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ORIGINAL PAPER

P. Silar · M. Rossignol · R. Tahar
Z. Derhy · A. Mazabraud

Informational suppressor alleles of the *eEF1A* gene, fertility and cell degeneration in *Podospora anserina*

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Abstract Mutations that increase readthrough at a UGA stop codon (informational suppressor mutations) were created in the gene (*AS4*) that encodes translation elongation factor eEF1A in the filamentous fungus *Podospora anserina*. The results strongly suggest that the net charge of the eEF1A protein controls the accuracy of translation. Physiological analysis of the mutant strains shows that some of the alleles dominantly increase life span, while only one drastically modifies fertility. This exceptional allele (*AS4-56*) causes a wide array of phenotypes, including a new growth cessation phenomenon that is different from Senescence or Crippled Growth, previously known degenerative syndromes that are both controlled by *AS4*. The data emphasise the fact that eEF1A exerts a complex control over cellular physiology.

Key words Translation elongation · Translational accuracy · Sexual reproduction · Ageing · *Podospora anserina*

Introduction

eEF1A is an essential G-protein that plays a major role in the elongation phase of cytosolic protein synthesis in all eukaryotic cells (Negrutskii and Elskaya 1998, for a review). Like its bacterial counterpart EF-Tu, eEF1A binds aminoacyl-tRNA and GTP to form a ternary complex that delivers the charged tRNA to the A site of the ribosome, at the expense of the hydrolysis of one GTP molecule. Several data suggest that the bacterial and the eukaryotic proteins differ with respect to how they perform this function (Negrutskii and Elskaya 1998), and the precise biochemical events that occur during the elongation step catalysed by eF1A are still unknown. Like its bacterial counterpart, and together with some ribosomal proteins and RNAs, eEF1A controls the accuracy of the mRNA decoding process (Sandbaken and Culberston 1988). Thus, it has been shown that it influences the levels of readthrough and frameshifting (Sandbaken and Culberston 1988; Dinman and Kinzy 1997; Farabaugh and Vimaladithan 1998). The precise mechanism by which eEF1A controls accuracy (especially frameshifting) is not yet fully understood.

eEF1A is a highly conserved protein. However, in several organisms, multiple genes encode different forms of eEF1A. These isoforms are differentially regulated under various conditions or in diverse tissues. A further level of complexity is added by the fact that eEF1A can be modified by phosphorylation, methylation or addition of glycerolphosphoryl-ethanolamine (Tuhackova et al. 1985; Merrick 1992; Kielbassa et al. 1995). Phosphorylation modifies the activity of the factor (Tuhackova et al. 1985), but no role has been detected for the other modifications. It is suspected that these isoforms and modifications allow optimisation of translation and/or of other events involving eEF1A.

Indeed, in addition to its role during translation, eEF1A exhibits numerous unrelated activities (Negrutskii and Elskaya 1998, for a review), including organisation of the cytoskeleton (see Durso and Cyr 1994; Condeelis 1995, for review), activation of phosphatidylinositol 4-kinase (Yang et al. 1993), and activation of the degradation of some proteins by the ubiquitin pathway (Gonen et al. 1994). All these properties suggest that eEF1A is likely to participate in the

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P. Silar (✉)
Institut de Génétique et Microbiologie de l'Université de Paris-Sud,
CNRS UMR 8621, Bât. 400,
91405 Orsay Cedex, France
E-mail: silar@igmors.u-psud.fr
Tel.: +33-1-69154658
Fax: +33-1-69157006

M. Rossignol · R. Tahar · A. Mazabraud
Centre de Génétique Moléculaire du CNRS,
91198 Gif-sur-Yvette Cedex, France

Z. Derhy
Institut d'Enzymologie du CNRS,
91198 Gif-sur-Yvette Cedex, France

co-ordination of translational activity with the demands of the cell, in response to various growth conditions.

In light of these characteristics, it is not surprising that eEF1A is essential for cell viability (Cottrelle et al. 1985; Silar et al. 2000). In the mouse, a mutation in the eEF1A2 isoform causes the "*wasted*" phenotype, characterised by a set of alterations leading to degeneration of the nervous and immune systems and death of the organisms (Chambers et al. 1998). Interestingly, eEF1a seems to be involved in numerous aspects of biology, including differentiation and ageing. Indeed, mutations affecting this factor may promote susceptibility to oncogenic transformation (Tatsuka et al. 1992) and a mutated eEF1A gene is found in some cancerous cells (Gopalkrishnan et al. 1999). During ageing, eEF1A activity diminishes in many cell types, and this is correlated with a decrease in the efficiency of translation (Webster 1985; Cavallius et al. 1986). Following this observation, Shepherd et al. (1989) overexpressed eEF1A in *Drosophila melanogaster* and observed an increase in longevity in adult males. However, subsequent studies on *Drosophila* led to contradictory conclusions on this point (Stearns and Kaiser 1993; Shikima et al. 1994). Nonetheless, the decrease in eEF1A activity with ageing was recently confirmed in *Drosophila* (Shikima and Brack 1996).

