Regulatory elements in eIF1A control the fidelity of start codon selection by modulating tRNA_i^{Met} binding to the ribosome

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eIF1A is the eukaryotic ortholog of bacterial translation initiation factor IF1, but contains a helical domain and long unstructured N-terminal tail (NTT) and C-terminal tail (CTT) absent in IF1. Here, we identify elements in these accessory regions of eIF1A with dual functions in binding methionyl initiator tRNA (Met-tRNA_i^{Met}) to the ribosome and in selecting AUG codons. A pair of repeats in the eIF1A CTT, dubbed Scanning Enhancer 1 (SE₁) and SE₂, was found to stimulate recruitment of Met-tRNA_i^{Met} in the ternary complex (TC) with eIF2·GTP and also to block initiation at UUG codons. In contrast, the NTT and segments of the helical domain are required for the elevated UUG initiation occurring in SE mutants, and both regions also impede TC recruitment. Remarkably, mutations in these latter elements, dubbed scanning inhibitors SI₁ and SI₂, reverse the defects in TC loading and UUG initiation conferred by SE substitutions, showing that the dual functions of SE elements in TC binding and UUG suppression are mechanistically linked. It appears that SE elements enhance TC binding in a conformation conducive to scanning but incompatible with initiation, whereas SI elements destabilize this conformation to enable full accommodation of Met-tRNA_i^{Met} in the P site for AUG selection.

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Identification of the translation initiation codon in eukaryotes typically occurs by a scanning mechanism in which the small (40S) ribosomal subunit recruits the methionyl initiator tRNA (Met-tRNA_i^{Met}) in a ternary complex (TC) with the GTP-bound form of eIF2 to form the 43S preinitiation complex (PIC). The 43S PIC then binds to the mRNA near the m⁷G-capped 5' end and scans the leader, using complementarity with the anticodon of the initiator as a key means of identifying the AUG start codon (Pestova et al. 2007). The mechanisms involved in ribosomal scanning and in distinguishing AUG from non-AUG triplets by the scanning PIC are not fully understood.

The factors eIF1 and eIF1A have been shown to stimulate scanning and assembly of a stable 48S PIC at AUG, and eIF1 also blocks recognition of near-cognate

triplets, in a reconstituted mammalian system (Pestova and Kolupaeva 2002). eIF1 appears to act with eIF1A to promote an open, scanning-conducive conformation of the PIC (Lomakin et al. 2000; Fekete et al. 2007; Passmore et al. 2007), and it impedes GTP hydrolysis by the TC in the absence of perfect base-pairing between the P-site codon and anticodon of Met-tRNA_i^{Met} (Unbehaun et al. 2004). Consistent with this, recognition of AUG elicits dissociation of eIF1 from the 40S subunit and accelerates P_i release from eIF2·GDP·P_i in a reconstituted yeast system (Algire et al. 2005; Maag et al. 2005).

The mechanisms of scanning and AUG selection are being dissected with genetic tools in budding yeast. Substitutions in the three subunits of eIF2, eIF5, and eIF1 were described that increase the frequency of initiation at the UUG start codon of *his4-301* mRNA, restoring the ability to synthesize histidine (His⁺ phenotype) (Yoon and Donahue 1992; Donahue 2000). A subset of such Sui⁻ (Suppressor of initiation codon mutant) substitutions affecting eIF1 (encoded by *SUI1*) appear to act simply by accelerating eIF1 dissociation from the PIC (Cheung et al. 2007). Conversely, overexpression of

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wild-type eIF1 suppresses the *Sui*[−] phenotypes of the *SUI5* and *SUI3-2* substitutions in eIF5 and eIF2β, respectively (Valasek et al. 2004; Fekete et al. 2007), conferring the *Ssu*[−] (Suppression of *Sui*[−]) phenotype. These findings support the notion that eIF1 is a “gate-keeper” that impedes start codon selection and whose inhibitory functions are eliminated at AUG codons, at least partly, by its dissociation from the 40S subunit. Presumably, overexpression of eIF1 prevents its release from the 40S subunit, which would otherwise occur at a higher frequency at UUGs in *Sui*[−] mutants, and thereby allows scanning to continue downstream.

The eIF1A contains an oligonucleotide/oligosaccharide-binding (OB) fold domain related to that present in bacterial translation initiation factor IF1, but additionally contains a helical domain comprised of helix α2 and a 3₁₀ helix connected by a short linker region (Lαα), plus structured N or C strands that pack against different surfaces of α2. eIF1A also contains long unstructured N-terminal tail (NTT) or C-terminal tail (CTT) (Fig. 1A; Battiste et al. 2000). The original genetic selections that yielded *Sui*[−] mutations affecting eIF1, eIF5, or subunits of eIF2 failed to identify *Sui*[−] mutations in *TIF11* encoding yeast eIF1A (Donahue 2000). However, we found previously that removing the unstructured CTT and the C strand, Lαα, and 3₁₀ elements of the helical domain by the C-terminal truncation Δ108–153 (ΔC) confers a *His*⁺/*Sui*[−] phenotype in the *his4-301* background. The ΔC truncation also impaired the ability of eIF1A to promote scanning in a reconstituted mammalian system, leading us to propose

that the C-terminal region of eIF1A acts to enhance scanning at non-AUG codons, such that its impairment by ΔC increases UUG initiation. Remarkably, alanine substitutions of residues 17–21 (17–21) in the unstructured NTT confer an *Ssu*[−] phenotype, leading to the proposal that the NTT acts to inhibit scanning and promote start codon recognition, such that its inactivation by 17–21 suppresses UUG initiation in *Sui*[−] mutants (Fekete et al. 2005, 2007). Thus, the NTT and C-terminal region of eIF1A appeared to have opposite effects on scanning and start codon recognition.

Interestingly, the ΔC truncation, or double-Ala substitution of eIF1A CTT residues Phe-131 and Phe-133, also impairs loading of the TC on 40S subunits in PIC assembly. A well-established manifestation of this defect is the perturbation of translational control of *GCN4* mRNA by four short upstream ORFs (uORFs). Ribosomes that have translated the 5'-most uORF in *GCN4* mRNA (uORF1) and resumed scanning can bypass the remaining three uORFs (uORF2–uORF4) and reinitiate at the *GCN4* AUG when the levels of TC are reduced by eIF2α phosphorylation by protein kinase Gcn2 in amino acid-starved cells. The reduced TC concentration enables a fraction of the reinitiating 40S subunits to rebind TC only after bypassing uORF2–uORF4, but before reaching the *GCN4* start codon. A similar shift in reinitiation from uORF2–uORF4 to the *GCN4* ORF also occurs in eIF mutants where the rate of TC loading on 40S subunits is impaired, constitutively derepressing *GCN4* translation independently of Gcn2 (the *Gcd*[−] phenotype) (Hinnebusch

Figure 1. Mapping the SE elements in the CTT of eIF1A. (A) Schematic showing the domains in eIF1A, indicating the residue number that begins each domain (above), and locations of the SEs in the CTT sequence (below). (Constructs a–p) Schematics indicating amino acids missing (red dashes) in different *TIF11* alleles. (Right) Relative growth, on a scale of 0–10, of *his4-301 tif11Δ* strains harboring the indicated *TIF11* alleles on *LEU2* plasmids under the conditions described in B. (B–D) *Slg*[−] and *His*⁺/*Sui*[−] phenotypes of strains harboring selected *TIF11* alleles, described in A, were determined by spotting serial 10-fold dilutions on synthetic complete medium lacking leucine (SC-L) supplemented with 0.3 mM His (+His) or 0.0003 mM His (−His) and incubating for 3 d (+His) or 6 d (−His) at 30°C.

