Fine tuning of translation termination efficiency in *Saccharomyces cerevisiae* involves two factors in close proximity to the exit tunnel of the ribosome

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In eukaryotes, release factor 1 and 3 (eRF1 and eRF3) are recruited to promote translation termination when a stop codon on the mRNA enters at the ribosomal A-site. However, their over-expression increases only moderately termination efficiency, suggesting that other factors might be involved in the termination process. In order to determine such unknown components, we performed a genetic screen in *Saccharomyces cerevisiae* that identified genes increasing termination efficiency when over-expressed. For this purpose, we constructed a dedicated reporter strain in which a leaky stop codon is inserted into the chromosomal copy of the *ade2* gene. Twenty-five anti-suppressor candidates were identified and characterized for their impact on readthrough. Among them, *SSB1* and *snR18*, two factors close to the exit tunnel of the ribosome, directed the strongest anti-suppression effects when over-expressed, showing that they may be involved in fine tuning of the translation termination level.
INTRODUCTION

Translation termination is the step that liberates the newly synthesised polypeptide from the ribosome, before recycling the translational machinery. Three triplets UAA; UAG (WEIGERT and GAREN 1965) and UGA (BRENNER et al. 1967) were identified as non-sense stop codons, and shown to serve in vitro as signals for release of polypeptide from the ribosome (TAKANAMI and YAN 1965). The misincorporation of an amino acid at the stop codon occurs at a frequency of around 10^{-4} and is called readthrough. The efficiency of this termination is modulated by cis and trans factors. In general, release factors efficiently recognize the termination codons, but in certain instances, near-cognate tRNAs over compete and lead to readthrough. tRNA decoding of a stop codon occurs more frequently when the stop codon is surrounded by a context that modifies the competition for stop codon recognition between release factor and near-cognate tRNA (ENGELBERG-KULKA 1981; FLUCK and EPSTEIN 1980; SALSER 1969). In *S. cerevisiae* both 5’ and 3’ sequences play a role on translation termination (BONETTI et al. 1995; NAMY et al. 2001; TORK et al. 2004). Several studies point to different elements that could be involved in the 5’ effect in *S. cerevisiae*: i) the tRNA located on ribosomal P-site (MOTTAGUI-TABAR and ISAKSSON 1998), ii) the mRNA structure shape due to the nucleotide sequence at the P site that could alter decoding through distortion of the ribosome structure (TORK et al. 2004) and iii) the chemical property of the amino acid at the penultimate position. Previous analyses have shown that the nucleotides 3’ of the stop have a predominant role on readthrough efficiency and that the 5’ context effect is dependent on the 3’ context (BONETTI et al. 1995; CASSAN and ROUSSET 2001; HOWARD et al. 1996; MOTTAGUI-TABAR and ISAKSSON 1998; NAMY et al. 2001; SKUZESKI et al. 1991). In particular, the nucleotide immediately following the stop is highly biased in prokaryotes and eukaryotes and it has been proposed that the stop signal could involve 4 nucleotides (BROWN
et al. 1990). Several studies have pointed to at least 3 nt upstream and 6 nt downstream of the stop to be involved in determining readthrough efficiency (Bonetti et al. 1995; Namy et al. 2001). Aminoglycosides can increase readthrough and have been shown to suppress premature stop mutations in several animal and cultured cell models (Barton-Davis et al. 1999; Bedwell et al. 1997; Manuvakhova et al. 2000; Bidou et al. 2004). These observations have opened the possibility to treat patients who bear non sense mutations with aminoglycoside antibiotics to express full length protein. Given the numerous human diseases caused by non sense mutation (Krawczak et al. 2000), it is thus imperative to determine the precise mechanism of translation termination in eukaryotes.

In eukaryotic cells, termination necessitates the recruitment of release factors eRF1 and eRF3 by the ribosomal machinery at the A site. eRF1 is involved in stop codon recognition but fully efficient termination needs interaction with the GTPase eRF3. In 1994, Frolova and coworkers showed that SUP45 protein of S. cerevisiae belongs to a highly conserved eukaryotic protein family, and corresponds most likely to the yeast eRF1 (Frolova et al. 1994). That assignment was subsequently experimentally demonstrated by Stansfield et al. (Stansfield et al. 1995). eRF1 comprises 3 domains: the N-terminal domain is involved in stop codon recognition (Bertram et al. 2000; Chavatte et al. 2001; Song et al. 2000); the M domain contains a GGQ motif highly conserved throughout evolution (Frolova et al. 1999) that is responsible for peptidyl transferase hydrolytic activity. These 2 domains form the functionally active “core” (Frolova et al. 2000), the C-terminal domain is involved in the interaction with the protein phosphatase PP2A (Andjelkovic et al. 1996) and with eRF3 (Stansfield et al. 1995; Zhouravleva et al. 1995). eRF3, encoded by SUP35 in S. cerevisiae, is made up of 3 domains. The N-terminal and M domains are not essential for viability and termination (Ter-Avanesyan et al. 1993). In S. cerevisiae, the N-terminus is asparagine- and glutamine- rich and underlies the conformational changes of eRF3 to
proteinase-resistant aggregates, leading to the [PSI+] phenotype, see review in (CHERNOFF 2001; COSSON et al. 2002; PATINO et al. 1996; PAUSHKIN et al. 1996). [PSI+] cells present a defect in translation termination characterized by an omnipotent nonsense suppression phenotype (LIEBMAN and SHERMAN 1979). The C-terminal domain carries GTPase activity (FROLOVA et al. 1996), it is essential for viability and termination and interacts with eRF1 and Upf1 (CZAPLINSKI et al. 1998; STANSFIELD et al. 1995; WENG et al. 1996). Recently, Bedwell and Salas-Marco showed that eRF3 mutants with a reduced GTPase activity lead to a decreased translation termination efficiency (SALAS-MARCO and BEDWELL 2004). Recent results suggest that a stable interaction between eRF1 and stop codon in the A-site stimulates eRF3 GTP hydrolysis, which leads to efficient release of the polypeptide from the ribosome by eRF1 (ALKALAEVA et al. 2006; SALAS-MARCO and BEDWELL 2004). In spite of genetic, biochemistry and crystallographic analyses on eRF1 and eRF3, questions about the translational termination mechanism remain. In particular, several factors have been demonstrated to interact with the termination process, either directly through contacts with release factors or indirectly, as demonstrated by genetic experiments. This is the case for the Upf1p factor that physically interacts with release factors eRF3 and eRF1. The two other Upf factors (Upf2p and Upf3p) are also connected with translational termination through a mechanism not well identified (CZAPLINSKI et al. 1998; WANG et al. 2001; WENG et al. 1996). An interaction of eRF1 with PABp has also been shown in Xenopus and human cells (COSSON et al. 2002) and could help recycling of translational components. In addition Itt1p (URAKOV et al. 2001) and PP2A (ANDJELKOVIC et al. 1996) have been described to interact with eRF1, but without clue on the mechanism of translational termination mediated by these interactions. Several observations also suggest a link between termination and the cytoskeleton. Sla1p is involved in the cytoskeleton and has been found to interact with the N-terminal domain of eRF3 (BAILLEUL et al. 1999). Actin mutants have been associated with
increased readthrough on UAA stop codon (KANDL et al. 2002) and a microtubule binding protein of the spindle pole body Stu2p has been identified in a genetic screen for translational termination efficiency modulating factors (NAMY et al. 2002).

