A, TC, and mRNA [43S•AUG complex]. The 9.12 mutant protein exhibits, at most, a twofold decrease in affinity, while the G107R mutant binds with wild-type affinity [Fig. 1F; Table 2, columns i-v]. The decreased rate of TC loading by the 9.12 mutant [Fig. 1E] cannot be due to its twofold or less 40S-binding defect because the eIF1 concentration used in the those assays [1 µM] is well above the measured K₉ of the mutant protein.

We investigated next whether the 9.12 and G107R mutations decrease TC recruitment in vivo. We began by examining their effects on translation initiation by measuring the polysome:monosome ratios [P/M] in the mutant cells. Consistent with its S¹g phenotype, the hc G107R/WT strain has a diminished P/M, indicating a reduced initiation rate [Fig. 2A, middle], and the P/M is even lower when hc G107R is the only SUI1 allele [Fig. 2A, right]. In contrast, the 9.12 mutant shows little decrease in polysome content [data not shown], consistent with its wild-type growth rate on SC medium. We then analyzed the levels of eIF2 and other MFC components associated with native PICs. Treating cells with formaldehyde cross-links factors to ribosomes in vivo, minimizing dissociation of PICs during sedimentation through sucrose gradients [Nielsen et al. 2004]. The hc G107R/WT mutant showed moderate reductions in amounts of eIF3 and eIF2 in the 40S fractions, but a higher than wild-type level of free 40S subunits [Fig. 2B,C]. Assuming that the excess free 40S subunits in the mutant are competent for PIC assembly, these data indicate that formation or stability of PICs is impaired by hc G107R. In contrast, the 9.12 mutant showed little or no reduction in 40S binding of eIF3 and eIF2 and no accumulation of free 40S subunits [Supplementary Fig. S1B,C].

Additional evidence that G107R reduces the stability of native PICs came from cross-linking analysis of the hc G107R mutant in the GCN2 background, which revealed an obvious decrease in 40S-bound eIF5 and accumulation of free subunits [Supplementary Fig. S1D,E]. Discrimination between eIFs cross-linked to 40S subunits and non-cross-linked factors present nonspecifically in 40S fractions can be achieved by resedimentation of the 40S fractions on a second gradient. This is particularly useful for eIF5 and eIF1, which often do not exhibit defined 40S peaks after a single separation
and found that GCN4
•
observed in values.
93 b Q84P
etics of TC loading in the reconstituted
•
(Gcd s >1 µM cannot be achieved in our experiments. The
were mixed with 40S subunits alone
9,12
•
D83G (nM)
mutations, excess unlabeled TC was added as a chase to
allele, which lacks an AUG start
mutant showed no defect in association
118 ± 31 9.6 ± 0.4 2.9 ± 0.7
values were calculated from fitting
phenotype and decreased
GCN4 K
9,12
rom unbound labeled TC.
93 Q84P
his4-303 (column iii); or 40S subunits, eIF1A, TC, and mRNA
and
2+
mutations increase UUG translation is much more sensitive
LUC
K
confers an 11-fold higher ratio of expression
K
(Gcd
nd to the complexes.
re-
mRNA.
phenotype (Fig. 3A; Table 1). Consistent with
−
measured by native gel assay by mixing preformed TC
−
reporters containing UUG versus
translation in cells starved with SM (e.g., Fig. 1D)
mutant, the presence of
93
mutant revealed higher than wild-type levels of co-
•
D83G D83G translation is much more sensitive
look for this complex.
Ni
eIF5 to 40S subunits in vivo.

to promote tight binding of eIF2, eIF3, and especially eIF5 to 40S subunits in vivo.
Further support for this last conclusion came from analysis of MFC integrity in cell extracts. Ni²⁺ affinity purification of His-tagged eIF1 revealed that G107R reduced copurification of eIF3, eIF2, and eIF5 with eIF1, demonstrating impaired interaction of eIF1 with all MFC components (Fig. 2F,G). In contrast, purification of the 9,12 mutant revealed higher than wild-type levels of copurifying eIFs, commensurate with the higher level of this mutant protein (Fig. 2H). Thus, consistent with its wild-type growth, polysome content, and native 43S PIC levels, the 9,12 mutant showed no defect in association with MFC components.
The nearly wild-type level of 40S-bound eIF2 in the 9,12 mutant extracts (Supplementary Fig. S1B) might seem at odds with the Gcd⁻ phenotype and decreased rate of TC loading in vitro produced by this mutation. However, GCN4 translation is much more sensitive than general translation to defects in TC recruitment (Hinnebusch 2005). Indeed, the strong induction of GCN4 translation in cells starved with SM [e.g., Fig. 1D] occurs with only a small reduction in 40S-bound eIF2 in extracts of cross-linked cells (Supplementary Fig. S1F). Thus, the partial derepression of GCN4 observed in 9,12 cells would not require an observable decrease in 40S-bound eIF2 in vivo. We propose that 9,12 reduces the rate of TC loading by an amount sufficient to partially derepress GCN4 translation but not enough to lower steady-state TC binding to bulk 40S subunits. The G107R mutation, in contrast, impairs MFC integrity and assembly or the stability of bulk PICs in addition to affecting the reinitiation events on GCN4 mRNA.