2005). The ΔC and the *F131A,F133A* mutations confer Gcd^- phenotypes by this mechanism, and also reduce the rate of TC loading on 40S subunits in the reconstituted yeast system (Olsen et al. 2003; Fekete et al. 2005, 2007). Thus, the eIF1A CTT appears to have dual functions in promoting TC binding and suppressing UUG initiation, but it was unclear whether these two functions are related.

While the *F131A,F133A* substitution in eIF1A increases the UUG:AUG initiation ratio measured using *HIS4-lacZ* reporters differing in these start codons, it does not confer the His^+/Sui^- phenotype observed for the ΔC truncation, suggesting that other residues removed by ΔC besides *F131,F133* act to enhance scanning and block initiation at UUG codons (Fekete et al. 2005). Hence, we set out to identify all residues in eIF1A that participate in this key initiation function. In the process, we discovered that two loosely conserved ~10-residue repeats in the CTT, dubbed scanning enhancers (SEs), are the critical elements that both inhibit UUG initiation and promote TC binding to the 40S subunit, and we provide genetic and biochemical evidence that these activities are mechanistically linked. We further demonstrate that segments of the helical domain of eIF1A function with the NTT to impede scanning and promote start codon recognition and also negatively regulate TC binding. Our results support a model in which SE elements stabilize a mode of TC binding that is conducive with scanning but incompatible with initiation, and thereby block selection of near-cognate codons, whereas scanning inhibitor (SI) elements antagonize this mode of TC binding to enable AUG recognition.

Results

Identification of partially redundant SE elements in the eIF1A CTT

The *F131A,F133A* mutation in the eIF1A CTT increases the UUG:AUG initiation ratio, but unlike the ΔC truncation, does not confer a His^+/Sui^- phenotype in *his4-301* cells (Fekete et al. 2005, 2007). To identify the other CTT residues responsible for the strong Sui^- phenotype of $\Delta 108-153$, we first constructed the set of nested deletions shown in Figure 1A (constructs b–f) with a common end point at the predicted N-terminal residue of the unstructured CTT (Asp-119) (Battiste et al. 2000). The *TIF11* alleles on a *LEU2* plasmid were introduced into a *his4-301 tif11 Δ ura3* strain harboring wild-type *TIF11* on a *URA3* plasmid, and the latter *TIF11^+* plasmid was evicted by counterselection on medium containing 5-fluoroorotic acid (5-FOA) (Boeke et al. 1987). Western analysis of whole-cell extracts (WCEs) of these and other mutants described below showed that none of their phenotypes could be attributed to reduced eIF1A expression (Supplemental Fig. S1). The deletion mutants were tested for His^+/Sui^- phenotypes by examining cell growth on (–His) medium containing only 0.1% of the amount of histidine added to +His medium to fully supplement His^- auxotrophs. Only the largest deletion ($\Delta 119-133$), which

removed *F131* and *F133*, produced a His^+ phenotype while also conferring a strong slow-growth (Slg^-) phenotype on +His medium (Fig. 1A [constructs a–f], B [lanes a,e,f]). Given that substituting *F131* and *F133* alone did not have a His^+ phenotype (Fekete et al. 2007), we surmised that residues between 119 and 130 are functionally redundant with *F131* and *F133*. We provisionally dubbed this hypothetical element Scanning Enhancer 1 (SE₁) and that containing *F131,F133* SE₂, and hypothesized that a strong Sui^- phenotype results only when SE₁ and SE₂ are impaired simultaneously.

To support the idea that SE₁ is functionally redundant with SE₂, we constructed another set of deletions with a common end point at *F133*, and thus all lacking *F131,F133* of SE₂ (Fig. 1A, constructs g–i). Examination of these mutants revealed that removing residues from *F133* to *S125* was required to evoke His^+ and Slg^- phenotypes (Fig. 1B [lanes i vs. h], summary in 1A [constructs g–i]). Comparing constructs i and h in Figure 1A suggested that the C-terminal boundary of SE₁ lies between *S125* and *E127*. To map the N-terminal boundary of SE₁, we deleted one residue at a time beginning with *D119* from construct $\Delta 128-133$, which lacks *F131, F133* of SE₂ (Fig. 1A, constructs j–l). Moderate His^+ and Slg^- phenotypes appeared only with removal of *F121* (Fig. 1C, lanes l vs. k). Together, these results suggested that SE₁ maps between *F121* and *E127* (Fig. 1A, schematic).

The aforementioned results for constructs e and f in Figure 1A suggested that *F131,F133* occupy the N terminus of SE₂. To map the C terminus of SE₂, we deleted residues from the extreme C terminus of eIF1A in a construct where SE₁ is removed by $\Delta 119-130$ (Fig. 1A, constructs m–p). Moderate His^+ and Slg^- phenotypes appeared only with the deletion of residues 134–136 in construct p (Fig. 1D, lanes p vs. o), thus suggesting that SE₂ extends from *F131* to *A136* (Fig. 1A, schematic).

To test directly the conclusion that SE₁ and SE₂ have overlapping functions in blocking UUG initiation, we constructed complete, multiple-Ala substitutions of SE₁ or SE₂. The *SE₁** mutation conferred only a slight His^+ phenotype (Fig. 2B, lanes a,b) and a correspondingly small increase in the UUG:AUG initiation ratio measured using *HIS4-lacZ* reporters (Fig. 2C, bars a,b). The *SE₂** mutation, in contrast, conferred Slg^- , a stronger His^+ phenotype (Fig. 2B, lanes b,c), and a much larger increase in the UUG:AUG initiation ratio (Fig. 2C, bars b,c). Strikingly, the *SE₁*,SE₂** double mutation is lethal, preventing eviction of the *TIF11^+* plasmid (data not shown). However, combining *SE₁** with an incomplete substitution of SE₂ in which *F131* is retained (mutation *SE₂** + *F131*) yields a viable mutant with strong Slg^- and His^+ phenotypes (Fig. 2B, lane d) and an even larger UUG:AUG initiation ratio than seen for *SE₂** alone (Fig. 2C, bars d vs. c). By assaying a pair of luciferase (*LUC*) reporters with UUG or AUG start codons, we confirmed that *SE₁*,SE₂** + *F131* provokes a larger increase in the UUG:AUG initiation ratio than does *SE₂** or *SE₁** (Fig. 2E, bars a–d). These findings support the idea that SE₁ and SE₂ are partially redundant elements that block initiation at UUG, and suggest that SE₂ is more crucial than SE₁ for this function.

Noting that SE₁ and SE₂ comprise a loosely conserved repeat of nine to 10 residues, each containing a pair of Phe residues (Fig. 2A), we sought to demonstrate that the Phe pairs are the critical residues in SE₁ and SE₂ blocking UUG recognition. First, we generated constructs combining a complete substitution of one SE element with an incomplete substitution of the other SE that retains both Phe residues and compared them with construct SE₁^{*}, SE₂^{*} + F₁₃₁ containing only one Phe residue. Both constructs retaining two Phe residues (Fig. 2B, lanes e,f) produce weaker Slg⁻ and His⁺ phenotypes (Fig. 2B) and lower UUG:AUG ratios (Fig. 2C) compared with that with only a single Phe (Fig. 2C, bar d). Furthermore, a construct (Fig. 2C, bar g) in which all of the residues in the two SEs are substituted except for the four Phe residues produces no Slg⁻ or His⁺ phenotype (Fig. 2B, lanes g vs. a) and only a slight increase in the UUG:AUG ratio compared with wild type (Fig. 2C, bars g vs. a). These results suggest that the number of Phe residues is the critical determinant of the Slg⁻ and Sui⁻ phenotypes of SE mutations.

