

eEF1A Controls Ascospore Differentiation Through Elevated Accuracy, but Controls Longevity and Fruiting Body Formation Through Another Mechanism in *Podospora anserina*

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ABSTRACT

Antisuppressor mutations in the eEF1A gene of *Podospora anserina* were previously shown to impair ascospore formation, to drastically increase life span, and to permit the development of the Crippled Growth degenerative process. Here, we show that eEF1A controls ascospore formation through accuracy level maintenance. Examination of antisuppressor mutant perithecia reveals two main cytological defects, mislocalization of spindle and nuclei and nuclear death. Antisuppression levels are shown to be highly dependent upon both the mutation site and the suppressor used, precluding any correlation between antisuppression efficiency and severity of the sporulation impairment. Nevertheless, severity of ascospore differentiation defect is correlated with resistance to paromomycin. We also show that eEF1A controls fruiting body formation and longevity through a mechanism(s) different from accuracy control. *In vivo*, GFP tagging of the protein in a way that partly retains its function confirmed earlier cytological observation; *i.e.*, this factor is mainly diffuse within the cytosol, but may transiently accumulate within nuclei or in defined regions of the cytoplasm. These data emphasize the fact that the translation apparatus exerts a global regulatory control over cell physiology and that eEF1A is one of the key factors involved in this monitoring.

ALTHOUGH intensively studied, the roles of the eukaryotic cytosolic translation elongation factor eEF1A (formerly EF-1 α) in cell physiology remain incompletely understood (see NEGRUTSKII and EL'SKAYA 1998 for a review). Like its bacterial counterpart EF1A (also called EF-Tu), this factor was first described as a G-protein that binds aminoacyl-tRNA to form an eEF1A:GTP:aa-tRNA ternary complex, which delivers the charged tRNA to the A site of the ribosome. As this task must be performed efficiently, but also accurately, it is not surprising that eEF1A controls the accuracy of the decoding process (SANDBAKEN and CULBERSTON 1988). Several data suggest that the bacterial and the eukaryotic proteins perform their functions differently, but the precise biochemical events during the elongation step catalyzed by eEF1A remain unknown (NEGRUTSKII and EL'SKAYA 1998). Moreover, the eukaryotic protein exhibits many additional properties and/or functions not displayed by the bacterial protein. For example, among others, eEF1A interacts with actin and tubulins (DURSO and CYR 1994 for a review), activates degradation of some proteins (GONEN *et al.* 1994), and is probably involved in signal transduction (YANG *et al.* 1993; KIM *et al.* 1999; CHEN *et al.* 2000) and cell cycle regulation (GANGWANI *et al.* 1998).

eEF1A is essential for cell viability (COTTRELLE *et al.* 1985; SILAR *et al.* 2000a). Some organisms contain several genes, which may encode slightly different isoforms, but there is little information concerning the *in vivo* significance for the presence of these different isoforms. For example, in mammals, two isoforms are present: eEF1A1 is expressed in all tissues and eEF1A2 is expressed in muscle and the immune and nervous systems. Despite the presence of eEF1A1 in all tissues, eEF1A2 is essential because a mutation in the mouse eEF1A2 isoform entails the "wasted" phenotype that consists of a set of alterations affecting the nervous and immune systems (CHAMBERS *et al.* 1998).

Interestingly, eEF1A is also involved in cell differentiation, apoptosis, and aging. Indeed, overexpression of this factor may promote susceptibility to oncogenic transformation (TATSUKA *et al.* 1992) and a mutated form of the human eEF1A gene is oncogenic (GOPALKRISHNAN *et al.* 1999). However, the exact mechanism(s) involved in these phenotypes is (are) not yet established. eEF1A is rapidly upregulated when cells are induced to enter apoptosis with H₂O₂ and transfection with eEF1A antisense RNA protects against H₂O₂-mediated cytotoxicity (CHEN *et al.* 2000 and references therein). During aging, eEF1A activity diminishes in numerous cellular types, correlating with a decrease in translation efficiency (WEBSTER 1985). Therefore, SHEPHERD *et al.* (1989) overexpressed eEF1A in *Drosophila melanogaster*. They observed an increased longevity in adult males.

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However, subsequent studies led to contradictory conclusions (STEARNS and KAISER 1993; SHIKIMA *et al.* 1994; SHIKIMA and BRACK 1996). Nevertheless, decreased eEF1A activity with aging was confirmed recently in *Drosophila* (SHIKIMA and BRACK 1996). Once again, the mechanism by which eEF1A may increase life span remains unclear.

We have studied the roles of eEF1A in the filamentous fungus *Podospora anserina* through a thorough genetic analysis. This filamentous fungus is well suited for such analysis. Like yeast, it is easy to handle for molecular genetic studies. Interestingly, it presents a more complex life cycle than yeast.

As in many filamentous ascomycetes, sexual reproduction in *P. anserina* is accompanied by elaborate cell differentiation processes that make possible the evaluation of eEF1A roles during differentiation (ZICKLER *et al.* 1995). The vegetative haploid mycelium is able to differentiate specialized male (conidia) and female (ascogonium) gametes. When the medium is exhausted, sexual reproduction takes place. Cell fusion of gametes during fertilization is not followed by nuclear fusion, but by nuclear proliferation to yield a multinucleated cell. Two compatible nuclei then migrate in a specialized cell, the ascogenous hyphae. This cell gives rise to a succession of dikaryotic cells by a complex process of mitosis and particular septum formation. Some of the produced cells undergo karyogamy. Meiosis and a postmeiotic mitosis immediately follow. Finally, four binucleated ascospores differentiate in an ascus. All these steps take place in a specialized fruiting structure, the perithecium. When mature, a perithecium contains several hundred asci that are ejected by turgor pressure.

Moreover, the presence of various cell degenerative processes allows assessment of the influence of eEF1A during cell aging in *P. anserina*. Two such processes have been described in *P. anserina*, Senescence and Crippled Growth (see SILAR *et al.* 2001 for a review). Senescence, which is present in all wild-type strains, results in cell death (this permits definition of longevity as the time elapsed between germination and death) and is correlated with drastic modification of the mitochondrial DNA (mtDNA). More precisely, amplification of three particular regions of the mtDNA (called senDNA α , senDNA β , and senDNA γ) correlates with Senescence, whereas the "young" mtDNA molecules disappear. The exact cause of Senescence, especially the relationship between mtDNA modification and Senescence at the present time is not clear (JAMET-VIERNY *et al.* 1999). Contrary to Senescence, Crippled Growth is present in a restricted set of strains, does not lead to cell death but to a severely impaired growth, and is not accompanied by mtDNA modification. It is associated with the presence of a nonconventional infectious element, called C, that is likely of an epigenetic nature (SILAR *et al.* 1999).