Apart from the above mentioned proteins one can envision that other factors able to modulate the termination process remain to be discovered. Indeed, over-expression of yeast eRF factors, Sup45p and Sup35p, increases translational termination efficiency no more than 2.6 fold (STANSFIELD et al. 1995; WILLIAMS et al. 2004). To identify anti-suppressors limiting near-cognate, tRNA-mediated suppression, we developed a screen for factors that would increase translational termination when over-expressed (multicopy anti-suppressors). For this purpose, we used a strain that carries an allele of the ADE2 gene, interrupted by an in frame UAG stop codon surrounded by sequences known to promote a readthrough level high enough to obtain white colonies. We screened for candidate DNA fragments able to confer a red colour to the colonies. Among those, SSB1 and snR18 sequences were found repeatedly and have been shown to actually decrease the readthrough level. The mechanism of SSB1 induced readthrough decrease has been further characterized.
MATERIALS AND METHODS

Yeast strains and media

The *S. cerevisiae* strains used for this work are:


- 74D694 (*MATα ade1-14 trp1-289 leu2-3,112 his3-200 ura3-52) [ψ-] ; [ψ+] (DERKATCH *et al.* 1998)

- MT557/3b (*MATα ade2-1 sup45-2 leu2-3,112 ura3-1 his5-2*) (STANSFIELD *et al.* 1995)

- FS1 (*MATα, ade2-592 lys2-201 leu2-3,112 his3-200 ura3-52*) (NAMY *et al.* 2001)

The modified FS1 strain used in the screen was constructed as follows: from the *ADE2* gene and its promoter cloned in a centromeric *URA3* vector (pFL38), a readthrough sequence derived from TMV (GGAACACAATAGCATTACAG) was cloned in the unique *HpaI* restriction site located within the coding sequence of the *ADE2* gene (NAMY *et al.* 2001). A homologous recombination in the FS1 strain at the *ADE2* locus was performed with this vector linearized by enzymatic restriction. The recombined white clones were selected on complete medium depleted in adenine due to the recovery of the activity of Ade2p protein synthesized. The correct integration was verified by sequencing of the genomic allele.

The strains were grown in minimal media supplemented with the appropriate amino acids to allow maintenance of the different plasmids after transformation.

Yeast transformations were performed by the lithium acetate method (ITO *et al.* 1983). Colour screening was performed on plates containing a drop-out medium, CSM™ (Bio 101), with all amino acids and 10 mg/l adenine. The colour intensity was checked after incubation for 5
days at 30°. 5-FOA was added at a final concentration of 1.5 mg/ml to select the loss of URA3 plasmids.

**Plasmids and molecular biology methods**

Yeast genomic DNA library was kindly provided by François Lacroute. It was constructed by partial restriction of genomic DNA by SauIIIA from S288c strain, and then fragments were ligated into BamHI site of the pFL44L multicopy vector (BONNEAUD et al. 1991). pAC derivatives were constructed by cloning the fragment of interest in the unique MscI site between LacZ and Luc open reading frames of pAC99 (BIDOU et al. 2000; STAHL et al. 1995).

The identification of the candidate genes were obtained by release of plasmid DNA from yeast as already described by Hoffman and Winston 1987 (HOFFMAN and WINSTON 1987) and used to transform *Escherichia coli* strain DH5α. Plasmid DNA was extracted from transformants and boundaries of the insert were sequenced using –21M13 and M13 reverse primers. This allowed to determine the coordinates of genomic region and identify the ORFs and genes present on the insert by comparison with data from Saccharomyces Genome Databank (SGD™).

The construction of mutated SSB1 coding sequence was realized as follow: a mutagenesis on pUC-SSB1cds using a high fidelity Taq DNA polymerase Pfu from Stratagene company was done with for SSB435 a couple of oligonucleotides (435w(CAAGAGAAGAACCTTTACTACAGTCGCTGACAACCAAACCACCGTTCAAT) and 435c (GAACGGTGGTTTGGTTGTCAGCGACTGTAGTAAAGGGTCTTCTTT)); for SSB436 (436w (GAGAAGAACCTTTACTACATGTAGTGACAACCAAACCACCGTTCAATTCCC) and 436c (GGGAATTGAACGGGTGGTTTGGTTGTCAGCGACTGTAGTAAAGGGTCTTCTTT)); for SSBCA (CAw (CCATCAAGAGAAGAACCTTTACTACATGTAGTACAGTCAGTGACAACCAAACCACCGTTCAAT
TCCC) and CAC (GGGAATTGAACGGTGGTTTGGTGTCACTGACTGTAGTAAAGGT TCTTCTCTTGATGG)). The mutated pUC-SSB1cds vectors after control of the sequence of the mutated region using as primer of sequence the oligonucleotide SSBseq2 have been digested by BglII and AgeI restriction enzymes to be cloned at the same sites in the pUC-SSB. The sequence of the mutated pUC-SSB was verified. Then the SSB mutated sequences under its own promoter were cloned in pFL44L vector following the same procedure as for wild type SSB1 under its own promoter.

