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# Suppressors of translation initiation defect in *hem12* locus of *Saccharomyces cerevisiae*✪

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**A system for the positive selection of transational initiation suppressors in *S. cerevisiae* has been developed. A mutant with an ATA initiation codon in the *HEM12* gene, encoding uroporphyrinogen decarboxylase, was used to select *cis*- and *trans*-acting suppressors. These suppressors partially restore growth on nonfermentable carbon sources, such as glycerol, but still allow the accumulation of porphyrins. All extragenic suppressors are mapped to the *SUI1* locus, encoding initiation factor eIF1. The effect of the *hem12* mutation is also partially reversed by the known *SUI3* suppressor encoding the $\beta$ subunit of eIF2. In contrast, the *sui2* suppressor encoding the $\alpha$ subunit of eIF2 does not affect the *hem 12* phenotype. The intragenic suppressors are able to restore the translation of *hem12* due to the generation of additional, in frame AUG codons upstream of the *hem12-14* mutation.**

**Mutational analysis of the *HEM12* leader sequence was also performed to determine the role of small open reading frames (uORFs) present upstream of the *HEM12* ORF. Studies on the expression of integrated *hem12-1/4-lacZ* fusion, devoid of all upstream ATGs, indicate a lack of regulatory effect of uORFs on *HEM12* translation.**

The initiation of protein synthesis in eukaryotic cells is a complex process that requires multiple initiation factors (eIFs) to stimulate the binding of mRNA and methionyl-initiator tRNA (Met-tRNA$_i$) to 40S ribosomes to form the 43S preinitiation complex [1]. The Met-tRNA$_i$ is delivered to 40S ribosomes in a ternary complex with eIF2 and

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**Abbreviations:** eIF, eukaryotic initiation factor; Met-tRNA$_i$, methionyl-initiator tRNA; ORF, open reading frame; SDS/PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; uOFR, upstream ORF.

GTP and binding of the 5′ end of mRNA to ribosomes is stimulated by eIF4F, eIF4A and eIF4B. The 43S preinitiation complex then scans the leader region of mRNA for the first downstream AUG codon that is a start site for translation in the majority of eukaryotic mRNAs. Once the AUG codon is found, eIF5 stimulates the hydrolysis of GTP bound to eIF2, the initiation factors are released and the 60S subunit joins the 40S subunit to form the 80S initiation complex and elongation of the peptide chain begins [1].

Eukaryotic ribosomes normally only select AUG codons as the start site for translation whereas prokaryotic translation can start by using alternative codons, such as GUG and UUG [2]. In yeast, each possible mutation of AUG abolishes initiation of translation of *HIS4* mRNA [3]. Suppressor mutations in three genes called *SUI1*, *SUI2*, *SUI3* were isolated that restore the $His^+$ phenotype of the *his4* mutant despite the absence of the AUG initiator codon [4]. The *SUI1* gene product encodes a translation factor corresponding to the mammalian homolog, eIF1 [5, 6]. It copurifies with eIF3 and plays a role in translational accuracy [7, 8]. *SUI2* and *SUI3* encode the $\alpha$ and $\beta$ subunits of the eIF2 complex, respectively [9, 10]. Mutations in the structural gene for eIF2$\gamma$ also influence the selection of the start site for protein synthesis [11]. Thus, eIF1 and eIF2 control the recognition of the start codon by the ribosome and they influence the functioning of $Met\text{-}tRNA_i$ that directs the scanning ribosome to the start site [12].

In many eukaryotic genes the first AUG in the mRNA sequence is not the translational start site of the main open reading frame (ORF). According to the most recent estimates, there are a few hundred genes in *S. cerevisiae* that have one or more small upstream ORFs (uORFs) that precede the main ORF [13]. The uORFs usually inhibit, but sometimes stimulate, downstream translation [2, 13]. A major paradigm of eucaryotic translation regulation *via* uORFs is the *GCN4* system of *S. cerevisiae* [14]. eIF2 plays an important role in this regulation.