These differentiation and degenerative processes are

strongly influenced by mutations located in genes coding for the cytosolic translation apparatus in a way that remains unclear. These mutations were selected as modifiers of the error level during the decoding process (see COPPIN-RAYNAL *et al.* 1988 for a review) either by increasing (suppressor mutations) or by decreasing this level (antisuppressor mutations). Several of these genes have been characterized at the molecular level and were proved to encode ribosomal proteins (DEQUARD-CHABLAT and SELLEM 1994; SILAR *et al.* 1997), termination factors (GAGNY and SILAR 1998), tRNAs (DEBUCHY and BRYGOO 1985), and eEF1A (SILAR and PICARD 1994). Evidence was provided that, in the case of the antisuppressor mutations located in the AS7 gene (a gene whose product is yet unknown), the ascospore production defect is likely due to the reduced error level (DEQUARD-CHABLAT and COPPIN-RAYNAL 1984). However, as other antisuppressor mutants are fertile, the relationship between errors and fertility is not yet fully understood. Strikingly, there is no correlation between longevity and translation error level (BELCOUR *et al.* 1991; SILAR *et al.* 1997; SILAR *et al.* 2001 for review). However, data suggest that elongation, not termination, of translation is a major step that regulates longevity (SILAR and PICARD 1994; GAGNY and SILAR 1998). Interestingly, some mutations in the translational genes also modify the mtDNA alteration pattern observed during Senescence through a mechanism that likely depends upon cytosolic translation (DEQUARD-CHABLAT and SELLEM 1994; SILAR *et al.* 1997). In contrast to Senescence, Crippled Growth is clearly promoted by decreased error level, but the involved mechanism is unknown (SILAR *et al.* 1999).

In *P. anserina*, eEF1A is encoded by a unique and essential gene called AS4 (SILAR and PICARD 1994). Several types of mutations were selected in AS4. (1) Deletion of the gene confirmed that the protein is essential for cell viability (SILAR *et al.* 2000a). However, eEF1A was shown to be dispensable for male gamete production and fertilization. (2) Increase of AS4 gene dosage did not result in a large increase of cellular eEF1A and in any physiological modification (SILAR *et al.* 2000a). (3) An *in vitro* mutagenesis followed by reintroduction of the mutant alleles in *P. anserina* allowed the recovery of informational suppressor alleles that increase readthrough (SILAR *et al.* 2000b). Four among the six strains bearing suppressor mutations display wild-type vegetative and sexual characteristics. However, two mutations display very peculiar properties. One, AS4-55, is lethal but dominantly increases longevity and the other, AS4-56, exhibits a very complex set of phenotypes including a new growth arrest syndrome. (4) A classical genetic screen allowed recovery of mutations that decrease the readthrough associated with tRNA suppressors. These antisuppressor mutants were shown to display a global increase in translation accuracy (PICARD-BENNOUN 1976; see Table 1). Most of these mutations are lethal but a

TABLE 1
Paromomycin resistance and antisuppression efficiencies of *AS4* nonlethal antisuppressor mutations

Strains	AAC ^a	Pm ^b (%)	<i>leu1-1</i> ^c	<i>193</i> ^d	<i>leu1-1</i> ^e		<i>193</i> ^f	
					<i>su4-1</i>	<i>su1-1</i>	<i>su1-1</i>	<i>su8-1</i>
<i>AS4</i> ⁺ <i>mat</i> ⁺		15	0	White	1.0	0.60	Dark green	Almost black
<i>AS4</i> ⁺ <i>mat</i> ⁻		15	0	White	1.0	0.45	Dark green	Almost black
<i>AS4-11 mat</i> ⁺	His27 → Arg	35	0	White	0.1 (2)	0.15 (5)	Dark green (6)	Light green (2)
<i>AS4-27 mat</i> ⁺	Thr107 → Ile	80	0	White	0.7 (4)	0.00 (1)	Light green (2)	Dark green (5)
<i>AS4-29 mat</i> ⁻	Gly51 → Asp	90	0	White	0.5 (3)	0.00 (1)	Light green (2)	White (1)
<i>AS4-30 mat</i> ⁺	Gly350 → Asp	2	0	White	0.0 (1)	0.15 (5)	Green (4)	Light green (2)
<i>AS4-43 mat</i> ⁺	Thr431 → Ala	30	0	White	1.0 (6)	0.05 (3)	White (1)	Dark green (5)
<i>AS4-44 mat</i> ⁺	Gly53 → Asp	85	0	White	0.9 (5)	0.10 (4)	Green (4)	Green (4)

^a Amino acid changes in the indicated mutants.

^b Paromomycin resistance is measured by the percentage of linear growth observed on medium containing 750 µg/ml of paromomycin compared to the growth on unsupplemented medium after 4 days of culture. The higher the ratio, the more resistant is the strain.

^c Lack of suppression as indicated by the ratio of growth rate on minimal medium *vs.* growth rate on minimal medium supplemented with leucine equal to 0 in the indicated *AS4* strains at the *leu1-1* mutation site.

^d Lack of suppression as indicated by the white color in the indicated *AS4* strains at the *193* mutation site.

^e The suppression efficiency at the *leu1-1* mutation site for *su4-1* and *su1-1* is the ratio of growth rate on minimal medium *vs.* growth rate on minimal medium supplemented with leucine. Diminution of the ratio is supposed to be proportional to the strength of the antagonistic effect (antisuppression) of the *AS4* mutations against the effect of the *su4-1* and *su1-1* suppressors. The ranking of the *AS4* mutation with respect to antisuppression efficiency (1 being the more efficient) is indicated in parentheses.

^f The suppression level at the *193* mutation site is indicated by the intensity of pigmentation of the ascospores. Diminution of the ascospores' pigmentation is proportional to the antisuppression strength of the *AS4* mutations against the *su1-1* and *su8-1* suppressors; the order of color is almost black > dark green > green > light green > white. The ranking of the *AS4* mutation with respect to antisuppression efficiency (1 being the more efficient) is indicated in parentheses.

few are not. Mutants with the latter class of mutations exhibit an extended life span and impairment in ascospore production (SILAR and PICARD 1994). Like all antisuppressor mutants, they also propagate the non-conventional infectious element that is responsible for the Crippled Growth (SILAR *et al.* 1999). All three phenotypes are recessive (SILAR and PICARD 1994; SILAR *et al.* 1999).

Clearly, suppressor and antisuppressor mutations in eEF1A entail a very complex set of phenotypes (pleiotropy) related to differentiation and aging in *Podospora*. Our aim is to uncover how mutations in eEF1A can trigger such a wide range of perturbations. Since the *AS4* antisuppressor mutations were recovered in a screen for mutants displaying increased translation accuracy, we have tried to determine the role of translation accuracy in ascospore formation and life span extension. The data reported in this article confirm that the defect of ascospore formation is due to elevated accuracy, but that longevity of extension is due to another process(es). However, analysis of additional mutants show that *AS4* also controls fertility through the production of perithecia by a pathway, which is independent from error level control.

MATERIALS AND METHODS

Strains, media, and genetic analysis: The *P. anserina* strains used in this study were all derived from the *S* strain, ensuring

a homogenous genetic background (RIZET 1952). Standard culture conditions, media, and genetic methods for this fungus have been described (ESSER 1974). Longevity was measured as described in SILAR and PICARD (1994) in at least nine independent cultures.