Finally, we compared substitutions of only the two Phe residues in SE₁ versus those in SE₂. F_{131,133}A provokes a stronger His⁺ phenotype (Figs. 2B, lanes h,i) and a larger increase in UUG:AUG ratio (Fig. 2D) compared with F_{121,123}A. Although neither double-Phe substitution confers a Slg⁻ phenotype, the quadruple-mutant F_{121,123,131,133}A is lethal (data not shown). Thus,

Figure 2. SE₁ and SE₂ elements functionally overlap in blocking UUG initiation. (A) Schematic of eIF1A showing sequence similarity between SE₁ and SE₂. Residues 121–127 were substituted with Ala in mutant SE₁^{*}, and residues 131–135 were similarly substituted in SE₂^{*}. (B) Slg⁻ and His⁺/Sui⁻ phenotypes of *his4-301* strains containing the indicated *TIF11* alleles determined as described in Figure 1B. (C,D) Strains from *B* harboring *HIS4-lacZ* reporter plasmids with an AUG (p367) or UUG (p391) start codon were cultured in SC lacking leucine and uracil (SC-LU) at 30°C, and β -galactosidase activities (nanomoles of o-nitrophenyl β -D-galactopyranoside cleaved per minute per milligram) were measured in WCEs. (E) *his4-301* strains with the indicated *TIF11* alleles and harboring the dual luciferase reporter plasmid pRaugFFuug, containing *LUC_{Renilla}* and *LUC_{firefly}* coding sequences with AUG or UUG start codons, and driven by the *ADH1* or *GPD* promoters, respectively, were cultured in SC-LU, and luminescence was measured in WCEs. The ratio of luminescence, in relative light units, for the UUG to AUG reporter was calculated and plotted. Error bars in C–E give the standard errors of the mean. Differences in bracketed mean values were judged to be significant by the Student's *t*-test, with *P* < 0.001 (***).

F121,F123 are less critical than F131,133, but become essential for blocking UUG initiation in the absence of F131,F133. (The fact that the F_{131,133}A mutant is His⁺ and Slg⁺ does not contradict our previous findings, as we had analyzed a Flag-tagged version of this allele. The Flag tag reduces the His⁺/Sui⁻ phenotypes and exacerbates the growth defects of various *TIF11* mutations [Fekete et al. 2007].) Together, the results in Figure 2 indicate that the phenylalanines are the critical residues in SE₁ and SE₂, which act redundantly to suppress UUG initiation and support cell growth.

Evidence that elimination of both SEs is lethal owing to an intolerable defect in scanning or non-AUG initiation

It is possible that precisely substituting both SEs is lethal because of an intolerable increase in non-AUG initiation. To test this idea, we asked whether the lethality could be overcome by the Ssu⁻ mutation 17–21 in the NTT of eIF1A or by overexpressing eIF1, both of which reduce UUG initiation in other Sui⁻ mutants. Indeed, 17–21 suppresses the lethality of SE₁^{*}, SE₂^{*}, although the resulting mutant retains Slg⁻ and His⁺/Sui⁻ phenotypes (Fig. 3A, +His and –His, segments c vs. a) and a high UUG:AUG ratio of 0.5 ± 0.05 . Importantly, additionally overexpressing eIF1 from a high-copy (hc) *SUI1* plasmid improved the growth rate and nearly eliminated the His⁺/Sui⁻ phenotype

Figure 3. SI element substitutions in eIF1A, or eIF1 overexpression, suppresses the Sui[−] phenotypes of SE substitutions. (A) *his4-301* strains with the indicated *TIF11* alleles plus hc *SUI1* plasmid YEpW-SUI1 or empty *TRP1* vector were streaked on SC lacking leucine and tryptophan (SC-LW) with 0.3 mM His (+His) or 0.0003 mM His (−His) and incubated for 3 d (+His) or 6 d (−His). (B) *his4-301* strains containing the indicated *TIF11* alleles or empty *LEU2* vector and harboring *TIF11*⁺ on a *URA3* plasmid (lanes a–f), and also hc *SUI1* or empty *TRP1* vector (lanes e, f), were replica-plated to either SC-LU (lanes a–d) or SC-LUW (lanes e, f), both containing 0.3 mM His (+His) or 0.0003 mM His (−His), and to either SC-L (lanes a–d) or SC-LW (lanes e, f), both containing 5.2 mM 5-FOA. Plates were incubated for 3 d (+His), 6 d (−His), or 8 d (5-FOA). (C) His⁺/Sui[−] phenotypes of *his4-301* strains containing the indicated *TIF11* alleles plus hc *SUI1* or empty vector were determined as in B. (D) *his4-301* strains with the indicated *TIF11* alleles, *TIF11*⁺ on a *TRP1* vector, and the AUG or UUG *HIS4-lacZ* reporters were analyzed as in Figure 2C except that cells were cultured in SC-LUW. (E) *his4-301* strains with the indicated *TIF11* alleles, hc *SUI1* or empty *TRP1* vector, and the AUG or UUG *HIS4-lacZ* reporters were analyzed as in D. (F) Schematic of eIF1A and the extent of C-terminal truncations in the indicated *TIF11* alleles, with a summary of phenotypes, as in Figure 1A. (G) Slg[−] and His⁺/Sui[−] phenotypes of *his4-301* strains containing the indicated *TIF11* alleles were determined as in Figure 1B. (H) *his4-301* strains with the indicated *TIF11* alleles and the AUG or UUG *HIS4-lacZ* reporters were analyzed as in Figure 2C.

of the *SE₁⁺SE₂⁺17-21* strain (Fig. 3A, +His and −His, segments d vs. c). Similarly, introducing either 17–21, hc *SUI1*, or both into the viable mutant *SE₁⁺SE₂⁺ + F₁₃₁* suppressed its His⁺/Sui[−] phenotype (Fig. 3A, −His, segments f–h vs. e) and (for 17–21) diminished the elevated UUG:AUG ratio in this viable Sui[−] mutant (Fig. 2E, bars e vs. d).

Consistent with the above findings, $\Delta 117-153$, which removes both *SE₁* and *SE₂*, is also lethal, and this lethality is suppressed by 17–21 (Fig. 3B, 5-FOA, lanes c vs. d). Interestingly, $\Delta 117-153$ confers a His⁺/Sui[−] phenotype in viable cells also harboring *TIF11*⁺, and this dominant His⁺/Sui[−] phenotype is suppressed by 17–21 or hc *SUI1* (Fig. 3B, −His, lanes c, d and e, f). As shown above for *SE₁⁺SE₂⁺17-21*, introducing hc *SUI1* into the viable $\Delta 117-153, 17-21$ mutant eliminates its residual His⁺/Sui[−] phenotype (Fig. 3C, lanes c vs. d).

The aforementioned results indicate that the lethality of eliminating both SEs involves an extreme defect in non-AUG initiation that is lessened by an Ssu[−] mutation

in eIF1A or overexpressing eIF1. The dominant Sui[−] phenotype of $\Delta 117-153$ further indicates that this defective protein can compete with wild-type eIF1A for incorporation into PICs, but then fails to block UUG initiation effectively. Given the proposed function of eIF1 in promoting the open, scanning conformation of the PIC, and the ability of eIF1 overexpression or the 17–21 mutation to suppress the lethality and Sui[−] phenotypes caused by eliminating both SE elements, we infer that the SE elements promote the open, scanning conformation of the PIC (hence, their designation as SEs). In contrast, the eIF1A NTT inhibits this open conformation and is regarded as an SI element, which we dubbed SI₁.