The construction of SUP45 on multicopy vector was realized as follow: from the pSP35-45 with SUP35 and SUP45 under control of their own promoter (Bidou et al. 2000). The SUP35 and SUP45 with their promoter were inserted into the multicopy pHS8 vector at the PvuII restriction site and were called pHS35-45. The SUP45 with its own promoter was purified from agarose gel after digestion of pHS35-45 by XbaI and cloned at the same restriction site into the pHS8 vector and this vector was called pHS-SUP45.

The construction of the eEF1Bα coding sequence under the cyc1 promoter were realized as follow: total RNA from Fy S. cerevisiae strain were extracted from 5 ml of exponential yeast culture (Schmitt et al. 1990) treated by 10U of RNase free DNase I (Boehringer) at 37° for 1H. DNase I was inactivated by heating at 90° for 5 mn, as recommended by manufacturer.

RNA was reserve transcribed with random primer by superscript II kit (Invitrogen) for amplification of eEF1Bα coding sequence with high fidelity Taq DNA polymerase Pfu from Stratagene company using eEF1Bα AUG (ATGGCATCCACCGATTTCTC) and eEF1Bα UAA (TTATAATTTTTCATAGCAG) as primers. The amplimer was cloned in pUC19 vector at the HincII restriction site and recombinant vector called pUC- eEF1Bα cds was sequenced using –21M13, M13 reverse primers. The eEF1Bα coding sequence cloned in pUC-SSB1cds vector was cut by PstI and Ecl136II restriction enzymes to be cloned at the same restriction site in pCM189 vector. The eEF1Bα coding sequence under cyc1 promoter
was then cloned in pFL44L vector at Smal site by enzymatic restriction of the pCM- eEF1Bα
cds vector with Eco47III and HindIII filled by Klenow enzyme. Recombinant clones in the
right orientation without intron were verified by amplification, enzymatic restriction and
sequencing of the junction site.

The snoRNA snR18 was also cloned under the cyc1 promoter as the eEF1Bα coding
sequence but using in first step snRw (TAAGCATCCACCGATTTCTCCAAGATTG) and
snRc (TTAGGTTGAACCATCTGGAGAATTTCTGGG) to amplify genomic DNA from Fy
S. cerevisiae strain with high fidelity Taq DNA polymerase Pfu from Stratagene company.

Sequencing. All constructs were verified by sequencing the region of interest using the Big
Dye terminator Kit and migrated on ABI310 automatic sequencer (Applied Biosystems).

Quantification of readthrough efficiency.

Luciferase and β-galactosidase activities were assayed in the same crude extract as previously
described (STAHL et al. 1995). All the quantification was the median of at least 5
independents measurements. The efficiency is defined as the ratio of luciferase activity to β-
galactosidase activity. To establish the relative activities of β-galactosidase and luciferase, the
ratio of luciferase activity to β-galactosidase activity from an in-frame control plasmid was
taken in reference. Efficiency of readthrough, expressed as percentage, was calculated by
dividing the luciferase/β-galactosidase ratio obtained from each test construct by the same
ratio obtained with in-frame control construct (BIDOU et al. 2000).
RESULTS

The genetic screen used was based on the ability to monitor termination efficiency through the expression of \textit{ADE2} gene which encodes the P-ribosyl-amino-imidazole-carboxylase (EC 4.1.1.21), responsible for the degradation of the red pigment amino imidazole ribotide. The screen was performed in a FS1 strain where the \textit{ade2} gene is interrupted by an in-frame UAG stop codon derived from the TMV leaky context (for the strain construction see Materials and Methods). This context promotes 15\% of readthrough that leads to a sufficient expression of Ade2p to degrade its red substrate and obtain white colonies (NAMY \textit{et al.} 2001). This strain was transformed with a \textit{S. cerevisiae} genomic library cloned on the multicopy pFL44L vector and plated on minimal medium supplemented with CSM containing a minimum quantity of adenine (10 mg/L) allowing healthy growth with the optimization of red colour. This allowed us to isolate “anti-suppressors” factors in a single step. Out of 52 000 transformants, 26 displayed a red colour after 5 days at 30\°. To check whether the anti-suppressor phenotype of the clones was due to the presence of an over-expressed gene, they were plated in the presence of 5-FOA that selects for cells that have lost the plasmid. All of the isolated candidates except \#17 reversed the phenotype after one week; this isolate was kept to serve as a negative control in further experiments. \textit{This result demonstrates that for the vast majority of the candidates, the effect was dependent on the continuous presence of the vector. This point is important since it ruled out the involvement of a cytoplasmic factor which might have been induced by an over-expressed gene.} For each of the 25 confirmed candidates, sequencing of the fragment boundaries was performed, allowing identification of the inserted genomic fragment. The complete list of the genes present on these 25 fragments is presented in table 1. Twelve are known to be involved in translation: ribosomal protein, translation termination factor, translation initiation factor, elongation factor, tRNA and Hsp70 chaperone.
Many irrelevant factors might modify the pigment accumulation in cells. Among these are the enzymes early in the adenine biosynthesis pathway, proteins involved in vacuole permeability where the pigment accumulates, factors involved controlling the efficiency of translation initiation, etc. In order to identify factors actually involved in translation termination, we used an independent reporter system and quantified readthrough efficiency in the presence or absence of the candidate plasmids.