Selection of the various *AS4* mutations used in this study was described by PICARD-BENNOUN (1976). Partial description of the physiological defects caused by the six nonlethal mutations, *AS4-11*, *AS4-27*, *AS4-29*, *AS4-30*, *AS4-43*, and *AS4-44*, was previously given by SILAR and PICARD (1994). Their sequences, as well as those of the three lethal mutations described here, were reported by SILAR *et al.* (2000b) and are reported in Table 1. Because *AS4* is closely linked to the mating-type locus, *AS4* mutations are available in only one mating type (either *mat*⁺ or *mat*⁻, depending on the mutation) except for *AS4-43*, which has been recombined with the mating-type locus and is thus available with both haplotypes. Strains carrying a deletion of *AS4* and an ectopic copy of *AS4-44*, allowing the recovery of this allele associated with both mating types, were previously described (SILAR *et al.* 2000a). These strains are designated $\Delta AS4 \{AS4-44\}$.

193 and *leu1-1* are UGA nonsense mutations that affect spore pigmentation and leucine biosynthesis, respectively. They are both efficiently suppressed and can be used to measure *in vivo* suppression levels. Both *su4-1* and *su8-1* suppressors are isoacceptor serine-inserting tRNAs that suppress the opal UGA termination codon (DEBUCHY and BRYGOO 1985). The *su1-1* mutation is an omnipotent suppressor mutation affecting the eRF3 release factor (GAGNY and SILAR 1998).

Paromomycin resistance: The paromomycin (Pm) resistance level is the ratio obtained by dividing the diameter of the thalli after 3 (or 4) days of growth on M2 medium supplemented with 750 µg/ml of paromomycin by the diameter of the thalli after 3 (or 4) days of growth on nonsupplemented M2 medium.

Antisuppression analysis: The antisuppression efficiency of the *AS4* mutations was measured according to the following strategies: (1) the *leu1-1* mutation is an UGA nonsense mutation preventing growth on M2 minimal medium and is suppressed by informational suppressors. The *leu1-1 su* strains grow on minimal medium at a suppressor-specific speed (COPPIN-RAYNAL 1981). The ratio of growth speed on M2 medium *vs.* growth speed on M2 supplemented with 50 $\mu\text{g}/\text{ml}$ leucine can be used to quantify the suppression level at the *leu1-1* mutation site. Diminution of this ratio in strains carrying an additional mutation is indicative of the efficiency of antisuppression of this mutation. (2) The *193* mutation is an UGA nonsense ascospore color mutation that is also suppressible (PICARD 1973). Wild-type ascospores are black and *193* ascospores are white. The suppression level at the *193* mutation site can be estimated by the intensity of the green pigmentation in *193 su* ascospores. The reduced coloration of the ascospores observed, if a supplementary mutation is present, gives an estimate of the antisuppression efficiency of this additional mutation.

DNA analysis: All DNA manipulation procedures were performed according to standard methods (AUSUBEL *et al.* 1987). mtDNA was extracted from senescent cultures of wild type and the six *AS4* mutant strains by the rapid method of LECÉLIER and SILAR (1994). Modifications of the mitochondrial genome in at least nine independent senescent strains per genotype were analyzed. mtDNA was digested with *HaeIII* restriction enzyme, run on a 1% agarose gel, and blotted onto nylon membrane. Specific probes for the *senDNA α* , *senDNA β* , and *senDNA γ* regions were used to analyze the various amplified regions (JAMET-VIERNY *et al.* 1997a).

Construction of *AS4*-GFP fusion and isolation of strains expressing the chimeric protein: A fragment consisting of the whole *AS4* coding sequence and the *AS4* promoter was amplified by PCR from pEF-1 α D (SILAR and PICARD 1994) using primers 4503 (5'-CTAAAGGGAACAAAAGCTG-3') and *AS4*3' (5'-CGG GATCCCGTTTCTTGCCAGCCTTGGCAGC-3'). The first primer anneals upstream of the multiple cloning site, in which the *AS4* gene is cloned. The second primer anneals at the end of the *AS4* coding sequence and introduces a *Bam*HI site in the place of the stop codon; two nucleotides were added in order for the GFP (green fluorescent protein) coding sequence to be in the same phase as *AS4*. The *AS4* gene was cloned in the pEGFP-1 (CLONTECH, Palo Alto, CA) through several steps. First, the PCR product was cloned in pBC-SK (Stratagene, La Jolla, CA) at the *Eco*RV site. Second, this construct was digested by *Xho*I and *Bam*HI and ligated with the pEFGP-1 that was linearized by the same enzymes, giving rise to plasmid pAS4-GFP. The *AS4* coding sequence present in this plasmid was completely sequenced to ensure the absence of mutation.

The *AS4-44* mutant strain was cotransformed with pAS4-GFP and pBC-hygro that carries a hygromycin resistance gene (SILAR 1995) as described in BRYGOO and DEBUCHY (1985). Several hundreds of hygromycin-resistant transformants were recovered and checked for fluorescence. Eighty transformants that exhibited fluorescence were crossed with the *AS4-43* mutant strain to look for the rescue of the ascospore formation defect. In most transformants, restoration of ascospore formation was observed, although not to the same level as in a cross between wild type and *AS4-43*. This suggested that in these transformants *AS4* activity was partially restored and argued for a functional expression of the *AS4-GFP* chimeric protein. Two such transformants, F4 and F7, were subjected to genetic analysis by crosses with wild type. Data showed a complete cosegregation of hygromycin resistance, restoration of ascospore formation, and fluorescence. The two integrations F4 and F7 were then recombined with *AS4*⁺ to obtain the F4

AS4⁺ and F7 *AS4*⁺ strains associated with the two mating types. The F4 *AS4*⁺ *mat*⁻ and F7 *AS4*⁺ *mat*⁻ strains were then crossed with the *leu1-1* Δ *AS4 mat*⁺/*leu1-1 AS4*⁺ *mat*⁻ heterokaryotic strain (SILAR *et al.* 2000a). In the progeny, F4 Δ *AS4 mat*⁺ and F7 Δ *AS4 mat*⁺ strains were recovered, showing that F4 and F7 are able to complement the lethal *AS4* deletion. Additional crosses between F4 *AS4*⁺ and F7 *AS4*⁺, on one hand, and *leu1-1* Δ *AS4 mat*⁻/*leu1-1 AS4*⁺ *mat*⁺, on the other hand, obtained F4 Δ *AS4 mat*⁻ and F7 Δ *AS4 mat*⁻ strains.

Cytological analyses: For immunofluorescence, asci were fixed in 7.4% paraformaldehyde at room temperature and crushed with a blunted hypodermic needle between a siliconized slide and a polylysine-coated coverslip, as described by THOMPSON-COFFE and ZICKLER (1994). Asci were incubated in anti- β -tubulin (1:1200; Amersham France) 12 hr at room temperature and in secondary antibody (FITC anti-mouse, Caltag, San Francisco) at 37° for 45 min. Chromatin was visualized by adding 0.5 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole (DAPI) dihydrochloride to the final rinse. Coverslips were mounted on 90% glycerol, 10% 100 mM phosphate, pH 8.7, with 10% w/v 1,4-diazabicyclo(2,2,2)octane (Sigma, St. Louis). Controls included the use of primary or secondary antibodies alone. *AS4*-GFP localization was analyzed using the FITC Zeiss filter set. Both sets of cells were observed on a Zeiss Axioplan microscope and images were captured by a CCD Princeton camera.

For light microscopy, specimens were fixed in fresh Lu's fixative (butanol, propionic acid, and 10% aqueous chromic acid, 9:6:2 v/v). After 10 min of hydrolysis at 70°, asci were stained in 2 drops of 2% hematoxylin mixed on the slide with 1 drop of ferric acetate solution.