Identification of a second SI element in the eIF1A helical domain

Our previous finding that deleting residues 108–153 from eIF1A (ΔC) confers a Sui[−] phenotype (Fekete et al. 2005) is consistent with the elimination of both SE elements, but

seems at odds with the lethality observed here for $\Delta 117-153$ and SE_1^*, SE_2^* , which likewise eliminate both SEs. Because the lethality of the latter mutations is suppressed by the 17–21 mutation in SI_1 , we reasoned that $\Delta 108-153$ is viable because it deletes a second SI element (provisionally dubbed SI_2) that is located just upstream of the SEs and would be left intact by the smaller truncation $\Delta 117-153$. Supporting this interpretation, extending the deletion in $\Delta 117-153$ by only the single codon for Asn-116 (producing $\Delta 116-153$) suppressed the lethality of $\Delta 117-153$ (data not shown). Furthermore, $\Delta 116-153$ confers a smaller increase in the UUG:AUG initiation ratio than does $\Delta 117-153$ when these alleles are compared in situations where $\Delta 117-153$ is not lethal, either in the presence of $TIF11^+$ (Fig. 3D) or $hc\ SUI1$ (Fig. 3E). $\Delta 116-153$ also confers weaker Slg^- and His^+/Sui^- phenotypes than does $\Delta 117-153$ in the presence of $hc\ SUI1$ (Supplemental Fig. S2A, lanes d vs. e). Thus, it appears that Asn-116 belongs to the putative SI_2 and its elimination by $\Delta 116-153$ suppresses the lethal, hyper- Sui^- phenotype of removing both SEs in $\Delta 117-153$.

To determine whether elimination of N116 fully inactivated SI_2 , we examined the Sui^- phenotypes of constructs that extend the deletion of C-terminal domain (CTD) residues further upstream to also remove P110, G107, or Q106 (Fig. 3F, constructs c–f). Compared with $\Delta 116-153$, these larger deletions improved the growth rate on complete medium and progressively reduced the Sui^- phenotype (Fig. 3G, +His and –His, lanes d–f vs. c)

and the UUG:AUG initiation ratio (Fig. 3H, bars c–e vs. b). (The weaker His^+ phenotype observed for $\Delta 116-153$ vs. $\Delta 110-153$ likely reflects the relatively poor growth of the $\Delta 116-153$ mutant evident on +His medium.) Thus, extending the deletion of CTD residues from N116 to Q106, removing the entire C strand, 3_{10} helix, and linker $L\alpha\alpha$ (Fig. 3F), constitutes a step-wise reduction in SI_2 function.

To confirm this last conclusion and also map the N-terminal boundary of SI_2 , we made nested Ala substitutions from residue 106 to 109 (the last residue of $\alpha 2$ and $L\alpha\alpha$ linker) in the lethal $\Delta 117-153$ truncation (Fig. 4A, “A” substitutions in constructs c–f). We reasoned that substitutions encroaching into the N terminus of SI_2 should confer an Ssu^- phenotype and suppress the lethal, hyper- Sui^- phenotype of $\Delta 117-153$. Indeed, substituting both Q106 and G107, but not Q106 alone, suppressed the lethality of $\Delta 117-153$, and the more extensive substitutions that include residues 108 and 109 conferred even higher growth rates (Supplemental Fig. S2B, 5-FOA, lanes c–f vs. b; results summarized in Fig. 4A, +His). Importantly, the 106–109 substitution ($106-109Ala$) also suppressed the lethality of the hyper- Sui^- mutation SE_1^*, SE_2^* (data not shown), and the residual His^+/Sui^- phenotype of the resulting viable $SE_1^*, SE_2^*, 106-109Ala$ mutant was eliminated by extending the Ala substitutions to encompass residues 106–116 in mutant $SE_1^*, SE_2^*, 106-116Ala$ (Fig. 4B, –His, sections c,d). Mutation $106-109Ala$ also reduced the Slg^- and Sui^-/His^+

Figure 4. Substitutions in region SI_2 , which packs against $\alpha 2$, confer an Ssu^- phenotype. (A) Mapping the N-terminal boundary of SI_2 . Schematic of eIF1A and the residues lacking (red dashes) or substituted with alanines (As) in the indicated $TIF11$ alleles, with a summary of phenotypes, as in Figure 1A. (B) Slg^- and His^+/Sui^- phenotypes of $his4-301$ strains containing the indicated $TIF11$ alleles were determined as in Figure 3A, except the cells were streaked on SC-L medium, and are summarized on the right. (C,D) Positions of conserved residues $E_{108}P_{110}E_{111}I_{115}N_{116}$ in human eIF1A and their probable contacts with the indicated residues in helix $\alpha 2$ helix (C), and of N strand residues $L_{26}Y_{28}K_{29}$ and their predicted contacts with $\alpha 2$ and $L\alpha\alpha$ residues (D). The coordinates of human eIF1A (PDB:1D7Q) were used to generate structural models using PyMOL software, showing selected side chains in stick representation.

phenotypes of the viable mutant $SE_1^*, SE_2^* + F_{131}$ (Fig. 4B, sections h vs. g). Together, the results suggest that SI_2 spans residues 107–117 and encompasses $L\alpha\alpha$, the 3_{10} helix, and most of the structured C strand in the eIF1A CTD.

Because all of the components of SI_2 belong to the helical domain (Fig. 4C), we asked whether altering residues in the $L\alpha\alpha$, 3_{10} helix, or C strand that interacts directly with $\alpha 2$ (Battiste et al. 2000) would also confer an Ssu^- phenotype by disrupting SI_2 function. We first examined the effects of Ala substitutions in five such C strand residues: Glu108, Pro110, Glu111, Ile115, and Asn116 (Fig. 4C). This mutation (*EPEIN-Ala*) suppressed the lethality of the hyper- Sui^- allele $\Delta 117-153$ (Supplemental Fig. S2B, 5-FOA, lanes b vs. g) as described above for Ssu^- mutation $106-109Ala$. Because the structured N strand of the N-terminal domain (NTD) (residues 26–33) also packs against $\alpha 2$, we then asked whether substituting N strand residues Leu26, Tyr28, and Lys29 that are predicted to contact residues in $\alpha 2$ or $L\alpha\alpha$ (Fig. 4D) would likewise confer Ssu^- phenotypes. Indeed, this triple-Ala substitution (*LYKAla*) suppressed the lethality of $\Delta 117-153$ (Supplemental Fig. S2B, 5-FOA, lanes b vs. h) and diminished the elevated UUG:AUG initiation ratio

conferred by $\Delta 116-153$, closely resembling the Ssu^- mutation $17-21$ (Fig. 3H, bars f,g vs. b). Consistent with these findings, the N and C strand mutations *LYKAla* and *106-109Ala* decreased the Slg^- and His^+/Sui^- phenotypes of the viable mutant $SE_1^*, SE_2^* + F_{131}$ (Fig. 5A, sections a–c vs. d), and, along with *106-116Ala*, also reduced the UUG:AUG ratio in $SE_1^*, SE_2^* + F_{131}$ cells (Fig. 5B, bars f–h vs. e). These results indicate that proper packing of both N and C strands against $\alpha 2$ is required for the function of SI_2 in supporting UUG initiation in Sui^- mutants of eIF1A.