These vectors (pAC) carry a dual lacZ-luc reporter interrupted by a unique cloning site at the junction of the 2 coding sequences where in frame stop codons in different context are inserted. The SV40 promoter, known to be active in both S. cerevisiae and mammalian cells (CAMONIS et al. 1990), drives the expression. β-galactosidase that originates from translation upstream of the stop codon is used as an internal control recapitulating the different levels where expression could be modulated. The firefly luciferase activity depends on translation downstream of the stop codon and allows precise quantification of readthrough (BIDOU et al. 2000; STAHL et al. 1995). In these conditions, the ratio of firefly luciferase to β-galactosidase activities reflects the readthrough efficiency without interference from other levels of control. To obtain absolute readthrough levels, the values obtained with the test constructs are normalized against results from a similar dual lacZ-luc reporter gene where the stop is replaced with a sense codon. Each of the 26 vectors was co-transformed, with a pAC vector bearing a UAG stop codon, into the FS1 strain. For each co-transformation, five independent assays with two independent clones were performed. The readthrough level was quantified and compared to that obtained in the presence of an empty pFL44L vector. As shown in Table 1, the readthrough level decreases in the presence of each of the 25 confirmed candidates. To assess if the difference of readthrough level between candidates and strain FS1 transformed by empty pFL44L vector is significant, we performed a non-parametric statistical test (Mann Whitney). Candidate #17, like the empty pFL44L cloning vector, did not affect readthrough,
which validated the screen. According to the Mann Whitney test, a difference of at least 20% on termination translation between the candidate and the empty vector is needed to consider an effect as significant. Two candidates do not exhibit a significant difference in readthrough efficiency compared to the negative controls: isolate #3 carrying the transcriptional activator gene *MSN1* and isolate #12 that bears *HEK2* gene involved in translation initiation. On the other hand, isolate #7 harboring *SUP35* (eRF3), isolate #9 carrying *RPL20B*, isolate #18 carrying *tRNA^ser* (IGA), the 5 isolates carrying the SSB1 gene and the 2 isolates carrying the eEF1Bα gene display a statistically significant difference. Altogether, 12 out of the 25 candidates directed a significant decrease of readthrough efficiency, which indicates that the screen based on ADE2 activity was highly stringent.

**eEF1Bα**

eEF1Bα is the β subunit of the eukaryotic translation elongation factor 1 (eEF1) which is highly conserved both functionally and structurally among species (Le Sourd *et al.* 2006). In yeast cells eEF1 is a heterotrimer containing three units, responsible for binding the aminoacylated tRNA to the ribosomal A site, and also participates in the proofreading of the codon-anticodon match; eEF1A is a classic G protein involved in the GTP-dependent binding of amino-acylated tRNA, and the eEF1Bα subunit, associated with the γ subunit, functions as a guanine exchange factor in vitro, and catalyzes the exchange of GDP for GTP on eEF1A to recycle it. The function of eEF1B has been described as critical in regulation of eEF1A activity, translational fidelity, translation rate and cell growth (Le Sourd *et al.* 2006). The *eEF1Bα* gene, in addition to encoding the eEF1Bα translation factor, contains an intervening sequence encoding the small nucleolar RNA snR18. In order to determine whether the increased expression of eEF1Bα protein or snR18, is involved in the decrease of readthrough, we have cloned two different versions of coding region in a multicopy vector pFL44L under
the strong Cyc1 promoter: i) the open reading frame of eEF1Bα without the intron, and ii) the intron containing snR18 surrounded by only 80 nt of eEF1Bα coding sequence and lacking the ATG. These constructs were transformed into the FS1 parental strain. As shown in Figure 1, only the construct carrying snR18 was able to restore the anti suppression phenotype. The readthrough efficiencies directed by these 2 constructs was quantified, using the three stop codons in the same surrounding context cloned in the pAC vector as targets. Strains co-transformed with the two pFL44L constructs were compared to strains co-transformed with the empty vector. Results presented in table 2 show that with the construct expressing only the snR18 matured from the eEF1Bα intron, there is a significant decrease of readthrough (31% to 20%, p = 0.03) on UAG stop codon and a slight decrease of readthrough on UAA and UGA stop codon (11% to 10% p = 0.045 and 15% to 10% p = 0.025 respectively). Interestingly, a strong increase of readthrough on UAA and UGA stop codons is observed upon over-expression of the eEF1Bα open reading frame without intron (from 11% to 27% p = 0.012 and 15% to 38% p = 0.006 respectively). This is reminiscent of previous observations by Carr-Schmid et al. (CARR-SCHMID et al. 1999). Altogether, we have observed that the increase of termination significantly for the UAG stop codon involves the small nucleolar RNA snR18, and not eEF1Bα.

**SSB1**

As mentioned above, the five candidates carrying the SSB1 gene and YDL228c were all able to efficiently decrease the readthrough level (from 35% to 51%) when co-transformed with pAC-TMG (compared to the empty vector pFL44L). To establish if this effect could be attributed to SSB1 or YDL228c over-expression, we cloned the SSB1 coding sequence in a centromeric vector under the strong Cyc1 promoter (pCMSSB1) and tested the readthrough efficiency in the presence of this construct in different S. cerevisiae strains. Results presented
in figure 2 show that over-expression of SSB1 reproduces the effect observed with the entire DNA fragment; although translational termination increases to a greater extent when expressed from the multicopy vector than from a centromeric vector (2 and 1.6 fold respectively in FS1 strain). Over-expression of SSB1 from a multicopy vector has a significant effect on termination in 74D694 [psi-] and FS1 strains (1.3 to 2 fold respectively), but surprisingly not in the [PSI+] state in the 74D694 strain (figure 2). This may be related to different levels of accumulation of the Sup35 protein in these two strains.