The 93–97, D83G, and Q84P mutations increase UUG selection in vivo and confer scanning defects in vitro
Sui⁻ mutations increase initiation at a UUG codon in the 5' end of the his4-303 allele, which lacks an AUG start codon, restoring growth on medium lacking histidine (~His). In addition to the previously described mutations D83G and Q84P, the 93–97 mutation produces a strong His⁰/Sui⁻ phenotype [Fig. 3A; Table 1]. Consistent with this, 93–97 confers an 11-fold higher ratio of expression of matched HIS4-lacZ reporters containing UUG versus AUG start codons [Fig. 3B]. Using a second set of UUG and AUG luciferase [LUC] reporters, we confirmed our findings for 93–97 and found that D83G and Q84P also produce large increases in the UUG/AUG initiation ratio [Supplementary Fig. S2].

Compared with the sc 93–97 mutant, the presence of

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Table 2. Effects of mutations on equilibrium binding of eIF1 to 40S subunits and kinetics of TC loading in the reconstituted system

<table>
<thead>
<tr>
<th>eIF1 mutations</th>
<th>40S</th>
<th>40S • 1A</th>
<th>43S</th>
<th>43S • AUG</th>
<th>43S • UUG</th>
<th>43S • AUG</th>
<th>43S • UUG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>11 ± 2</td>
<td>1 ± 0.1</td>
<td>6.9 ± 1.7</td>
<td>219 ± 9</td>
<td>57 ± 2</td>
<td>7.8 ± 0.6</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>FDPF9,12ADPA [Gcd⁺]</td>
<td>22 ± 11</td>
<td>0.9 ± 0.3</td>
<td>9.9 ± 2.2</td>
<td>450 ± 17</td>
<td>19 ± 9</td>
<td>0.37 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>G107R [mof2–1] [Gcd⁻]</td>
<td>11 ± 1</td>
<td>0.7 ± 0.2</td>
<td>7.3 ± 3.8</td>
<td>232 ± 3</td>
<td>24 ± 8</td>
<td>0.6 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>ISQLG93–97ASQAA [Gcd⁻] [Sui⁺]</td>
<td>62 ± 2</td>
<td>18 ± 0.5</td>
<td>20 ± 2.3</td>
<td>966 ± 12b</td>
<td>118 ± 31</td>
<td>9.6 ± 0.4</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>D83G [sui-1] [Sui⁺]</td>
<td>≥5000</td>
<td>≥4400</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q84P [sui-17] [Sui⁺]</td>
<td>≥5000</td>
<td>≥4100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


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* TAMRA-labeled wild-type or mutant eIF1 proteins with mutations 9,12, G107R, or 93–97 were mixed with 40S subunits alone (column i), 40S subunits and eIF1A (column ii), 40S subunits, eIF1A, and TC (column iii), or 40S subunits, eIF1A, TC, and mRNA containing AUG (column iv) or UUG (column v) start codons and allowed to reach equilibrium. The increase in fluorescence anisotropy of the labeled protein was measured, yielding the fraction bound to the complexes. Kₐ values were calculated from fitting with either hyperbolic or quadratic binding curves. Binding of the D83G and Q84P mutants was performed by competition assay, wherein TAMRA-labeled wild-type eIF1 was prebound to 40S [in the presence or absence of eIF1A and TC], and unlabeled mutant eIF1 was added to compete for binding of labeled eIF1. The decrease in fluorescence anisotropy of the labeled wild-type protein as a function of the concentration of unlabeled proteins yielded the Kₐ values.

* This value was derived by curve fitting, as concentrations of 43S complexes >1 µM cannot be achieved in our experiments. The Kₐ cannot be significantly lower than 1000 nM or the binding would have approached saturation; hence, we regard 966 nM as a lower limit for this complex.

* The observed first-order rate constant for TC binding to 40S ribosomes was measured by native gel assay by mixing preformed TC (eIF2•GDPNP•t5S-Met-tRNA,Met with 15 nM 40S ribosomal subunits and saturating amounts of mutant or wild-type eIF1, eIF1A, and mRNA with an AUG (column vi) or UUG (column vii) start codon. At different times, excess unlabeled TC was added as a chase to stop assembly, and reactions were loaded on a native gel to separate bound from unbound labeled TC.
93–97 on a hc plasmid greatly improved growth on +His medium but decreased growth somewhat on −His medium (Fig. 3A), suggesting that the Sui− phenotype was partially suppressed by overexpressing 93–97. Consistent with this, overexpressing 93–97 decreased the UUG/AUG expression ratio for both reporters [3B; Supplementary Fig. S2], consistent with previous studies (Cui et al. 1998). Moreover, we found that hc G107R, as well as 93–97, is synthetically lethal with the dominant Sui− mutation in eIF5 encoded by SUI5 [data not shown], as shown previously for D83G (Valášek et al. 2004). These findings suggest that G107R also increases UUG initiation in vivo. Perhaps a His+/Sui− phenotype is masked by the strong Slg− phenotype of this mutant.