UV mutagenesis: To select for secondary mutations that rescue the *AS4* ascospore formation defect, crosses between *AS4-44 mat*⁺ and *AS4-29 mat*⁻ were set up on 60 petri plates. Just before spermatization, the plates were irradiated with 200 J/m² of UV 254 nm. After fertilization, ~1200 perithecia/plate were obtained. Among these, 4 were able to discharge ascospores within 10 days of incubation.

Three-dimensional model: The model for *P. anserina* eEF1A was made by using the SWISS-MODEL server accessible at <http://www.expasy.ch/swissmod/SWISS-MODEL.html>. The resulting model was analyzed with the Weblab ViewerLite from Molecular Simulations.

RESULTS

The mechanisms causing life span extension and ascospore formation defects were investigated in the six viable eEF1A mutants: *AS4-11*, *AS4-27*, *AS4-29*, *AS4-30*, *AS4-43*, and *AS4-44*.

Longevity extension and sporulation defects: In *P. anserina*, senescence is accompanied by accumulation of specific mtDNA rearrangements (see DUJON and BELCOUR 1989 for a review). Some mutations affecting the translational apparatus modify the spectrum of the amplified rearranged mtDNA molecules (BELCOUR *et al.* 1991; SILAR *et al.* 1997). Therefore, we analyzed the status of the mitochondrial genome upon appearance of senescence in the six mutant strains. Figure 1 shows a typical result for the wild-type and *AS4-30* mutant strains. Similar data were observed for the five other mutant strains. In all studied senescent cultures, no particular mtDNA rearrangements were detected. Thus, the *AS4* mutants display a delayed accumulation of mito-

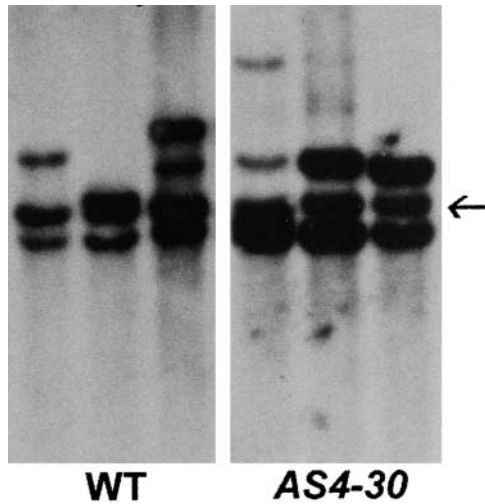


FIGURE 1.—mtDNA modification during senescence. A typical result for the analysis of senDNA α and senDNA β in wild-type and *AS4-30* senescent cultures is presented. mtDNA was extracted from three independent wild-type senescent cultures (left) or three independent *AS4-30* senescent cultures (right). DNA was analyzed by Southern blot, as indicated in MATERIALS AND METHODS. Filters were hybridized with a probe made with DNA fragments originating from the senDNA α and senDNA β regions. Both types of culture exhibit the same pattern of mtDNA modification during Senescence. The arrow points toward the 2.5-kb band that is characteristic for senDNA α (the same band hybridized when a probe corresponding to the senDNA α region is used). This senDNA α is always present and exhibits the same structure in all wild-type senescent cultures. It is also always present in the *AS4-30* senescent cultures. The other bands reveal the variable senDNA β (JAMET-VIERNY *et al.* 1997a). This senDNA was found as frequently in wild-type and *AS4-30* senescent cultures.

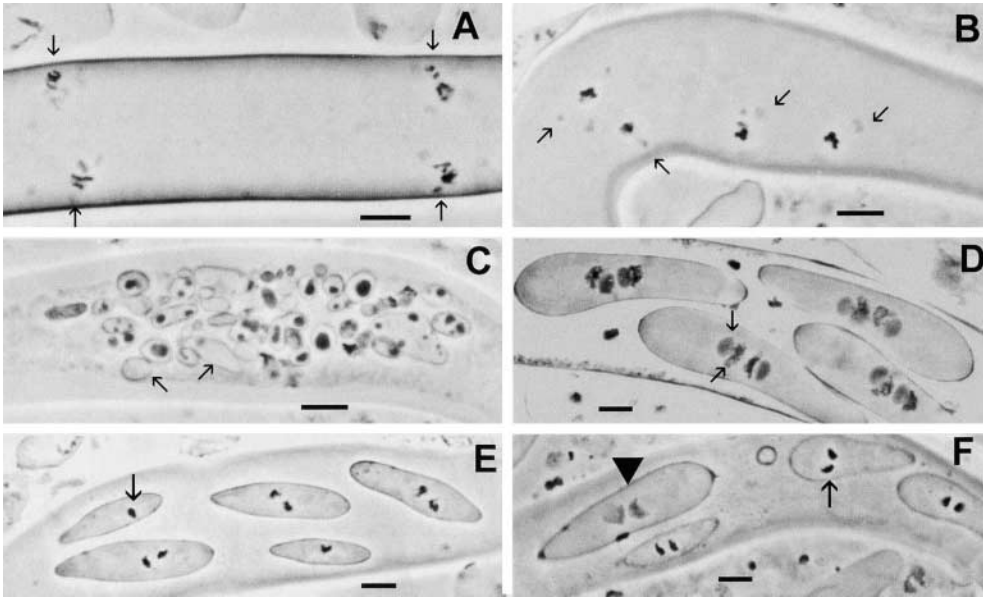
chondrial DNA alterations typical of the classical senescent state.

To learn when and how the *AS4* mutants were impaired in the formation of ascospores, we crossed these strains with every compatible combination (see MATERIALS AND METHODS). Perithecia from crosses involving *AS4-11* and especially *AS4-30* were not completely barren and did yield a small progeny. No progeny were obtained from the crosses involving the four other mutants. Cytological analyses of perithecia from *AS4-43* \times *AS4-43*, $\Delta AS4$ {*AS4-44*} \times $\Delta AS4$ {*AS4-44*}, and *AS4-27* \times *AS4-43* crosses showed that sporulation was abnormal in several respects. (1) Sporulation in filamentous ascomycetes follows a postmeiotic mitosis (PMM) in which proper spindle alignment is crucial for correct ascospore formation (THOMPSON-COFFE and ZICKLER 1994). In four-spored species like *P. anserina*, formation of binucleate ascospores also requires extensive nuclear migration: First, meiotic spindles are regularly spaced along the ascus and the four PMM spindles are grouped in widely separated pairs across the long axis (Figure 2A). Second, paired nuclei issued from the PMM must turn and migrate along the ascus and then to the cell membrane in order to be isolated in the forming asco-

