Translation initiation at non-AUG codons mediated by weakened association of eukaryotic initiation factor (eIF) 2 subunits

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The heterotrimeric eukaryotic initiation factor (eIF) 2 binds the initiator methionyl-tRNA in a GTP-dependent mode and delivers it to the 40 S ribosomal subunit. In the present study, we have identified amino acid residues in eIF2 β required for binding to eIF2 γ in yeast. Alteration of six residues in the central region of eIF2 β abolished this interaction, as determined by GST-pull down and two-hybrid assays, and leads to cell lethality. Substitution of ¹³¹Tyr and ¹³²Ser by alanine residues (¹³¹YS), although abolishing the binding to eIF2 γ in these assays, resulted in a functional but defective protein *in vivo*, imparting a temperature-sensitive growth phenotype to cells. A dramatically weakened association of this mutant protein with eIF2 γ *in vivo* was shown by co-immunoprecipitation. The ¹³¹YS mutation in eIF2 β allows translation to initiate at non-AUG codons, as

defined by the ability of cells carrying an initiator codon mutation in the HIS4 mRNA to grow in the absence of histidine. The combination of this mutation with the ²⁶⁴Ser → Tyr alteration, a previously isolated suppressor of initiator codon mutations which has been shown to increase the spontaneous GTP hydrolysis in the ternary complex, caused a recessive lethality, suggesting additive defects. Thus the impaired interaction of these two subunits represents a novel type of defect in eIF2 function, providing *in vivo* evidence that the strength of interaction between eIF2 β and eIF2 γ defines the correct usage of the AUG codon for translation initiation.

Key words: eIF2 β mutations, non-AUG recognition, subunit interaction determinants, translation initiation.

INTRODUCTION

Protein synthesis initiation in eukaryotes requires the participation of numerous factors which, acting in a precise timely fashion, help to position the ribosomes in the correct AUG codon in the mRNA to engage in peptide-chain elongation (for reviews see [1,2]). A ternary complex of eukaryotic initiation factor (eIF) 2, GTP and the initiator methionyl-tRNA (Met-tRNA^{Met}) associates with the 40 S ribosomal subunit carrying eIF3 and eIF1A. This 43 S complex binds to the 5'-end of eukaryotic mRNA through interactions mediated by components of the cap-binding factor eIF4F with both the cap structure and 40 S-bound eIF3 and possibly eIF1 and eIF5 [2]. This initial coupling is followed by the migration of the ribosomal subunit on the mRNA in a process called 'scanning'. Upon encountering the initiator AUG codon, usually the first from the 5'-end, eIF5 stimulates the hydrolysis of the eIF2-bound GTP molecule, leading to the release of all initiation factors, while the Met-tRNA^{Met} remains bound to the ribosomal P site. A second GTP molecule, bound to eIF5B, is hydrolysed, allowing the joining of the 60 S ribosomal subunit and leading to the elongation cycle of protein synthesis [3].

eIF2 is a heterotrimeric factor whose main functions are to deliver Met-tRNA^{Met} to the 40 S subunit and to ensure that the correct codon is used for initiation (for review see [2]). This latter function seems to be directly related to its ability to maintain the bound state of the Met-tRNA^{Met}, as shown in Sui⁻ mutants in yeast which act as suppressors of initiator codon mutations at the HIS4 locus [4–7]. Thus mutations in the β subunit of

eIF2 in yeast, encoded by the *SUI3* gene, which lead to increased spontaneous GTP hydrolysis independently of the ribosome and eIF5, result in initiation of translation in a non-AUG codon due to the premature release of Met-tRNA^{Met} [8]. A mutation in the γ subunit, encoded by *GCD11*, which increases the dissociation rates of Met-tRNA^{Met} in the absence of GTP hydrolysis, also results in non-AUG codon recognition [8]. The α subunit of eIF2 is required for mediating and regulating the interaction of eIF2 with the guanine nucleotide exchange factor eIF2B, which stimulates the exchange of GDP to GTP on eIF2 [2]. Yeast cells lacking the α subunit were found to be viable as long as the β and γ subunits of eIF2 and the initiator tRNA were overexpressed [9]. The same requirements were determined for cells lacking eIF2B. *In vitro*, it has been shown that yeast eIF2 has an intrinsic ability for the GDP–GTP exchange reaction [9]. It appears, then, that the β and γ subunits of eIF2 provide the basic structural components for eIF2 function, which are Met-tRNA^{Met} and GTP binding and the coupling of this complex to the 40 S subunit. However, it is not known how these activities are distributed between the two subunits. eIF2 γ has a high degree of sequence conservation with the bacterial translation elongation factor EF1A (formerly known as EF-Tu), including G-domain elements known from X-ray crystallography to directly interact with guanine nucleotides, thus leading to the hypothesis that the binding site for GTP is located in the γ subunit. This functional similarity has been probed and confirmed genetically and biochemically with mutations in the *GCD11* gene [7,10]. However, the isolated subunit has not yet been shown to be capable of binding GTP or Met-tRNA^{Met} in a

Abbreviations used: aIF, archaeal initiation factor; eIF, eukaryotic initiation factor; eIF2 β , β subunit of eIF2; ¹³¹YS, eIF2 β mutant in which Tyr¹³¹ and Ser¹³² have been changed to alanine; ¹³³ELL, eIF2 β mutant in which Glu¹³³, Leu¹³⁴ and Leu¹³⁵ have been changed to alanine; ¹³⁴LL^{138F141L}, eIF2 β mutant in which Leu¹³⁴, Leu¹³⁵, Phe¹³⁸, Ile¹⁴¹, Leu¹⁴² and Leu¹⁴⁹ have been changed to alanine; ¹⁴¹IL, eIF2 β mutant in which Ile¹⁴¹ and Leu¹⁴² have been changed to alanine; ¹⁴⁹L, eIF2 β mutant in which Leu¹⁴⁹ has been changed to alanine; eIF2 γ , γ subunit of eIF2; GAD, Gal4-activation domain; GAP, GTPase-activating protein; GBD, Gal4 DNA-binding domain; GST, glutathione S-transferase; Met-tRNA^{Met}, initiator methionyl-tRNA; SUP, *SUI3-2* suppressor allele, which carries the mutation ²⁶⁴Ser → Tyr (²⁶⁴S → Y).

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GTP-dependent mode. Both the β and γ subunits have been shown to cross-link to guanine nucleotides and Met-tRNA_i^{Met} [11,12]. The β subunit is involved in several interactions with other translation factors. Its N-terminal sequences bind to eIF5, the GTPase-activating protein (GAP) for eIF2, and to the ϵ subunit of the eIF2B catalytic subcomplex [13]. This same region, involving three lysine repeats, has been shown to bind RNA *in vitro* [14]. The C-terminal region of eIF2 β contains a C2–C2 motif, where the suppressor mutations that result in increased spontaneous GTPase activity map [8,15]; this region seems to be capable of binding RNA *in vitro* [14]. The β subunit has also been implicated in binding the δ subunit of the regulatory subcomplex of eIF2B [16]. Thus eIF2 β is a major mediator of contacts with several other molecules that participate in the initiation of translation.