The filamentous fungus *Podospora anserina* is a model organisms that is extensively used in ageing studies (Jamet-Vierny et al. 1999; Osiewacz and Kimple 1999). We have shown that mutations in *AS4*, the sole gene that encodes eEF1A in *P. anserina* drastically increase life span, diminish fertility and permit the propagation of a non-conventional infectious element responsible for a growth alteration called Crippled Growth (Silar and Picard 1994; Silar et al. 1999). The mutations located in the *AS4* gene were all recovered because they antagonise the deleterious effect on sporulation of a UGA suppressor tRNA and were shown globally to decrease accuracy since they also antagonise the effect of an omnipotent informational suppressor (antisuppressor mutations, Picard-Bennoun 1976). Whereas increased translational accuracy promotes the effects of the *AS4* mutations on the development of Crippled Growth (Silar et al. 1999), it is yet not clear what causes fertility impairment and life span extension.

Here, we report the phenotype caused by new mutations located in eEF1A that were obtained following in vitro mutagenesis. These were based either on mutations already known to act as suppressor mutations in yeast (Sandbaken and Culberston 1988) or designed to change the amino acid at positions modified in the previously obtained antisuppressor *AS4* mutants. The mutations cause a dominant increase in the incidence of UGA readthrough (suppressor effect). Surprisingly, some increase life span in a dominant fashion. However, with the exception of *AS4-56*, they do not significantly perturb sexual reproduction. Interestingly, this new allele displays several unexpected properties, including the triggering of a new type of growth arrest. These data emphasise the complexity of the role of eEF1A in cell physiology. They also show that the net charge of the eEF1A protein controls the accuracy of translation.

Materials and methods

Strains, mutations, growth conditions and genetic analysis

The *P. anserina* strains used in this study were all derived from the S strain (Rizet 1953), thus ensuring a homogenous genetic background. *193* and *leu1-1* are UGA nonsense mutations that affect spore pigmentation and leucine biosynthesis, respectively. These alleles are used to test the level of in vivo readthrough (Picard 1973; Coppin-Raynal 1981), and hence serve to measure the translation error rate (see below).

Standard culture conditions, as well as general genetic methods for this fungus, have been described previously (Esser 1974).

DNA manipulations

DNA manipulations were performed as recommended by Ausubel et al. (1987). The various antisuppressor *AS4* alleles were cloned by screening minibanks of genomic DNA from the corresponding mutant strains. Mitochondrial DNA (mtDNA) was extracted by the rapid method of Lecellier and Silar (1994). Modifications of the mitochondrial genome in at least 9 senescent strains per genotype were analyzed by restriction analysis with *Hae*III, as described (Jamet-Vierny et al. 1997).

Construction of strains carrying *AS4* suppressor alleles

The $AS4^{+}$ gene was mutagenised in vitro by conventional methods (Ausubel et al. 1987) in order to change only one amino acid within the protein. These alleles should thus be expressed from the *AS4* promoter at the same level as the wild-type allele. Each mutant allele was then sequenced to ensure that only the desired mutation had been introduced. This allowed the recovery of the mutant alleles *AS4-55* to *AS4-61* listed in Table 1. DNA fragments containing the various alleles were then cloned into pMOcosX (Orbach 1994) or pBC-Hygro (Silar 1995) and transformed into a

Table 1 Amino acid replacements in eEF1A

Allele	Amino acid change	Remarks
AS4-4	Lys_{42}→Glu	–
AS4-11	His_{27}→Arg	–
AS4-24	Arg_{68}→His	–
AS4-27	Thr_{107}→Ile	–
AS4-29	Gly_{51}→Asp	–
AS4-30	Gly_{350}→Asp	–
AS4-33	Ala_{433}→Val	–
AS4-43	Thr_{431}→Ala	–
AS4-44	Gly_{53}→Asp	–
AS4-55	Glu_{287}→Lys	Equivalent to yeast *TEF2-1*
AS4-56	Gly_{53}→Lys	Same position as *AS4-44* but opposite charge
AS4-57	Thr_{143}→Ile	Equivalent to yeast *TEF2-7*
AS4-58	Glu_{41}→Lys	Equivalent to yeast *TEF2-3*
AS4-59	Glu_{163}→Gln	Difference in the two mammalian isoforms
AS4-60	Gly_{51}→Lys	Same position as *AS4-29* but opposite charge
AS4-61	Gly_{350}→Lys	Same position as *AS4-30* but opposite charge