We next examined whether the eIF1 mutations alter AUG recognition in a reconstituted mammalian system where eIF1 is needed for efficient scanning to the AUG start codon of /H9252-globin mRNA, using inhibition of primer extension to map the leading edge of the ribosome in the 48S PICs (toeprinting) (Pestova et al. 1998). As eIF5 is omitted, these assays reveal defects in the...
ability of eIF1 to promote the open, scanning conformation of the PIC independent of GTP hydrolysis and P_i release from eIF2-GDP→GTP_i. With all human eIFs present except eIF1, most 48S PICs formed aberrantly near the 5’/H11032 end (complex I), whereas addition of human or yeast eIF1 allowed the majority to reach the AUG codon (complex II) (Fig. 3C). Smaller proportions of complexes formed 7 nucleotides (nt) upstream of complex II (designated complex III) with both factors, and wild-type yeast eIF1 allowed slight complex formation at the near-cognate GUG upstream of the AUG. (The nature of complex III is currently under investigation.) The previously described Sui^− mutations Q84P and D83G, and the 93–97 Sui^− mutation isolated here, allowed higher levels of aberrant complex I and GUG complexes, indicating defects in scanning and the ability to suppress recognition of a near-cognate triplet [Fig. 3C]. The G107R mutation produced smaller increases in complex I and the GUG complex, and the N-terminal Gcd^− mutation 9,12 was even more similar to wild-type yeast eIF1 in both respects. (Note that eIF1 was added at a concentration [17 µM] high enough to compensate for the 40S binding defects [Table 2] of all mutants.)

We analyzed a second mRNA containing an upstream AUG located only 1 nt from the cap. Human and yeast wild-type eIF1 suppressed 48S assembly at the upstream AUG, as expected (Pestova and Kolupaeva 2002), whereas Q84P, D83G, and 93–97 allowed significant recognition of the upstream AUG (Fig. 3D). The 9,12 and G107R mutations conferred little or no complex assembly at the upstream AUG. Thus, 93–97 and the previously isolated Sui^− mutations impair the ability of eIF1 to promote scanning past an AUG codon located close to the mRNA cap.

The 93–97 mutation impairs eIF1 association with the MFC and 40S subunits in vivo

We sought next to elucidate the biochemical basis for the Sgi^− and Sui^− phenotypes of the 93–97 mutant. Consistent with its growth defect, the 93–97 mutation greatly decreases the P/M ratio [Fig. 4A] and copurification of other MFC components with His-tagged eIF1 [Fig. 4B,C]. Consistent with the latter, 93–97 impairs binding between recombinant eIF1 and the eIF3c N-terminal segment [Supplementary Fig. S3A], the strongest interaction linking eIF1 to the MFC (Asano et al. 2000). 93–97 also reduces the amounts of MFC components that cosediment with 40S subunits in extracts of cross-linked cells [Supplementary Fig. S3B]. These defects in eIF binding to native PICs were also observed in the resedimentation
protocol, as greatly reduced levels of all MFC constituents, including eIF1 itself, cosedimented with 40S subunits from 93–97 extracts (Fig. 4D, panels i,ii).

Interestingly, the 40S-binding defects were partially suppressed by overexpressing the 93–97 allele from a hc plasmid (Fig. 4D, panels ii,iii), which correlates with suppression of its Slg− and Sui− phenotypes by overexpression (Fig. 3A,B). Nearly wild-type 40S binding of eIF1 was achieved, whereas 40S binding of other eIFs remained below wild-type levels in cells overexpressing the 93–97 allele (Fig. 4D, panel iii). (As noted above, the eIF1 in the upper fractions after resedimentation likely represents molecules from the 40S fractions of the first gradient that were not cross-linked in vivo or lost their cross-links in vitro; this behavior is exacerbated by eIF1 overexpression.) Importantly, association of all MFC components with polysomes [representing the PICs bound to mRNAs being translated by 80S ribosomes] was substantially recovered in hc 93–97 cells (Supplementary Fig. S3C), in accordance with the nearly complete rescue of polysome content and growth rate. Presumably, 43S PICs remain less stable, while 48S PICs (bound to mRNA) achieve nearly wild-type stability in cells overexpressing the 93–97 mutant. Together, our data indicate that 93–97 impairs translation initiation, MFC integrity, and PIC assembly in a manner partially rescued by increasing the concentration of mutant eIF1. We showed above that overexpression of the Q84P and D83G alleles suppresses their Slg− and Sui− phenotypes (Fig. 3A). Analysis of extracts from cross-linked cells revealed that overexpression likewise rescues 40S binding by these eIF1 mutants (Supplementary Fig. S3D; data not shown).
Sui− mutations in eIF1 elicit more rapid dissociation of eIF1 from reconstituted PICs

