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The products of the *SUP45* (eRF1) and *SUP35* genes interact to mediate translation termination in *Saccharomyces cerevisiae*

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The product of the yeast *SUP45* gene (Sup45p) is highly homologous to the *Xenopus* eukaryote release factor 1 (eRF1), which has release factor activity *in vitro*. We show, using the two-hybrid system, that in *Saccharomyces cerevisiae* Sup45p and the product of the *SUP35* gene (Sup35p) interact *in vivo*. The ability of Sup45p C-terminally tagged with $(His)_6$ to specifically precipitate Sup35p from a cell lysate was used to confirm this interaction *in vitro*. Although overexpression of either the *SUP45* or *SUP35* genes alone did not reduce the efficiency of codon-specific tRNA nonsense suppression, the simultaneous overexpression of both the *SUP35* and *SUP45* genes in nonsense suppressor tRNA-containing strains produced an antisuppressor phenotype. These data are consistent with Sup35p and Sup45p forming a complex with release factor properties. Furthermore, overexpression of either *Xenopus* or human eRF1 (*SUP45*) genes also resulted in antisuppression only if that strain was also overexpressing the yeast *SUP35* gene. Antisuppression is a characteristic phenotype associated with overexpression of both prokaryote and mitochondrial release factors. We propose that Sup45p and Sup35p interact to form a release factor complex in yeast and that Sup35p, which has GTP binding sequence motifs in its C-terminal domain, provides the GTP hydrolytic activity which is a demonstrated requirement of the eukaryote translation termination reaction.

Keywords: release factor/*Saccharomyces cerevisiae*/*SUP35*/*SUP45*/translation termination

Introduction

In mRNAs the three codons UAA, UAG and UGA are almost universally employed to signal termination of translation. In bacteria this process is catalysed by one of two release factors, RF1 at UAA and UAG codons and RF2 at UAA and UGA (Scolnick *et al.*, 1968). A third release factor, RF3, showing some homology to bacterial elongation factor EF-G, enhances the rate of RF1 and RF2 catalysed termination in a GTP-dependent and codon-independent manner (Milman *et al.*, 1969; Grentzmann *et al.*, 1994; Mikuni *et al.*, 1994). Following peptidyl-tRNA hydrolysis to free the nascent peptide, a ribosome release factor (RRF) is employed to release the ribosomal subunits from the mRNA, enabling them to participate in new rounds of initiation (Hirashima and Kaji, 1972).

In eukaryotes the process of translational termination has been defined less equivocally: it is known that a single polypeptide, the eukaryote release factor (eRF) will catalyse termination *in vitro* at all three stop codons in a GTP-dependent manner (Goldstein *et al.*, 1970; Konecki *et al.*, 1977). An additional stimulatory factor 's' was identified, but not further characterized (Konecki *et al.*, 1977). Attempts to clone the gene coding for eRF resulted in isolation of a gene with 89% amino acid identity to bovine tryptophanyl-tRNA synthetase (TrpRS; Lee *et al.*, 1990). However, it was subsequently shown that the TrpRS polypeptide purified from rabbit reticulocyte lysates does not have eRF activity, but rather co-purifies with a protein that does (Frolova *et al.*, 1993). Using an *in vitro* peptidyl release assay, originally employed to identify components of the bacterial termination complex (Caskey *et al.*, 1968, 1973), Frolova *et al.*, (1994) showed that the eRF protein had release factor activity in response to all three termination codons. N-Terminal sequencing identified the protein as a product of the *SUP45* gene family coding for closely-related polypeptides, including the *Saccharomyces cerevisiae* Sup45p (Himmelfarb *et al.*, 1985), *Xenopus* C11 (Tassan *et al.*, 1993) and human TB3-1 (Grenett *et al.*, 1992) proteins. Frolova *et al.* (1994) showed that these latter two proteins also have omnipotent release factor activity.

The yeast *SUP45* gene codes for a protein (Sup45p) that is tightly associated with polysomal ribosomes (Stansfield *et al.*, 1992) and present at a low level in the cell, with a molar ratio to ribosomes of $<1:20$ (Stansfield *et al.*, 1992), an abundance typical of the *Escherichia coli* release factors RF1 and RF2 (Klein and Capecchi, 1971). Mutant alleles of the *SUP45* gene which exhibit either omnipotent suppressor (*sup1*, Inge-Vechtomov and Andrianova, 1970; *sup45*, Hawthorne and Leupold, 1974) or allosuppressor phenotypes (*sal4*, Cox, 1977) are known. Allosuppressors are selected on the basis of an ability to enhance the suppressor efficiency of the weak ochre suppressor tRNA *SUQ5*, while omnipotent suppression is thought to result when the limited nonsense suppressor ability of some wild-type tRNAs is enhanced (Stansfield *et al.*, 1995). Both the omnipotent and allosuppressor phenotypes can

be associated with a single *sup45* allele (Stansfield *et al.*, 1995), as would be expected of a gene encoding a protein with release factor activity.

Although the release factor activity of yeast Sup45p protein has not been formally demonstrated using the *in vitro* biochemical termination assay, it seems extremely likely that it does perform this role *in vivo*, for two reasons: first, the yeast Sup45p polypeptide exhibits a high degree of amino acid identity with the *Xenopus* (Cl1) and human (TB-3) eRF1 proteins (68 and 66% respectively; Frolova *et al.*, 1994); second, the *Xenopus*, human and Syrian hamster eRF1 genes can be used to functionally replace the *S.cerevisiae SUP45* gene *in vivo* (B.Urbero, L.Eurwilaichitr, I.Stansfield, M.Philippe, M.Kress and M.F.Tuite, in preparation).