P. anserina S mat+ strain. These two vectors carry a hygromycin resistance gene, which allows one to screen for transformants and follow the segregation of the transgenes in crosses. For each allele, six transformants were crossed with a *leu1-1 ΔAS4 mat-*/*leu1-1 AS4*$^+$ *mat+* heterokaryotic strain (Silar et al. 2000). Among the progeny of these crosses, we could recover homokaryotic strains with the following genotypes: *leu 1-1* {*hygR, AS4*$^-$} Δ*AS4 mat-*, {*hygR, AS4*$^-$} Δ*AS4 mat-*, *leu1-1* {*hygR, AS4*$^-$} *AS4*$^+$ *mat+* and {*hygR, AS4*$^-$} *AS4*$^+$ *mat+*, where {*hygR, AS4*$^-$} designates the mutant transgenic copy. Except in the case of *AS4-59* and *AS4-55*, two independent integrated copies derived from two different primary transformants that were able to ensure cell viability were then selected for further studies. The strains were then crossed with *S mat-* in order to obtain {*hygR, AS4*$^-$} *AS4*$^+$ *mat-* strains. These were crossed with a heterokaryotic *leu1-1 ΔAS4 mat+*/*leu1-1 AS4*$^+$ *mat-* strain (Silar et al. 2000). Among the progeny of these crosses, we recovered *leu1-1* {*hygR AS4*$^-$} Δ*AS4 mat+*, {*hygR, AS4*$^-$} Δ*AS4 mat+*, *leu1-1* {*hygR, AS4*$^-$} *AS4*$^+$ *mat-*, and {*hygR, AS4*$^-$} *AS4*$^+$ *mat-* strains. The crosses thus allowed us to obtain strains that carry either the Δ*AS4* allele at the endogenous locus and a transgenic copy of the mutant allele or the *AS4*$^+$ allele at the endogenous locus and a transgenic copy of the mutant allele (partial diploid heterozygous strains). All the desired strains were then subjected to phenotypic analysis.

For *AS4-59*, only one integrated copy was selected and analysed as described, since this allele was found to display the same properties as wild type. In the case of *AS4-55*, none of the six integrated copies first tested were able to promote viability when combined with the deletion (i.e., ascospores with the genotype *leu1-1* {*hygR, AS4*$^-$} Δ*AS4 mat-* or {*hygR, AS4*$^-$} Δ*AS4 mat-* were not recovered), suggesting that this allele was lethal. We thus crossed 41 additional transformants with the *193* tester strain. Among these crosses, two clearly yielded green spores. This was taken as an indication that the two transformants carried an expressed copy of *AS4-55*. Attempts to recombine these copies with the deletion failed, indicating that *AS4-55* is unable to restore viability. Thus only the partial diploid heterozygous strains that carry *AS4*$^+$ and *AS4-55* were analysed.

Correct expression of the *AS4-56*, *AS4-57*, *AS4-58*, *AS4-60* and *AS4-61* mutant alleles was verified as previously described by examining total protein extracts from the various mutants and from wild type fractionated on polyacrylamide gels, followed by quantification using the using Scion Image software (Silar et al. 2000). The data (not shown) indicated that all mutant alleles are expressed at the same level as the wild-type form.

Physiological analysis

Longevity was measured on M_2 minimal medium at 27 °C in the dark as described (Belcour and Begel 1980). The longevity given for a particular strain is expressed as the average length (in cm ± standard deviation) reached by independent cultures before the onset of senescence. At least nine cultures derived from at least two different ascospores of the appropriate genotype were analysed for each strain (i.e., the values listed in Table 2 are derived from at least $4 \times 9 = 36$ independent cultures). The fertility of a particular *AS4* mutant strains was measured after fertilization with the conidia of a strain of the opposite mating type that carried the same *AS4* allele(s), as described (Coppin et al. 1993).

Suppression analysis

The suppression efficiency of the new *AS4* alleles was measured according to the following strategies. (1) The *193* mutation is a suppressible UGA nonsense mutation that affects ascospore colour (Picard 1973). Wild-type ascospores are black and *193* ascospores are white. The readthrough level at the *193* mutation site can be estimated in the progeny of a {*hygR, AS4*$^-$} Δ*AS4* × *193* {*hygR, AS4*$^-$} *AS4*$^+$ cross based on the intensity of the green pigmentation in *193* {*hygR AS4*$^-$} Δ*AS4* and *193* {*hygR AS4*$^-$} *AS4*$^+$ ascospores. The *leu1-1* mutation is a UGA nonsense mutation that prevents growth on M2 minimal medium and is suppressed by informational suppressors (Coppin-Raynal 1981). The *leu1-1* {*hygR, AS4*$^-$} Δ*AS4* or *leu1-1* {*hygR, AS4*$^-$} *AS4*$^+$ strain can grow on minimal medium at a specific rate. The ratio of the growth rate on M_2 medium to that on M_2 supplemented with 50 mg/l leucine can thus be used to quantify the level of readthrough level at the site of the *leu1-1* mutation.