Little is known about the subunit connections and their functions in the complex. We have shown previously [17] that, in the eIF2 complex, the γ subunit acts as a bridge between the α and β subunits, with eIF2 β binding only to eIF2 γ . The region in eIF2 β that is involved in contacting eIF2 γ was mapped to the central portion of the molecule, involving residues 128–205 of the yeast protein. This region is conserved in the β subunit from all organisms analysed from archaea to mammals. The archaeal protein begins exactly in the region that we have defined in eIF2 β to be involved in the interaction with the γ subunit. The N-terminal region of eIF5 contains extensive sequence similarity to eIF2 β and archaeal initiation factor (aIF) 2 β . However, this eIF5 sequence diverges from the β subunit in a few residues at the N-terminal extremity of the protein, corresponding to the location of residues that have been shown to be required for its GAP activity [18,19], and eIF5 does not interact with eIF2 γ [17].

In the present study, we have probed in detail the residues of eIF2 β that are required for its binding to eIF2 γ in *Saccharomyces cerevisiae*. We show that the strength of the binding of eIF2 β to eIF2 γ determines the correct utilization of the AUG codon for initiation of translation *in vivo* in eukaryotes.

MATERIALS AND METHODS

Yeast strains and genetic methods

Standard yeast genetic techniques and media have been described previously [20]. Yeast strain 167-3C (*MAT α* , *ura3-52*, *leu2-3,-112*, *his4*^{UG}-306, *ino1-13*) and BCV59 (*MAT α* /*MAT α* , *ura3-52*/*ura3-52*, *leu2-3,-112*/*leu2-3,-112*, *his4*^{UG}-306/*his4*^{UG}-306, *SUI3/sui3::URA3*) have been described elsewhere [15]. The yeast strain used for the two-hybrid assays was PJ69-4A (*MAT α* , *trp1-901*, *leu2-3,-112*, *ura3-52*, *his3-200*, *gal4 Δ* , *gal80 Δ* , *GAL2-ADE2*, *LYS2::GAL1-HIS3*, *met2::GAL7-lacZ*) [21]. Function of the mutant proteins was assessed in ascospore derivatives of strain BCV59 transformed with pSB32(*LEU2CEN4*)-based plasmids containing the mutated alleles of *SUI3*. Growth of cells was analysed by spotting 5 μ l of a 10-fold serial dilution of a cell suspension at $D_{600} = 0.5$ on to plates containing YPD-rich medium or minimal SD medium supplemented with the appropriate amino acids.

Construction of deletions and site-directed mutations in eIF2 β

The cloning of the wild-type sequence of *SUI3* (plasmid pBE260), the segment of eIF2 β corresponding to amino acid residues 128–205 (plasmid pBE309) as well as the sequence encoding eIF2 β lacking the region corresponding to amino acid residues 96–179 (plasmid pBE297) in pGAD-C1 [21] have been described previously [17]. For cloning of the sequence encoding amino acid

residues 128–179 in pGAD-C1, a *Bam*HI–*Eco*RV fragment of 159 bp was isolated from plasmid pBE296, which encodes a Gal4-activation domain (GAD) fusion with amino acids 128–285 of eIF2 β , as described previously [17]. The fragment was then inserted into pGAD-C1 digested with *Sal*I, blunt-ended with Klenow polymerase and digested with *Bam*HI to produce plasmid pBE372. For the expression of amino acid residues 128–159 fused to GAD, plasmid pBE296 was digested with *Eco*RI, blunt-ended with Klenow polymerase and digested with *Bam*HI. A resulting fragment of 100 bp was isolated and inserted into pGAD-C1 prepared as above to generate plasmid pBE373. The protein lacking residues 141–179 fused to GAD was obtained by transferring a *Mlu*I–*Age*I fragment from plasmid pBE220 (a *LEU2CEN4*-based plasmid containing, in a *Hind*III fragment, the *SUI3* gene with a *Ssp*I–*Eco*RV internal fragment deleted) to plasmid pBE262 {a plasmid containing the complete eIF2 β fused to the Gal4 DNA-binding domain (GBD) in plasmid pGBD-C1 [21]}, digested with *Mlu*I and *Age*I to produce plasmid pBE356. The *Bam*HI fragment of this plasmid was transferred to pGAD-C1 to generate plasmid pBE359. Similarly, the construction of the deletion of residues 180–205 in pGAD-C1 (pBE361) was made by swapping the *Mlu*I–*Age*I fragment of pBE262 with that in plasmid pBE224 (which contains the *SUI3* gene from which the internal *Eco*RV–*Hinc*II fragment was removed) and then transferring the *Bam*HI fragment to pGAD-C1. The fusion of eIF2 γ to the GBD was as described previously [17].

Mutations in the central region of eIF2 β were obtained by PCR using a *Bam*HI fragment from plasmid pBE149 [14] as a template for the amplification of a sequence encoding residues 128–285 of eIF2 β and, as the reverse primer, the oligonucleotide BC 49 (5′-GGGGATCCACAAGATAAACGAAATCCG-3′), which is complementary to the 3′ untranslated region (‘UTR’) of *SUI3*. The forward primers for the introduction of the alanine substitutions were: BC101 (5′-GGGGATCCGGCCTACCTGCTGCAGAGTTATTA-3′) to replace Tyr¹³¹ and Ser¹³² (131YS); BC102 (5′-GGGGATCCGGCCTACCTTATTCAGCTGCAGCTTCTAGATTTTCAAT-3′) to replace Glu¹³³, Leu¹³⁴ and Leu¹³⁵ (133ELL); BC103 (5′-GGGGATCCGGCCTACCTTATTCAGAGTTATTATCTAGATTTTCAATGCTGCAAGAACTAACAATCCA-3′) to replace Ile¹⁴¹ and Leu¹⁴² (141IL); BC104 (5′-GGGGATCCGGCCTACCTTATTCAGAGTTATCTAGATTTTCAATATTCTAAGAATAACAATCAGAAAGCTGCTGGTGATAGA-3′) to replace Leu¹⁴⁹ (149L); and BC105 (5′-GGGGATCCGGCCTACCTTATTCAGAGGCTGCATCTAGAGCTTTCAATGCTGCAAGAACTAACAATCCAGAAGCTGCTGGTGATAGA-3′) to replace Leu¹³⁴, Leu¹³⁵, Phe¹³⁸, Ile¹⁴¹, Leu¹⁴² and Leu¹⁴⁹ (134LL¹³⁸F¹⁴¹IL¹⁴⁹L). The PCR-generated mutant fragments were digested with *Bam*HI and cloned into the *Bam*HI site of pGAD-C1, generating plasmids pBE377, pBE378, pBE379, pBE380 and pBE381 respectively. The amplified fragments were sequenced completely.