The *HEM12* gene encodes uroporphyrinogen decarboxylase (Hem12p) [15], the fifth enzyme of the heme biosynthesis pathway [16]. Molecular analysis of our collection of *hem12* mutants revealed that the *hem12-14* allele contains a mutation in the translation start codon ATG→ATA. This causes a lack of detectable Hem12p whereas a normal amount of *hem12* mRNA is present [17]. This defect results in the lack of growth of cells on media containing a nonfermentable carbon source, such as glycerol ($gly^-$ phenotype) and the accumulation of large amounts of porphyrins in the cell. Porphyrins are easily observed because of the red fluorescence ($rf^+$ phenotype) of cells under UV light [18].

In this report, we describe the isolation and characterization of extragenic and intragenic suppressors that can partially suppress the $gly^-$ phenotype of *hem12-14* mutant. Mutational analysis of the *HEM12* gene was also initiated to determine if uORFs present in the *HEM12* leader sequence have a regulatory effect on *HEM12* expression.

## MATERIALS AND METHODS

### Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this study were: BJ4627 [17]; AH201, S150-2B and WS17-5D/hem12Δ [19]; 117-8AR4, 117-8AR20 and 117-1AR7 [10]; TZ21A *MAT***a** *trp1 leu2-3,118 hem12-14*; TZ21C *MATα trp1 ura3 hem12-14*; TZ21A/21C *MAT***a**/*MATα trp1/trp1 leu2-3,118/LEU2 URA3/ura3 hem12-14/hem12-14*. Other yeast strains were derived from these, as described in the text.

Yeast transformation was performed by the lithium acetate method [20]. The transformants were recovered on glucose selective medium and the phenotype of the strains tested on YPGly medium [21]. For biochemical analysis, cells were grown at 28°C in YPG

medium, supplemented with heme (15 mg/liter) or Tween 80 (0.2%) and ergosterol (30 mg/liter) for heme deficient mutants. For $\beta$-galactosidase assays, cells were grown in a selective medium containing 2% ethanol and 0.5% glucose. The X-gal indicator medium was prepared as previously described [22].

To isolate revertants, strains TZ21A or TZ21A/21C were plated on YPGly medium at approximately $10^7$ cells/plate. Spontaneous revertants that fluoresce under UV at 366 nm were isolated after 3–4 days of incubation at 28°C.

### DNA preparation and manipulations

*Escherichia coli* strain DH5$\alpha$ and standard protocols were employed for DNA preparation, cloning and propagation [25]. Yeast plasmid DNA for the transformation of *E. coli* was isolated as described by Rose *et al.* [21]. All PCR amplifications were carried out with *Taq*I polymerase (Promega). DNA probes were radiolabeled by the random priming method with [$\alpha$-$^{32}$P]dCTP (Amersham) using a kit from Boehringer. DNA sequencing was performed using an automatic ALF sequencer (Pharmacia).

### Construction of yeast genomic library

Genomic DNA was isolated from RFR11-1A [21] and partially digested with endonuclease *Sau*3A to yield a maximum of fragments in the 6–10 kb range. The purified fragments were cloned into the *Bam*HI site of the shuttle vector YCp50 [23]. The resulting plasmid pools were used to transform *E. coli* by electroporation. After propagation on plates, plasmid DNA was extracted by alkaline lysis. Genomic DNA from other *RFR* mutants was digested with *Sph*I and *Hin*dIII and fractionated by agarose gel electrophoresis. DNA fragments of approximately 2–3 kb in length were isolated and inserted into pUC18. Plasmids containing the *HEM12* inserts were detected by *in situ* hybridization to a digoxigenin-labeled *HEM12* probe using DIG DNA Labeling and Detection Kit (Boehringer).