A eukaryote gene family has thus been identified which codes for proteins with omnipotent release factor activity. However, the translation termination process is known to be GTP dependent in rabbit reticulocyte systems (Goldstein *et al.*, 1970; Konecki *et al.*, 1977), yet the *SUP45* gene family members show no homology to any known GTP binding sequence motifs (I.Stansfield and M.F.Tuite, unpublished data). This raises the question of whether eRF1 (Sup45p) is the only component of the eukaryotic release factor. One potential candidate protein with GTP binding sequence motifs, which by virtue of its mutant phenotypes may play a role in translation termination, is the product of the *SUP35* gene (Sup35p; Tuite and Stansfield, 1994). Like the *SUP45* gene, the *SUP35* gene was identified through both allosuppressor and omnipotent suppressor mutational screens and, like Sup45p, Sup35p is also closely associated with the ribosome at an approximate stoichiometry of 1 mol Sup35p to 20 mol ribosomes (Didichenko *et al.*, 1991). In the following work we confirm that Sup35p and Sup45p interact both *in vivo* and *in vitro* and that they act in concert to form a functional termination complex *in vivo* in *S.cerevisiae*.

Results

Sup35p and Sup45p can interact in vivo

The yeast *SUP35* and *SUP45* genes have a number of properties in common. Both genes were identified through screens for omnipotent suppressor mutations (Inge-Vechtomov and Andrianova, 1970; Hawthorne and Leupold, 1974) and for allosuppressor mutations (Cox, 1977) and both gene products Sup35p and Sup45p are associated with polysomal ribosomes, with a probable location on the 40S subunit (Didichenko *et al.*, 1991; Stansfield *et al.*, 1992). Two observations indicate that these two proteins either participate in a common process or interact directly: first, a *sal3 sal4* (*sup35 sup45*) double allosuppressor mutant is inviable (Cox, 1977); second, an increased dosage of the *SUP45* gene relieves the degree of temperature sensitivity of some *sup35* conditional-lethal mutations, while overexpression of *SUP35* partially complements a *sup45* temperature-sensitive mutation (Ter-Avanesyan *et al.*, 1984, 1993).

In order to confirm that Sup35p and Sup45p interact *in vivo*, the *GAL4*-based two-hybrid system was employed as a direct assay of protein–protein interaction *in vivo* (Fields and Song, 1989; Chien *et al.*, 1991). The *SUP35*

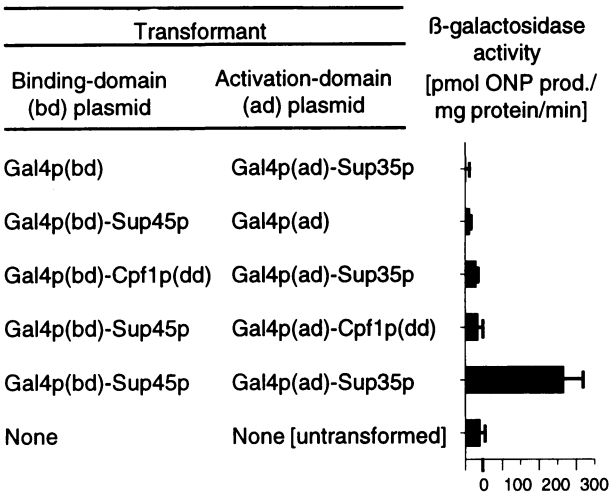

Fig. 1. Two-hybrid analysis of Sup35p interaction with Sup45p. β-Galactosidase activities of cell lysates of strain Y526 both untransformed and transformed with pair-wise combinations of the two-hybrid vectors were determined (where background activity represented by the untransformed Y526 strain was taken as 0 pmol/min/mg protein). β-Galactosidase activities are the mean of three determinations. Error bars represent ± 1 SD. Domains of proteins expressed, in terms of amino acid numberings, are given in Table II.

and *SUP45* genes were cloned in-frame downstream of the *GAL4* activation and binding domains respectively (see Materials and methods). The two plasmids thus generated, together with a number of control constructs detailed below, were transformed in different pair-wise combinations into the *S.cerevisiae* host strain Y526 (Bartel *et al.*, 1993a), carrying the *GAL1–lacZ* gene fusion to assay Gal4p activation of *GAL1* transcription (Fields and Song, 1989). The activity of the β-galactosidase reporter was assayed initially by colony colour, using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). For each of the different classes of transformant detailed in Figure 1, five independent double transformants were screened, including a number of pair-wise control combinations of plasmids. The results showed that neither Sup45p–bd nor Sup35p–ad fusion proteins alone interacted with the complementary *GAL4* domains, the colonies of these double transformants remaining white on X-gal medium (not shown). There was also no non-specific interaction of Sup45p–bd or Sup35p–ad fusions with other proteins, exemplified by *GAL4* domain fusions with the dimerization domain of transcription factor *CPF1* (Dowell *et al.*, 1992). Only double transformants expressing both bd–Sup45p and ad–Sup35p fusions (pUKC601/pUKC605) gave a blue colony colour with X-gal (not shown).

These results were confirmed using the β-galactosidase assay (Figure 1), with all control cell lysates exhibiting β-galactosidase activities approximately equivalent to the background levels of activity measured in the untransformed Y526 host strain lysate. However, the β-galactosidase activity of the lysate expressing the bd–Sup45p plus ad–Sup35p protein fusions was measured at 227 pmol/min/mg protein above background, strong evidence that Sup45p and Sup35p interact directly *in vivo*. Moreover, this activity was twice that measured in a positive control transformant expressing both the ad and bd fusions of

Snf1p and Snf4p respectively (not shown), two proteins whose interaction has been previously demonstrated using the two-hybrid system (Fields and Song, 1989).

Sup45p and Sup35p also interact in vitro

To confirm that Sup45p and Sup35p specifically interact, the ability of immobilized Sup45p to precipitate Sup35p from a post-ribosomal supernatant (PRS) was tested. The immobilization of Sup45p onto nickel (Ni–NTA)–agarose beads was achieved by tagging the polypeptide at its C-terminus with a $(His)_6$ peptide (see Materials and methods).

The multicopy plasmid pUKC625 encoding the Sup45p$(His)_6$ construct was transformed into yeast strain MT552/36d and a PRS prepared. Since Sup35p and Sup45p are both known to bind to the 40S ribosomal subunit and 80S ribosomes (Didichenko *et al.*, 1991; Stansfield *et al.*, 1992), the preparation of a PRS fraction by removal of ribosomal material from the Sup45p$(His)_6$ lysate was felt to be essential for assessing interactions between the two polypeptides in solution.