For the expression of glutathione S-transferase (GST) fusion proteins, the *Bam*HI fragments from these plasmids were transferred to the *Bam*HI site of pGEX2T [22], generating plasmids pBE383, pBE384, pBE385, pBE388 and pBE386 respectively. The plasmid constructions for the expression of GST fusion proteins of the wild-type eIF2 β (pBE149) and residues 128–285 (pBE192) of eIF2 β have been described previously [17].

To construct a complete version of eIF2 β containing the site-directed mutations, an *Eco*RI fragment of 990 bp was isolated from pBE32 [15] and digested with *Hae*III. The resulting fragment of 896 bp comprises 515 bp of the 5′ upstream sequences plus nucleotides in the open reading frame corresponding to the amino acid residue at position 128 (*Hae*III site). A *Bam*HI fragment of approx. 533 bp was isolated from the mutants

cloned in pGAD-C1. This fragment was then digested with *Hae*III and the 527 bp fragment containing the C-terminal region of eIF2 β was isolated. The two fragments were ligated into pBluescript-SK(+) digested with *Eco*RI and *Bam*HI, restoring the complete sequence of *SUI3* for all mutations and generating plasmids pBE392, pBE393, pBE394, pBE395 and pBE396 in the same order as given previously. The *Bam*HI–*Hind*III fragment from each of these plasmids was isolated and inserted into the pSB32 (*LEU2CEN4*) plasmid, which had been digested with *Bam*HI and *Hind*III, to produce plasmids pBE397, pBE398, pBE399, pBE400 and pBE401 respectively.

For the construction of the double mutants, a *Bgl*II–*Hind*III fragment of approx. 500 bp was isolated from the *SUI3*-2 suppressor allele (SUP) present in pBE66 [15]. A 1700 bp *Pvu*I–*Bgl*II fragment was isolated from each of the mutant alleles present in the pSB32 vector. The two fragments were ligated with pSB32 digested with *Pvu*I and *Hind*III. These constructions generated plasmids pBE410, pBE411, pBE412, pBE413 and pBE414, in the same order given above for the mutations in the interacting domain.

Cloning and expression of aIF2

The coding sequences for the β and γ subunits of aIF2 from the archaea *Methanococcus jannaschii* [23] were obtained by PCR from total genomic DNA. For the β subunit, the oligonucleotides MJ2A (5'-GGGATCCAGCGACCTTGAAAATATTGAC-3') and MJ2B (5'-GGTCGACATCCTCTATGGTATTTAAATC-3') were used. For the γ subunit, the oligonucleotides MJ3A (5'-GGGATCCCATTTCACAAAATTATATATTG-3') and MJ3B (5'-GGTCGACTTAAAATTATCCCTCAATTGTC-3') were used. The PCR product for the β subunit was digested with *Bam*HI and *Sal*I and ligated into pBluescript SK(+), which had been digested with *Bam*HI and *Sal*I. The γ subunit sequence was cloned directly into the same vector digested with *Eco*RV and poly(T) was added to the extremities. Both inserts were sequenced. The coding sequences were transferred to the two-hybrid system by cloning the *Bam*HI–*Sal*I fragments into pGAD-C1 or pGBD-C1 vectors digested with the same enzymes, creating plasmids pBE340 (aIF2 β fused to GBD), pBE344 (aIF2 β fused to GAD), pBE343 (aIF2 γ fused to GBD) and pBE339 (aIF2 γ fused to GAD). To construct a hybrid β subunit containing the N-terminal region from eIF2 β (amino acids 1–128) and the remaining sequences comprising the complete aIF2 β , a *Hae*III fragment from the *SUI3* gene present in plasmid pBE32 was isolated containing the coding sequences for the initial 128 amino acid residues and all upstream sequences required for the expression of the gene in yeast. This fragment was cloned in the blunt-ended *Xho*I site of plasmid pET14b, creating plasmid pBE325. This plasmid was digested with *Bgl*II, blunt-ended with Klenow and digested with *Bam*HI. The resulting fragment and a *Bam*HI–*Pvu*II fragment from pBE326 were inserted into pSB32 digested with *Hind*III and treated with Klenow to generate plasmid pBE330 coding for the hybrid protein. This hybrid was transferred to pGAD-C1 and pGBD-C1 by isolating the *Bam*HI–*Xba*I fragment from pBE265 and the *Xba*I–*Sal*I fragment from pBE330 and cloning both in pGAD-C1 or pGBD-C1 digested with *Bam*HI and *Sal*I. The resulting plasmids were called pBE347 and pBE348 respectively.

GST-pull down and immunoprecipitation

Cell extracts were prepared from logarithmically growing cells by agitation with glass beads in a buffer containing 10 mM Tris/HCl (pH 7.5), 100 mM NaCl, 30 mM MgCl₂ and 1 mM PMSF. The

purification of the GST fusion proteins from *Escherichia coli* was performed essentially as described previously [17]. For the GST-pull down assays, 2 μ g of purified GST fusion proteins and 100 μ g of whole-cell extract, prepared from strain 167-3C over-expressing the α and γ subunits of eIF2 from the high-copy number plasmid pBE189 carrying the *SUI2* and *GCD11* genes [14], were incubated in a buffer containing 20 mM Tris/HCl (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 7 mM 2-mercaptoethanol, 1 mM PMSF and 0.05 % Igepal CA-630 in a final volume of 200 μ l. Glutathione–Sephadex beads (Amersham Biosciences) were added (20 μ l of a 1:1 suspension in the same buffer) and, after 15 min of incubation at 4 °C, the beads were washed three times with 0.5 ml of PBS, resuspended in 20 μ l of 2 $\times$ Laemmli buffer [40 mM Tris/HCl (pH 6.5), 1 % (w/v) SDS, 5 % (v/v) glycerol and 2.5 % (v/v) 2-mercaptoethanol] and subjected to SDS/PAGE (10 % gels). The proteins were blotted on to a Hybond-C membrane and probed with an antiserum raised against a GST–eIF2 γ fusion protein, followed by incubation with Protein A–peroxidase and visualization by enhanced chemiluminescence (ECL[®]; Amersham Biosciences). For immunoprecipitation assays, antibodies raised against each of the three subunits of eIF2 were adsorbed on to Protein A–Sephadex beads (Amersham Biosciences) for 1 h at 23 °C in buffer containing 20 mM Tris/HCl (pH 7.0), 50 mM KCl, 0.1 % Triton X-100 and 1 mM PMSF in a total volume of 200 μ l. The beads were washed three times with the same buffer and 20 μ l of beads (1:1 suspension) were added to whole-cell extracts (50 μ g of total protein) from the indicated strains prepared in a buffer containing 200 mM Tris/HCl (pH 7.0), 100 mM KCl, 30 mM MgSO₄ and 1 mM PMSF. After incubation at 4 °C for 1 h, the beads were washed three times in the same buffer, resuspended in 20 μ l of 2 $\times$ Laemmli buffer and subjected to SDS/PAGE (10 % gels). The proteins were transferred on to Hybond-C and probed simultaneously with antisera against GST–eIF2 β and GST–eIF2 γ , followed by Protein A–peroxidase and visualization by ECL[®].