### Mutagenesis of *HEM12* leader sequence

The ATG initiation codons of the *HEM12* uORFs were mutated by PCR mutagenesis. The *Sac*I–*Eco*RV *HEM12* fragment, encompassing 898 bp of the promoter and the first 243 bp of the coding region [15], was cloned into pBluescript KS and served as a template for reverse PCR amplification. In uORF1, the CAATGT (–279 to –274) sequence was replaced by AGATCT, introducing a *Bgl*II restriction site. In uORF2 the ATGAGG (–212 to –207) sequence was replaced by CCCGGG, introducing a *Sma*I site. In uORF3 the GAAATG (–184 to –179) sequence was replaced by GAATTC, introducing an *Eco*RI site. In uORF4 the GTGATG (–146 to –141) sequence was replaced by CTGCAG, introducing a *Pst*I site. Two specific primers were used in the first PCR reaction, in which ATG codons of uORF2,3,4 were changed. The PCR product was cut with *Eco*RI, ligated and transformed into *E. coli*. Isolated plasmids, verified by restriction analysis, were used as a template in the second reverse PCR reaction to mutate uORF1. The PCR product was cut with *Bgl*II and ligated, giving pBShem12-1/4. The *Sph*I–*Eco*RV 1116-bp fragment from this plasmid was inserted into the integrative *lacZ* fusion vector YIp358R [24] between *Sph*I and *Sma*I sites. The resulting plasmid, YIphem12-1/4, was integrated into the *URA3* locus of the S150-2B strain. Transformants were tested for $\beta$-galactosidase activity on X-gal indicator medium and in cell-free extracts as described [21].

### RNA isolation and analysis

Total RNA (20 $\mu$g) was isolated as described [19] and fractionated by electrophoresis, transferred to nylon membranes and hybridized with radiolabelled probes by standard protocols [25]. The *HEM12* probe was the 1.27

kb PCR product [17]. The same blot was hybridized with the *Xho*I–HindIII fragment of the *ACT1* gene encoding actin for internal control of the amount of RNA loaded onto the gels. The autoradiograms were quantified by densitometry with an LKB UltroScan XL.

### Low temperature spectra of whole cells and determination of porphyrin concentrations

Low temperature spectra of whole cells and porphyrins accumulated in the cells and excreted into the growth medium were determined as described previously [26].

### Immunodetection of Hem12p

Total proteins (40 $\mu$g) extracted from yeast cells [27] were resolved by SDS-PAGE and transferred to nitrocellulose membranes. An alkaline phosphatase-coupled secondary antibody (Promega) was used to detect the anti-Hem12p antibody [28].

## RESULTS

### Isolation of external suppressors of *hem12-14*

Spontaneous reversion analysis was performed with the *hem12* yeast strain with the ATA initiator codon. $Gly^+$ colonies that fluoresce under UV light ($rf^+$) were identified. This phenotype reflects partial Hem12p deficiency. The true revertants, ATA to ATG, have $gly^+$ $rf^-$ phenotype, as does the wild type strain. The frequency of spontaneous reversion to $gly^+$ $rf^+$ was $5 \times 10^{-6}$, compared to the $1 \times 10^{-9}$ frequency of spontaneous reversion to the wild type. Twenty $gly^+$ $rf^+$ revertants were isolated, they are all recessive (Fig. 1) and constitute one complementation group, *rfr1* (for red fluorescent revertant). The *hem12-14 rfr1* suppressor strains accumulate less porphyrins than the *hem12-14* mutant, as determined by low temperature spectra of whole cells and by porphyrin extraction analysis (not shown). A detailed genetic analysis of one revertant *hem12-14 rfr1-1* confirmed that *rfr1* is a single second site suppressor mutation and this strain was used in further studies.

### The *rfr1* is allelic to *sui1* and *hem12-14* is not suppressed by *sui2-1*

To compare the properties of the *rfr1* mutation to known translation initiation suppressors, recessive *sui1, sui2* and dominant *SUI3,* genetic and biochemical analyses were performed. The strain *hem12-14 rfr1* was crossed to strains 117-8AR4 *sui1-1,* 117-8AR20 *sui2-1* and 117-1AR7 *SUI3-3* bearing *sui* and *his4-303* (ATT) mutations and the integrated *ura3-52::his4* (AUU)-*lacZ* reporter fusion containing ATT mutation at the *HIS4* translation start codon. Analysis of diploid progeny indicated that *rfr1* suppresses the *his4-303* mutation ($His^+$ phenotype) and allows the expression of the *his4-lacZ* fusion as determined by the $\beta$-galactosidase activity test. The efficiency of suppression of *his4-lacZ* by *rfr1* was similar to *sui1-1* and *sui2-1*. Tetrad analysis also showed that *rfr1* is allelic to *sui1* and segregates independently of *sui2* and *SUI3*. *rfr1* does not have a temperature sensitive pheno-