spore wall. In the *AS4-43* \times *AS4-43*, $\Delta AS4$ {*AS4-44*} \times $\Delta AS4$ {*AS4-44*}, and *AS4-27* \times *AS4-43* crosses, non-four-spored asci were the rule mainly due to faulty meiotic and especially PMM spindle alignment and to impaired nuclear migrations (Figure 2B). The issuing ascospores were either anucleate or polynucleate or badly shaped or aborted, and most asci contained unenclosed nuclei that remained free in the cytoplasm (Figure 2C). (2) After spore closure, wild-type nuclei divide and the rounded resting nuclei show characteristic decondensed chromatin and prominent nucleolus (Figure 2D). Mutant nuclei had the same appearance just after spore closure, but then often became highly condensed in the same time that the ascospores degenerated (Figure 2E). Sometimes all eight nuclei of an ascus condensed, but sometimes only part of them did so (Figure 2F). However, the proportion of normal *vs.* abnormal or degenerated asci was different from one mutant to the other. In *AS4-43* \times *AS4-43*, 10% of the asci showed a wild-type sporogenesis and, although not efficiently ejected from the perithecium, the corresponding ascospores were viable; 30% displayed abnormal spores with mostly abnormal numbers of nuclei (one or three or more, instead of two). The remainder 60% of the asci were blocked either during meiosis or during PMM with four or eight condensed nuclei. A similar but more severe phenotype was observed in $\Delta AS4$ {*AS4-44*} \times $\Delta AS4$ {*AS4-44*}, and defects were even stronger in *AS4-27* \times *AS4-43*, with only rare ripe spores formed. Crosses involving *AS4-29* showed phenotypes similar to those involving *AS4-44*. On the basis of the severity of the ascospore formation defect, the following classification of the *AS4* mutant can be proposed: (1) *AS4-30* and *AS4-11*, which display a moderate defect and are able to eject some mature ascospores; (2) *AS4-43*, which is able to produce mature ascospores but not able to eject them out of the perithecium; (3) *AS4-44* and *AS4-29*, which do produce a few ripe but abnormal ascospores; and (4) *AS4-27*, which do not produce any mature ascospores.

To study the involvement of the cytoskeleton in the defects observed in the three crosses, we analyzed their microtubule arrays during sporulation by antitubulin immunofluorescence. When compared to wild type, both cytoplasmic and spindle microtubules were normal (Figure 3, A and B). However, unlike the regularly spaced wild-type spindles, in the three mutants, the two meiotic and/or the four PMM spindles were mostly randomly oriented across the ascus (Figure 3, C and D).

The sporulation phenotypes seen for the *AS4* mutants resemble the *AS7* mutant phenotypes, for which ascospore impairment was shown to be due to increased accuracy (DEQUARD-CHABLAT and COPPIN-RAYNAL 1984). However, as these authors reported only spindle defects and no nuclear condensation, we decided to perform a more detailed cytological analysis of an *AS7-2* \times *AS7-2* cross. Like the *AS4* mutants, *AS7-2* showed both abnor-



(arrow). All eight nuclei are highly condensed. (F) Same cross as E but, in this ascus, one ascospore carries two normal nuclei (arrowhead; compare with D), whereas the three others carry condensed nuclei (vertical arrow). Bar, 5 μ m.

mal ascospore formation and condensed nuclei. In addition, even when normally delimited, ascospore maturation was stopped. Like *AS4* mutants, *AS7* mutants also exhibit an increased life span (BELCOUR *et al.* 1991; P. SILAR, unpublished results), but it was not determined whether accuracy was involved in life span extension. In the view of the similar defect in *AS4* and *AS7* mutants, we decided to determine whether increased accuracy was involved in ascospore formation but also whether it controls life span extension in *AS4* mutants.

Ascospore formation, but not wild-type longevity, is restored in the presence of paromomycin: Paromomy-

cin is an aminoglycoside antibiotic known to increase the translation misreading frequency *in vitro* (PALMER and WILHEM 1978; SINGH *et al.* 1979). Most *P. anserina* strains carrying a suppressor mutation have an increased sensitivity whereas most *P. anserina* strains carrying an antisuppressor mutation have a decreased sensitivity to the antibiotic (COPPIN-RAYNAL 1981). The resistance to 750 μ g/ml of paromomycin triggered by the six viable *AS4* mutations was measured and compared with that of the wild type (Table 1). Three mutants are clearly more resistant (*AS4-27*, *AS4-29*, and *AS4-44*) and two are slightly more resistant (*AS4-11* and *AS4-43*), whereas

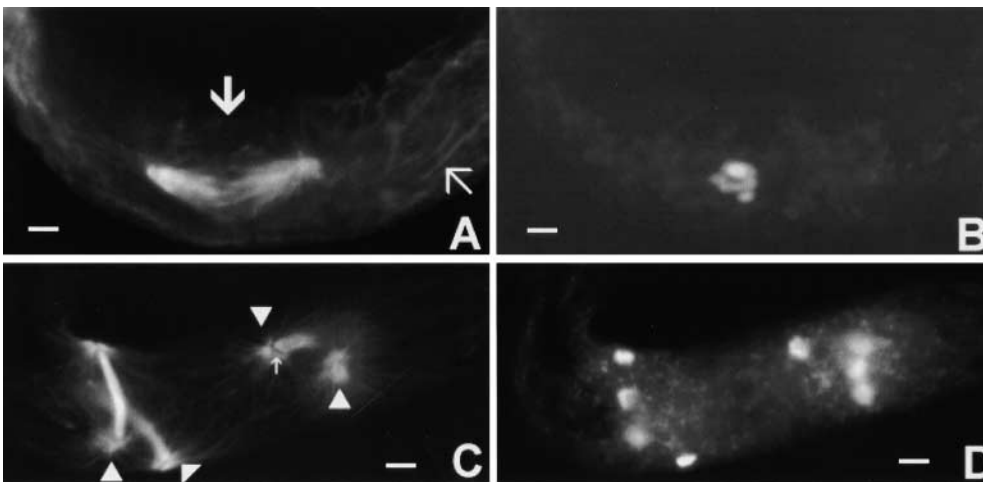


FIGURE 3.—Microtubule organization in *AS4* mutant crosses. (A) A typical first meiosis division metaphase of a $\Delta AS4 \{AS4-44\} \times \Delta AS4 \{AS4-44\}$ cross shows that cortical microtubules (slanting arrow) are normal as well as the spindle microtubule (vertical thick arrow indicates the position of the chromosomes). (B) DAPI staining corresponding to A. (C) *AS4-43* \times *AS4-27* PMM anaphase showing abnormal orientations of the four spindles (indicated by arrowheads). Vertical arrow indicates the position of a spindle pole body with astral microtubules to the left and intranuclear microtubules to the right. (D) Corresponding DAPI staining of eight nuclei. Bar, 5 μ m.