RESULTS

Mutations in conserved residues of eIF2 β lead to defects in the interaction with eIF2 γ

Previous results [17] have indicated that the interaction of eIF2 β with eIF2 γ is mediated by the central region of eIF2 β , comprising, at most, residues 128–205 of the *S. cerevisiae* protein, a region where eIF2 β sequences significantly differ from eIF5 (Figure 1). In order to define more precisely this interacting domain, additional deletions of yeast eIF2 β were constructed and assayed for binding to eIF2 γ in two-hybrid assays. As shown in Figure 1, this deletion analysis narrowed down the residues involved in this interaction to a region comprising amino acids 128–159, which was necessary and sufficient to maintain wild-type levels of binding to the γ subunit and suggested that all determinants were found between residues 128 and 140.

To determine the residues responsible for this interaction, we performed a mutational analysis in this region, where both conserved and non-conserved residues in all β subunit sequences were substituted with alanine residues (Figure 1C). The mutant proteins comprising residues 128–285 were tested in two-hybrid (Figure 1C) and GST-pull down (Figure 1D) assays. Substitution of residues that are highly conserved in the β subunits of all species abolished interactions in these two assays (¹³¹YS, ¹³³ELL and ¹³⁴LL¹³⁸F¹⁴¹IL¹⁴⁹L), whereas substitutions of residues that are less conserved (¹⁴¹IL) led to a decrease in the interaction observed in the two-hybrid assay. In the GST-pull down assay, the latter mutation also appeared to affect the association when

Figure 1 Mutations in conserved residues of eIF2 β abolish interaction with eIF2 γ

(A) The relevant region of eIF2 β where mutations were introduced is shown as an alignment of β subunit sequences and the N-terminal region of eIF5 derived from several organisms. S.c., *S. cerevisiae*; H.s., *Homo sapiens*; D.m., *Drosophila melanogaster*; T.a., *Triticum aestivum*; C.e., *Caenorhabditis elegans*; M.j., *M. jannaschii*; A.f., *Archaeoglobus fulgidus*; P.h., *Pyrococcus horikoshii*; and Z.m., *Zea mays*. Conserved residues are indicated in black boxes. The residues in eIF5 that are implicated in its GAP activity [18] are underlined. (B) Two-hybrid assays between eIF2 β sequences fused to GAD and eIF2 γ fused to GBD. The location of the proteins within the complete eIF2 β protein is shown schematically on the left. In the complete protein (residues 1–285), K represents the runs of lysine residues and C, the cysteine residues that form the zinc finger. The black-shaded area represents the region that is conserved in the β subunits of eukaryotes and archaea, whereas the grey-shaded area is the region found exclusively in the eukaryotic β subunits. The numbering corresponds to the amino acid residues included in the proteins. The protein containing residues 128–205 and the protein with residues 96–179 deleted have been described previously [17] and were included as controls. On the right, interactions were detected as growth of PJ69-4A cells transformed with each pair of plasmids on medium containing histidine (+HIS), medium lacking histidine (–HIS) or medium lacking histidine and supplemented with 1 mM 3-aminotriazole (–HIS, +AT). (C) On the left, the mutations introduced in residues 128–153 of the eIF2 β sequence are shown by the arrows. In the bottom sequence, the multiple substitutions made are indicated by dots. On the right, the results of two-hybrid assays with the mutant eIF2 β sequences fused to GAD and eIF2 γ to GBD are shown with growth conditions being the same as in (B). n.s., not shown. (D) *In vitro* interaction of the mutant eIF2 β proteins with eIF2 γ was determined by GST-pull down assays. Whole-cell extract (100 μ g of total protein), prepared from strain 167-3C

Figure 2 Function of eIF2 β mutant proteins

Growth of haploid cells expressing eIF2 β mutated in the interaction domain, indicated by their modified residues (**A**) and expressing the double mutant proteins (eIF2 β mutated in the interaction domain and carrying the SUP mutation $^{264}\text{S} \rightarrow \text{Y}$) (**B**) as the sole source of eIF2 β . These strains are Ura⁺, Leu⁺ ascospore derivatives of the diploid strain BCV59 carrying *LEU2CEN4*-based plasmids expressing the mutant eIF2 β proteins. As controls, ascospore derivatives carrying *LEU2CEN4* plasmids expressing the wild-type *SUI3* gene (WT) or SUP were included. Cell suspensions ($D_{600} = 0.5$) were spotted in three serial 10-fold dilutions on YPD plates and were grown at 23 °C or 37 °C. n.v., non-viable ascospores. L, ^{149}L ; ELL, ^{133}ELL ; IL, ^{141}IL ; YS, ^{131}YS ; LLFILL, $^{134}\text{LL}^{138}\text{F}^{141}\text{IL}^{149}\text{L}$. (**C**) The steady-state protein levels of the ^{131}YS mutant protein (YS) compared with the SUP $^{264}\text{S} \rightarrow \text{Y}$ mutant and wild-type (WT) proteins. Identical number of cells from strains expressing only these forms of eIF2 β from a plasmid, isolated as described in (**A**) and grown overnight at the indicated temperatures, were pelleted and, after boiling in Laemmli buffer, subjected to SDS/PAGE. The proteins were transferred on to Hybond-C and incubated with antibodies directed against hexokinase (HXK) and eIF2 β . The ratio between the hexokinase and the wild-type eIF2 β proteins was given a value of 1 and used as the baseline for the ratios obtained for hexokinase and the mutant eIF2 β proteins. (**D**) The steady-state levels of the lethal double mutants were analysed from Ura⁺, Leu⁺ ascospore-derived strains, isolated as described in (**B**) and also expressing the wild-type protein to maintain cell viability. Cells were grown overnight in minimal SD medium supplemented with uracil and histidine at 23 °C and processed as described in (**C**), except that antibodies against eIF2 γ and eIF2 β were used. The ratios between the two proteins in the double mutants were calculated relative to the ratio obtained for the SUP-expressing strain.

the levels of the pulled-down eIF2 γ were normalized to the amounts of GST fusion proteins bound to the beads. Substitution of a non-conserved residue (^{149}L) did not affect the association in the two-hybrid (results not shown) and GST-pull down assays (Figure 1D), as determined by normalization to the bound fusion protein.