The PRS, containing Sup45p$(His)_6$ in high salt concentration (0.8 M KCl) lysis buffer, was repeatedly passed through Ni–NTA–agarose, ensuring preferential binding of Sup45p$(His)_6$ to the resin and limiting non-specific binding of other proteins. The majority of non-specifically bound proteins were removed by washing the resin under stringent conditions of low pH and high salt and glycerol (Materials and methods). This resulted in a preparation of partially purified Sup45p$(His)_6$ bound to the nickel resin. A control nickel resin was prepared in the same way using a PRS prepared from untransformed strain MT552/36d. This control resin had the same non-specific complement of PRS proteins bound to it, but lacked any bound Sup45p$(His)_6$ detectable by immunoblot assay.

In order to investigate the Sup45p–Sup35p interaction, the Sup45p$(His)_6$–resin and the control resin were tested for their ability to precipitate Sup35p from solution. Sup35p was overexpressed in yeast strain MT422/1c transformed with plasmid pUKC606, from which a PRS (Sup35p enriched) was prepared. The Sup35p-rich PRS was then incubated with either the Sup45p$(His)_6$–resin or the control resin for 2 h in a low salt buffer to promote protein–protein interactions. Both control resin and Sup45p$(His)_6$–resin were then returned to columns to facilitate washing.

Samples of the Sup35p-rich PRS were analysed by SDS–PAGE and Western blotting before and after incubation with the two resin preparations, as were, after extensive washing, samples of the Sup45p$(His)_6$–resin and the control resin. The Western blots were probed with antibodies to Sup35p and Sup45p (Figure 2b and c). The results show that the Sup45p$(His)_6$ was immobilized on the Ni–NTA resin, whilst no Sup45p was detectable on the control resin (Figure 2b). The small amounts of Sup45p present in the Sup35p PRS, derived from expression of the genomic copy of *SUP45*, were not detectable under the conditions used.

Sup35p was detectable in the Sup35p-rich PRS (Figure 2c) and to a lesser extent in the supernatants following incubation with the two resin types. This loss of Sup35p from the PRS was due to non-specific adsorbtion of Sup35p onto the control resin (data not shown) and specific interaction of Sup35p with Sup45p on the Sup45p$(His)_6$–

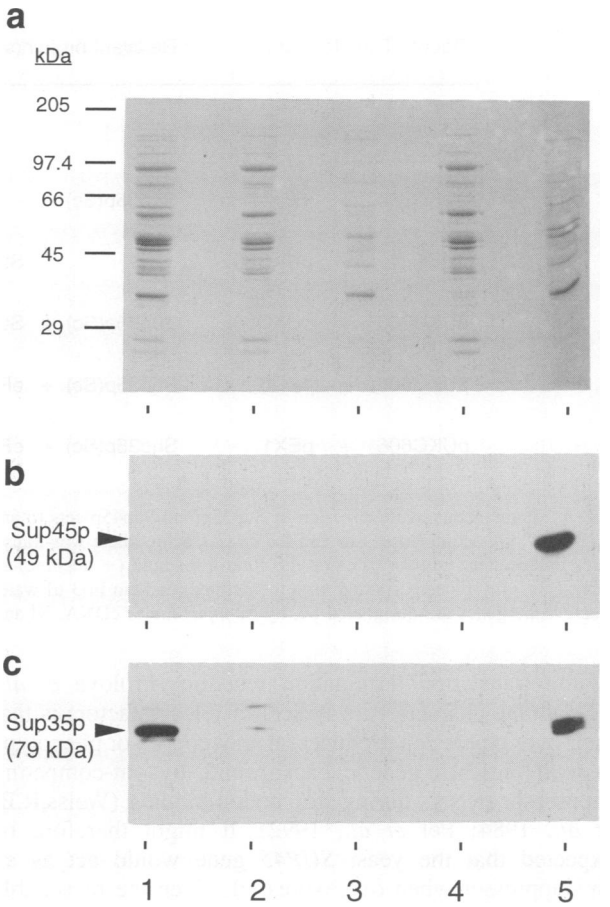

Fig. 2. Precipitation of Sup35p from a post-ribosomal supernatant (PRS) by immobilized Sup45p $(His)_6$. The PRS fraction from a yeast strain overexpressing Sup35p was incubated with either Ni–NTA resin loaded with Sup45p$(His)_6$ or a Sup45p-free control resin (Materials and methods). Following washing, equal quantities of the two resins and post-incubation supernatant fractions were assayed for total protein by Coomassie blue staining of SDS–PAGE gels (**a**) and the presence of Sup45p (**b**) or Sup35p (**c**) detected on immunoblots probed with Sup45p and Sup35p affinity-purified antibodies (Materials and methods). Lane 1, Sup35p-enriched PRS; lane 2, supernatant following incubation with control resin; lane 3, control resin following washing; lane 4, supernatant following incubation with Sup45p$(His)_6$–resin; lane 5, resin-bound Sup45p$(His)_6$ following washing. The positions of migration of molecular mass markers are shown, as are the positions of the bands representing Sup45p and Sup35p.

resin. This difference in the nature of Sup35p interaction with the two resins was clearly demonstrated by the fact that while no Sup35p was retained on the control resin following washing, Sup35p remained tightly bound to the Sup45p$(His)_6$–resin (Figure 2c). These data confirm that Sup35p interacts tightly and specifically with Sup45p in solution. In a complementary experiment which employed the reverse strategy, a glutathione S-transferase–Sup35p fusion protein bound to glutathione–agarose beads was used to precipitate Sup45p from a cell lysate, further confirming that Sup35p and Sup45p interact *in vitro* (data not shown).