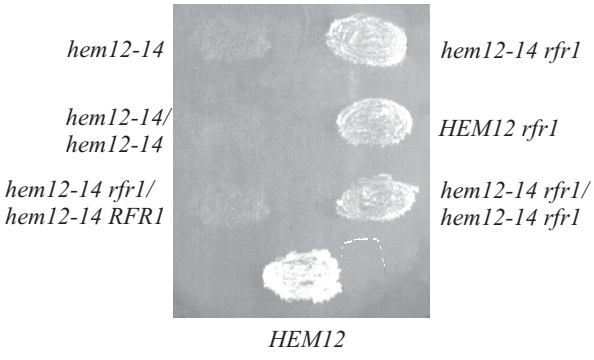


**Figure 1. *rfr1* partially suppresses the growth defect of *hem12-14* and is recessive.**

The growth of wild type (*HEM12*), mutant (*hem12-14*, diploid *hem12-14/hem12-14*) and suppressor (*hem12-14 rfr1*, *HEM12 rfr1*; diploids *hem12-14 rfr1/hem12-14 RFR1* and *hem12-14 rfr1/hem12-14 rfr1*) strains on YPGly medium after 4 days of incubation at 28°C.

type, characteristic for *sui1-1*, but the *rfr1 SUI3-3* double mutant is synthetically lethal, as observed for *sui1-1 SUI3-23* [4]. By contrast, pair combination of *rfr1 sui2-1* does not have a lethal effect. Furthermore, *sui1-1* and *SUI3-3* suppress *hem12-14*. The *sui2-1* suppressor is unable to suppress the *hem12-14* translation initiation defect, hence the *hem12-14 sui2-1* mutant does not grow on glycerol-containing medium ($gly^-$). *rfr1* and *sui1-1* do not affect the steady state level of *HEM12* mRNA (Fig. 2, lanes 1–5). In the *HEM12*

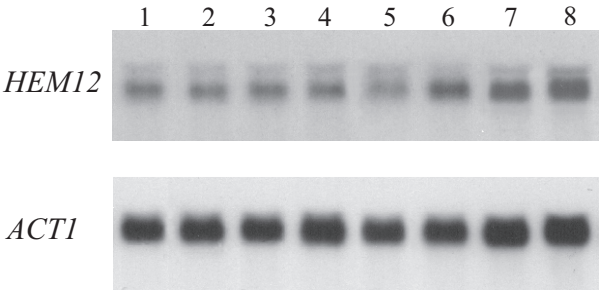


**Figure 2. The *rfr1* and *RFR11* suppressor mutations do not affect the steady-state level of *HEM12* mRNA.**

Northern blot of total RNA (20 $\mu$g) hybridized with a *HEM12* probe. The *ACT1* probe was used as an internal control for RNA loading. Lane 1, *HEM12*; lane 2, *hem12-14 rfr1*; lane 3, *hem12-14 sui1*; lane 4, *hem12-14*; lane 5, *HEM12 rfr1*; lane 6, *hem12-14/hem12-14 RFR11*; lane 7, *hem12-14 RFR11*; lane 8, *hem12-14 RFR11 sui1*.

background, *rfr1* and *sui1-1* do not essentially affect the amount of Hem12p. In the *hem12-14* background, the amount of enzyme is below the level of detection (Fig. 3, lanes 1–9).