Table 2 Characteristics of *AS4* suppressor alleles

Genotype[a]	Mutation	Phenotypic parameter				Suppressor activity		
		Morphology	Longevity[b]	Fertility[c]	CG[d]	*193*[e]	*leu1-1*[e]	Pm resistance[f]
AS4$^+$	–		9.5 ± 1.0	+ + +	No	White	0	70%
AS4$^+$ *AS4-55*	E287K	Slightly altered	16.5 ± 2.2	+ +	No	Light green	0	55%
AS4$^+$ *AS4-56*	G53K	WT	15.7 ± 2.3	+ + +	Yes	White to dark green	< 0.05	40%
AS4$^+$ *AS4-57*	T143I	WT	9.9 ± 1.9	+ + +	No	Almost white	0	70%
AS4$^+$ *AS4-58*	E41K	WT	9.6 ± 1.0	+ + +	No	Almost white	0	70%
AS44$^+$ *AS4-59*	E163Q	WT	9.4 ± 0.9	+ + +	No	White	0	70%
AS4$^+$ *AS4-60*	G51K	WT	10.8 ± 1.5	+ + +	No	Green	0	40%
AS4$^+$ *AS4-61*	G350K	WT	12.8 ± 1.7	+ + +	No	Almost white	0	70%
Δ *AS4 AS4-56*	G53K	Very altered	?[g]	–	ND	White to dark green	ND	0%
Δ *AS4 AS4-57*	T143I	WT	10.1 ± 0.9	+ + +	No	Very light green	0	70%
Δ *AS4 AS4-58*	E41K	WT	9.8 ± 1.0	+ + +	No	Very light green	0	65%
Δ *AS4 AS4-59*	E163Q	WT	10.0 ± 0.8	+ + +	No	White	0	70%
Δ *AS4 AS4-60*	G51K	WT	11.1 ± 1.4	+ +	No	Green	0	30%
Δ *AS4 AS4-61*	G350K	WT	13.1 ± 2.0	+ + +	No	Very light green	0	70%

[a] The first allele refers to the one present at the *AS4* locus, the second one to the nature of the transgenic allele carried at an ectopic position

[b] Longevity is expressed in cm ± SD

[c] Fertility was estimated for self-crosses of the strain with the indicated genotype

[d] Presence of Crippled Growth (CG) was checked as described by Silar et al. (1999)

[e] Suppression at the *193* and *leu1-1* sites was measured as described in Materials and methods

[f] Paromomycin resistance (Pm) is expressed as the percentage of linear growth observed on medium containing 500 mg/l of paromomycin relative to that on unsupplemented medium after 3 days of culture

[g] See text for details

ND: not determined, see text

Paromomycin resistance

The paromomycin resistance level of a particular strain is the ratio obtained by dividing the diameter of the thalli after 3 days of growth on M_2 medium supplemented with 500 mg/l of paromomycin by the diameter of the thalli after 3 days of growth on non-supplemented M_2 medium.

Results

We have previously shown that antisuppressor mutations in the *AS4* gene that encodes eEF1A in *P. anserina* increase longevity, impair ascospore formation and permit the development of Crippled Growth (Silar and Picard 1994; Silar et al. 1999). It was thus of interest to test the effect on these phenotypes of suppressor mutations also located in *AS4*. Unlike the antisuppressor mutation, these would decrease translational accuracy. Mutations with this latter property have already been described in *Saccharomyces cerevisiae* (Sandbaken and Culberston 1988).

Suppressor alleles that were designed to mimic the *S. cerevisiae* ones could perhaps display slightly different properties compared to the yeast alleles and may modify life span.

In order to perform site-directed mutagenesis on the *AS4* gene, three of the known suppressor alleles of *TEF2*, one of the two genes that encode eEF1A in *S. cerevisiae*, were chosen, *TEF2-1*, *TEF2-3* and *TEF2-7*. These alleles display various effects on frameshifting, readthrough and sensitivity to inhibitors of translation elongation (Sandbaken and Culberston 1988; Dinman and Kinzy 1997; Farabaugh and Vimaladithan 1998). In each case, the corresponding mutation was constructed in vitro in *AS4* (see Table 1) to yield the alleles *AS4-55*, *AS4-57* and *AS4-58*, respectively. These alleles were then transformed into the *P. anserina S* strain and two independent transformants were selected for further analysis (see Materials and methods), in order to take position effects into account. Transformants were crossed with the heterokaryotic strains carrying a deletion of *AS4* (Silar et al. 2000), allowing the recovery of strains carrying the ectopically inserted mutant alleles in association with either $AS4^+$ or $\Delta AS4$. Phenotypic and translational parameters were then measured (Table 2).

In no instance were differences detected between the two integrated copies; similarly no difference due to the mating type was seen. For each allele, the data described below are thus a compilation of the results observed for the four different combinations, e.g., the two integrated copies associated with either of the mating types.