Functional defects of the mutations in eIF2 β

To address the effects of these mutations on the function of eIF2 β *in vivo*, the mutations were transferred to an intact *SUI3* gene in a *LEU2CEN4* plasmid. The mutant *SUI3* alleles were tested initially for their ability to rescue a lethal disruption of the chromosomal *SUI3* gene in ascospore derivatives of strain BCV59. Surprisingly, only the mutant allele involving alterations in six residues ($^{134}\text{LL}^{138}\text{F}^{141}\text{IL}^{149}\text{L}$) resulted in non-viable ascospores, indicating loss of function of the mutant eIF2 β (results

not shown). The other mutations, which in the two-hybrid or GST-pull down assays were non-functional, gave rise to viable cells. However, the ascospores carrying the allele with the ^{131}YS mutation showed a severe defect in germination, resulting in microcolonies (results not shown). To look for other effects of these mutations, the haploid segregants carrying the mutant alleles in the plasmid and the chromosomal disruption of the *SUI3* gene (Ura⁺, Leu⁺ segregants) were tested for growth at 37 °C (Figure 2A). A haploid segregant carrying a plasmid copy of SUP, which carries the mutation $^{264}\text{Ser} \rightarrow \text{Tyr}$ ($^{264}\text{S} \rightarrow \text{Y}$) in the C2-C2 motif and has been shown to impart a high rate of spontaneous GTP hydrolysis to the eIF2 complex [8], was also included as a control. The ^{131}YS mutant showed a severe temperature-sensitive phenotype for growth. Interestingly, the SUP $^{264}\text{S} \rightarrow \text{Y}$ mutant also resulted in a temperature-sensitivity for growth, with this phenotype being most severe on rich medium (see below). As shown in Figure 2(C), the steady-state

overexpressing the eIF2 γ and eIF2 α subunits from the high-copy plasmid pBE189, was incubated with 2 μg of either GST alone or purified GST-eIF2 β fusion proteins, comprising the complete wild-type β protein (WT), residues 128–285 of the wild-type β protein ($\text{wt}^{128-285}$) or the mutant proteins ^{149}L (L), ^{133}ELL (ELL), ^{141}IL (IL), ^{131}YS (YS) and $^{134}\text{LL}^{138}\text{F}^{141}\text{IL}^{149}\text{L}$ (LLFILL.L.L), and glutathione-Sepharose beads. Sepharose-bound material and whole-cell extract alone (WCE; 10 μg of total protein) were subjected to SDS/PAGE, followed by immunoblotting using antibodies raised against a GST-eIF2 γ fusion, which also recognize the GST-eIF2 β proteins. In the right-hand panel, an independent set of GST-pull down assays is shown with the GST-eIF2 $\beta^{128-285}$ ($\text{wt}^{128-285}$) and ^{149}L mutated proteins to ascertain whether this mutation alters the association with eIF2 γ . The arrowheads indicate GST and GST fusion proteins, whereas the arrows indicate pulled-down eIF2 γ and eIF2 γ present in the whole-cell extract.

Figure 3 Association defect of the ¹³¹YS and ¹³³ELL mutant proteins

eIF2 subunits were immunoprecipitated from whole-cell extracts, prepared from ascospore-derived strains grown at 23 °C, isolated as described in Figure 2, expressing the wild-type eIF2 β protein (WT), ¹³¹YS (YS) or ¹³³ELL (ELL) mutant proteins. Whole-cell extracts (50 μ g of total protein) were incubated with antibodies against eIF2 α , eIF2 β or eIF2 γ and bound to Protein A–Sepharose beads as indicated. The bound material (P) and one-tenth of the unbound material left in the supernatant (S) were subjected to Western blot using sera directed against GST–eIF2 β and GST–eIF2 γ . In, input (one-tenth of the whole-cell extract used in the assays). n.s., immunoprecipitation using non-specific antiserum.

level of the ¹³¹YS mutant protein was similar to the SUP²⁶⁴S $\rightarrow$ Y mutant protein in cells grown at 23 °C and 37 °C, both being somewhat lower than the wild-type eIF2 β . Thus these results indicate that the temperature-sensitivity for growth does not appear to be due to extensive degradation of these two proteins at 37 °C.

The ability of the ¹³¹YS mutant protein to form an eIF2 complex *in vivo* was also determined by co-immunoprecipitation assays. Extracts from the haploid segregants carrying only the plasmid-borne wild-type *SUI3* or the ¹³¹YS mutant allele of *SUI3* were prepared from cells grown at 23 °C and incubated with antibodies directed against each of the three subunits of eIF2. Protein A–Sepharose-bound material was detected by Western blotting with sera directed against the β and γ subunits. As shown in Figure 3, the ¹³¹YS mutation led to a drastic reduction in the β subunits associated with the complex, as detected in the immunoprecipitates with the anti-(eIF2 α) and anti-(eIF2 γ) sera, with most of the mutant protein remaining in the supernatant fractions. When compared with the wild-type eIF2 complexes, only 10–15% of the mutant eIF2 β was associated with the other two subunits. In the immunoprecipitations using the anti-(eIF2 β) antibodies, no eIF2 γ was detected associated with the mutant protein. In this case, it is possible that the anti-(eIF2 β) antibodies may have displaced the γ subunit from an unstable complex. These results thus provide further support for the weakened association caused by this mutation. The ¹³³ELL mutation, although not imparting a growth defect, also showed a similar defect in association with eIF2 γ , as determined by co-immunoprecipitation with anti-(eIF2 β) antibodies, thus reflecting the results of the two-hybrid and GST-pull down assays. Using antibodies directed against the α subunit, it appeared that more ¹³³ELL mutant protein was detected forming the complex when compared with the ¹³¹YS mutation, perhaps explaining their phenotypic differences. Considering the conservation of these residues (Figure 1) and the nature of the substitutions introduced,

it is expected that the alterations in ¹³¹YS should be functionally more deleterious than those involving ¹³³ELL.

Weakened interaction between eIF2 β and eIF2 γ results in a recessive suppressor phenotype

Previous reports [11,12] suggesting that eIF2 β is in close proximity with both GTP and Met-tRNA^{Met} and the apparent inability of eIF2 γ to bind these molecules on its own indicate that the β subunit might stabilize the ternary complex. Given that a premature release of Met-tRNA^{Met}, due to either increased rates of dissociation of the tRNA or spontaneous GTP hydrolysis is correlated with initiation events at non-AUG codons, as shown for the suppressor mutations characterized so far in eIF2 γ and eIF2 β respectively [8], it is likely that a weakened association of eIF2 β with eIF2 γ might result in instability of the ternary complex and thus in suppression of initiator codon mutations. In order to test this possibility, the ascospore derivatives of strain BCV59 carrying the mutant alleles as the sole source of eIF2 β , isolated as described above, were tested for growth in the absence of histidine, with the derivative expressing the suppressor (SUP)²⁶⁴S $\rightarrow$ Y mutant protein as a control (Figure 4A). Strain BCV59 carries in both copies of the *HIS4* gene the mutation that alters the initiation codon AUG to UUG, and thus all ascospore derivatives are unable to grow in the absence of histidine (His[−] phenotype). Therefore the presence of a suppressor form of eIF2 β in these derivatives should result in growth in medium lacking histidine (His⁺ phenotype). Also, from the same dissection, Ura[−], Leu⁺ segregants carrying both the wild-type chromosomal *SUI3* allele and the plasmid-borne mutant alleles were tested for their His phenotype. The ¹³¹YS mutation, which abolished interaction *in vitro* and resulted in a temperature-sensitive phenotype *in vivo*, indicating a weakened interaction with eIF2 γ , led to a suppressor phenotype (His⁺), as determined by the growth of these cells on media lacking histidine. This phenotype was recessive, as determined by the inability of cells carrying also a wild-type copy of *SUI3* to grow in the absence of histidine (results not shown). Since this protein is expressed at the same levels as the SUP²⁶⁴S $\rightarrow$ Y mutant protein, which has a co-dominant suppressor phenotype, these results give further support for an *in vivo* impairment of the association of the ¹³¹YS mutant protein with eIF2 γ .