The facts that sui1 mutations D83G, Q84P, and 93–97 reduce eIF1 binding to native PICs and that their Sui− phenotypes are diminished by overexpression suggest that increased UUG initiation in these mutants results from weak eIF1 binding to the 40S subunit. This model is consistent with our previous findings using the reconstituted yeast system that AUG stimulates eIF1 dissociation from the PIC and that eIF1 dissociation and P3 release from eIF2•GDP•P3, occur with similar kinetics (Algire et al. 2005; Maag et al. 2005b). Hence, eIF1 mutations that accelerate its dissociation would be predicted to increase selection of non-AUG triplets and confer a Sui− phenotype.

We tested this hypothesis by measuring the effects of the Sui− mutations on the Kd for eIF1 binding to 40S subunits using the binding assays described above. Remarkably, the D83G and Q84P Sui− mutations produce an ~500-fold increase in the Kd of eIF1 for free 40S subunits and 43S PICs (Table 2, columns i and iii). The 93–97 mutation increases the Kd by fourfold to 15-fold for free 40S, 40S•1A, and 43S•AUG complexes (Fig. 1F; Table 2), suggesting a moderate reduction in eIF1 affinity for the PIC. The Kd of wild-type eIF1 for the 48S PIC is reduced when UUG replaces AUG, reflecting the known higher affinity of eIF1 for 48S PICs with non-AUGs in the P-site (Maag et al. 2005b). The 93–97 mutation decreased eIF1 affinity for the 43S•UUG complex as well (Fig. 5A; Table 2), suggesting that it weakens eIF1 association with the PIC regardless of the P-site triplet.

To confirm this last conclusion, we examined the effect of 93–97 on eIF1 dissociation kinetics using stopped-flow fluorometry of 43S•AUG PICs assembled with TAMRA-labeled eIF1 and fluorescein-labeled eIF1A (eIF1A-fl). eIF1A-fl acts as the energy donor in fluorescence resonance energy transfer (FRET) between eIF1A-fl and eIF1-TAMRA in these PICs. AUG evokes biphasic loss of FRET and an attendant increase in eIF1A-fl fluorescence, with the slower phase of the reaction corresponding to eIF1 dissociation. The slow phase for wild-type eIF1 has a rate constant of 0.26 sec−1, in accordance with previous results (Maag et al. 2005b), whereas the 93–97 mutation increases the rate constant fivefold to 1.29 sec−1, suggesting a moderate reduction in eIF1 affinity for 43S•AUG complexes (Fig. 1F; Table 2), suggesting a moderate reduction in eIF1 affinity for the PIC. The Kd of wild-type eIF1 for the 48S PIC is reduced when UUG replaces AUG, reflecting the known higher affinity of eIF1 for 48S PICs with non-AUGs in the P-site (Maag et al. 2005b). The 93–97 mutation decreased eIF1 affinity for the 43S•UUG complex as well (Fig. 5A; Table 2), suggesting that it weakens eIF1 association with the PIC regardless of the P-site triplet.

Figure 5. Sui− mutation 93–97 increases the rates of eIF1 dissociation and P3 release from eIF2•GDP•P3 in reconstituted 48S PICs. (A) Reduced affinity of the 93–97 mutant for 43S•UUG complexes demonstrated in vitro as described in Table 2. (B) 93–97 accelerates dissociation of eIF1 from 43S•mRNA complexes. 43S complexes reconstituted with TC, eIF1A-fl, and mutant or wild-type eIF1-TAMRA were mixed rapidly with mRNA (AUG) and excess unlabeled eIF1, and the increase in eIF1A-fl fluorescence (caused by decrease in FRET efficiency) was monitored by stopped-flow fluorometry. (C, D) 93–97 accelerates the P3-release phase of GTP hydrolysis. 43S complexes (with [γ-32P]GTP in the TC) were mixed with eIF5, excess unlabeled GDP, and model mRNA containing AUG (C) or UUG (D) start codons in a rapid quench apparatus, and reactions were quenched at the indicated times with EDTA. The extent of GTP hydrolysis was measured by separating free [γ-32P]GTP by gel electrophoresis followed by PhosphorImager analysis. (E) FL-17–21 mutation in eIF1A decreases the rate of eIF1 dissociation from 43S•mRNA complexes. Kinetic experiments conducted as in B using mutant or wild-type forms of eIF1A-fl and wild-type eIF1-TAMRA in 43S•mRNA [AUG] complexes. For wild-type eIF1A, the rate constants (k) and amplitudes (amp) of the fast [conformational change] and slow [eIF1 release] phases, respectively, are k1 = 9.2 ± 0.3 sec−1, amp1 = 0.16 ± 0.002 and k2 = 0.32 ± 0.003 sec−1, amp2 = 0.83 ± 0.003. The corresponding values for FL-17–21 mutant are k1 = 1.0 ± 0.4 sec−1, amp1 = 0.19 ± 0.03 and k2 = 0.15 ± 0.007 sec−1, amp2 = 0.81 ± 0.09.
1.2 sec\(^{-1}\) [Fig. 5B]. 93–97 had a similar effect in accelerating eIF1 dissociation from 43S\(\text{UUG}\) complexes, in this case by a factor of 2.5 [data not shown]. These findings support the idea that 93–97 increases initiation at UUG at least partly by increasing the eIF1 off-rate from the PIC.