We asked next whether mutations affecting SI_2 can suppress the Sui^- phenotype of *SUI3-2*, encoding the S264Y substitution in eIF2 β . Remarkably, *106-116Ala* and *LYKAla* both diminish the Sui^-/His^+ phenotype of *SUI3-2* cells (Fig. 5C; data not shown) and reduce the elevated UUG:AUG initiation ratio conferred by *SUI3-2* (Fig. 5D), mimicking the Ssu^- mutation $17-21$ in SI_1 in both respects. Interestingly, the *DEAR-AASA* mutation described previously (Fekete et al. 2007), which substitutes Asp98, Glu99, and Arg101 of $\alpha 2$ itself and eliminates contacts with C or N strand residues (Fig. 5E), also confers an Ssu^- phenotype in *SUI3-2* cells (Fig. 5C,D). Thus, the integrity of the entire helical domain is required

Figure 5. SI substitutions suppress the Sui^- phenotype of *SUI3-2*. (A) Slg^- and His^+/Sui^- phenotypes of *his4-301* strains containing the indicated *TIF11* alleles were determined as in Figure 4B. (B) *his4-301* strains with the indicated *TIF11* alleles plus the dual luciferase reporter pRauGFFuug were assayed as described in Figure 2E. (C) Slg^- and His^+/Sui^- phenotypes of *his4-301* strains with the indicated *TIF11* alleles and containing either plasmid pRSSU13-S264Y-W harboring *SUI3-2* (lanes 2–7) or empty vector (lane 1) were determined as in Figure 1B, except that the cells were spotted on SC-LW medium. (D) Strains described in C harboring the AUG or UUG *HIS4-lacZ* reporters were analyzed as in Figure 2C. (E) Predicted interaction of eIF1A residues D₉₈E₉₉A₁₀₀R₁₀₁ with C and N strand residues is depicted as in Figure 4C.

to support the elevated UUG initiation rate in *SUI3-2* cells. Finally, eliminating the entire NTT by the ΔN mutation confers a stronger *Ssu*[−] phenotype compared with 17–21 (Fig. 5D), indicating that 17–21 only partially impairs *SI*₁.

Gcd[−] phenotypes of *Sui*[−] mutations in *SE*₁*SE*₂ are suppressed by *Ssu*[−] mutations in *SI*₂

We showed previously that the ΔC and *F*_{131,133A} mutations impair TC binding to 40S subunits. When introduced into Flag-tagged eIF1A, these substitutions derepress translation of *GCN4* mRNA independently of eIF2 α phosphorylation by GCN2. This *Gcd*[−] phenotype was attributed to a reduced rate of TC loading on 40S subunits that have translated uORF1 and resumed scanning, allowing them to bypass uORF2–uORF4 and reinitiate at *GCN4* without any decrease in TC formation (Olsen et al. 2003; Fekete et al. 2005, 2007). Consistent with these findings, the *SE*₂^{*} mutation (which eliminates F131,F133)

confers a pronounced *Gcd*[−] phenotype, derepressing by ~10-fold the *GCN4-lacZ* reporter containing all four uORFs in nonstarved *GCN2* cells (Fig. 6A, Nonstarvation, bars c vs. a). The *SE*₁^{*} mutation confers a smaller derepression of *GCN4-lacZ*, and adding to it the Ala substitutions of all *SE*₂ residues except F131 (mutant *SE*₁^{*},*SE*₂^{*}+*F*₁₃₁) evokes a stronger derepression than does *SE*₁^{*} alone (Fig. 6A, Nonstarvation, bars d vs. b). None of these mutations increases expression of a *GCN4-lacZ* construct lacking all four uORFs in nonstarvation conditions (Supplemental Fig. S3A), confirming that they diminish the translational repression imposed by the uORFs. Importantly, co-overexpressing all three eIF2 subunits and tRNA^{Met}, the components of TC, from an *hc* plasmid (*hc TC*) reduces the derepression evoked by the *SE*₁^{*}, *SE*₂^{*}, and *SE*₁^{*},*SE*₂^{*}+*F*₁₃₁ mutations (Fig. 6C). This supports the idea that their *Gcd*[−] phenotypes arise from slower TC loading on reinitiating 40S subunits scanning downstream from uORF1, which is overcome by mass action at higher concentrations of TC.

Figure 6. In vivo and in vitro TC loading defects of SE substitutions are suppressed by SI substitutions. (A) *GCN2* strains containing the indicated *TIF11* alleles and *GCN4-lacZ* reporter plasmid p180, depicted at the top, were cultured in repressing (Nonstarvation) medium (SC-LU) or derepressing (Starvation) medium (SC-LU lacking isoleucine and valine and supplemented with 0.5 μ g/mL sulfometuron), and β -galactosidase activities were assayed in WCEs. (B) *GCN2* strains containing the indicated *TIF11* alleles were analyzed as in A under nonstarvation conditions. (C) *GCN2* strains harboring the indicated *TIF11* alleles, *GCN4-lacZ* reporter plasmid p180, and either empty vector, *hc SUI1*, or *hc TC* plasmid p4385 were assayed under repressing conditions as in A, except that cells were grown in SC-LUW. (D) *GCN2* strains harboring the indicated *TIF11* alleles plus the AUG or UUG *HIS4-lacZ* reporters and either empty vector, *hc SUI1*, or *hc TC* were analyzed as in Figure 3D. (E) *GCN2* strains harboring the indicated *TIF11* alleles, *GCN4-lacZ* reporter plasmid p180, and either empty vector or *SUI3-2* were assayed under repressing conditions as in C. (F) Binding of TC to 40S subunits as a function of the concentration of 40S subunits (K_d), or time (k_{obs}), measured as the fraction of [³⁵S]-Met-tRNA^{Met} associated with 40S subunits in a native gel assay containing saturating eIF1 (1 μ M), model mRNA(AUG), and wild-type or mutant eIF1A proteins at 1 μ M. Errors are mean deviations of at least three measurements. (G) Effects of eIF1A substitutions on the rate of ribosomal subunit joining. Kinetics of subunit joining measured using purified 40S and 60S subunits at 100 nM and 200 nM, respectively, and wild-type or mutant eIF1A proteins at 1 μ M by monitoring the change in intensity of light scattering over time. Kinetics were biphasic for wild type, 106–116Ala, and 17–21, but monophasic for the other three eIF1A mutants. (k_1 and k_2) Rate constants for the first and second phases, respectively; (α_1/α_2) ratio of amplitudes of the first to second phases; (na) not applicable owing to monophasic reactions. Errors are mean deviations of at least three measurements.

The Ssu^- mutations *106–109Ala* and *106–116Ala* in SI_2 do not provoke Gcd^- phenotypes; rather, *106–116Ala* has the opposite effect of reducing *GCN4-lacZ* derepression under starvation conditions, indicating a Gcn^- phenotype (Fig. 6A, Starvation, bars e,f vs. a). Ssu^- mutations in SI_1 , including ΔN , 17–21, and *G21D*, confer comparable Gcn^- phenotypes (Supplemental Fig. S3B). Importantly, the Ssu^- mutations *106–109Ala*, *106–116Ala*, 17–21, and *LYKAla* all diminish the derepression of *GCN4-lacZ* expression conferred by $SE_1^*, SE_2^* + F_{131}$, with *106–116Ala* having the greatest effect (Fig. 6B, bars c–f vs. b). The Sui^- mutation in eIF2 β *SUI3-2* has a Gcd^- phenotype (Williams et al. 1989), and, remarkably, the Ssu^- mutations ΔN , 17–21 and *106–116Ala* all greatly suppress the derepression of *GCN4-lacZ* in *SUI3-2* cells (Fig. 6E). Together, these findings indicate that the SE and SI elements have opposite effects on *GCN4* translational control. Whereas SE mutations appear to reduce the rate of TC loading and confer Gcd^- phenotypes, the Gcn^- phenotypes of SI mutations could indicate that they enhance TC binding to reinitiating 40S subunits scanning the leader of *GCN4* mRNA.