Multicopy expression phenotypes of the SUP45 and SUP35 genes

The rabbit, *Xenopus* and human homologues of yeast Sup45p have peptidyl release activity in an *in vitro* assay

Double Transformant			Relevant protein(s) expressed			Three independent double-transformants grown on defined medium (-ura -leu)	
						+adenine	-adenine
pUKC606	+	YEp24	Sup35p(Sc)		-		
pRS425	+	pUKC802	-		Sup45p(Sc)		
pUKC606	+	pUKC802	Sup35p(Sc)	+	Sup45p(Sc)		
pUKC606	+	pEO6	Sup35p(Sc)	+	eRF1(Hs)		
pUKC606	+	pEX1	Sup35p(Sc)	+	eRF1(Xl)		

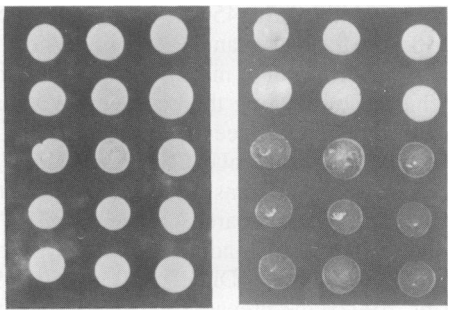

Fig. 3. Simultaneous overexpression of Sup35p and Sup45p acts to antisuppress the ochre suppressor tRNA *SUP4*. Strain MT422/1c carrying the *SUP4-o* tRNA ochre suppressor and the *ade2-1* ochre allele was transformed with the pair-wise combinations of plasmids indicated and growth of three independent transformants on the control medium (+ adenine) compared with growth on defined medium lacking adenine. Each yeast 'colony' represents ~1×10^5 cells spotted onto the defined medium in 3 μl water and allowed to grow for 3 days at 30°C. Expressed proteins coded for by *S.cerevisiae* genes are designated Sc, by *Xenopus laevis* cDNA, Xl and by human cDNA, Hs.

of the translation termination reaction (Frolova *et al.*, 1994). One characteristic typical of release factors is that their overexpression produces an antisuppressor phenotype in a *SUP* tRNA genetic background, by out-competing suppressor tRNAs during stop codon binding (Weiss,R.B. *et al.*, 1984; Pel *et al.*, 1992). It might therefore be expected that the yeast *SUP45* gene would act as an antisuppressor when overexpressed, given the reasonable assertion that it too encodes a protein with eRF1 activity (Tassan *et al.*, 1993; B.Urbero, L.Eurwilaichitr, I.Stansfield, M.Philippe, M.Kress and M.F.Tuite, in preparation). However, overexpression of the yeast *SUP45* gene from a multicopy plasmid does not generate an antisuppressor phenotype (I.Stansfield and M.F.Tuite, unpublished data) The *SUP35* gene also does not act as an antisuppressor when overexpressed, instead causing increased suppression of nonsense codons (Chernoff *et al.*, 1993). We therefore examined whether simultaneous overexpression of both *SUP35* and *SUP45* genes could produce an antisuppressor phenotype: a positive result would indicate that *in vivo* Sup35p and Sup45p form a complex with release factor properties.

To test this hypothesis we employed two multicopy plasmids to overexpress the Sup35p and Sup45p proteins simultaneously in a yeast strain carrying the efficient ochre suppressor tRNA mutation *SUP4*, testing for the antisuppressor phenotype diagnostic for overexpressed release factors. The *SUP4* yeast strain used also carried the *ade2-1* ochre mutation, which causes adenine auxotrophy in a SUP^+ background. Suppression of the *ade2-1* mutation by *SUP4* tRNA ordinarily generates an adenine prototrophy (Ade^+) phenotype in a yeast strain of genotype *SUP4 ade2-1*; antisuppression of the *SUP4* tRNA by overexpression of any release factor (complex) should result in a reversion to adenine auxotrophy (Ade^-).

Overexpression of either the *SUP35* or *SUP45* genes in isolation had no antisuppressor effect on *SUP4*-mediated suppression, while overexpression of both *SUP35* and *SUP45* genes in the same strain produced a clear antisuppressor phenotype, indicated by the Ade^- phenotype of the double transformant (Figure 3). This result indicates that Sup45p and Sup35p act in concert as components of a release factor complex to mediate translation termination in yeast, with neither protein alone being sufficient to outcompete the suppressor tRNA.

Xenopus and human SUP45 genes can couple effectively with yeast SUP35 to antisuppress an efficient tRNA suppressor

To confirm that Sup35p and Sup45p act together in a complex to mediate translation termination, a *SUP4 ade2-1* yeast strain carrying the yeast multicopy *SUP35* gene was transformed with a second plasmid expressing either the *Xenopus* or human *SUP45* (eRF1) homologues and tested for antisuppressor phenotype. The results show that overexpression of *Xenopus* eRF1 (Sup45p/Cl1) and *SUP35* generated an antisuppressor phenotype, as did overexpression of *SUP35* with human eRF1 (TB3-1; Figure 3). Neither overexpression of *Xenopus* eRF1 alone nor human eRF1 alone generated an antisuppressor phenotype (data not shown). The antisuppressor phenotype in the two heterologous combinations appeared qualitatively to be as efficient as that generated by the combined overexpression of yeast Sup45p and yeast Sup35p (Figure 3).

This result confirms that human and *Xenopus* eRF1 proteins can interact functionally with the yeast Sup35p protein producing a phenotype typical of that expected from release factor complex overexpression. The findings also lend further credence to the assertion that yeast Sup45p represents the *S.cerevisiae* eRF1 protein, since in multicopy the yeast *SUP45*, *Xenopus* Cl1 and human TB-3 genes all generate a similar antisuppression phenotype with the yeast *SUP35* gene.

Antisuppression due to co-expression of SUP35 and SUP45 is not restricted to a single suppressor tRNA species and is effective against ochre, amber and UGA suppression

To confirm that the simultaneous overexpression of *SUP35* and *SUP45* could act to antisuppress suppressor tRNAs

Table I. Simultaneous overexpression of Sup35p and Sup45p acts to antisuppress all three stop codons

Termination codon	Percentage stop codon readthrough[a]			Fold-reduction in suppressor efficiency[b]
	None (pEMBLYe23)	Sup45p (pVK62)	Sup45p and Sup35p (pVK63)	
UAA	4.6 ± 0.4	4.5 ± 0.34	1.77 ± 0.25	2.6
UAG	0.66 ± 0.02	0.65 ± 0.05	0.29 ± 0.04	2.3
UGA	0.23 ± 0.03	0.27 ± 0.06	0.18 ± 0.03	1.4