### Isolation of dominant suppressors of *hem12-14*

In search for new components of the translation initiation complex, the dominant suppressors of *hem12-14* were isolated in the homoallelic *hem12-14/hem12-14* diploid strain obtained by the cross of TZ21A and TZ21C. Three diploids, RFR8, RFR9 and RFR11 of phenotype $gly^+$ $rf^+$ were analyzed in detail. They all accumulate less porphyrins when compared to parental strain by low temperature spectra of whole cells (not shown). Analysis of progeny of RFR diploids revealed a tight linkage of suppressor mutations to *hem12-14* (43, 32 and 60 tetrads analyzed, respectively). The haploid strain RFR11-1A was crossed to strains 117-8AR4, 117-8AR20 and 117-1AR7 that bear *sui* mutations. Analysis of spore clones from these crosses confirmed that *RFR11* and *sui* are not allelic and showed that *RFR11* and *sui1-1* or *SUI3-3* have additive effects. More Hem12p is observed by Western blot analysis in double mutant *RFR11sui1-1* than in the respective single mutants (Fig. 3, lanes 5, 10–12). Consequently, *RFR11sui1-1* and *RFR11SUI3-3* do not fluoresce ($rf^-$). *RFR11* suppresses neither *his4-303* nor *his4-lacZ* fusion, therefore is specific to *hem12-14*. *RFR11* does not affect the steady-state level of *HEM12* mRNA (Fig. 2, lanes 6–8).

### Molecular analysis of suppressors linked to *hem12*

To characterize the *RFR11* mutation, a genomic bank was prepared from DNA obtained from the RFR11-1A strain and plasmids complementing the $gly^-$ phenotype of *hem12-14* mutant were isolated. All of four independent plasmids pKTE1, 3, 8, 9 contain the *hem12* gene and only the subcloned fragments that contain the full *hem12* gene complement the *hem12-14* mutation. Sequencing of plasmid pKTE8 from –1016 to +266 nucleotides (+1 is A of ATG starting codon) of the *hem12* gene identified an ATT → ATG mutation at nucleotide –78 (Fig. 4). This mutation generates a new translational starting codon in frame with the downstream *HEM12* ORF that extends the Hem12p N-terminus by 26 amino acids and is responsible for suppression.

The mutations responsible for suppression in the RFR8 and RFR9 strains were determined after respective *hem12* alleles were cloned, identified by *in situ* hybridization and

sequenced. These mutations generate upstream additional ATG codons at positions with respect to the main ORF. The uORF1 and uORF2 are in frame with the main ORF. To

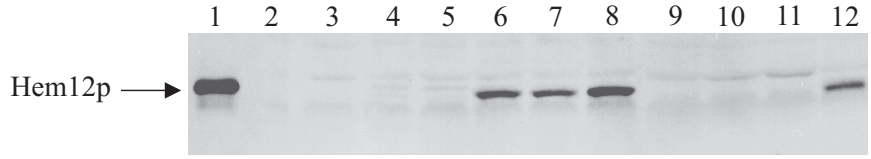


**Figure 3. Hem12p is not detectable in strains bearing single suppressors of *hem12-14*.**

Western blot of whole cell protein extracts (40 $\mu$g) probed by anti-Hem12p antibody. Lane 1, *hem12::URA3* [YEp-HEM12]; lane 2, *hem12::URA3*; lane 3, *hem12-14*; lane 4, *hem12-14 rfr1*; lane 5, *hem12-14 sui1*; lane 6, *HEM12 rfr1*; lane 7, *HEM12 sui1*; lane 8, *HEM12*; lane 9, *hem12-14 /hem12-14*; lane 10, *hem12-14 /hem12-14 RFR11*; lane 11, *hem12-14 RFR11*; lane 12, *hem12-14 RFR11 sui1*.

–26 and –96, respectively, that are in frame with the downstream *HEM12* ORF and allow the synthesis of longer forms of Hem12p (Fig. 4).