In yeast, *TEF2-1* promotes a strong dominant increase in frameshifting and moderate UAG/UAA readthrough; no effect is detected on UGA readthrough (Sandbaken and Culberston 1988; Farabaugh and Vimaladithan 1998). The mutant allele also increases misreading and causes a strong reduction in resistance to paromomycin (Sandbaken and Culberston 1988). We could not obtain a homozygous strain carrying the corresponding mutant allele (*AS4-55*) in *P. anserina*. A similar situation was also reported for the *TEF2-1* allele in yeast (Dinman and Kinzy 1997). The analysis was thus performed on heterozygous, partial diploid strains carrying *AS4-55* and $AS4^+$. As described in Table 2, these strains exhibit increased longevity and slightly reduced fertility. Clearly, the mutation promotes UGA readthrough at the *193* mutation site but not at *leu1-1*. Like the *TEF2-1* mutation, it also promotes a marked reduced sensitivity to paromomycin.

In yeast, *TEF2-3* causes a dominant increase in frameshifting but has no effect on readthrough (Sandbaken and Culberston 1988; Dinman and Kinzy 1997). It does not promote any modification of sensitivity to paromomycin (Sandbaken and Culberston 1988; Dinman and Kinzy 1997). The corresponding mutation in *P. anserina* is called *AS4-58*. Homozygous or heterozygous strains carrying this mutation do not present any phenotypic modification. They display a very small increase in UGA readthrough, which is more pronounced in the homozygous strain. Note that, unlike the yeast strain carrying *TEF2-3*, strains homozygous for *AS4-58* show a small decrease in paromomycin resistance. This is clearly visible on medium containing 750 mg/l paromomycin, since at this concentration the wild-type *S* strain grows at 10% of the rate on medium without paromomycin, whereas the *AS4-58* homozygous strains do not grow at all.

The *TEF2-7* mutation causes a dominant increase in frameshifting and UAG readthrough (Sandbaken and Culberston 1988; Dinman and Kinzy 1997). It is also associated with increased resistance to paromomycin when homozygous (Dinman and Kinzy 1997). The corresponding mutation in *P. anserina*, *AS4-57*, does not promote any phenotypic alteration. As observed for *AS4-58*, it promotes, at the *193* locus only, a very small increase in readthrough, that becomes more pronounced when the mutation is homozygous. This mutation does not modify the resistance/sensitivity of the strains to paromomycin.

An additional allele was designed following a similar trans-species rationale. Comparison of the two mammal isoforms of eEF1A reveals several polymorphic positions. Some of these could affect the degree of translational fidelity (Silar 1994). We thus modified *AS4* at one of these positions; the glutamic acid at position 163 was changed into a glutamine, to obtain *AS4-59*. Analysis of this mutant allele revealed that it does not promote any modification in the parameters of translation or any obvious phenotypic alteration.

Electrostatic interactions may be involved in the control of translational accuracy

Because *AS4-55* displays increased life span, we decided to test additional suppressor alleles to confirm this phenotype. However, we changed the rationale for the design of the mutations. There is a striking correlation

between the net charge of eEF1A and translational accuracy. In the yeast mutants, eight out of the nine yeast suppressor alleles change a negatively charged amino acid to a positively charged one (Sandbaken and Culberston 1988). To confirm this, the nine available *P. anserina* antisuppressor mutant alleles were cloned and sequenced (Table 1). Four out of the nine *P. anserina* antisuppressor alleles (*AS4-4*, *AS4-29*, *AS4-30* and *AS4-44*) display the same correlation between charge and translational fidelity (Table 1). To ascertain whether charge controls accuracy, we designed three more alleles that change the amino acid glycine to lysine at the three positions where it is changed to aspartic acid in *AS4-29*, *AS4-30* and *AS4-44*, yielding *AS4-60*, *AS4-61* and *AS4-56*, respectively (Table 1). These were then analysed as described for the previous alleles (Table 2). In all three cases, we were able to obtain the alleles associated with the $AS4^+$ or the Δ*AS4* allele, showing that these alleles are viable. In all three cases, the mutations caused a suppressor phenotype, as measured by the level of readthrough at the *193* locus (Table 2). Note that the presence of a wild-type copy of *AS4* in association with the mutant copy reduces the suppression efficiency in the three cases. This confirms the role of electrostatic interactions in the control of translational accuracy.

For *AS4-60*, suppression is moderate at the *193* locus but none is detected at the *leu1-1* mutation site. Paromomycin sensitivity is strongly increased. Longevity and mycelium morphology are the same as in wild type. However, fertility is slightly diminished.

For *AS4-61*, suppression is barely detectable at the *193* locus and none is detected at the *leu1-1* locus. Paromomycin sensitivity is not affected. It is noteworthy that the corresponding antisuppressor mutation (*AS4-30*), from which *AS4-61* was derived, surprisingly shows increased sensitivity to this antibiotic (Coppin-Raynal 1981). Longevity is slightly increased –to the same extent in the homozygous and heterozygous strains. Otherwise, *AS4-61* mutant strains are indistinguishable from wild type.

Strikingly, the *AS4*-56 allele is associated with very unusual phenotypes that are described in the next section.