[a]Strain 5V-H19/DBY746 carrying the SUQ5 suppressor tRNA was transformed with either plasmid pEMBLYe23 (control), pVK62 to overexpress *SUP45* or pVK63 to overexpress both *SUP35* and *SUP45*, in different pairwise combinations with either pUKC815-L, pUKC817-L, pUKC818-L or pUKC819-L, to quantify nonsense suppression levels (Materials and methods). β-Galactosidase activities in each of the pUKC817-L, pUKC818-L or pUKC819-L transformants, representing the levels of UAA, UAG and UGA suppression respectively, were expressed as a percentage of the β-galactosidase activity in the pUKC815-L transformant. Values are the means of three independent assays ± 1 SD.
[b]Fold reduction in suppressor efficiency occuring with Sup45p and Sup35p co-overexpression.

other than *SUP4*, the experiment was repeated in a [*psi*$^+$] diploid strain (5V-H19/DBY746) heterozygous for *SUQ5*, which encodes the weak ochre suppressor tRNA^{Ser}. A [*psi*$^+$] strain was selected so as to elevate the ordinarily inefficient *SUQ5*-mediated suppression to more easily detectable levels (Cox, 1965).

To accurately quantify any antisuppressor effect, the plasmids pUKC815-L, pUKC817-L, pUKC818-L and pUKC819-L (Stansfield *et al.*, 1995; Materials and methods) were introduced into the *SUQ5/suq5*$^+$ [*psi*$^+$] strain overexpressing *SUP35* and *SUP45*. This was achieved by mating 5V-H19[pVK63] with DBY746 transformed with different pUKC-815 series vectors. pUKC815-L consists of a *PGK1–lacZ* gene fusion, while the pUKC817-L, pUKC818-L and pUKC819-L plasmids are identical to pUKC815-L except that one of the three termination codons, TAA, TAG and TGA respectively, is cloned in-frame at the junction of the *PGK1* and *lacZ* genes (Stansfield *et al.*, 1995). Any suppression of the in-frame premature stop codons will result in β-galactosidase activity and the levels of β-galactosidase activity can therefore be used to quantify suppressor or antisuppressor phenotypes.

In the [*psi*$^+$] *SUQ5/suq5*$^+$ strain the level of ochre codon suppression in a pUKC817-L transformant was 4.6% of control β-galactosidase activity of a pUKC815-L transformant (Table I). Overexpression of *SUP45* in a pVK62 transformant had no effect on stop codon suppression levels (Table I). However, simultaneous overexpression of *SUP35* and *SUP45* using plasmid pVK63 resulted in a 2.6-fold decrease in UAA suppression. The suppression of UAG and UGA termination codons observed in the [*psi*$^+$] *suq5*$^+$/*SUQ5* strain and measured using plasmids pUKC818-L and pUKC819-L was also reduced 2.3- and 1.4-fold respectively in the pVK63 transformants (Table I). We propose that this suppression derives from the action of natural suppressor tRNAs (Stansfield *et al.*, 1995), including a UGA suppressing tRNA^{Trp} (Tuite and McLauchlin, 1982), a UAG suppressing tRNA$^{Gln}_{CAG}$ (Pure *et al.*, 1988) and tRNA$^{Gln}_{CAA}$ capable of suppressing UAA (Weiss,W.A. and Frieberg, 1987).

To further confirm that the antisuppressor phenotype was effective against UGA suppression, the adenine prototroph strain MT576/5c, essentially *SUP4*^{UGA} *ade2*^{UGA}, was transformed with the different pair-wise combinations of plasmids detailed in Figure 4. These resulted variously in either *SUP35* or *SUP45* overexpression alone or simultaneous *SUP35* and *SUP45* overexpression. The results show that of all combinations only overexpression of Sup35p and Sup45p together is effective in antisuppressing the UGA suppressor tRNA, indicated by the adenine auxotrophy phenotype of this double transformant (Figure 4).

Taken together, the results presented in Table I and Figure 4 demonstrate clearly that overexpression of Sup35p and Sup45p together produces an antisuppressor phenotype effective against suppression at all three stop codons. This directly infers that the Sup35p–Sup45p protein complex acts to catalyse termination at all three stop codons.

The results additionally confirm that the antisuppressor phenotype produced when Sup35p and Sup45p are overexpressed together can act to antisuppress both a seryl-tRNA (*SUQ5*) and a tyrosyl-tRNA (*SUP4*^{UGA}) and that antisuppression is therefore not restricted to limiting the suppressor efficiency of a single tRNA species.

Discussion

Members of the *SUP45* (eRF1) gene family have release factor activity and can catalyse peptide chain release at all three stop codons (Frolova *et al.*, 1994), yet the eukaryote translation termination process is GTP dependent (Goldstein *et al.*, 1970; Konecki *et al.*, 1977). We have therefore proposed that *SUP35*, encoding a protein with C-terminal homology to translation elongation factor EF-1α, including concensus GTP binding sequence motifs (Kushnirov *et al.*, 1988; Wilson and Culbertson, 1988), might supply the necessary GTP hydrolytic activity (Tuite and Stansfield, 1994). Like *SUP45*, mutant alleles of the *SUP35* gene can be isolated with either an omnipotent suppressor or allosuppressor phenotype (Inge-Vechtomov and Andrianova, 1970; Cox, 1977), an indication that Sup35p may play a role in translation termination. It is also likely that homologues of the *SUP35* gene will be found in a wide variety of organisms; for example a human cDNA has been isolated encoding a protein with 52.3% identity to *S.cerevisiae* Sup35p (Hoshino *et al.*, 1989) and *SUP35* homologues have been identified in the yeast *Pichia pinus* (Kushnirov *et al.*, 1990) and in *Xenopus laevis* (Zhouravleva *et al.*, 1995)

We show here, using the two-hybrid system, that the yeast eRF1(Sup45p) protein interacts *in vivo* with Sup35p (Figure 1) This interaction was also demonstrated *in vitro*