Substituting SE_1SE_2 impairs TC binding in vitro in a manner suppressed by Ssu^- substitutions in SI elements

To test our prediction that SE and SI substitutions have opposite effects on TC loading, we measured their effects on TC binding to 40S subunits in the yeast reconstituted system (Algire et al. 2002; Acker et al. 2007). We first examined the effects of SE and SI substitutions on the equilibrium binding constant of eIF1A for 40S subunits in the presence of saturating concentrations of wild-type eIF1, using a previously established assay (Maag and Lorsch 2003). The results in Supplemental Fig. S4A indicated that only the two mutants harboring the *LYKAla* substitution displayed an appreciable reduction in eIF1A affinity for 40S subunits. To compensate for these defects in subsequent TC-binding assays, we used eIF1 and eIF1A at concentrations of 1 μM , >80-fold above the K_d values measured for all mutant eIF1A proteins.

To assay TC loading, preformed TC containing [^{35}S]-labeled Met-tRNA Met was incubated with purified 40S subunits, a model mRNA with an AUG start codon, eIF1, and either wild-type or mutant eIF1A, and TC binding to the 40S subunit was monitored by an electrophoretic mobility shift (Kolitz et al. 2009). Importantly, the SE_1^*, SE_2^* compound substitution markedly decreased the affinity (greater than eightfold increase in K_d) and rate (>35-fold decrease in k_{obs}) of TC binding to the PIC (Figs. 6F; Supplemental Fig. S4B). The individual SE_1^* and SE_2^* substitutions also reduced the k_{obs} for TC loading, but by smaller amounts—less than twofold and twofold, respectively (Fig. 6F). These results fit with the fact that SE_1^* and SE_2^* produce moderate and strong Gcd^- phenotypes, respectively (Fig. 6A), whereas SE_1^*, SE_2^* is lethal in vivo.

In contrast, the Ssu^- substitutions *LYKAla*, *106–109Ala*, and *106–116Ala* all increased the rate of TC loading in vitro compared with that seen for wild-type

eIF1A (Fig. 6F). Remarkably, all three Ssu^- substitutions also greatly reduced the deleterious effects of SE_1^*, SE_2^* on the affinity and rate of TC loading, restoring the K_d values to <0.5 nM and increasing k_{obs} by factors of 7–11 compared with the SE_1^*, SE_2^* mutant alone (Fig. 6F). The SI_1 substitution 17–21 also strongly suppressed the defective rate of TC binding conferred by SE_1^*, SE_2^* , although it did not suppress the reduction in TC-binding affinity (Fig. 6F). These results suggest that both SI_1 and SI_2 negatively regulate the rate of TC loading, and that substitutions in these regions compensate for the impaired TC binding conferred by SE substitutions. The fact that SI mutations cosuppress the TC-binding defects in vitro and the Gcd^- and Sui^- phenotypes in vivo of SE mutations strongly suggests that all of these defects are linked mechanistically to the rate and stability of TC binding.

Substituting the SE elements stabilizes a closed conformation of the 40S subunit competent for subunit joining

Cryo-electron microscopy (cryo-EM) reconstructions of the 40S subunit in the presence or absence of eIF1 and eIF1A demonstrated that the two factors synergistically stabilize the open conformation of the subunit, and biochemical experiments strongly suggested that TC initially binds to this open state (Passmore et al. 2007). Accordingly, we hypothesized that the eIF1A SEs might stimulate the rate of TC loading, at least in part, by maintaining the open conformation of the 40S subunit. To test this possibility, we exploited the observation that eIF1 and eIF1A synergistically impede joining of the 60S with the 40S subunit, consistent with the idea that the open conformation is not receptive to subunit joining and is instead optimized for scanning, whereas subunit joining occurs in the closed conformation following AUG recognition and eIF1 release (Acker et al. 2006). Hence, if mutating the SE elements shifts the equilibrium from the open to closed conformation of the 40S subunit, this should stimulate the rate of 60S joining with the 40S subunit in vitro.

Spontaneous joining of the 40S and 60S subunits follows biphasic kinetics, with the fast and slow phases having roughly equal amplitudes. These two phases likely correspond to different conformational states of the subunit; the fast phase may be 60S joining to the closed state of the 40S subunit and the slow phase may be the conversion of the open to closed state of the 40S subunit (Acker et al. 2006). In the presence of the SE_1^*, SE_2^* mutant, the kinetics of subunit joining were completely monophasic, with a rate constant (0.05 s $^{-1}$) similar to that of the fast phase of joining observed for wild-type eIF1A (Fig. 6G; Supplemental Fig. S4C). This is consistent with the idea that substituting the SE elements shifts the conformational equilibrium of the 40S subunit in favor of the closed state, which is competent for subunit joining but not for the initial stage of TC loading. In contrast, the SI substitutions 17–21 and *106–116Ala* have the opposite effect of increasing the amplitude of the slow phase of the subunit joining reaction

(Fig. 6G), which would be expected if they stabilize the open conformation as a means of enhancing the rate of TC binding. Strikingly, the SI substitutions 17–21 and 106–116Ala also partially suppress the effect of SE_1^*, SE_2^* by reducing the rate of subunit joining compared with that given by SE_1^*, SE_2^* alone (Fig. 6G; Supplemental Fig. S4C). The fact that these SI mutations do not fully restore subunit joining to wild-type behavior is consistent with the fact that they only partially suppress the TC loading defects of the SE_1^*, SE_2^* substitution. These findings strongly suggest that the SE and SI elements regulate the open-to-closed transition of the 40S in opposite directions, which likely contributes to their opposing effects on the rate of TC loading.

Discussion

Previously, we provided evidence that the CTT and NTT of eIF1A differentially regulate the open and closed conformations of the 40S subunit as the means of ensuring stringent selection of AUG as a start codon (Maag et al. 2006; Fekete et al. 2007). Here, we provide additional evidence for this model, but, importantly, we also demonstrate that the SE elements in the CTT and SI elements in the NTT and helical domain regulate scanning and initiation by their differential effects on TC binding to the 40S subunit. By fine-structure mutational analysis, we identified a segment in the unstructured CTT containing loosely conserved 10-amino-acid repeats that act in a partially redundant manner to suppress initiation at the UUG start codon at *his4-301*. We dubbed these repeats SE_1 and SE_2 because they appear to promote the open conformation of the PIC that facilitates continued scanning when a non-AUG codon occupies the P site. In-depth analysis revealed that these elements contain a pair of Phe residues—F121, F123 and F131, F133, respectively—as critical constituents, and that SE_2 is more important than SE_1 in blocking UUG initiation.

We showed previously that the $\Delta 108-153$ (ΔC) truncation of the entire CTT, C strand, 3_{10} helix, and part of the $L\alpha\alpha$ linker, produces a viable Sui^- mutant (Fekete et al. 2005). Remarkably, we found here that truncations smaller than ΔC that eliminate both SEs without encroaching extensively into the C strand (e.g., $\Delta 117-153$) are lethal, as is precisely substituting both SEs by the SE_1^*, SE_2^* mutation. Because the dominant Sui^- phenotypes and lethality of these mutations are suppressed by overexpressing eIF1 (hc *SUI1*) or the Ssu^- mutation 17–21, which also suppress the Sui^- phenotypes of mutations in eIF5 (*SUI5*) and eIF2 β (*SUI3-2*), we can infer that the lethality of removing both SEs involves an intolerably high level of initiation at UUG or other near-cognate start codons that can be mitigated by eIF1 overexpression or NTT substitutions. Although no *TIF11* mutations were isolated in the original selections that yielded Sui^- mutations (Donahue 2000), our results show that substituting both Phe pairs in the eIF1A SEs increases UUG initiation comparably with the strongest Sui^- mutations of eIF1, eIF5, or eIF2. Aligning eIF1A sequences from different species indicates that F121, F131, and F133 are

conserved among lower eukaryotes, but only F121 and F131 are conserved in animals (Supplemental Fig. S5). Our results indicate that both F131 and F133 contribute to the function of SE_2 (Fig. 2), but F121 might suffice for SE_1 function.