We investigated next whether 93–97 accelerates GTP hydrolysis and \(P_\text{r}\) release from eIF2\(\text{GDP}\text{P}_\text{i}\). 43S PICs formed with \([\gamma^\text{32P}]\text{GTP}\) in the TC were mixed with saturating eIF5 and mRNA using a rapid quench device. When eIF5 binds, the GTP is hydrolyzed until an internal equilibrium is reached between GDP\(\text{P}_\text{i}\) and GTP of ~0.3 \([K_{\text{m}} = |\text{GDP}\text{P}_\text{i}|/|\text{GTP}|]\), which occurs rapidly and is only modestly stimulated by AUG. Dissociation of eIF1 on AUG recognition allows \(P_\text{r}\) release, driving GTP hydrolysis to completion, and this second phase of the reaction occurs more slowly (~0.6 sec\(^{-1}\) for wild-type eIF1) and is strongly stimulated by AUG [Algire et al. 2005]. In accordance with previous findings, analysis of GTP hydrolysis in the 43S\(\text{AUG}\) complex with wild-type eIF1 revealed a fast phase [establishment of the internal equilibrium between GTP and GDP\(\text{P}_\text{i}\) of amplitude 0.25 and rate constant of 13 sec\(^{-1}\)], and a slow phase [governed by \(P_\text{r}\) release] of amplitude 0.37 and rate constant 0.8 sec\(^{-1}\). With the 93–97 mutant, the first phase is essentially the same wild type (amplitude of 0.29 and 12 sec\(^{-1}\)), but the second phase is faster (amplitude of 0.33 and 1.6 sec\(^{-1}\)) [Fig. 5C]). [This twofold increase in rate constant for the 93–97 protein was observed in multiple experiments.] These results agree with the more rapid dissociation of mutant 93–97 from the 43S\(\text{AUG}\) complexes observed above.

For the 43S\(\text{UUG}\) complex and wild-type eIF1, the slow phase of the reaction \([P_\text{r}\) release], but not the fast phase, is reduced for the UUG versus AUG complex (0.45 sec\(^{-1}\) vs. 0.8 sec\(^{-1}\)), as expected. Importantly, 93–97 increases the rate of the second phase by sixfold (2.7 sec\(^{-1}\) vs. 0.45 sec\(^{-1}\)) [Fig. 5D]). [Curiously, the amplitude of the fast phase is decreased by 93–97, suggesting that it perturbs the position of the internal equilibrium between GTP and GDP\(\text{P}_\text{i}\).] Thus, 93–97 accelerates the rate of \(P_\text{r}\) release with AUG or UUG in the P-site. This supports the idea that 93–97 generates a Sui\(^-\) phenotype at least partly by increasing the rate of GTP hydrolysis and \(P_\text{r}\) release at UUG codons because of accelerated eIF1 dissociation.

A hyperaccuracy mutation in eIF1A reduces the rate of eIF1 dissociation in vitro

To provide additional evidence that the rate of eIF1 dissociation is a critical determinant of AUG selection, we asked whether mutations in another factor that affect AUG selection in vivo alter the rate of eIF1 dissociation in vitro. We recently described a clustered-alanine substitution in residues 17–21 of the NTT of Flag-tagged eIF1A [FL-17–21] that confers a hyperaccurate phenotype, suppressing the increased UUG initiation in Sui\(^-\)mutants of eIF5 and eIF2B [Fekete et al. 2007]. [Both the N-terminal Flag and Ala substitutions contribute to this phenotype.] We predicted that these mutations would slow down, rather than accelerate, eIF1 dissociation from initiation complexes. After introducing the FL-17–21 mutations into eIF1A-II, we conducted stopped-flow fluorometry of 43S\(\text{AUG}\) PICs assembled with wild-type eIF1-TAMRA. The results indicate that FL-17–21 significantly decreases the rate of eIF1 dissociation [Fig. 5E]. Interestingly, it reduces both the rapid phase, which involves a conformational change that separates the C termini of eIF1A and eIF1, and the slow phase of the reaction corresponding to eIF1 dissociation [see legend for Fig. 5]. These findings provide evidence that the rapid conformational change and subsequent dissociation of eIF1 are important steps in AUG recognition in vivo, which are modulated by the eIF1A recognition in vivo.