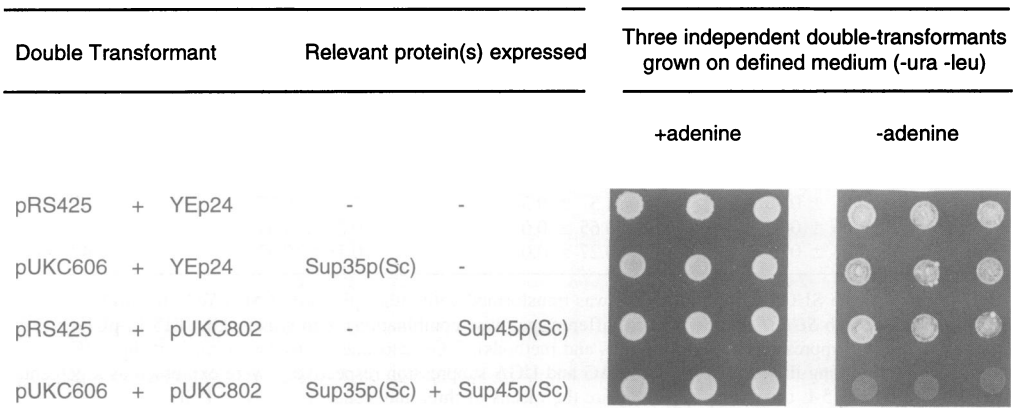

Fig. 4. Simultaneous overexpression of Sup35p and Sup45p also acts to antisuppress the UGA suppressor tRNA $SUP4^{UGA}$. Strain MT576/5c carrying the $SUP4^{UGA}$ tRNA suppressor and the $ade2^{UGA}$ allele was transformed with the pair-wise combinations of plasmids indicated and growth of three independent transformants on the control medium (+ adenine) compared with growth on defined medium lacking adenine. Each yeast 'colony' represents ~1×10^5 cells spotted onto the defined medium in 3 μl water and allowed to grow for 3 (+ adenine plate) or 5 days (–adenine plate) at 30°C.

using immobilized Sup45p to precipitate Sup35p (Figure 2). We have subsequently used gel filtration to demonstrate that Sup35p and Sup45p exist as a heterodimer in yeast cell lysates (data not shown), again confirming that the two proteins form a complex *in vivo*. That this interaction is functionally significant is demonstrated by the anti-suppression phenotype accompanying simultaneous over-expression of the *SUP35* and *SUP45* genes (Figures 3 and 4): this phenotype is typical of and diagnostic for release factor genes, having been demonstrated for the *prfA* and *prfB* genes encoding *E.coli* release factors RF1 and RF2 respectively (Weiss,R.B. *et al.*, 1984) and the *S.cerevisiae MRF1* gene encoding the mitochondrial release factor mRF1 (Pel *et al.*, 1992). In each case increasing the cellular levels of the release factor in relation to the suppressor tRNA acts to out-compete the tRNA species for stop codon binding. Furthermore, we show here that this antisuppression phenotype is effective against UAA, UAG and UGA suppressors (Table I and Figure 4), inferring that Sup35p and Sup45p together form an omnipotent release factor complex. This finding corroborates the results of genetic studies, where mutations in either the *SUP35* or *SUP45* gene resulted in an omnipotent suppressor phenotype (Inge-Vechtomov and Andrianova, 1970; Hawthorne and Leupold, 1974; Stansfield *et al.*, 1995). The omnipotence of the yeast release factor complex, observed *in vivo*, parallels the *in vitro* situation in *Xenopus*, where Sup35p is able to enhance, in a GTP-dependent manner, the peptidyl release activity of Sup45p at all three stop codons (Zhouravleva *et al.*, 1995).

The case for Sup35p and Sup45p together forming a release factor complex is strengthened by our finding that either of the higher eukaryote *SUP45* homologues, *Xenopus* Cl1 and human TB3-1, both with demonstrated eRF1 activity (Frolova *et al.*, 1994), can act in this putative release factor complex and couple functionally with yeast Sup35p, as indicated by their ability to act as multicopy antisuppressors in a strain overexpressing *SUP35* (Figure 3).

While *Xenopus* and human eRF1 (Sup45p) proteins have demonstrated release factor activity *in vitro* (Frolova *et al.*, 1994), it is not clear whether the level of termination activity demonstrated in the *in vitro* assay would be sufficient to out-compete any nonsense suppressor tRNA cognate for one of the stop codons if added to the assay, i.e. can Sup45p (eRF1) mediate efficient antisuppression alone *in vitro*? We would argue not, based on the results presented here, proposing instead that Sup45p is necessary, but not sufficient, for efficient *in vivo* termination. Rather, both the Sup45p and Sup35p proteins are required to generate the levels of release factor complex activity necessary to out-compete suppressor tRNAs and for normal cellular translation.

Confirmation that Sup35p represents a second component of the eukaryote release factor comes from evidence that in the *in vitro* termination assay Sup35p can stimulate eRF1 (Sup45p) activity in a GTP-dependent manner (Zhouravleva *et al.*, 1995). Nevertheless, the findings presented here raise questions about the mechanism of eukaryotic translation termination and its relationship to the corresponding prokaryote process, as defined by studies with *E.coli*.

A number of differences between the two are immediately apparent. Sup35p could, with GTP binding capability, perform a role analogous to that of the prokaryotic termination factor RF3. RF3, encoded by a non-essential gene, stimulates RF1- and RF2-mediated activity in a GTP-dependent manner (Milman *et al.*, 1969) and shows sequence homology to elongation factor EF-G (equivalent to the eukaryote elongation factor EF-2; Grentzmann *et al.*, 1994; Mikuni *et al.*, 1994). In contrast, Sup35p is encoded by an essential gene and shows considerable C-terminal homology to elongation factor EF-1α (Kushnirov *et al.*, 1988; Wilson and Culbertson, 1988). The homology of RF3 and Sup35p to different elongation factors argues for a fundamental mechanistic difference between the prokaryote and eukaryote termination processes. Perhaps as important in this respect is the observation that over-expression of both Sup45p and Sup35p is required to enhance the efficiency of termination (Figure 3), again a point of difference with prokaryotes, where overexpression of RF1 or RF2 alone is sufficient to generate an antisuppressor phenotype (Weiss,R.B. *et al.*, 1984).