### Translation of *HEM12* ORF is not affected by uORFs

Examination of the *HEM12* sequences reveals the presence of four uORFs in the 5′ leader region. The longest transcript of the *HEM12* gene contains a 22aa uORF1, an overlapping short 5aa uORF2, 11aa uORF3 and 7aa uORF4, that are positioned upstream determine whether uORFs regulate *HEM12* expression, the ATG initiation codons –277, –212, –181 and –143 bp were mutated by PCR and *hem12-1/4* allele generated. A plasmid carrying the *hem12-1/4-lacZ* translational fusion was integrated into yeast wild type strain. A strain containing the *HEM12-lacZ* integrated fusion was used as a positive control for *lacZ* expression. Independent transformants were tested for *lacZ* expression on X-gal indicator medium. Since the colony phenotypes of transformants were the same, the pooled cell-free extracts of twelve transformants were assayed *in vitro* for

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          -130 ↓                          -100
hem12-14  GGTAACATAG AGTGATCGAT AGGTTCTTCA ATTCATCAAG CATTATCTCA GAATTGTGTT TGGTAAGCTC TCTTCGAGAA
HEM12     GGTAACATAG AGTGATCGAT AGGTTCTTCA ATTCATCAAG CATTATCTCA GAATTGTGTT TGGTAAGCTC TCTTCGAGAA
RFR8      GGTAACATAG AGTGATCGAT AGGTTCTTCA ATTCATCAAG CATTATCTCA GAATTGTGTT TGGTAAGCTC TCTTCGAGAA
RFR11     GGTAACATAG AGTGATCGAT AGGTTCTTCA ATTCATCAAG CATTATCTCA GA ATG GTG TTT GGT AAG CTC TCT TCG
                                                                  M   V   F   G   N   L   S   S
RFR9      GGTAACATAG AGTGATCGAT AGGTTCTTCA ATTC ATG AAG CAT TAT CTC AGA ATT GTG TTT GGT AAG CTC TCT TCG
                                            M   K   H   Y   L   R   I   V   F   G   N   L   S   S

          -50                                                +1
hem12-14  AAGGAAGAAC AAAAAAAAAA AAAATAAAGC AAAGAACAGG TTATAACGCT ATAGGTAACT TTCCAGCT
HEM12     AAGGAAGAAC AAAAAAAAAA AAAATAAAGC AAAGAACAGG TTATAACGCT ATG GGT AAC TTT CCA GCT
                                                              M   G   N   F   P   A
RFR8      AAGGAAGAAC AAAAAAAAAA AAA ATG AAG CAA AGA ACA GGT TAT AAC GCT ATA GGT AAC TTT CCA GCT
                                    M   K   Q   R   T   G   Y   N   A   I   G   N   F   P   A
RFR11     AGA AAA GGA AGA ACA AAA AAA AAA AAA ATA AAG CAA AGA ACA GGT TAT AAC GCT ATA GGT AAC TTT CCA GCT
          R   K   G   R   T   K   K   K   K   I   K   Q   R   T   G   Y   N   A   I   G   N   F   P   A
RFR9      AGA AAA GGA AGA ACA AAA AAA AAA AAA ATA AAG CAA AGA ACA GGT TAT AAC GCT ATA GGT AAC TTT CCA GCT
          R   K   G   R   T   K   K   K   K   I   K   Q   R   T   G   Y   N   A   I   G   N   F   P   A
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**Figure 4. Intragenic suppressors of *hem12-14* generate new translational start codons.**

DNA sequences of the 5′-region of the *hem12* alleles. The ATG start codons are in bold. The mutant initiator codon of the *hem12-14* allele is underlined. An arrow marks the position of the major initiation site of *hem12* transcription. The first base of the normal ATG start codon is designated +1.

$\beta$-galactosidase activity. Mutations introduced in the *HEM12* leader do not influence the $\beta$-galactosidase activity since *hem12-1/4-lacZ* mutants yielded activities of 9-14U, which were equivalent to that observed for the control (11U).