In order to better characterize the effect of SSB1 protein we tested a panel of recoding targets corresponding to different stop codons and surrounding sequences in FS1 strain: the UAG stop codon targets corresponding to the TMV termination context, a derived sequence (TMG) where CAA on each side of the stop were replaced by CAG, and the MoMuLV context including the UAG stop and the downstream pseudoknot. In all cases, a 2-fold increase of translation termination is observed upon SSB1 over-expression (table 2). The effect of SSB1 over-expression on termination was also examined on the three stop codons in the TMV context. Although an effect is observed in all cases, its extent varies, from 1.5 fold increase with UGA, to 4 fold with UAA.

We also tested the effect of over expressing the paralogous SSB2 gene under its own promoter cloned in a multicopy vector pYESSB2 and compare to the same vector expressing SSB1 (kindly provided by S. Rospert) on the same set of readthrough targets. As shown in Table 2 the effect on the level of translation termination was significantly less pronounced for Ssb2p than for Ssb1p.

As mentioned above, over-expression of release factors in yeast has been shown to have only a moderate effect on translation termination. To compare the extent of the effect directed by Ssb1p and release factors over-expression, we cloned both SUP45 and SUP35 genes on the same multicopy vector and quantified the readthrough efficiency. As for Ssb1p, a three-fold
increase was obtained upon Sup35p and Sup45p over-expression on the UAA stop codon in the TMV readthrough context. This confirms the relatively weak effect of the over-expression of both factors on termination.

To evaluate whether Ssb proteins are actually involved in the termination process, we used the MT556/3b strain that carries a sup45 thermo-sensitive allele and determined whether Ssb1p over-expression could revert the phenotype. The readthrough level was quantified at 30° in the presence or absence of the over-expressed release factor eRF1 or/and the Ssbp chaperones. The Sup45 thermo-sensitive *S. cerevisiae* strain was first transformed with multicopy URA3 vectors carrying either SUP45 alone, or SUP45 and SUP35. The MT556/3b strain was then transformed with pYES1 or pYES2 or the empty pFL44L vector. Figure 3 shows that a very high readthrough level is obtained with all three stop codons in the TMV context in the MT557/3b strain (27% on UGA, 33% on UAA and 69% on UAG stop codon). The readthrough efficiency decreases about 10-fold for all stop codons in the presence of over-expressed wild type Sup45p protein, but is not affected by Ssb1p or Ssb2p over-expression (Figure 3). We then examined the thermo sensitive phenotype. At 30° all transformants grow on liquid culture with a generation time of 2 hrs. At 37° only cells transformed with SUP45, with SUP45 and SUP35 or with SSB1 are able to grow, but no growth is detected in cells transformed with pFL44L or SSB2. The generation time is 8 hrs with SSB1 and 2 hrs with SUP45 at non-permissive temperature (table3). Thus, over-expression of Ssb1p, but not of Ssb2p, allows a partial recovery of the thermo-resistance in the Sup45 thermo-sensitive strain (figure 4). To determine if suppression of the thermo-sensitive phenotype is a general chaperone effect of Ssb1p, we tested in the same manner ability of Ssb1p to revert thermo-sensitivity of a cdc25 thermo-sensitive mutant in the strain OL556. We observe no reversion of thermo-sensitive phenotype in this strain in presence of Ssb1p over-expression (data not shown).
The difference in the ability of Ssb1p and Ssb2p to revert the sup45p thermo-sensitivity allows determining if one or more of the amino acids that differ between the two proteins could play a role in the ability to revert the thermo-sensitivity. An aspartate is found in Ssb1p and a glutamine is found in Ssb2p at position 49 in the ATPase domain. Three others are found in the polypeptide binding domain, and correspond to: methionine at position 413, cysteine at position 435 and alanine at position 436 in Ssb1p compared to isoleucine, valine and serine at the corresponding positions in Ssb2p (figure 5). Methionine and isoleucine are neutral amino acids, but cysteine in SSB1 could form a disulfide bridge which is not possible in Ssb2p. The cysteine in position 435 was mutated to valine or the alanine in position 436 was mutated to serine, in Ssb1p protein. A third mutant was constructed with both mutations. Over-expression of any of these mutated forms is not able to revert the t.s. phenotype (figure 4).
DISCUSSION

In this work we identify $eEF1B\alpha$ and $SSB1$ genes as able to increase translation termination efficiency when over-expressed. Since the $eEF1B\alpha$ gene that encodes the $\beta$ subunit of the eukaryotic translation elongation factor 1 also carries an intron encoding the snR18 snoRNA, we uncoupled expression of these two factors and show that the effect on termination is directed by the snR18. In the following sections we shall discuss the results obtained with the two genes independently, although they may have similar mode(s) of action since both potentially affect the peptide exit tunnel region of the ribosome.

snR18

The maturation of pre-ribosomal RNA of the translational machinery involves a large number of cleavage events which frequently follow alternative pathways. In addition, rRNAs are extensively modified, with the methylation of the 2'-hydroxyl group of sugar residues and conversion of uridines to pseudouridines being the most frequent modifications, although the extent of the modification event (i.e. the proportion of modified ribosomes) is unknown and possibly variable (GROSJEAN 2005). In particular, the degree of modification has been shown to vary with growth temperature in certain Archaea, plants and trypanosomes (BROWN et al. 2003; OMER et al. 2003; ULIEL et al. 2004). In human, it has been shown that the 5.8S rRNA is hypo-2'O-methylated in neoplastic tissues (MUNHOLLAND and NAZAR 1987). Both cleavage and modification reactions of pre-rRNAs are assisted by a variety of an abundant class of trans-acting RNAs, small nucleolar RNAs (snoRNAs), which function in the form of ribonucleoprotein particles (snoRNPs). The majority of snoRNAs acts as guides directing site-specific 2'-O-ribose methylation or pseudouridine formation by base pairing near target sites. Eukaryotic rRNAs display a complex pattern of ribose methylations. Ribose
methylations of eukaryotic rRNAs are each guided by a cognate small RNA, belonging to the family of box C/D antisense snoRNAs, through transient formation of a specific base-pairing at the rRNA modification site. Over one hundred RNAs of this type have been identified to date in vertebrates and the yeast *S. cerevisiae*. Many snoRNAs are produced by unorthodox modes of biogenesis including salvage from introns of pre-mRNAs, as for snR18 in yeast, or of non-protein-coding transcripts. In yeast however, numerous snoRNAs are generated from independent transcription units.