AS4-56 strains display a variegated suppressive effect, a new growth arrest phenotype and show Crippled Growth

We observed that the *AS4-56* allele promotes pronounced and dominant suppression of *193*. Most surprisingly, suppression is highly variable, since ascospore colour ranges from white to dark green, most being green. This is not due to the presence of another, cryptic, mutation but is likely to be caused by a novel epigenetic effect, akin to variegation. (1) No Mendelian segregation of the ascospore colour is observed. (2) *193* Δ*AS4 AS4-56* or *193* $AS4^+$ *AS4-56* mycelium derived from white ascospores yields ascospores with the full spectrum of colour when crossed with *193*. This was observed in two consecutive generations. (3) The same phenomenon is observed with the ascospores derived from dark green ascospores. (4) The positions of transgene integration is unlikely to play a role, since the two copies tested map at different locations and both display the phenomenon.

Unlike the *AS4* suppressor alleles previously examined, suppression by *AS4-56* is not restricted to the *193* site, since *leu1-1* is also suppressed in the $AS4^+$ *AS4-56* background (we were not successful in constructing a *leu1-1* Δ*AS4* strain, which prevents us from testing the suppression efficiency when *AS4-56* is homozygous). The mutation also promotes a dominant increase in sensitivity to paromomycin.

$AS4^+$ *AS4-56* strains live longer than wild type but have wild-type morphology and fertility. In contrast, Δ*AS4 AS4-56* strains present a very altered mycelium morphology, grow very slowly, and are unable to form perithecia (Fig. 1).

Δ*AS4 AS4-56* ascospores present an interesting pattern of germination and growth. A few form a germi-

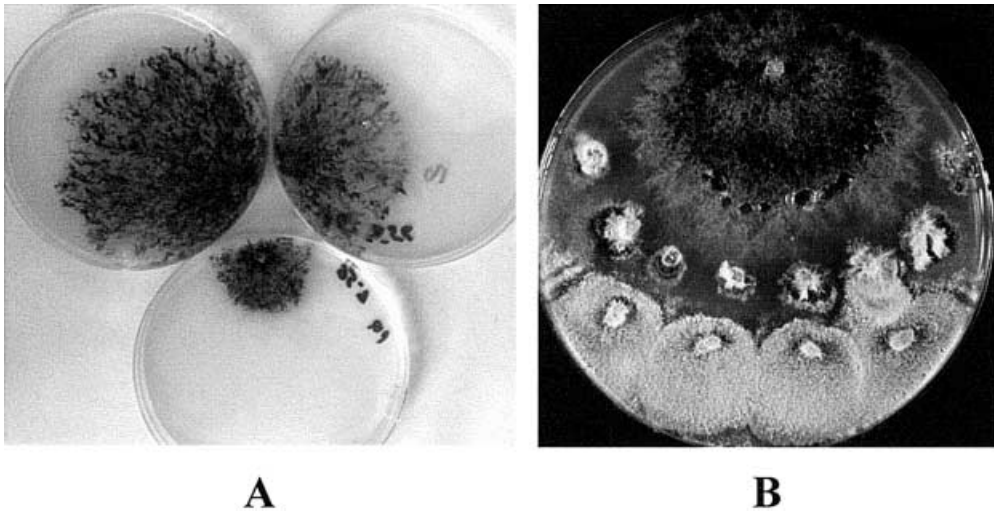

Fig. 1 A The three *AS4* Δ*AS4-56* cultures depicted in the Figure were obtained from three independent ascospores and were inoculated onto M2 medium on the same day. The resulting cultures showed a range of growth potential despite having the same genotype. **B** A *AS4* Δ*AS4-56* culture was inoculated on M2 medium and incubated until it stopped growing. Mycelium explants from a wild-type *S* mycelium were then added and the culture was incubated for another 4 days. Explants placed near the *AS4* Δ*AS4-56* culture showed an inhibition of growth, whereas those located farther away did not

nation tube, but do not grow further. The others stop growing at various times, generating a range of cultures with various diameters (see Fig. 1 for typical examples). Although reminiscent of the Senescence exhibited by *P. anserina*, this phenomenon is not due to the same cause. Indeed, several lines of evidence suggest that this is a novel growth type of growth arrest. (1) Upon reinoculation, all the explants taken either from the centre or from the edge of the arrested cultures regenerate a short culture. (2) This occurs with both short-grown and long-grown cultures. (3) Because mtDNA is rearranged during Senescence in *P. anserina* (Osiewacz and Kimple 1999 for review), we purified mtDNA from arrested *AS4-56* cultures and subjected it to molecular analysis. The analysis of arrested cultures showed no obvious mtDNA rearrangements (in contrast, mtDNA modifications classical of Senescence are detected in senescent, heterozygous, $AS4^+$ *AS4-56* cultures, as well as in all strains carrying any of the alleles *AS4-55*, *AS4-57*, *AS4-58*, *AS4-59*, *AS4-60* or *AS4-61*). (4) Arrested cultures are not able to transmit Senescence to young wild-type mycelia, under conditions where wild-type senescent cultures do so.