TABLE 2
Effect of paromomycin and informational suppressors on the life span of *AS4* mutant strains

	Control ^a	+Pm ^b	<i>su1-1</i> ^c	<i>su8-1</i> ^c	<i>su4-1</i> ^c	<i>su3-8</i> ^c	1976 ^c
<i>AS4</i> ⁺ <i>mat</i> ⁺	9.5 ± 1.0	16.0 ± 3.0	8.5 ± 0.5	9.0 ± 0.5	8.0 ± 0.5	30.0 ± 3.0	11.0 ± 1.5
<i>AS4</i> ⁺ <i>mat</i> ⁻	9.5 ± 1.0	15.5 ± 1.5	8.5 ± 1.5	8.5 ± 0.5	8.0 ± 0.5	27.5 ± 4.0	12.5 ± 1.5
<i>AS4-11 mat</i> ⁺	18.0 ± 3.0	20.0 ± 1.5	15.0 ± 1.0	NT	NT	64.5 ± 25.5	22.0 ± 2.5
<i>AS4-27 mat</i> ⁺	23.5 ± 7.0	17.5 ± 3.0	23.0 ± 2.5	22.5 ± 4.5	24.5 ± 3.5	18.0 ± 4.0	19.0 ± 3.0
<i>AS4-29 mat</i> ⁻	20.5 ± 3.5	NT	17.5 ± 2.5	NT	NT	NT	24.5 ± 4.0
<i>AS4-30 mat</i> ⁺	19.0 ± 3.0	28.0 ± 3.5	16.0 ± 3.5	NT	NT	>200 ^d	27.5 ± 3.5
<i>AS4-43 mat</i> ⁺	13.0 ± 2.0	13.0 ± 2.0	11.0 ± 1.0	12.0 ± 1.5	12.5 ± 1.5	22.0 ± 3.5	11.0 ± 1.0
<i>AS4-44 mat</i> ⁺	21.5 ± 3.5	23.5 ± 4.5	17.0 ± 3.5	21.5 ± 1.5	15.5 ± 2.5	20.0 ± 2.0	14.0 ± 0.5

Because during its life span a culture is able to invade a defined portion of the medium, longevity is conveniently measured as a distance of growth between the germination point of the ascospore and the point at which growth stops; longevity is thus given in centimeters ± standard deviation; NT, not tested.

^a Longevity with wild-type background except for the indicated *AS4* mutation on M2 medium.

^b Longevity with wild-type background except for the indicated *AS4* mutation on M2 medium supplemented with 500 µg/ml of paromomycin.

^c Longevity in the indicated informational suppressor and *AS4* antisuppressor backgrounds on M2 medium.

^d Experiment was stopped; at that time, two out of nine cultures presented Senescence at about 1.5 m.

one was more sensitive (*AS4-30*) than wild type. This latter phenotype was also reported previously (COPPIN-RAYNAL 1981).

If a phenotype is caused by increased accuracy, it should be phenotypically suppressed by paromomycin. Therefore, we analyzed ascospore formation and longevity of *AS4* strains grown on medium to which 500 µg/ml of paromomycin was added (with this concentration of paromomycin, all strains grew with a significant speed). All *AS4* crosses performed on such medium were as fertile as wild-type crosses on unsupplemented medium. This is in sharp contrast to the wild-type crosses, which were sterile on the medium supplemented with paromomycin. This result strongly suggests that the ascospore formation defect seen in *AS4* mutants is due to reduced error level. Strikingly, the longevity of the *AS4* cultures is not restored to the wild-type level on medium with or without the antibiotics (Table 2), suggesting that elevated accuracy is not responsible for increased life span.

An omnipotent informational suppressor, but not UGA tRNA suppressors, seems to display the same effect as paromomycin on the *AS4* mutations: To confirm the phenotypic suppression of the *AS4* ascospore formation defect by paromomycin, we introduced, by crosses, various suppressor mutations in the *AS4* strains. As for paromomycin, if translation accuracy is involved in the determination of the phenotype, this phenotype should be suppressed. We first tested the *su1-1* omnipotent suppressor that is located in the eRF3 release factor subunit (GAGNY and SILAR 1998). Double mutant strains containing *su1-1* and either one of the *AS4* mutations were constructed. All the resulting strains had a slightly improved fertility when compared with the corresponding *AS4* mutant strains. All had a slightly reduced longevity (Table 2). However, since *su1-1* seems to decrease lon-

gevity by itself (Table 2; BELCOUR *et al.* 1991), it was not possible to know whether the *su1-1* effect was brought about through decreased accuracy or through an indirect effect.

We similarly tested the UGA tRNA suppressors *su8-1* and *su4-1*. Clearly, these tRNA are not able to restore ascospore formation and have no effect on life span, except when *su4-1* is associated with *AS4-44* (Table 2).

Suppressors of the sporulation defect of the *AS4* mutations confirm that translation accuracy causes ascospore formation defects but has no effect on life span extension: Because the effect with the informational suppressors was not clear-cut, we decided to search for suppressors that would restore ascospore formation in the *AS4* mutants. If ascospore formation impairment is indeed due to elevated accuracy, a subset of these suppressors should be informational suppressors.

One suppressor was recovered spontaneously in one of our *AS4-44* stocks and four others were recovered after UV mutagenesis of *AS4-44* × *AS4-29* crosses (see MATERIALS AND METHODS). The five suppressors were crossed with the 193 tester strain and, for two of them, green spores were recovered in the progeny. The fact that an informational suppressor was present in two of these strains indicates that accuracy indeed is involved in the control of ascospore formation. Mapping of these two informational suppressors showed that one was a new allele of the *su3* gene (now called *su3-8*). The other could not be mapped to any of the known suppressor loci and may thus define a new gene (for the present article the mutant allele is called 1976). Both *su3-8* and 1976 were associated with the various *AS4* mutations and both fertility and longevity were measured. Although fertility was found increased in all strains, none restored a wild-type longevity level (Table 2). Several kinds of interactions were observed depending on the mutants'

combination, suppressive as seen in *AS4-27 su3-8* or additive as seen in *AS4-11 su3-8*. Interestingly, in one of the double mutants (*AS4-30 su3-8*) a synthetic effect was observed and life span was so tremendously increased that the strain became almost immortal since it lived more than 20 times the life span of wild type before the experiment was stopped! This confirmed the data obtained with the paromomycin; *i.e.*, increased accuracy is not involved in life span extension.

Among the three suppressors that did not yield green ascospores when crossed with 193, two (N1 and N3) were unlinked to *AS4*. Both exhibited a 90% second division segregation frequency but segregated independently. The N1 suppressor was not very efficient in restoring fertility and did not act in crosses involving *AS4-27*, whereas the N3 suppressor was very efficient and restored ascospore formation in all *AS4* mutant strains. The two mutations did not modify translation accuracy because we did not detect any modification of the levels of suppression or the resistance to paromomycin in strains carrying these mutations. Confirmation was given by the fact that *AS4-44* N1 and *AS4-44* N3 did present a Crippled Growth phenotype while *AS4*⁺ N1 and *AS4*⁺ N3 did not. The N1 suppressor had a reduced fertility and strains carrying N1 lived slightly longer (14.5 ± 1.9 for *AS4*⁺ N1 compared to 9.5 ± 1.0 for *AS4*⁺ and 31.0 ± 4.5 for *AS4-44* N1 compared to 21.5 ± 3.5 for *AS4-44*), whereas the N3 suppressor did not entail any obvious phenotype.

The fifth suppressor (N6) was so closely linked to *AS4-44* that we could not recombine *AS4*⁺ with it. It is not an intragenic revertant because no additional mutation was found when the *AS4* allele was sequenced. The informational status of this extragenic suppressor (called N6) is not clear. First, it did not modify the antisuppression efficiency of the *AS4-44* mutation at the 193 mutation site in the presence of the *su8-1* suppressor. Second, it did not modify the paromomycin resistance of the *AS4-44* mutation. However, the *AS4-44* N6 strain did not present Crippled Growth anymore, as expected for an informational suppressor. It is also noteworthy that the *AS4-44* N6 strain had a slightly diminished longevity (17.0 ± 1.5 cm) when compared to the *AS4-44* strain.