The snoRNA snR18 is 102 nucleotides long and guides the methylation at the sites corresponding to the A647/C648 positions of the 25S rRNA (LOWE and EDDY 1999). We have shown here that over expression of snR18 induces increased termination efficiency. Different mechanisms may account for this observation. If the effect is direct, it could act through a hyper modification of the A647/C648 position. This would imply that, in the normal situation, not all ribosomes are methylated at this position and that modified ribosomes are more efficient terminators than unmodified ones. Although the proportion of ribosomes methylated at A647/C648 is not known, it has been shown for other positions that only a fraction of ribosomes are modified (GROSJEAN 2005). Alternatively, the effect of snR18 over-expression might be indirect. A possibility would be that it acts through titration of a general factor(s) — perhaps even the methylase Nop1p — involved in the biogenesis or action of methylation snoRNP, which could result in producing fewer snoRNP complexes, less active snoRNP complexes, and/or less stable snoRNP complexes. Such an effect might lead to hypomethylation of other position(s). The precise role of the different methylated nucleotides in the rRNA has not yet been deciphered, precluding further speculations.

While trying to identify the portion of the eEF1Bα gene region involved in the effect on readthrough, we made the interesting observation that overexpression of the eEF1Bα gene, devoid of its snR18 encoding intron, actually decreases termination efficiency. This is in full
agreement with the work of Carr-Schmid et al. who have previously shown that eEF1Bα mutants exhibit an antisuppressor phenotype. They interpreted this effect as a more efficient competition for recognition of the stop codon by release factors due to an increased ratio of release factor to active eEF1A (CARR-SCHMID et al. 1999). This interpretation is strongly supported by the results presented here. Finally, it might be significant that two factors acting in an opposite way on termination are co-expressed as a single RNA, preventing an imbalance in termination efficiency.

**SSB1**

We show that the *SSB1* gene is able to increase translation termination efficiency when over-expressed. This increase is effective on the three stop codons, although to different extents, and on several stop codon contexts. Ssb1p is one of the Hsp70 homologues present in the *S. cerevisiae* genome. It is closely related to its Ssb2p paralogous gene. Ssb1p and Ssb2p share identical function and similar level of expression; they differ by 4 amino acids (BOORSTEIN et al. 1994). Rakwalska and Rospert (RAKWALSKA and ROSPERT 2004) showed previously that the lack of functional Ssb1/2 in yeast caused severe problems in translational fidelity, which were strongly enhanced by paromomycin and correlated with growth inhibition. Since *SSB1* and *SSB2* transcript levels are regulated independently of those of genes encoding ribosomal proteins (see Discussion in MULDOON-JACOBS and DINMAN 2006), not all ribosomes would be associated with functional chaperone complement and over-expression of Ssb proteins would improve the functioning of the ribosome.
The quantification of readthrough in the strain with a conditional-lethal mutant allele of *SUP45* (sup45-2) demonstrates an extremely high level of readthrough on the UAG stop codon and 30% readthrough on UAA and UGA stop codon in the same surrounding environment. Ssb1p over-expression does not decrease the readthrough level in the *SUP45* thermo-sensitive strain but specifically allows a partial recovery of the thermo-resistance phenotype, which suggests that this effect could be associated to the chaperone role of Ssb1p protein on the folding of the Sup45p protein. It would be however insufficient to allow a significant effect on the termination capacity of the protein, since the mutation specifically affects this activity. Interestingly, over-expression of Ssb2p protein was unable to reverse the Sup45p thermo-sensitivity phenotype. The proteins differ only by four amino acids. Out of these four amino acids, 3 are located in the polypeptide binding domain. We analysed the role of cysteine 435 and alanine 436 found in the Ssb1p protein by mutating them to the amino acids found in the corresponding residues of the Ssb2p protein. The mutant Ssb1p proteins are not able to reverse the thermo-sensitivity of the mutant SUP45 strain. This demonstrates that the polypeptide-binding domain is responsible for the specific effect of Ssb1p on the thermo-sensitive phenotype of the strain with allele sup45-2. Since the effect was recapitulated by mutation of only the cysteine residue, it could be dependent on the formation of a specific disulfide bridge with misfolded Sup45p. Although we cannot exclude an additional effect of the polymorphism located in the ATPase domain, this effect should be limited, since the mutants of the polypeptide binding domain recapitulate the observed difference between the two isoforms.

**Possible involvement of the polypeptide exit tunnel of the ribosome in translation termination**
Ssbp is associated to the ribosome when it is actively synthesizing proteins (NELSON et al. 1992), and it interacts both with the ribosome and directly with the nascent chain as it emerges from the ribosome. Ssb1p and Ssb2p are actually in close proximity to a variety of nascent polypeptides (GAUTSCHI et al. 2002; PFUND et al. 1998; ROPPERT et al. 2002). This suggests that Ssbp functions as a chaperone for polypeptide chains during translation. Such a role on emerging polypeptides could be to facilitate the successful folding of newly synthesized proteins (BECKMANN et al. 1990; EGGERS et al. 1997; FRYDMAN et al. 1994; HARDESTY et al. 1995). An alternative explanation of the role of Ssb chaperone would be through a direct role in the decoding process. As proposed by Muldoon-Jacobs and Dinman (MULDOON-JACOBS and DINMAN 2006), Ssb chaperone activity might help nascent peptides to back up into the exit tunnel and thus would participate to efficient accommodation of the aa-tRNA in the ribosomal A-site. Whatever the mechanism involved, it is significant that absence of the RAC complex components, Ssz1 and zuotin, decrease translational accuracy, reinforcing the role of the ribosome exit tunnel associated chaperone on decoding (RAKWALSKA, M., and S. ROPPERT, 2004). Whether over-expression of Ssz1 and zuotin also increases termination efficiency would be interesting to determine.