Growth arrest of the Δ*AS4 AS4-56* strains could be due to several factors; among these are exhaustion of some nutrient in the growth medium or modification of this medium in such a way that it can no longer sustain growth. In order to test the first of these possibilities, we increases the concentration of the nutrients up to 1.2 times that in the classical medium, while maintaining the volume constant. This results in a uniform set of the cultures that display a short life span. Clearly, nutrient deficiency is not the cause of the growth arrest and increasing the concentration of nutrients actually decreases the growth potential. Secondly, we increased the volume of the medium in the Petri dishes without increasing the amounts of nutrients. This results in a set of cultures with a long life span, as if dilution of some substance allows the hyphae to grow longer. Strikingly, we observe inhibition of the growth of explants of wild-type mycelium that are inoculated close to an arrested Δ*AS4 AS4-56* mycelium (see Fig. 1 for a typical result). In contrast, explants located further away from such an arrested mycelium do grow. These data indicate that dying *AS4 AS4-56* mycelia release some poisonous substance that may cause their growth arrest.

Because the *AS4-56* mutation causes a variegated suppression effect and the mutant strains show a range of life span, we investigated the relationship between these two phenomena. The longevity of white or dark green *193 AS4-56* ascospores was measured. No correlation was observed between the two phenotypes: both white and dark green ascospores yielded strains with the full range of longevity. Because there is a strong effect of culture conditions (see above) on the growth potential, the most likely explanation to account for the range of life span is some variability in medium composition between the individual petri plates.

One final striking phenotype caused by the *AS4-56* mutation is observed in the $AS4^+$ *AS4-56* strains. Upon exit from stationary phase these strains exhibit the Crippled Growth alteration. The alteration is not stable and the cultures revert to Normal Growth after a few centimetres of expansion. Because their growth is very altered, we could not reliably test this phenotype in Δ*AS4 AS4-56* strains. Note that none of the *AS4* suppressor mutations exhibits this effect.

Discussion

The complex phenotypes associated with eEF1A mutations in *P. anserina*

Our previous analysis of eEF1A antisuppressor mutations showed that these types of mutations cause three main phenotypes: an increase in life span, impairment of sporulation and the potential for the Crippled Growth phenomenon to develop (Silar and Picard 1994; Silar et al. 1999). Note that, in the original screen, numerous additional mutations were described as lethal (Picard-Bennoun 1976). These mutations are recessive with respect to the three phenotypes (Silar and Picard 1994; Silar et al. 1999).

Here, we have described the effects of six suppressor mutations located in *AS4*. These display a set of phenotypes that vary depending on the mutation considered.

First, at the translational level, all promote suppression of *193*, although with different efficiencies. Indeed, only with three of them is moderate suppression of *193* achieved. Note that these same mutations also trigger an increase in sensitivity to paromomycin. *AS4-56* deserves special mention since (1) it presents a variegation for suppression of *193*; (2) it is the only one of the six suppressors that is also able to suppress the *leu1-1* mutation; and (3) the $AS4^+$ *AS4-56* strains present the Crippled Growth alteration. This latter phenotype is quite unexpected for a suppressor mutation, because we have presented convincing evidence (Silar et al. 1999) to show that this phenotype is caused by an increase in translational accuracy. We thus favour the idea that *AS4-56* may display very unusual properties with respect to suppression, in the sense that it may decrease or increase accuracy depending on the nucleotide context that surrounds the decoded codon in the mRNA. Such opposite effects on accuracy at different codons have already been observed when the level of eEF1A is varied (Farabaugh and Vimaladithan 1998).

Second, at the physiological level, four out of the six suppressor mutations (*AS4-57*, *AS4-58*, *AS4-60* and *AS4-61*) cause no or only very limited extension of life span, and wild-type or near-wild-type fertility. *AS4-55* proved to be lethal, and to cause a significant, dominant, increase in life span when heterozygous. Strikingly, *AS5-56* displays a very complex set of phenotypes that differ depending on whether a wild-type copy of the gene is present or not.

When heterozygous, its main phenotype is increased life span. Note that the same phenotype is promoted by

AS4-55 and, to a lesser extent, by *AS4-61*. The increase in longevity associated with *AS4-56* and other suppressor alleles is dominant, unlike that promoted by the *AS4* antisuppressor alleles. We thus propose that increase in longevity may be caused by different mechanisms in the suppressor and antisuppressor strains. This is supported by the following observation. When the same amino acid is changed to lysine in the suppressor or to aspartic acid in the antisuppressor mutations the results on longevity can be very different; for example, *AS4-29* doubles life span (Silar and Picard 1994) whereas *AS4-60* does not (Table 2). In the suppressor, it is unlikely that the error rate per se is involved because the level of suppression of *193* associated with the different alleles is not correlated with extension of life span (see Table 2). We have obtained data (Silar et al., unpublished results) that demonstrate that the error rate per se is also not involved in the life span extension associated with *AS4* antisuppressor alleles. This is consistent with previous findings that the error level is not involved in defining life span (Belcour et al. 1991; Silar et al. 1997).