The fact that $\Delta 117-153$ is lethal but the more extensive truncation $\Delta 108-153$ (ΔC) is viable was the critical clue that the latter disrupts an element whose elimination compensates for the lethality of deleting both SEs. By systematically testing additional mutations for suppression of the lethality of $\Delta 117-153$, we determined that this new element includes the $L\alpha\alpha$, 3_{10} helix, and residues in the structured N and C strands that contact $\alpha 2$ in the helical domain. Similar to the 17–21 mutation in the NTT, disrupting the integrity of the helical domain suppresses Sui^- mutations in the SE elements and in eIF2 β (*SUI3-2*), conferring the Ssu^- phenotype. Hence, we conclude that $\alpha 2$ and its associated N and C strands comprise a functional unit, dubbed SI_2 , that acts in conjunction with SI_1 to arrest scanning and promote start codon selection.

Interestingly, mutations affecting SE_1 and SE_2 also confer strong Gcd^- phenotypes that can be suppressed by overexpressing TC, indicating reduced rates of TC loading during reinitiation on *GCN4* mRNA. Just as in blocking UUG initiation, SE_2 is more critical than SE_1 in promoting TC loading and repressing *GCN4* translation. Our biochemical analysis in the reconstituted system supports this conclusion by revealing reduced rates of TC binding that are more severe for SE_2 versus SE_1 substitutions, and of greatest severity when both elements are lacking. This last result indicates that SE_1 and SE_2 have overlapping functions in TC loading in addition to blocking UUG initiation. The strong correlation between the effects of different SE mutations on the degree of Sui^- phenotypes in vivo and the severity of Gcd^- phenotypes and TC binding defects in vitro provides evidence that the increased UUG initiation in SE mutants is linked mechanistically to their defective TC binding.

Remarkably, Ssu^- mutations in either SI element diminish the Gcd^- phenotypes of both SE mutations and the eIF2 β Sui^- mutation *SUI3-2*, thus suggesting that the Ssu^- mutations compensate for defects in TC binding. Our biochemical data support this conclusion, as defects in TC binding provoked by the SE_1^*, SE_2^* substitution are partially suppressed by multiple Ssu^- substitutions in SI_1 or SI_2 , all of which elicit an increased rate of TC binding when present in otherwise wild-type eIF1A. Together, these results provide compelling evidence that the ability of SI mutations to suppress UUG initiation in Sui^- mutants involves their ability to compensate for TC loading defects. This leads us to the surprising deduction that the wild-type SI elements act to oppose continued scanning and promote start codon recognition by negatively regulating TC binding.

How might the defect in TC binding provoked by SE mutations lead to increased UUG initiation? We begin by proposing that the SEs stabilize TC binding to the PIC in a conformation that is compatible with scanning but incompatible with initiation. For example, the SEs could

bind Met-tRNA^{Met} in a way that prevents it from being fully engaged with the P site—a mode of binding we dub the “P_{out}” state, which would be associated with the open, scanning conformation of the PIC (Fig. 7A). The SEs could promote the P_{out} mode of TC binding directly (e.g., by interacting with the anticodon stem-loop [ASL] of the initiator), or indirectly by stabilizing the open conformation of the 40S subunit, to which TC initially binds (Passmore et al. 2007). In either case, entry of AUG into the P site and its perfect complementarity with the anticodon of the initiator would be required to overcome the SEs and enable Met-tRNA^{Met} to be fully accommodated in the P site and trigger downstream steps in the initiation pathway. We dub this second conformation the “P_{in}” state, and envision that it is characteristic of the closed, scanning-incompatible conformation of the PIC (Fig. 7A). These two states could be identical to the two states that bind TC weakly or stably, respectively, that we detected previously in kinetic studies (Kolitz et al. 2009).

As SE substitutions would impair the ability of eIF1A to stabilize the P_{out} conformation in which TC initially binds, this would account for their deleterious effect on TC binding to the 40S subunit in vitro and their Gcd[−] phenotypes in vivo. By destabilizing P_{out}, SE mutations would also facilitate the P_{out}-to-P_{in} transition at UUG codons, which occurs without a perfect codon–anticodon match, and this would help explain their Sui[−] phenotypes (Fig. 7B). Thus, although SE substitutions reduce the rate of TC loading, once TC is bound to the 40S subunit, the transition from P_{out} to P_{in} would occur more frequently at near cognate codons. We found that TC overexpression suppresses the Gcd[−] phenotypes of SE mutants (Fig. 6C) but does not reduce the elevated UUG:AUG ratio in

SE₁[−], SE₂[−] + F₁₃₁ cells (Fig. 6D). These findings are consistent with our model, as increasing TC levels should boost the rate of TC binding to the P_{out} state, reducing the Gcd[−] phenotype, but should not mitigate the increased probability of P_{out}-to-P_{in} transitions at UUG codons that, in our model, contribute to the Sui[−] phenotypes of SE mutants.

Our proposal that SE elements stabilize TC binding in a conformation that facilitates scanning but is incompatible with initiation predicts that Met-tRNA^{Met} binds in the P site of the scanning PIC (the P_{out} state) in a manner distinct from that seen in crystal structures of bacterial 70S-mRNA-tRNA complexes (Berk et al. 2006; Korostelev et al. 2006; Selmer et al. 2006). In fact, this prediction is strongly supported by results of directed hydroxyl radical mapping of the mammalian eIF1A in reconstituted 43S PICs (Yu et al. 2009). This work revealed that the CTT extends into the P site, threading under the Met-tRNA^{Met}, in a configuration that would obstruct Met-tRNA^{Met} binding to the P site in the manner observed in bacterial 70S complexes. Hence, it was concluded that AUG recognition and formation of the closed complex would likely require removal of the eIF1A CTT from the P site. Indeed, we argued previously that the eIF1A CTT would be ejected from the P site on AUG recognition (Fekete et al. 2007), based on its physical displacement from eIF1 in the PIC (Maag et al. 2005) and its AUG-dependent functional interaction with eIF5 (Maag et al. 2006). It seems likely, therefore, that the SEs sterically block the P_{in} mode of TC binding in addition to stabilizing the P_{out} conformation (Fig. 7A). This idea is very attractive because SE mutations would then facilitate the P_{out}-to-P_{in} transition at UUGs (conferring Sui[−] phenotypes) in two ways: by destabilizing P_{out} and also removing a steric impediment to P_{in} (Fig. 7B).

Figure 7. (A) Model describing the positive and negative effects of the SE and SI elements of eIF1A, respectively, on TC binding in the P_{out} conformation, which is conducive to scanning, and the second function of the SEs in blocking TC binding in the P_{in} conformation, which is incompatible with scanning and permissive for initiation. (B) SE inactivation destabilizes P_{out}, reducing TC loading and conferring the Gcd[−] phenotype and also enhancing the P_{out}-to-P_{in} transition at UUGs to confer the Sui[−] phenotype. The P_{out}-to-P_{in} transition and UUG initiation is further stimulated by loss of the inhibitory effect of the SEs on P_{in}. (C) SI inactivation stabilizes P_{out}, promoting TC loading and replacing Gcd[−] phenotypes with Gcn[−] phenotypes, and suppressing the P_{out}-to-P_{in} transition at UUGs to confer the Ssu[−] phenotype. (D,E) Hypothetical model depicting the different conformations of initiator tRNA in the P_{out} (D) and P_{in} (E) states and the proposed roles of the eIF1A

SE elements in stabilizing initiator binding in P_{out}, where the initiator is not fully accommodated in the P site, and impeding initiator binding in P_{in}, where the initiator is more fully engaged with the P site. On AUG recognition, the SEs are ejected from the P site to allow greater accommodation of the initiator in the P_{in} state.