Remarkably, positions A647/C648 that are methylated by the snoRNA snR18 were included in the sequences identified as approaching the surface around the lumen of the polypeptide exit tunnel in the large ribosomal subunit (NISSEN et al. 2000) (see Figure 6). Although the precise mechanism of this effect could not be inferred from the study reported here, the fact that both Ssb1p and snR18 are somehow linked to the exit tunnel, might be significant regarding the termination mechanism.

ACKNOWLEDGMENTS
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Table 1

Selected clones

<table>
<thead>
<tr>
<th>Name</th>
<th>Insert size (bp)</th>
<th>ORF or genes name</th>
<th>Readthrough decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4732</td>
<td>MRPL32-YCP4-CIT2-YCR006</td>
<td>-15%</td>
</tr>
<tr>
<td>2</td>
<td>3490</td>
<td>VPS8-SNR18-eEF1Bα</td>
<td>-26%</td>
</tr>
<tr>
<td>3</td>
<td>5462</td>
<td>MSN1-RR12-YOL118c-MCH4</td>
<td>-9%</td>
</tr>
<tr>
<td>4</td>
<td>5764</td>
<td>SSB1-YDL228c-HO</td>
<td>-46%</td>
</tr>
<tr>
<td>5</td>
<td>5434</td>
<td>PRC1-YMR298w-YMR299c-ADE4-ATML</td>
<td>-37%</td>
</tr>
<tr>
<td>6</td>
<td>4572</td>
<td>STB4</td>
<td>-11%</td>
</tr>
<tr>
<td>7</td>
<td>3945</td>
<td>SUP35-ARG82-HMO1-</td>
<td>-25%</td>
</tr>
<tr>
<td>8</td>
<td>3882</td>
<td>YBR225w - YBR226c - MCX1-SLX1</td>
<td>-12%</td>
</tr>
<tr>
<td>9</td>
<td>4292</td>
<td>RPL20B-SPS4-YOR314w-YOR314w A-5'YOR315w</td>
<td>-20%</td>
</tr>
<tr>
<td>10</td>
<td>4092</td>
<td>BNA4 -BRN1-YBL096c-YBL095w</td>
<td>-17%</td>
</tr>
<tr>
<td>11</td>
<td>6574</td>
<td>PTP1-SSB1- YDL228c- HO</td>
<td>-43%</td>
</tr>
<tr>
<td>12</td>
<td>6033</td>
<td>STU1-RIB1- HEK2 - SHE1</td>
<td>-13%</td>
</tr>
<tr>
<td>13</td>
<td>4000</td>
<td>VTS1-PDE2-PRT1</td>
<td>-10%</td>
</tr>
<tr>
<td>14</td>
<td>4636</td>
<td>SSB1-YDL228c-HO</td>
<td>-35%</td>
</tr>
<tr>
<td>15</td>
<td>3928</td>
<td>ILV2-YMR107w-YMRWdelta15-YMRcdelta14</td>
<td>-20%</td>
</tr>
<tr>
<td>16</td>
<td>5429</td>
<td>YAP1-GIS4-ARNtS(AGA)</td>
<td>-35%</td>
</tr>
<tr>
<td>17</td>
<td>5140</td>
<td>ATM1-PRP12</td>
<td>-12%</td>
</tr>
<tr>
<td>18</td>
<td>5140</td>
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<td>19</td>
<td>4088</td>
<td>TUS1-YLRCdelta26</td>
<td>-21%</td>
</tr>
<tr>
<td>20</td>
<td>4531</td>
<td>HFA1-ERG12-YMR209c</td>
<td>-5%</td>
</tr>
<tr>
<td>21</td>
<td>9430</td>
<td>VPS67-YKR021W-YKR022C-YKR023W-DBP7-RPC37</td>
<td>-10%</td>
</tr>
<tr>
<td>22</td>
<td>5170</td>
<td>eEF1Bα - SNR18 - VPS8</td>
<td>-56%</td>
</tr>
<tr>
<td>23</td>
<td>5724</td>
<td>PTP1-SSB1- YDL228c-HO</td>
<td>-40%</td>
</tr>
<tr>
<td>24</td>
<td>6257</td>
<td>PIN4-SAS3-YBL053w-YBL054w</td>
<td>-20%</td>
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<tr>
<td>25</td>
<td>3680</td>
<td>SSB1-YDL228c-HO</td>
<td>-51%</td>
</tr>
<tr>
<td>26</td>
<td>5735</td>
<td>MPH2-YDL246C</td>
<td>-8%</td>
</tr>
</tbody>
</table>

List of the 25 clones selected with the name of the genes present on the DNA fragments. FS1 strain was co-transformed with pFL44L clones and pAC vector bearing a UAG stop codon.

The readthrough decrease was expressed as the percentage of readthrough decrease referred to empty pFL44L vector.
Table 2

Readthrough efficiency in the presence of over-expressed proteins

<table>
<thead>
<tr>
<th></th>
<th>TAA</th>
<th>TAG</th>
<th>TGA</th>
<th>TMG</th>
<th>IXR1</th>
<th>MoMuLV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty vector</td>
<td>11%</td>
<td>31%</td>
<td>15%</td>
<td>4%</td>
<td>3%</td>
<td>4%</td>
</tr>
<tr>
<td>SSB1</td>
<td>2%</td>
<td>19%</td>
<td>9%</td>
<td>2%</td>
<td>1%</td>
<td>2%</td>
</tr>
<tr>
<td>SSB2</td>
<td>5%</td>
<td>23%</td>
<td>19%</td>
<td>2%</td>
<td>2%</td>
<td>4%</td>
</tr>
<tr>
<td>eEF1βα coding sequence</td>
<td>27%</td>
<td>36%</td>
<td>38%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>snR18</td>
<td>10%</td>
<td>20%</td>
<td>10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUP35 and SUP45</td>
<td>3%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUP45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4%</td>
</tr>
</tbody>
</table>