Discussion

In this study, we provide several important findings regarding the mechanism of eIF1 function in PIC assembly and AUG selection. First, we identified surface-exposed residues in eIF1 involved in the ability of the factor to stimulate TC recruitment to the 40S subunit, a critical step in 43S PIC assembly. The 9,12, 93–97, and G107R mutations all produce Gcd\(^-\) phenotypes and partially de-repress GCN4 expression in the absence of elevated eIF2\(\alpha\) phosphorylation. This phenotype signifies impaired TC loading in vivo, as 40S subunits that resume scanning after translating uORF1 fail to rebind TC quickly enough to ensure reinitiation at uORFs 2–4, allowing a fraction to continue scanning and reinitiate downstream at GCN4. Both 9,12 and G107R greatly reduced the rate of TC binding to reconstituted PICs in vitro. As neither mutation significantly reduces the affinity of eIF1 for 43S or 48S PICs, the 9,12 and G107 residues are required primarily for a function of eIF1 in TC loading rather than 40S binding of eIF1 itself.

Biochemical analysis of native PICs in the 9,12 mutant revealed no obvious defects in MFC integrity, formation or stability of PICs, or polysome assembly. As explained in Results, these findings do not contradict the moderate Gcd\(^-\) phenotype of this mutant because GCN4 translation is much more sensitive than general initiation to reductions in TC loading. The hc G107R mutant also shows little decrease in 40S-bound eIF2, although a reduction in MFC integrity and less stable 40S association of all MFC components other than eIF1 was detected in hc G107R extracts. Only 40S binding of eIF5 was dramatically decreased in the hc G107R extracts, so the strong initiation defect in this mutant [Slg\(^-\) phenotype] might result primarily from diminished eIF5 function in promoting MFC binding to 40S subunits [Jivotovskaya et al. 2006] or in stimulating hydrolysis of GTP in the TC.

The 93–97 mutation in eIF1 produces a Gcd\(^-\) phenotype and decreases binding of eIF2 and other MFC components to native PICs, but does not reduce the rate of TC loading on 40S subunits in vitro. There are several consequences of this mutation in vivo that might account for these results. First, 93–97 reduces 40S binding of eIF1, and this could limit its ability to stimulate TC
loading on 40S subunits in vivo. In contrast, its 40S binding defect was overcome in the TC loading assays in vitro by using a saturating concentration of eIF1. Second, 93–97 impairs eIF1 binding to eIF3c and decreases MFC integrity, and optimal 40S binding of eIF2 is enhanced by MFC assembly in vivo (Jivovskaya et al. 2006). Thus, 93–97 might indirectly impair TC loading in vivo by destabilizing the MFC. As eIF3 was not present in the TC loading assays, the effect of weakened eIF1–eIF3c interaction conferred by 93–97 would not be revealed in these in vitro experiments.

Even though the 93–97 mutation lowers TC occupancy of 40S subunits in vivo, it still does not fully derepress GCN4 translation. This can be explained by noting that 93–97 also impairs the efficiency of scanning, as judged by in vitro toeprint assays. A reduced rate of scanning between uORF1 and uORF4 is expected to compensate for the decreased rate of TC recruitment, restoring reinitiation at uORFs 2–4 and diminishing the Gcd− phenotype (Hinnebusch 2005). The sui mutations D83G and Q84P do not produce Gcd− phenotypes despite their pronounced defects in 40S binding and PIC assembly in vitro and in vivo. Presumably, their defects in scanning fully compensate for the defective TC recruitment to maintain repression of GCN4 in nonstarvation conditions.

A similar situation was described previously for mutations in eIF1A (Fekete et al. 2005) and eIF5 (Yamamoto et al. 2005) that produce compound defects in PIC assembly and scanning/AUG recognition.

Our findings on the D83G, Q84P, and 93–97 Sui− mutations provide strong in vitro and in vivo evidence supporting the model that eIF1 dissociation from the PIC is an important step in AUG selection. All three mutations dramatically increase initiation at UUG in vivo and confer scanning defects in the reconstituted mammalian system, increasing the frequency at which PICs fail to scan from the 5′ cap, or arrest at an upstream GUG or an AUG located too close to the 5′ end. Accordingly, we propose that these mutations stabilize the scanning-arrested conformation of the PIC at non-AUG triplets and at AUGs lacking optimal 5′ flanking sequences. The D83G and Q84P mutations dramatically impair eIF1 binding to native PICs (Supplementary Fig. S3D; Valašek et al. 2004). While less severe in degree, 93–97 also weakens 40S binding of eIF1 in cell extracts. These in vivo results are commensurate with the increases in Kd for eIF1 binding to 40S subunits or reconstituted PICs of more than two orders of magnitude for D83G and Q84P, and threefold to 18-fold for the 93–97 mutation. Importantly, overexpression of all three mutants diminished their Sui− phenotypes and restored higher than wild-type translation. This can be explained by not releasing at UUGs. Fulfilling this prediction, doi and P release at UUGs. Fulfilling this prediction, doi do not produce Gcd− release and promoting con-