Combination of two suppressor mutations in eIF2 β leads to recessive lethality

If the recessive suppressor phenotype of the ¹³¹YS mutation does indeed lead to a defect in the maintenance of Met tRNA^{Met} bound to eIF2, either through an increased dissociation of the tRNA, independent of GTP hydrolysis, or through an increased spontaneous GTP hydrolysis, it is possible that this mutation would be lethal in conjunction with the suppressor mutation ²⁶⁴S $\rightarrow$ Y, which maps to the C2–C2 region. To test this, the mutation derived from the *SUI3*-2 allele was introduced in all of the mutants constructed in the region that binds eIF2 γ , and these double mutants were tested for both maintenance of cell viability in ascospores of strain BCV59 and the suppressor phenotype. The combination of the two suppressor mutations led to non-viable ascospores when this double mutant allele was the sole source of eIF2 β in the cells (results not shown). With the exception of the combination with the ¹³⁴LL¹³⁸F¹⁴¹IL¹⁴⁹L mutant, which was lethal on its own, the other double mutants were functional (Figure 2B). The ¹³³ELL, ¹⁴¹IL and ¹⁴⁹L mutations,

Figure 4 Suppressor phenotypes

Growth in the presence (+HIS) or absence of (−HIS) histidine and at different temperatures of the Ura⁺, Leu⁺ ascospore derivatives expressing eIF2 β mutated in the interacting domain (**A**) or the double mutant proteins (eIF2 β mutations in the interacting domain and the SUP mutation ²⁶⁴S → Y) (**B**), as shown in Figures 2(**A**) and 2(**B**) respectively. Cells were grown as described in Figure 2. n.v., non-viable ascospores. (**C**) Growth in the presence (+HIS) or absence (−HIS) of histidine and at various temperatures of Ura⁺, Leu⁺ ascospore derivatives expressing the double mutant proteins and the chromosomal wild-type copy of *SUI3*.

which had no previously detectable phenotype *in vivo*, when combined with the ²⁶⁴S → Y mutation in the same protein appeared to abolish or alleviate the temperature-sensitivity of the latter for growth (Figure 4B). It is possible that the mutations in the interacting region might provide an increased stability for the

proteins containing the ²⁶⁴S → Y mutation. These double mutants were capable of expressing the suppressor phenotype associated with ²⁶⁴S → Y at 23 °C; however, at higher temperatures, the ¹³³ELL mutation eliminated the suppressor phenotype. The presence in the same cells of the wild-type protein in conjunction

Figure 5 Interactions mediated by the *M. jannaschii* aIF2 subunits

The *M. jannaschii* β and γ subunits were tested in two-hybrid assays in the presence (+HIS) or absence (−HIS) of histidine for interactions with the eukaryotic subunits. *hybβ* indicates the protein containing 128 amino acids from the N-terminal region of the yeast eIF2 β and the remaining sequences from aIF2 β . The β proteins were fused to the GAD domain and the γ subunits to the GBD domain of Gal4. Growth conditions were essentially those described in Figure 1.

with the double mutants abolished suppression in all cases, except for the ¹⁴⁹L mutation, which did not alter the co-dominance of the ²⁶⁴S → Y mutation (Figure 4C). These phenotypes are consistent with the observed behaviour of the mutant proteins in the GST-pull down or two-hybrid assays, suggesting that the amino acid alterations affect the binding of the proteins to the γ subunit *in vivo*, and thus they would be out-competed by the wild-type version of eIF2 β in the formation of the eIF2 factor.

The steady-state levels of the non-functional double mutant proteins (¹³¹YS-SUP and ¹³⁴LL¹³⁸F¹⁴¹IL¹⁴⁹L-SUP) were determined by quantitative Western blots from cells grown in minimal media carrying the lethal mutations along with the chromosomally encoded wild-type protein (Ura[−], Leu⁺ ascospore-derived strains) (Figure 2D). No significant degradation was detected at 23 °C when compared with the SUP ²⁶⁴S → Y mutant protein in the presence of the wild-type protein, suggesting therefore that the lethality is due to impaired function, rather than to low levels of the proteins.

The archaeal β subunit associates with eIF2 γ and aIF2 γ

Given the degree of divergence found in the sequence of the archaeal β subunit around the region identified in the present study to mediate the binding to the γ subunit, we decided to determine if this protein was capable of interacting with eIF2 γ , thus defining any role of the less-conserved residues in this region in maintaining the interaction with the γ subunit. This approach would also be relevant in addressing the question of whether the sequence annotated in the genomic sequencing of these organisms was truly a subunit of aIF2. Since Archaea seem to lack an orthologue of eIF5, it is possible that this sequence is that of a potential eIF5-like protein and not a subunit of aIF2. Thus the sequences encoding the β and γ subunits of aIF2 were cloned from *M. jannaschii* and initially tested for interaction with the eukaryotic subunits using two-hybrid assays. As shown in Figure 5, the aIF2 β sequence was able to interact with eIF2 γ , thus providing further support for the role of the conserved residues mapped previously in eIF2 β , and especially the tyrosine residue, in maintaining the interaction with eIF2 γ . However, no interaction was detected with aIF2 γ in either orientation of the system (results not shown). A hybrid protein was then constructed containing the N-terminal region derived from eIF2 β (residues

1–128) and the remaining sequences derived from the complete archaeal β subunit. This protein was now capable of binding to the archaeal γ subunit (Figure 5). These data suggest that this protein is a *de facto* subunit of an archaeal trimeric complex and not a GAP-like eIF5. We employed this hybrid protein further to test its ability to function in yeast by rescuing the lethal disruption of *SUI3*. The results indicated that cells carrying this hybrid molecule were not viable and, when present alongside the wild-type eIF2 β protein, did not lead to a suppressor phenotype (results not shown).