The readthrough efficiency was expressed as the ratio of luciferase activity to β-galactosidase activity referred to similar ratio from in-frame control in presence or absence of over-expressed proteins Ssb1p, Ssb2p, eEF1βα coding sequence, the snoRNA SnR18 and Sup45p alone or with Sup35p. Columns correspond to the different stop codon targets tested, TAA, TAG and TGA correspond to the 3 stop codons in the same TMV surrounded context (CAA stop CAA), TMG corresponds to TAG stop codon surrounded by CAG on each side of the stop, IXR1 corresponds to TAA stop codon from *IXR1 S. cerevisiae* coding sequence and MoMuLV corresponds to TAG stop codon from the Gag-Pol junction of the Moloney Murine Leukemia Virus. All the tests were performed in the FS1 strain.
Table 3

Growth of MT557/3b strain in presence of over-expressed proteins at permissive and non permissive temperature

<table>
<thead>
<tr>
<th>MT557/3b transformed by</th>
<th>Generation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°</td>
</tr>
<tr>
<td>Empty vector</td>
<td>2H</td>
</tr>
<tr>
<td>SUP45</td>
<td>2H</td>
</tr>
<tr>
<td>SSB1</td>
<td>2H</td>
</tr>
<tr>
<td>SSB2</td>
<td>2H</td>
</tr>
<tr>
<td>Ssb1.435</td>
<td>2H</td>
</tr>
<tr>
<td>Ssb1.436</td>
<td>2H</td>
</tr>
<tr>
<td>Ssb1.CA</td>
<td>2H</td>
</tr>
</tbody>
</table>

The generation time at 30° and 37° was established from liquid culture of MT557/3b strain in presence or absence of over-expressed proteins Sup45p, Ssb2p, Ssb1p and Ssb1p mutants.
Figures Legends

Figure 1
FS1 modified strain with \textit{ADE2} locus reporter was transformed by pFL44L vector empty, pFL44L\,-\,eEF1B\(\alpha\) or pFL44L\,-\,snR18 and spread on CSM minimal medium. The colour intensity was checked after incubation for 5 days at 30\(^\circ\).

Figure 2
FS1 and 74D694 [psi-] or [PSI+] strains were co-transformed with pAC\,-\,TMG vector bearing a UAG stop codon and SSB1 expressed either from centromeric (pCMSSB1) or multicopy (pYESSB1) vector. The readthrough level was expressed as the percentage of readthrough decrease referred to empty vector.

Figure 3
The readthrough level in MT557/3b strain was quantified by the dual reporter system pAC with the 3 stop codons targets TAA, TAG or TGA in presence or not of over-expressed Sup45p, Ssb1p or Ssb2p.

Figure 4
The MT557/3b strain transformed or not by over-expressed proteins Sup45p, Ssb2p, Ssb1p or \textit{ssb1p mutated} was incubated at 30\(^\circ\) and 37\(^\circ\) for 3 days.
Figure 5

Four amino acid differ between *S. cerevisiae* SSB1 and SSB2. These proteins show an ATPase Domain from amino acid 1 to 400 and a Polypeptide Binding Domain from amino acid 401 to 507.

Figure 6

snR18 guides modification to sites in the wall of the polypeptide exit tunnel.

(A) The large ribosomal subunit viewed down the polypeptide exit tunnel, with the start of the tunnel in the front of the image and the end, where the nascent polypeptide emerges, in the back. Proteins (brick) and RNA as ribbon representation. The nucleotides targeted for 2′-O-methylation due to complementarity between the rRNA and snR18 are highlighted by showing their van der Waals radii (green). Starting in the canonical ‘crow view’, the subunit (of *T. thermophilus*; pdb code 2j01) has been rotated forward slightly around the horizontal axis and slightly counter-clockwise around the vertical axis. The fragment of A-site tRNA visible in the complex is pink; a complete P-site tRNA (purple) and E-site tRNA (cyan) is observed. Importantly, the targetd sites occur in the conserved core of the large subunit (GERBI 1996), validating examining structures from other species. (B) Cross-section of the large ribosomal subunit (a 13.2 Å thick slab) as viewed from the side to feature the polypeptide exit tunnel. Details are as in part (A), except the tRNAs are not shown. This view is obtained by rotation 90° about the vertical axis from the crown view and rotating the subunit backwards slightly around the horizontal axis.
Figure 1

Over-expression in FS1 ADE2::TMG
Figure 2

Readthrough decrease upon SSB1 over-expression in different strains
Figure 3

Readthrough in MT557/3b

TAA

Sup45  pFL44L  SSB1  SSB2

3%  33%  39%  28%

TAG

Sup45  pFL44L  SSB1  SSB2

6%  69%  77%  56%

TGA

Sup45  pFL44L  SSB1  SSB2

2%  27%  32%  32%
Figure 4

Test of thermosensitivity of the MT557/3b strain transformed by over-expressed proteins

![Image of bacterial colony growth at 37°C and 30°C with different proteins](image)

Figure 5

Amino acid aligment between SSB1 and SSB2 from *S. cerevisiae*

<table>
<thead>
<tr>
<th>ATPase Domain</th>
<th>Polypeptide binding Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. c SSB1 MAEG---FTP---EER---VAP---SLGVGMQ---GDMFG---IVV---P---FT---CADNQ---NITI---SR</td>
<td></td>
</tr>
<tr>
<td>S. c SSB2 MAEG---FTP---QER---VAP---SLGVGMQ---GDI---FG---IVV---P---FT---TS---DNQ---NITI---SR</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1</th>
<th>49</th>
<th>400</th>
<th>413</th>
<th>435</th>
<th>436</th>
<th>507</th>
<th>613</th>
</tr>
</thead>
</table>
Figure 6

Localisation of snR18 in the ribosome. The exit tunnel is visible in the center of the ribosome.