Combining the results of Yu et al. (2009) with our finding that SE elements promote TC binding to the scanning-competent conformation of the PIC, we propose that, in the P_{out} state, the SEs occupy the P site in a manner that blocks accommodation of the initiator ASL without preventing the codon–anticodon interactions required during scanning (Fig. 7D). The SEs might interact directly with the anticodon or ASL of the initiator to stabilize TC bound in this scanning conformation of the PIC. Pairing with the AUG start codon would lead to ejection of the SEs from the P site and allow more extensive P-site engagement of the initiator ASL in the P_{in} conformation, as depicted in Figure 7E. The P_{in} conformation might resemble the “30S P/I” state visualized in a cryo-EM model of the bacterial 30S initiation complex (Simonetti et al. 2008), and further conformational changes would presumably occur on subunit joining before reaching the classical P-site binding of tRNA seen in bacterial 70S structures (Berk et al. 2006; Korostelev et al. 2006; Selmer et al. 2006). Our model in Figure 7, D and E, fits with the notion that accommodating initiator tRNA in different intermediate states is a key feature of the small ribosomal subunit (Simonetti et al. 2008).

We can readily incorporate the functions of eIF1A SI elements into our model simply by proposing that they oppose the SEs and destabilize the P_{out} state to which TC initially binds (Fig. 7A). This can explain our finding that SI mutations partially suppress the defective TC binding conferred by the SE_1^*, SE_2^* mutant in vitro and, consistently, suppress the Gcd^- phenotypes of SE mutations in vivo (Fig. 7C). At the same time, SI mutations would shift the equilibrium back from P_{in} to P_{out} , promoting the scanning conformation of the PIC and suppressing UUG initiation in Sui^- mutants; i.e., their Ssu^- phenotypes (Fig. 7C). The function of the wild-type SI elements in destabilizing the P_{out} state can be viewed as a driving force that facilitates the P_{out} -to- P_{in} transition required for AUG selection. Because the NTT also appears to interact directly with the P site (Yu et al. 2009), it might play a direct role in antagonizing initiator binding to the P_{out} state (Fig. 7D).

Results of our previous studies suggested that TC binds initially to the open conformation of the PIC, stabilized synergistically by eIF1 and eIF1A (Passmore et al. 2007). Hence, it is plausible that the SEs could stimulate TC loading by stabilizing the open conformation of the 40S rather than interacting directly with the initiator. Indeed, we found that inactivation of the SEs accelerates the rate of 60S subunit joining, which is thought to proceed only from the closed conformation of the 40S. Remarkably, SI substitutions had the opposite effect and partially suppressed the more rapid 60S joining conferred by the SE substitutions, as would be expected if the wild-type SIs antagonize the open conformation as the means of promoting start codon recognition. Thus, it appears likely that the SEs and SIs regulate the rate of TC loading, at least partly, by their opposing effects on the open-to-closed conformational transition of the 40S subunit. However, it is noteworthy in this connection that eIF1 overexpression did not suppress the Gcd^- phenotype of an SE mutation even though it suppressed the Sui^-

phenotype (Fig. 6C,D). As eIF1 promotes the open conformation, one might expect that overexpressing eIF1 would rescue TC binding in SE mutants if this defect arises only from reduced occupancy of the open state. Hence, the SEs likely stimulate TC loading by a second, possibly direct, mechanism in addition to promoting the open conformation of the 40S subunit.

In its simplest formulation, our model posits that the SEs in eIF1A help to recruit TC in the open, scanning conformation and block initiator accommodation at non-AUGs, whereas SIs drive the transition to the closed state in which the initiator is fully engaged in the P site at AUG codons (Fig. 7). IF1, the bacterial ortholog of eIF1A, lacks the CTT, NTT, and helical domain (Supplemental Fig. S6; Carter et al. 2001), whereas the archaeal ortholog (aIF1A) lacks only the C strand and unstructured CTT (Supplemental Figs. S7, S8). Similar to bacteria, it appears that many mRNAs in archaea use the Shine-Delgarno (SD) sequence upstream of the start codon to recruit the 30S subunit directly to the initiation region (Dennis 1997; Londei 2005), obviating the scanning mechanism. Thus, in bacteria and archaea, the presence of SD-facilitated AUG selection is correlated with the absence of SEs, consistent with the role of these eukaryotic elements in promoting a scanning-competent intermediate in the initiation pathway (Fig. 7D). Considering that archaea resemble eukaryotes in employing a TC with aIF2-GTP for initiator recruitment (Pedulla et al. 2005), the helical domains in aIF1A/eIF1A might also play a role in promoting TC binding in the P_{in} state. Recent in vitro findings suggest that bacterial IF1 functions in stabilizing a conformation of the 30S initiation complex that is incompatible with subunit joining, which can be overcome by a favorable SD sequence (Milon et al. 2008). Thus, although IF1 lacks the SE elements, it carries out one of the functions we ascribe to the SE, of stabilizing a small subunit conformation incompatible with subunit joining. This raises the possibility that a region of the OB fold—the main structural element shared between eIF1A and IF1—could augment this aspect of SE function.

Materials and methods

Plasmids and yeast strains

Plasmids and yeast strains used in this study are listed in Supplemental Tables S1 and S2, respectively. Descriptions of plasmid constructions and site-directed mutagenesis are given in the Supplemental Material. The *his4-301* yeast strain H3582 [*MATa ura3-52 trp1Δ63 leu2-3, leu2-112 his4-301 (ACG) tif11Δ p3392 <TIF11, URA3>*] was transformed with single-copy or *hc LEU2* plasmids harboring various *TIF11* alleles on SC-L medium, and the resident *TIF11⁺ URA3* plasmid p3392 was evicted by selection on 5-FOA medium to obtain the mutant strains listed in Supplemental Table S2.

Biochemical assays with yeast extracts

Assays of β -galactosidase activity in WCEs were performed as described previously (Moehle and Hinnebusch 1991). Measurements of luminescence in WCEs were conducted essentially as described (Dyer et al. 2000). For Western analysis, WCE extracts

were prepared by trichloroacetic acid extraction as described previously (Reid and Schatz 1982), and immunoblot analysis was conducted as described (Olsen et al. 2003).

Biochemical assays in the reconstituted yeast system

Reagent preparation is described in the Supplemental Material. Fluorescence anisotropy measurements to determine K_d values for 40S binding of eIF1A were performed as described previously using wild-type eIF1A Fluorescein-labeled at the C terminus and competing its 40S binding with unlabeled eIF1A mutants (Maag and Lorsch 2003; Maag et al. 2006). For all experiments, buffer conditions were 30 mM HEPES (pH 7.4), 100 mM potassium acetate (pH 7.4), 3 mM $MgCl_2$, and 2 mM dithiothreitol. TC binding was measured by gel mobility shift assays as described previously (Algire et al. 2002). Kinetics of ribosomal subunit joining in the presence of wild-type or mutant eIF1A was measured by light scattering on an SX.180MV-R stopped-flow fluorometer (Applied Photophysics) (Acker et al. 2009).

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Erratum

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Regulatory elements in eIF1A control the fidelity of start codon selection by modulating tRNA^{Met} binding to the ribosome

Adesh K. Saini, Jagpreet S. Nanda, Jon R. Lorsch, and Alan G. Hinnebusch

In the above-mentioned article, the label for the third row of data in Figure 6G, " α_1/α_2 ," is incorrect. Instead, it should read " α_2/α_1 ," the ratio of amplitudes of the second (slow) phase to the first (fast) phase of the subunit joining reaction. The descriptions and interpretations of these data in the Results and Discussion sections require no corrections.

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