***AS4* mutations reveal complex effects on *in vivo* misreading:** To more precisely correlate accuracy increase and ascospore formation defect, we analyzed the antisuppression efficiency of the six *AS4* mutants. To do this, we measured the *in vivo* readthrough at either the *leu1-1* or 193 sites promoted by two different types of suppressors, tRNA suppressors (*su4-1* or *su8-1*) and an omnipotent suppressor (*su1-1*) resulting from a mutation in the eRF3 release factor subunit. Although this does not measure all kinds of translation error, it should give a good indication of the *AS4* mutations' behavior. The data obtained are reported in Table 1 and can be summarized as follows: First, all *AS4* mutations acted on

both types of suppressors. Second, the efficiency of a given *AS4* allele varied with both the mutation site and the type of suppressor in such a way that the four combinations (*leu1-1 su1-1*, *leu1-1 su4-1*, 193 *su1-1*, and 193 *su8-1*) resulted in different orders in the strength of antisuppression (see DISCUSSION).

As *P. anserina* crosses yield dikaryotic ascospores, we were able to observe the recessivity/dominance of the *AS4* translational effect by the color of the heterokaryotic 193 *su AS4*⁺/193 *su AS4*⁻ ascospores. Two situations were found. First, for all six mutations, the 193 *su1-1 AS4*⁺/193 *su1-1 AS4*⁻ ascospores had the same color as the 193 *su1-1 AS4*⁺ ascospores, demonstrating that the *AS4* mutations were recessive when they antagonized the *su1-1* omnipotent suppressor. Second, for all six mutations, the 193 *su8-1 AS4*⁺/193 *su8-1 AS4*⁻ ascospores exhibited a color that was intermediate between the color of the 193 *su8-1 AS4*⁺ and the color of the 193 *su8-1 AS4*⁻ ascospores, indicating that the mutations were semi-dominant when they antagonized the *su8-1* tRNA suppressor.

These results show that the various *AS4* alleles could not be ordered on the criterion of antisuppression at a particular locus. Similarly, no obvious correlation between paromomycin resistance and antisuppression could be detected.

***In vivo* localization of eEF1A by GFP tagging:** Localization of eEF1A was determined using a reporter-tagged version of the protein carrying a carboxy-terminal addition of GFP (see MATERIALS AND METHODS for details). The *AS4-44* mutant strain was transformed by a plasmid carrying the eEF1A-GFP fusion gene. Among the transformants, which expressed GFP and restored the *AS4-44* sporulation defect, two (F4 and F7) were selected for further analysis. Each carried a transgene located at a different position in the genome. Although these transgenes restored viability of a strain bearing a deletion of *AS4* (see MATERIALS AND METHODS for experimental design), and thus showed that they expressed a fusion protein able to perform eEF1A functions, restoration of *AS4-44* fertility was only partial. This result suggested that the GFP-fusion protein was less active than the wild-type eEF1A. Therefore, one could expect the fusion protein to be endowed with an antisuppressor phenotype. We could not test this directly. Nevertheless, this idea was supported by the fact that the $\Delta AS4$ F4 and $\Delta AS4$ F7 strains exhibited the following phenotypes: (1) ascospore formation defects; (2) increased longevity (24.4 ± 4.9 cm and 23.1 ± 3.5 cm, respectively; note that this life span extension is recessive); (3) Crippled Growth; (4) resistance to paromomycin similar to *AS4-44*; and (5) a very low production of perithecia. All these phenotypes are characteristics of *AS4* antisuppressor mutants, suggesting that the chimeric gene was indeed an antisuppressor allele.

eEF1A-GFP localization was analyzed in both F4 $\Delta AS4$ and F7 $\Delta AS4$ strains. Hyphae taken from the growing

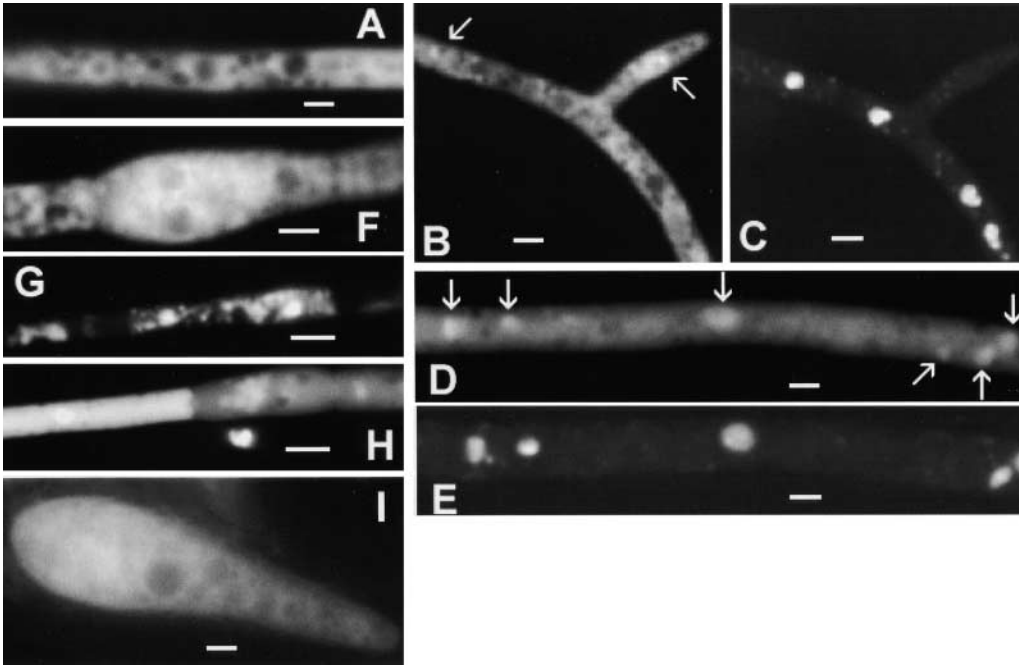


FIGURE 4.—*In vivo* localization of eEF1A. Fluorescence emitted by the eEF1A-GFP fusion was observed as described in MATERIALS AND METHODS. (A) Hyphae from the growing edge. (B and D) Hyphae showing the accumulation of eEF1A in some regions of the cell. Slanting arrows point toward eEF1A accumulation outside nuclei. Vertical arrows point toward eEF1A accumulation inside nuclei. (C and E) Corresponding DAPI staining. (F–H) Hyphae taken 5 cm away from the growing edge (*i.e.*, cells have stopped growing for ~ 1 wk). (I) Nonmature ascospore.

edge were always brightly fluorescent (Figure 4A). The staining heterogeneity visible in most cells was mainly due to the fact that organelles and vacuoles remained unstained. Some cells also showed a few brighter foci. These most often localized in the nuclei (Figure 4, D and E), while others, sometimes in the same hyphae, did not co-localize with nuclei (Figure 4, B–E). This focal localization was not observed in the GFP control (*i.e.*, ectopic integration of GFP alone expressed from the constitutive GPD promoter of *Aspergillus nidulans*). Hyphae taken from stationary phase cultures exhibited an even more variable pattern of eEF1A-GFP fluorescence (Figure 4, F–H). While a few cells were uniformly stained like those of hyphae from the growing edge (Figure 4F), others showed highly heterogeneous staining (Figure 4G). Surprisingly, two neighboring cells could present a completely different pattern of staining (Figure 4H), and the same was true for the GFP-control hyphae. This suggests that the staining is not representative of a particular localization for eEF1A-GFP in stationary phase mycelia. This could be due either to the fact that those hyphae are in various physiological conditions or that they correspond to eEF1A-GFP dislocalization.