## DISCUSSION

We have developed a genetic system designed to identify factors, acting either in *cis* or in *trans,* that suppress the effect of the mutant ATA initiation codon of the *hem12* gene. The *trans*-acting *rfr1* suppressors isolated are mapped to the *SUI1* locus, encoding initiation factor eIF1. Mutant *SUI3-3,* encoding eIF2$\beta$, is also a suppressor of *hem12-14*. In contrast, *sui2-1,* another known suppressor of translation initiation defects, is unable to suppress the *hem12-14* allele. *SUI2* encodes eIF2a. All *cis*-acting suppressors analyzed partially restore the translation of *hem12-14* by the generation of new, in frame AUG codons upstream of the *hem12-14* mutation. We also determined that uORFs present in the *HEM12* leader do not influence the efficiency of translation of the main *HEM12* ORF.

Mutations in the *SUI1* gene were first shown to affect start site selection, allowing translation to initiate at the non-AUG codon [5], but also to increase programmed –1 ribosomal frameshifting [8] and recently were shown to affect nonsense-mediated mRNA decay [29]. Sui1p is suggested to contain an RNA-binding domain [30] and may function as a general regulator for RNA recognition in the processes of translation and mRNA decay. The mechanism of suppression by *sui* is common and results in an altered initiation start site [3, 5, 12], the UUG codon located at amino acid position three in the *HIS4* coding region. Similar mechanism of suppression could be predicted for *rfr1* and *hem12-14*; probably translation starts at the downstream UUG codon at amino acid position 11.

Why does *sui2-1* not suppress *hem12-14*? The simplest explanation is that *sui2* is unable to suppress *hem12-14* because it is the weakest *sui* suppressor (8% of wt) [4]. The other possibility may result from the special regulatory role of Sui2p. *sui2-1* contains a mutation at the N-terminus of the $\alpha$ subunit of eIF2 [9]. Ser-51 of eIF2$\alpha$ is phosphorylated by protein kinase Gcn2 and this phosphorylation mediates gene-specific translational control of *GCN4* [31]. Hyperphosphorylation of eIF2$\alpha$ at Ser-51 leads to down-regulation of global protein synthesis. Carboxyl-terminal serines of eIF2$\alpha$ are phosphorylated by casein kinase II and this modification is required for optimal function of eIF2 [32]. It is possible that the *hem12* mutant contains an abnormal phosphorylation status of the mutated form of eIF2$\alpha$ that does not allow suppression.

The *hem12-14* mutation can be suppressed by intragenic mutations generating new, upstream AUG codons in frame with the *HEM12* ORF. The suppression is partial, probably because of the suboptimal context around suppressor AUGs. The effect of suppressor AUGs is enhanced by the *rfr1/sui1* suppressor and these two suppressors may operate independently or *rfr1/sui1* further increases the initiation of translation from a new AUG.

Generally, translation initiates at the most 5′-proximal AUG codon but translation initiation at the downstream AUG codon is possible by bypassing (leaky scanning) or reinitiation. These two processes depend on the position of the upstream AUG codons, the context of the two AUG codons [33] and the context of the uORF downstream sequences, respectively [13]. Translation initiation from the upstream AUG codon with an optimal context can have a dramatic influence on translation initiation from the +1 AUG codon. The *HEM12* initiator region 5′-ACGCUAUGGGU-3′ corresponds rather weakly to the yeast consensus start region, 5′-AA/YAA/UAAUGUCU-3′ [34]. At least the start region of uORF2, 5′-AAAAAAUGAGG-3′ fits better to the yeast

consensus and should efficiently initiate translation. However, we did not find an inhibitory effect of uORFs on translation of the main *HEM12* ORF. As *HEM12* mRNA has multiple 5′ends, spanning positions –297 through –270 and– 148 through –94 [19], the presence of uORFs can be limited to a subclass of mRNAs with longer 5′ leader regions. The longer transcripts account for about 5% of total expression in the wt strain. Therefore, the effect of upstream ORFs on translation starting from the normal initiatior AUG codon could be limited and not physiologically significant. We cannot exclude its importance under some growth conditions.

In summary, translational suppressors of *hem12-14* could function by at least two distinct mechanisms. These include initiating at codons other than AUG and generating a new AUG initiation codon upstream of the initial mutation.

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