DISCUSSION

In the present study, we have determined the domain of eIF2 β that mediates its association with the γ subunit and have identified residues in this region that are involved in binding eIF2 γ . We also showed that a weakened association between these two subunits caused by the substitution of ¹³¹Tyr and ¹³²Ser by alanine residues resulted in translation initiation at a non-AUG codon. The serine residue is not conserved in the β subunit sequences of organisms shown in the sequence alignment (Figure 1A). On the other hand, the tyrosine residue is found in all β subunit sequences and must therefore be a major determinant of this interaction, since its substitution by alanine imposes severe defects to the protein and, moreover, to cells expressing this mutant form of eIF2 β . The ¹³³ELL mutation resulted in similar biochemical defects, but no growth impairment or non-AUG initiation events were detected. We have detected a slightly improved ability of this protein to form an eIF2 complex, as compared with the ¹³¹YS mutant protein, which could account for the differences in phenotypes. *In vivo*, an association defect of the ¹³³ELL mutant protein was obvious from the genetic analysis. It is possible that the two leucine residues are required for conferring a hydrophobic environment favouring subunit association and their substitution by alanine residues, by not changing the characteristic hydrophobic nature of the region, still allowed this interaction to occur *in vivo* at levels similar to those of the wild-type protein. This interaction may also have been stabilized further by other factors. In contrast, the tyrosine to alanine substitution, by changing both the chemical nature and the structure of the residue, had more drastic effects. Thus the residues mutated here are clearly involved in maintaining the association with the γ subunit.

On the basis of the *in vitro* results, it is surprising that these proteins still allow the survival of the cells. A possible interpretation is that, in intact cells, the two subunits might be able to maintain their stable association by virtue of their interactions with other components of the initiation complex. However, the maintenance of the mutant β subunit associated with the γ subunit through indirect interactions must be very weak, since in the immunoprecipitation assays the subunits dissociate very easily. An alternative explanation to account for the apparent discrepancy between the viability results of the ¹³¹YS and ¹³³ELL mutants and the co-immunoprecipitation data is that, in the latter assay, a larger complex containing the associated subunits may not be able to be precipitated, either because it is not recognized by the antibodies, due to a masking of the subunits by other components of the translational apparatus, or because it is too large and thus not stably bound to the antibody–Protein A–Sepharose bead complex. Thus only the eIF2 molecules that are not associated with this larger complex are detected bound to the beads. This interpretation also requires the binding of the mutant eIF2 β and eIF2 γ to be stabilized by the interaction with other factors. The results shown in the present study for these mutations suggest that the binding between the two subunits

may not be as tight as previously thought and implied for this trimeric factor. It is not known, however, how much of the subunits are indeed associated at all times in the cells. For several reasons this might be difficult to assess experimentally. First, in the past, the factor, was isolated on the basis of its activity of GTP-dependent Met-tRNA^{Met} binding, which requires the two subunits. Secondly, more recently, it has been isolated by using tags to one of its subunits, usually from cells overexpressing all three subunits, a condition that does not reflect their natural status in the cells. Finally, because the subunits interact with several other participants of translation initiation, including the ribosomes, the determination of the extent of their direct constant association by co-immunoprecipitation assays cannot be addressed accurately.

In a previous deletion analysis from our laboratory [17], a protein comprising residues 1–186 was not able to interact with eIF2 γ in the presence of aminotriazole at levels detectable in the two-hybrid assay, whereas, in the present study, we determined that the protein comprising residues 128–159 was sufficient for maintaining this strong interaction. Because the mutational and *in vivo* data support the notion that this short region contains the necessary determinants for interaction with eIF2 γ , the reason for the non-interaction of residues 1–186 is not clear. However, we have observed that the strength of the His⁺ phenotype in the two-hybrid assays involving only the central portion of eIF2 β was always stronger than that of the complete protein and of most constructs containing large portions of eIF2 β . It is likely that the binding of other protein factors to eIF2 β , such as eIF5 and eIF2B, might inhibit the migration of the GAD fusion protein to the nucleus in the two-hybrid assay. In this scenario, the central minimal-binding region described in the present study would not be capable of other interactions and would thus result in a strong His⁺ phenotype in the two-hybrid assays. However, instability problems with some of the fusion proteins cannot be ruled out.

The residues in eIF2 β found in the present study to mediate its interaction with eIF2 γ are substituted in eIF5 for residues that are implicated in its GAP activity [18]. The conservation of eIF2 β and eIF5 sequences begins immediately after the minimal segment of eIF2 β mapped in the present study as necessary for its binding to eIF2 γ . Thus it is clear that eIF5 cannot bind to eIF2 γ , at least at levels comparable with eIF2 β . Indeed, no association between these two proteins has been detected employing several different approaches, such as two-hybrid [17] and overlay [24] assays. However, the possibility that they might interact transiently *in vivo* cannot be excluded. The β subunit is present early in evolution, as a similar sequence is found in Archaea and, as we have shown in the present study, it seems to be a *de facto* subunit of aIF2. The observation that this protein was capable of interacting with eIF2 γ , given that it differs in several positions, including the presence of a small gap, gives strong support for the conclusion that the residues identified in this analysis for eIF2 β are indeed the major points of contact with the γ subunit, since they are conserved in aIF2 β .

The suppressor mutations mapping in eIF2 have been shown previously to be the result of different defects [8]. The eIF2 γ Asn¹³⁵Lys mutant shows a somewhat increased intrinsic GTP hydrolysis, as the use of the non-hydrolysable GTP analogue GppNp stimulates binding of the initiator tRNA. However, this same study suggested that this mutation results in an increased dissociation rate of the initiator tRNA from the complex. On the other hand, the SUP mutation ²⁶⁴S → Y in eIF2 β seems to result in an increased intrinsic GTPase activity, although also showing a small defect in tRNA binding in the presence of the non-hydrolysable GTP analogue. Therefore two defects might be associated with non-AUG initiation events,

both leading to the premature release of the initiator tRNA at wrong codons: (a) increased GTPase activity independent of eIF5; and (b) increased spontaneous dissociation rates of the initiator tRNA independent of GTP hydrolysis. A simple tRNA-binding defect may not be entirely sufficient to allow translation at non-AUG codons, since the mutation in eIF2 γ changing residue ¹⁴²Tyr → His causes a dramatic reduction in tRNA binding, but does not have a suppressor phenotype [10]. Furthermore, the observations that the binding of the tRNA provides stability for GTP suggest that these two events may be intrinsically linked in the correct recognition of the start site for translation in eukaryotes. Thus, due to a defective interaction, the suppressor phenotype of the ¹³¹YS mutation could be the result of either one of the two events or both. The combination of the ¹³¹YS and the ²⁶⁴S → Y suppressor mutations resulted in lethality to the cells. Since this protein was as stable as either one of the two proteins containing the independent mutations, it seems that the defect is functional and additive, perhaps causing a high rate of non-AUG initiation events incompatible with cell viability. Alternatively, this protein might form a ternary complex that is too unstable to support any translation initiation. Unfortunately, we were unable to purify the eIF2 complex containing the ¹³¹YS mutation for *in vitro* binding assays, since the subunits fall apart rather easily, as can be inferred from the results shown in the present study. Nevertheless, the suppressor phenotype directly points to an *in vivo* role for the eIF2 β subunit in maintaining the stability of the initiator tRNA bound in the ternary complex, a function that has been biochemically inferred *in vitro* from the requirement of eIF2 β in the GTP-dependent binding by the purified eIF2. It will certainly be important to determine whether eIF2 β contacts the tRNA and/or GTP or if it only stabilizes the structure of eIF2 γ for this to bind the two molecules.