For example, the transition Sui− might indirectly impair TC loading in vivo by de-

K i and release with UUG in the P-site. We suggest that the rate constant for eIF1 dissociation, or the rate constant or amplitude of the AUG-dependent conformational change (Maag et al. 2005b). Thus, addition of eIF3 to reconstituted PICs might be expected to modulate the quantitative effects of the eIF1 Sui− mutations but should not alter their overall mechanisms of action.

To explain the partial suppression of the Sui− phenotypes of the 93–97, D83G, and Q84P mutants by overexpression, we propose that increasing the cellular concentrations of the mutant eIF1 proteins allows them to rebinding more rapidly after inappropriate dissociation at a UUG codon, preventing P release and promoting continued scanning to the next triplet. The same mechanism can be invoked to explain our previous finding that overexpressing wild-type eIF1 partially suppresses the Sui− phenotypes of mutations in eIF3c, eIF5, and eIF2B (Valašek et al. 2004). The fact that overexpressing the Sui− mutants only partially suppresses their Sui− phenotypes, even though they restore wild-type levels of 40S binding, might be explained by proposing that the rate of eIF1 rebinding to a scanning PIC from which eIF1 has dissociated is not rapid enough to completely prevent UUG selection. Another explanation for these genetic characteristics is that more rapid eIF1 dissociation is not the only defect conferred by these mutations. They might also impair eIF1 function in scanning, increasing the dwell time of the PIC and probability of P release at non-AUG codons. Such a defect that facilitates UUG selection with eIF1 still bound to the scanning PIC can be invoked to explain the moderate Sui− phenotype of the G107R mutant, as this protein dissociates more slowly than wild-type eIF1 from reconstituted PICs (Aligire et al. 2005). Finally, although eIF1 and P release are accelerated in vitro by the 93–97 mutation with AUG or UUG in the P-site, initiation at UUG codons is enhanced relative to initiation at AUG in vivo. To explain why increasing the rate of eIF1 release has a smaller effect on initiation at AUG versus UUG, we suggest that other steps in AUG selection—for example, the transition from the open, scanning conformation to the closed complex—become limiting when the rate of eIF1 release exceeds the level achieved by the wild-type factor at AUG.

A model of eIF1 bound to the PIC has been generated by hydroxyl radical mapping of the eIF1-binding site on the 40S subunit (Lomakin et al. 2003). None of the Sui− mutations alter residues in the predicted eIF1–40S inter-
face (Supplementary Fig. S4), suggesting that they impair 4OS binding by an indirect mechanism. The fact that overexpressing the mutant eIF1 proteins eliminates their slow-growth phenotypes shows that the mutations do not produce grossly misfolded proteins. As the mutated residues lie on exposed surfaces of eIF1 in the model, they could also affect interactions of eIF1 with TC, other MFC components (including eIF5), or eIF1A, and thereby modulate PIC assembly, scanning, or eIF5 function.

Finally, it is notable that the FL-17–21 mutations in the NTT of eIF1A, which decrease selection of both UUG and AUG triplets in vivo [Fekete et al. 2007], reduce the rates of two reactions involving eIF1 that are triggered by AUG recognition: (1) a conformational change that increases eIF1A–eIF1 separation in the 48S complex and (2) subsequent release of eIF1 from the 40S subunit. This provides strong evidence that both events contribute to AUG selection and are modulated by the eIF1A NTT in vivo. In contrast to eIF1, which binds less tightly to the complex, eIF1A binds more tightly with AUG in the P-site [Maag et al. 2005a]. Moreover, the FL-17–21 mutations eliminate eIF1A’s tighter interaction with the PIC upon AUG recognition [Fekete et al. 2007]. Thus, tighter binding of eIF1A through its NTT likely promotes the conformational change that separates eIF1A from eIF1 and enhances eIF1 release upon AUG recognition.

Materials and methods

Yeast strain constructions

Deletion of the chromosomal SUI1 gene to construct strains CHY01 and JCY03 is described in the Supplemental Material. The resulting strains were transformed with various His-SUI1 alleles cloned in sc or hc LEU2 plasmids and plated on SC-UL. The resident SUI1 URA3 plasmid (p1200) was evicted by selection on 5-FOA medium to obtain the relevant mutant strains listed in Supplementary Table S3.