The identification of Sup35p as an essential protein interacting with Sup45p (eRF1) is thus an important first step in the characterization of the components of the

Table II. Plasmids used in this study

Plasmid[a]	Protein expressed[b]	Source
pUKC802	Sup45p	Akhmaloka (1991)
pUKC606	Sup35p	This study
pVK62	Sup45p	This study
pVK63	Sup45p and Sup35p	This study
pEX1	*Xenopus* eRF1 (Sup45p) (yeast Sup45p1–10, *Xenopus*, 11–437)	M.Philippe
pEO6	Human eRF1 (Sup45p) (yeast Sup45p1–10, human, 11–437)	M.Kress
Two-hybrid vectors		
pUKC601	Gal4p bd (1–147)–Sup45p(10–437)	This study
pUKC605	Gal4p ad (768–881)–Sup35p(41–685)	This study
pG–DD	Gal4p ad (768–881)–Cpf1p(266–351)	J.Mellor
p13–DD	Gal4p bd (1–147)–Cpf1p(266–351)	J.Mellor
pGBT9	Gal4p bd (1–147)	S.Fields
pGAD424	Gal4p ad (768–881)	S.Fields

[a]All plasmids in the table carry the 2μ origin of replication and are therefore multicopy in yeast.
[b]Figures in brackets refer to the amino acids of each protein expressed, numbered with the initiator methionine residue as 1.

eukaryote termination process and will expedite further definition of the mechanisms involved. The findings presented here also have a direct bearing on related and developing issues, such as the suggestion that Sup35p may be a prion-type protein, able to adopt different conformations: it is proposed that each conformation exhibits distinct activities with respect to translation termination, giving rise to the yeast non-Mendelian genetic phenomenon [*psi*] (Cox, 1994; Tuite, 1994; Wickner, 1994). Finally, we forsee further interest in the notion of regulation of translation at the level of termination, with the recent finding that the *SAL6* gene, mutant alleles of which can act to enhance the activity of *sup35* and *sup45* omnipotent suppressors (Song and Liebman, 1987), codes for a protein, Sal6p, with homology to serine/threonine phosphatases (Vincent *et al.*, 1994).

Materials and methods

Strains and media

The *S.cerevisiae* strains used in this study were: MT552/36d (MATα *ura3-1*); MT422/1c (MATα *ura3-1 leu2-3,112 his5-2 ade2-1 SUP4-o*); 5V-H19 (MATa *ade2-1 can1-100 leu2-3,112 ura3-52 SUQ5* [psi^+]); DBY746 (MATα *his3-1 leu2-3,112 ura3-52 trp1-289*); MT576/5c (MATα $SUP4^{UGA}$ $ade2^{UGA}$ *ura3-1 leu2-3,112*); Y526 (MATa *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 can-1 gal4-542 gal80-538 ura3::GAL1-lacZ*). Y526 (Bartel *et al.*, 1993a) was a gift from S.Fields (State University of New York at Stony Brook). *Escherichia coli* strain DH5α (F′/*endA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1 recA1 gyrA* (Nal^R) *relA1* Δ(*lacZYA-argF*) U169 (ϕ*80dlac* Δ(*lacZ*)*M15*) was used for all cloning experiments. Yeast cultures were grown using standard conditions (Sherman, 1991) in YEPD liquid medium (2% w/v Bactopeptone, 1% w/v yeast extract and 3% w/v glucose). Yeast transformants were grown in 2% w/v glucose, 0.67% w/v yeast nitrogen base without amino acids (Difco), supplemented with the required amino acids and co-factors. Bacteria were grown in LB broth (Sambrook *et al.*, 1989).

Plasmid construction and DNA manipulation

All DNA manipulations and plasmid construction techniques were carried out using standard protocols (Sambrook *et al.*, 1989). The salient features of the plasmids used in this work are outlined in Table II.

Plasmid pEX1 consists of the yeast *SUP45* promoter and the first 10 codons of the yeast *SUP45* coding sequence ligated to the C-terminal 428 codons of the *Xenopus SUP45* (eRF1) gene. Plasmid pEO6 is identical, except the sequence coding for the C-terminal 427 codons of the human eRF1 (*SUP45*) gene replaces the *Xenopus* sequence of pEX1. These plasmids were generously donated by Dr M.Kress (Laboratoire d'Oncologie Moléculaire, Villejuif, France) and Dr M.Philippe (University of Rennes, Rennes, France). Plasmid pUKC606 consists of the yeast *SUP35* coding sequence and promoter on an *Xho*I–*Not*I fragment derived from plasmid pSM138 (Doel *et al.*, 1994) ligated into the multicopy vector pRS425 (Christiansen *et al.*, 1992) cut with *Xho*I and *Not*I. Plasmid pUKC802 was made by cloning the yeast *SUP45* promoter and gene into the multicopy vector YEp24 (Akhmaloka, 1991). Plasmid pUKC601 is a derivative of pGBT9 (Bartel *et al.*, 1993b) and consists of the yeast *SUP45* gene sequence coding for amino acids 10–437 cloned in-frame and downstream of the *GAL4* binding domain. pUKC601 was constructed by cloning the *SUP45* coding sequence and promoter on a *Sal*I–*Xho*I fragment cut from pUKC802 (Akhmaloka, 1991) into pBluescript IIKS+ cut with *Sal*I, generating pUKC600. The *SUP45* sequence coding for amino acids 10–437 was excised from pUKC600 on a *Bgl*II–*Sal*I fragment and ligated into pGBT9 cut with *Bam*HI and *Sal*I. Plasmid pUKC605 is derived from pGAD424 (Bartel *et al.*, 1993b) and was constructed by cloning the *SUP35* coding sequence and promoter on a *Xba*I fragment excised from pSM138 (Doel *et al.*, 1994) into plasmid pSP73 (Promega Corporation) cut with *Xba*I, generating pSP73SUP35. The *SUP35* coding sequence representing amino acids 41–685 was excised from pSP73SUP35 on a *Pst*I fragment and ligated into pGAD424 cut with *Pst*I, downstream of and in-frame with the *GAL4* activation domain, creating pUKC605.

Plasmid pUKC625, a derivative of pUKC802 (Akhmaloka, 1991), contains the *SUP45* gene with an adaptor inserted between the *Bam*HI and *Xho*I sites coding for a C-terminal affinity tag of six histidine residues.