The results shown in the present study, besides defining structural elements of eIF2 β that mediate its interaction with eIF2 γ , also provide *in vivo* evidence that the strength of the interaction of eIF2 β with eIF2 γ determines the correct utilization of AUG as an initiator codon in eukaryotes, probably by stabilizing the GTP/Met-tRNA^{Met} bound in the ternary complex.

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REFERENCES

- 1 Hershey, J. W. B. and Merrick, W. C. (2000) Pathway and mechanism of initiation of protein synthesis. In *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds.), pp. 33–88, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- 2 Hinnebusch, A. G. (2000) Mechanism and regulation of initiator methionyl-tRNA binding to ribosomes. In *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds.), pp. 185–243, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- 3 Pestova, T. V., Lomakin, I. B., Lee, J. H., Choi, S. K., Dever, T. E. and Hellen, C. U. T. (2000) The joining of ribosomal subunits in eukaryotes requires eIF5B. *Nature* (London) **403**, 332–335.
- 4 Donahue, T. F., Cigan, A. M., Pabich, E. K. and Castilho-Valavicius, B. (1988) Mutations at a Zn(II) finger motif in the yeast eIF-2 β gene alter ribosomal start-site selection during the scanning process. *Cell* (Cambridge, Mass.) **54**, 621–632.
- 5 Cigan, A. M., Pabich, E. K., Feng, L. and Donahue, T. F. (1989) Yeast translation initiation suppressor *sui2* encodes the α subunit of eukaryotic initiation factor 2 and shares sequence identity with the human α subunit. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2784–2788.

- 6 Castilho-Valavicius, B., Yoon, H. and Donahue, T. F. (1990) Genetic characterization of the *Saccharomyces cerevisiae* translational initiation suppressors *sui1*, *sui2* and *SUI3* and their effects on *HIS4* expression. *Genetics* **124**, 483–495
- 7 Dorris, D. R., Erickson, F. L. and Hannig, E. M. (1995) Mutations in *GCD11*, the structural gene for eIF-2 γ in yeast, alter translational regulation of *GCN4* and the selection of the start site for protein synthesis. *EMBO J.* **14**, 2239–2249
- 8 Huang, H., Yoon, H., Hannig, E. M. and Donahue, T. F. (1997) GTP hydrolysis controls stringent selection of the AUG start codon during translation initiation in *Saccharomyces cerevisiae*. *Genes Dev.* **11**, 2396–2413
- 9 Erickson, F. L., Nika, J., Rippel, S. and Hannig, E. M. (2001) Minimum requirements for the function of eukaryotic translation initiation factor 2. *Genetics* **158**, 123–132
- 10 Erickson, F. L. and Hannig, E. M. (1996) Ligand interactions with eukaryotic translation initiation factor 2: role of the γ -subunit. *EMBO J.* **15**, 6311–6320
- 11 Bommer, J. A., Kraft, R., Kurzchalia, T. V., Price, N. T. and Proud, C. G. (1991) Amino acid sequence analysis of the β - and γ -subunits of eukaryotic initiation factor eIF-2. Identification of regions interacting with GTP. *Biochim. Biophys. Acta* **1079**, 308–315
- 12 Gaspar, N. J., Kinzy, T. G., Scherer, B. J., Humbelin, M., Hershey, J. W. and Merrick, W. C. (1994) Translation initiation factor eIF-2. Cloning and expression of the human cDNA encoding the γ -subunit. *J. Biol. Chem.* **269**, 3415–3422
- 13 Asano, K., Krishnamoorthy, T., Phan, L., Pavitt, G. D. and Hinnebusch, A. G. (1999) Conserved bipartite motifs in yeast eIF5 and eIF2B ϵ , GTPase-activating and GDP-GTP exchange factors in translation initiation, mediate binding to their common substrate eIF2. *EMBO J.* **18**, 1673–1688
- 14 Laurino, J. P., Thompson, G. M., Pacheco, E. and Castilho, B. A. (1999) The β subunit of translation initiation factor eIF2 binds mRNA through the lysine repeats and a region comprising the C₂–C₂ motif. *Mol. Cell. Biol.* **19**, 173–181
- 15 Castilho-Valavicius, B., Thompson, G. M. and Donahue, T. F. (1992) Mutation analysis of the Cys-X₂-Cys-X₁₉-Cys-X₂-Cys motif on the β subunit of eukaryotic translation initiation factor 2. *Gene Expression* **2**, 297–309
- 16 Kimball, S. R., Heinzinger, N. K., Horetsky, R. L. and Jefferson, L. S. (1998) Identification of interprotein interactions between the subunits of eukaryotic initiation factors eIF2 and eIF2B. *J. Biol. Chem.* **273**, 3039–3044
- 17 Thompson, G. M., Pacheco, E., Melo, E. O. and Castilho, B. A. (2000) Conserved sequences in the β subunit of archaeal and eukaryal translation initiation factor 2 (eIF2), absent from eIF5, mediate interaction with eIF2 γ . *Biochem. J.* **347**, 703–709
- 18 Das, S., Ghosh, R. and Maitra, U. (2001) Eukaryotic translation initiation factor 5 functions as a GTPase-activating protein. *J. Biol. Chem.* **276**, 6720–6726
- 19 Paulin, F. E., Campbell, L. E., O'Brien, K., Loughlin, J. and Proud, C. G. (2001) Eukaryotic translation initiation factor 5 (eIF5) acts as a classical GTPase-activator protein. *Curr. Biol.* **11**, 55–59
- 20 Guthrie, C. and Fink, G. R. (1991) *Guide to Yeast Genetics and Molecular Biology*, Methods in Enzymology, vol. 194, Academic Press, San Diego
- 21 James, P., Halladay, J. and Craig, E. A. (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144**, 1425–1436
- 22 Smith, D. B. and Johnson, K. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**, 31–40
- 23 Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., FitzGerald, L. M., Clayton, R. A., Gocayne, J. D. et al. (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* (Washington, D.C.) **273**, 1058–1073
- 24 Das, S., Maiti, T., Das, K. and Maitra, U. (1997) Specific interaction of eukaryotic translation initiation factor 5 (eIF5) with the β -subunit of eIF2. *J. Biol. Chem.* **272**, 31712–31718

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