When associated with Δ*AS4*, *AS4-56* determines drastic alterations in growth, which stops at various times. This growth arrest syndrome is different from the classical Senescence presented by *P. anserina* (Rizet 1953). Our data strongly suggest that the Δ*AS4 AS4-56* strains release into the medium a toxic substance that may be responsible for cessation of their own growth. Several compounds are good candidates for this poison. Indeed, some *P. anserina* are known to produce anserinones, which are antifungal, antibacterial and cytotoxic compounds (Wang et al. 1997). Like its close relative *Sordaria araneosa*, *P. anserina* may also produce (a) Sordarin-like toxin(s), which are specific antifungal agents that act on the translational apparatus (Hauser and Sigg 1971).

AS4-56 thus displays very peculiar phenotype both at the translational and physiological levels. *AS4-60*, which involves the same amino acid change two amino acids away (Gly51→Lys in *AS4-60* and Gly53→Lys in *AS4-56*), or *AS4-44* (Gly53→Asp), from which *AS4-56* was derived, does not cause such a drastic phenotype. Note that position 53 is in a region of the eEF1A protein that does not align well with the EF-Tu eubacterial protein (Cousineau et al. 1992). This region is very conserved in eukaryotes and may thus have evolved to fulfil a function specific of the eukaryotic cell. At the present time it is premature to speculate upon the nature of this function, but as stated in the Introduction many eEF1A functions are potential candidates.

Thus, overall, our data show that modifications in eEF1A may perturb many cellular processes, resulting in a large array of phenotypes. Two hypotheses may be proposed. First, all the effects could be due to specific effect on translation of some key proteins. The different mutations in eEF1A may modify the translation of these proteins, which would result in the observed phenotype. Alternatively, perturbation of some of the several known functions of eEF1A may cause the phenotypes. One mutation may indeed specifically perturb some but not all of these processes, leading to a complex effect on physiology. Note that the fact that increased longevity may be caused by different mechanisms in the antisuppressor and suppressor strains agrees well with our estimate of the number of genes involved in longevity control (Rossignol and Silar 1996), which suggests that many mechanisms are involved in defining longevity.

Role of electrostatic interactions in defining translational accuracy

We have detected an effect of the net charge of the eEF1A protein on translational fidelity: the more negatively charged the protein is, the more accurate is translation. It is noteworthy that the mutations concerned are scattered all over the protein. This relationship has been explored and confirmed by changing three positions in the *P. anserina* eEF1A protein. The same charge relation has already been observed for the S7 ribosomal protein of *P. anserina* (Dequard-Chablat 1986). This protein is encoded by the *su12* gene in *P. anserina* (Silar et al. 1997). We propose that a mechanism that involves electrostatic interactions is implicated at the decoding center in checking the fidelity of the translation process. One possibility is that electrostatic interactions participate in the binding of the eEF1A:GTP:aa-tRNA complex to the ribosome. Rejection of the incoming complex could be in part based on repulsion of negative charges carried both by the ribosome and the elongation complex. If these repulsive forces prove to be stronger than the codon/anticodon interactions, the complex could be rejected. Under these conditions, increasing the negative charge of the ribosome or eEF1A would result in a higher rejection rate and hence in increased accuracy. By combining appropriate pairs of *AS4* and *su12* alleles, we can now test this proposal.

Relationship between translational accuracy and sensitivity to paromomycin

We introduced three mutations into the *P. anserina* eEF1A gene by using data obtained with the yeast *S. cerevisiae*. While the overall effect of the mutations is conserved between *Podospora* and yeast, we did detect some differences in the sensitivity to paromomycin. Paromomycin is known to bind to the 16S rRNA in *E. coli* (Blanchard et al. 1998). However, its potential interactions with elongation factor eEF1A are unknown. Classically, increased paromomycin sensitivity is associated with an increase in translation error rate (Palmer and Wilhelm 1978; Singh et al. 1979). In *P. anserina*, it has been known for a long time that this relationship is not absolute (Coppin-Raynal 1981). Recent data obtained with the *TEF2-7* allele in yeast also show that decreased accuracy may be associated with increased

paromomycin resistance (Dinman and Kinzy 1997). In *P. anserina*, the corresponding mutation (*AS4-57*) is not associated with any modification of paromomycin sensitivity. However, there is a situation in *P. anserina* that is similar to that seen with *TEF2-7*, since the *AS4-30* alleles decrease both the error rate and the paromomycin resistance (Coppin-Raynal 1981). The *TEF2-7* and *AS4-30* mutations are located in different regions of the protein. It is thus likely that complex interactions are involved between the ribosome, the elongation complex and paromomycin that define translational accuracy and paromomycin resistance. Our data suggest that the details of these interactions may not be conserved during evolution.

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