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5′-GATCCAGACATCACCATCACCATCACTAAGCTTGAGC-3′
    3′-GTCTGTAGTGGTAGTGGTAGTGATTCGAACTCGAGCT-5′
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Plasmids p13-DD and pG-DD consist of the DNA sequence coding for the dimerization domain (amino acids 266–351) of transcription factor *CPF1* cloned in an in-frame fusion with either the *GAL4* binding or activation domains (Dowell *et al.*, 1992); both p13-DD and pG-DD were generous gifts of Dr Jane Mellor (University of Oxford, UK).

Plasmid pVK62 was constructed by ligating the *Xba*I–*Bam*HI fragment from pYsup1-1 (Breining *et al.*, 1984) into pEMBLYE23 (Cesareni and Murray, 1987) cleaved using *Xba*I and *Bcl*I. Plasmid pVK63 was constructed by cloning the *Bgl*II–*Bam*HI fragment from pSTR4 (Telckov *et al.*, 1986) containing the complete *SUP35* coding sequence and promoter into pVK62 cut with *Bam*HI.

Plasmids pUKC815-L, pUKC817-L, pUKC818-L and pUKC819-L were used in this work and are derived from pUKC815, pUKC817, pUKC818 and pUKC819 respectively (Stansfield *et al.*, 1995). The 'L' in the pUKC815-L series vectors used in this work designates replacement of the plasmid selectable marker *URA3* on a *Sma*I–*Sal*I fragment with the *LEU2* gene on a *Sma*I–*Sal*I fragment derived from plasmid pJJ250 (Jones and Prakash, 1990).

β-Galactosidase assays of yeast strains transformed with the pUKC815/817/818/819 series vectors

β-Galactosidase assays were performed according to the method of Finkelstein and Strausberg (1983).

Yeast transformation with plasmid DNA

Yeast were transformed by electroporation according to the method of Becker and Guarente (1991), using BioRad Gene-pulser equipment according to the instructions of the manufacturer.

Preparation of post-ribosomal supernatants

Yeast cultures were grown to a cell density of 3.5×10^7 cells/ml. Harvested cells were washed with lysis buffer (25 mM Tris–HCl, pH 7.2, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 25 mM KCl) containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine, 10 μM leupeptin and 10 μM pepstatin A to limit proteolytic degradation. The concentration of KCl in the lysis buffer was increased to 0.8 M if the PRS was to be subsequently used in the purification of Sup45p$(His)_6$. Following washing in lysis buffer, harvested cells were resuspended in a minimum volume of the same buffer. Cells were lysed by vortexing with glass beads and cell debris removed by centrifugation at 13 000 r.p.m. for 15 min in a benchtop microcentrifuge, producing a post-mitochondrial supernatant (PMS). The PMS was centrifuged in a Beckman TL100.3 rotor at 50 000 r.p.m. for 75 min, resulting in the sedimentation of polysomal ribosomes, 80S ribosomes and 40S and 60S subunits, with the generation of a PRS.

Immobilization of Sup45p$(His)_6$

The following operations were performed at room temperature unless otherwise stated and columns were allowed to flow by gravity.

A 500μl column of Ni–NTA resin (Qiagen) was equilibrated with 30 mM bis-Tris–HCl, pH 6.8, 0.8 M KCl, 5 mM 2-mercaptoethanol, 30 mM imidazole (buffer A). The PRS containing Sup45p$(His)_6$ was passed three times through the resin, which was then washed with 10 column vol. buffer A followed by 10 vol. buffer containing 30 mM bis-Tris–HCl, pH 6.2, 1 M KCl, 5 mM 2-mercaptoethanol, 20% glycerol, 30 mM imidazole. This procedure resulted in partial purification and immobilization of Sup45p$(His)_6$ on the Ni–NTA resin.

In order to assess the binding of Sup35p to the immobilized Sup45p$(His)_6$, the resin was equilibrated with 10 column vol. lysis buffer containing 25 mM KCl. A PRS prepared from cells overexpressing Sup35p (transformed with plasmid pUKC606) was then incubated with a suspension of the resin in 25 mM KCl lysis buffer for 2 h on a shaking platform at 4°C. After this time the resin was centrifuged briefly, the supernatant removed and retained and the resins returned to columns for washing with 10 column vol. 25 mM KCl lysis buffer containing 0.15% w/v Tween-20 detergent.

Protein gel electrophoresis and Western blot analysis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to standard protocols (Laemmli, 1970). SDS–PAGE gels contained 10% w/v acrylamide. Western blotting onto nitrocellulose was performed according to standard protocols (Towbin *et al.*, 1979; Harlow and Lane, 1988). Western blots were probed with either an anti-Sup45p polyclonal rabbit antibody used at 1:100 dilution (Stansfield *et al.*, 1992) or a polyclonal rabbit anti-Sup35p antibody used at 1:2000 dilution (Didichenko *et al.*, 1991). Bound antibody was detected using the Amersham ECL system according to the manufacturer's instructions.

Identification and quantification of two-hybrid system protein–protein interactions

Strain Y526 (Bartel *et al.*, 1993a), transformed with pair-wise combinations of two-hybrid vectors, was tested for β-galactosidase activity initially using X-gal staining of yeast colonies grown on nitrocellulose laid on agar medium, according to the method of Breeden and Nasmyth (1985). β-Galactosidase activities were further quantified by growing the transformants in liquid medium to a cell density of 4×10^7 cells/ml. A cell lysate was prepared in Z-buffer (Finkelstein and Strausberg, 1983) containing 1 mM phenylmethylsulphonyl fluoride, using glass bead lysis. Lysates were made up to 1 ml using Z-buffer and 200 μl *o*-nitrophenyl galactoside (ONPG; 4 mg/ml in water) added. The enzyme reaction rate was monitored at 420 nm and enzyme activity calculated using an exctinction coefficient for ONP of 0.0045 nmol/ml. β-Galactosidase activities were expressed as pmol ONP produced/min/mg protein. Protein concentrations were determined using standard protocols (Lowry *et al.*, 1951).

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