Plasmid constructions and site-directed mutagenesis

Details of the construction of pCFB01 containing the sui1Δ::hisG-URA3-hisG disruption cassette, and LEU2 plasmids p4389 and pCFB03 containing His-SUI1, are described in the Supplemental Material. Mutations were introduced into the His-SUI1 allele by PCR fusion or the GencTailor site-directed mutagenesis system [Invitrogen] using the mutagenic primers listed in Supplementary Table S2.

Plasmid pRaugFFaug was derived from a dual luciferase reporter plasmid described previously [Harger and Dinman 2003], modified to express Renilla and firefly luciferase as separate messages, with LUC<sub>renilla</sub> under the control of the ADH1 promoter and HIS terminator, LUC<sub>firefly</sub> under the GPD promoter and CYC1 terminator, and the start codon of the LUC<sub>firefly</sub> ORF altered to TTG.

Biochemical assays with yeast extracts

Assays of β-galactosidase activity in WCEs were performed as described previously [Moeche and Hinnebusch 1991]. Measurements of luminescence in WCEs were conducted essentially as described [Dyer et al. 2000]. Analyses of polysome profiles and fractionation of native PICs in WCEs from HCHO cross-linked cells, including reedemediation analysis, were conducted essentially as already described [Nielsen et al. 2004, Fekete et al. 2005, Ivivotovskaya et al. 2006]. For Western analysis of WCEs, extracts were prepared by breaking the cells in 1:1 [v/v] cracking buffer (20 mM Tris-HCl at pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 5 mM NaF with 7 mM β-mercaptoethanol, 1 mM PMSF, Complete protease inhibitor cocktail tablets freshly added) and vortexing with glass beads. WCEs were also prepared by trichloroacetic acid extraction as previously described [Reid and Schatz 1982]. Ni<sup>2+</sup> chelation chromatography with WCEs was conducted as previously described [Valašek et al. 2001]. WCEs were incubated with 4 µL of 50% Ni<sup>2+</sup>-NTA-silica resin (Qiagen) suspended in 200 µL of buffer A for 2 h at 4°C, followed by washing and elution. In vitro GST pull-down assays were conducted as already described [Asano et al. 1998].

Biochemical assays in the reconstituted yeast system

Reagent preparation is described in the Supplemental Material. Fluorescence anisotropy measurements of equilibrium binding constants were performed as previously described using C-terminally TAMRA-labeled eIF1 or mutants of eIF1 [Maag and Lorsch 2003, Maag et al. 2005b]. For all experiments, buffer conditions were 30 mM HEPES (pH 7.4), 100 mM potassium acetate (pH 7.4), 3 mM MgCl₂, and 2 mM dithiothreitol. 43S complex formation was measured by gel mobility shift assays as described previously [Algire et al. 2002] and in the Supplemental Material.

FRET experiments were carried out as previously described [Maag et al. 2005b]. GPase assays were conducted by preformingly 43S complexes with [γ<sup>32</sup>P]GTP and initiating the reaction by addition of saturating eIF5 and mRNA using a rapid quench apparatus [KinTek], as previously described [Algire et al. 2005]. Component concentrations were 810 nM Met-tRNA<sub>Met</sub>, 800 nM elF2, 830 nM elF1, 800 nM elF1A, 300 nM 40S subunits, 10 µM mRNA(AUG) or 25 µM mRNA(UUG), and 1 µM elF5.

Toeprinting analysis in the reconstituted mammalian system

48S complexes were assembled in 40-µL reactions containing 3 pmol 40S subunits, 9 pmol elF2, 9 pmol elF3, 5 pmol elF4F, 10 pmol elF4A, 10 pmol elF4B, 9 pmol elF1A, 680 pmol elF1 (recombinant human or yeast mutants), 5 pmol Met-tRNA<sub>Met</sub>, 2 pmol β-globin or CAUG(CAA) GUS mRNA, 20 mM Tris-HCl (pH 7.5), 100 mM potassium acetate, 2.5 mM magnesium chloride, 1 mM DTT, 0.25 mM spermidine, 1 mM ATP, 0.2 mM GTP, and 2 U/mL ribonuclease inhibitor (RNAseOUT, Invitrogen). Primer extension and polyacrylamide gel electrophoresis of cDNA products were performed as described [Pestova et al. 1998, Pestova and Kolupaeva 2002].

Acknowledgments

We thank Azadeh Esmaili for technical assistance, Tom Donahue, Ernie Hannig, and Jon Warner for strains and antibodies, Assan Marintchev for help with the eIF1-30S model, and Tom Dever for many helpful suggestions. This work was supported in part by the Intramural Program of the NIH. T.V.P. and J.R.L. acknowledge support from NIH Grants GM59660 and GM62128, respectively.

Note added in proof

We found recently that hCG107R is not lethal but confers Slg<sup>−</sup> and Gcd<sup>−</sup> phenotypes in the gcn2A strain lacking SUI1<sup>−</sup>, as we observed in the presence of SUI1<sup>+</sup>.
References