Because *AS4* mutant strains fail to produce ascospores and/or female structures, we observed young ascospores issued from crosses of F4 $\Delta AS4$ and F7 $\Delta AS4$ by wild type (Figure 4I). These ascospores displayed a uniform distribution of eEF1A within their cytosol.

Since *AS4* mutants promote a modification of life span, we checked whether the localization of eEF1A was changed in senescent cells. Hyphae taken from the growing edge of senescent F4 $\Delta AS4$ and F7 $\Delta AS4$ cultures also displayed a highly heterogeneous fluores-

cence pattern. In this case, such a pattern may be related to the fact that senescent cells show a drastically rearranged cytoplasm (DELAY 1963).

Three additional *AS4* antisuppressor alleles: The *AS4-4*, *AS4-24*, and *AS4-33* alleles were recovered in the same screen as the *AS4-11* to *AS4-44* alleles and were categorized as lethal alleles (PICARD-BENNOUN 1976). We analyzed these three alleles again 25 years later. *AS4-24* was indeed lethal. However, we were able to recover homokaryotic strains carrying *AS4-4* or *AS4-33*. Both were very slow growing and unable to differentiate perithecium (although they produced ascogonia). Crosses involving these strains as the male parent and the other *AS4* mutant strain as the female parent were all barren, as observed in crosses involving the other *AS4* antisuppressor strains. Note that the growth of both strains resembles Crippled Growth too much, so as to prevent us from checking if these strains are able, like the other *AS4* antisuppressor strains, to develop the Crippled Growth alteration. Strains carrying *AS4-4* could not be analyzed for longevity because reversion of their phenotype toward a wild-type phenotype occurs rapidly. However, longevity was measured for the strains carrying *AS4-33*. Their life span is extended as observed for the other antisuppressor strains (16.5 ± 3.5 cm; wild-type longevity is 9.5 ± 1.0 cm). Like the other *AS4* antisuppressor alleles, these three alleles are recessive since strains carrying a transgenic copy of *AS4⁺* have a wild-type fertility and longevity. However, the *AS4-4* strain carrying the ectopic copy of *AS4⁺* has a slightly increased longevity (12.4 ± 1.5 cm) as described for the *AS4-44* strain carrying the same transgene.

We next analyzed whether the inability to differentiate perithecium and the slow growth of the *AS4-33* strain

was an extreme effect due to increased accuracy. Clearly, neither impairment in perithecium production nor normal growth was restored on paromomycin nor when either one of the *su1-1*, *su3-8*, or *1976* informational suppressors were associated with the mutation. Hence, sterility of this strain is not due to increased accuracy. Interestingly, this strain is resistant to paromomycin. A similar analysis with respect to longevity confirmed that longevity was not controlled by accuracy. For example, longevity of this strain on medium containing 500 $\mu\text{g}/\text{ml}$ paromomycin is 27.5 ± 5.7 cm compared to 9.5 ± 1.0 for wild type on nonsupplemented medium.

DISCUSSION

Considering the numerous eEF1A activities, it is not surprising that mutations in eEF1A are pleiotropic (SILAR and PICARD 1994; SILAR *et al.* 2000b). What is not clear is the exact mechanism(s) involved in each of the phenotypes. In this study, we try to clarify this pleiotropy with respect to some antisuppressor mutations, which present three main phenotypes: increased life span, fertility defect, and Crippled Growth. Because these mutations were selected with a screen connected with translation accuracy, we could expect that part of their effects were related to translation accuracy and/or other translation parameters. Another argument pointing toward this inference is the fact that the molecular characterization of the mutations (SILAR *et al.* 2000b) showed them to be scattered all over the primary sequence of the protein (all regions of the protein are necessary for translation). Indeed, if one of the other known eEF1A functions was more specifically involved in the mutant phenotype, it would be expected that the mutations cluster in this region. To confirm that the amino acid changes map to a different part of the protein, we have modeled the 3D structure of *P. anserina* eEF1A on the basis of the recently determined structure of the yeast eEF1A in complex with eEF1B α (ANDERSEN *et al.* 2000). As seen on Figure 5, the six viable amino acid changes map in a different part of the protein, either in domain I or in domain III. *AS4-29* and *AS4-44* map in the same loop of domain I and are characterized by the same amino acid change (glycine to aspartic acid). Interestingly, they both display phenotypes with similar strength (similar yield of ascospores, similar longevity, similar level of paromomycin resistance, and an effect on readthrough associated with all suppressors; see below), suggesting that they may act through modification of the same mechanism. On the contrary, the four other mutations are localized in different regions of eEF1A, resulting in different quantitative effects. Additionally, the eEF1A-GFP fusion gene presents the same three characteristic phenotypes associated with the other viable antisuppressor mutants. This is anticipated

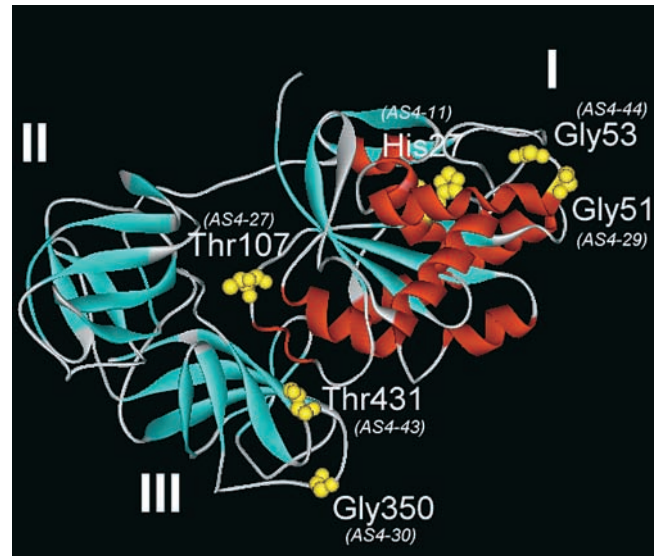


FIGURE 5.—Ribbon structure for *P. anserina* eEF1A. Domains I, II, and III are labeled. Position of the amino acid changes of the indicated alleles are highlighted by giving the depiction of their side chain.

if one assumes that the fusion protein is less efficient during translation than the wild-type one.

We already proposed a model for the control of Crippled Growth, in which a translation error was involved (SILAR *et al.* 1999). Indeed, we observed a clear relationship between accuracy and propagation of the *C* element that is involved in the development of this cell degenerative process whereas wild type and suppressors do not (SILAR *et al.* 1999). We have recently discovered one exception in one *AS4* suppressor mutant, but this can be related to the special characteristic of this mutant (SILAR *et al.* 2000b). It is likely that mutations in *AS4*, like the other antisuppressor mutations, promote Crippled Growth through their effect on translation fidelity.

The relation between fertility and translation accuracy is less clear. On one hand, many high fidelity mutants have a defect in ascospore formation, but on the other hand, some others do not. Here, we show that the defect presented by the *AS4* mutations is similar to the defect presented in the *AS7* mutants (DEQUARD-CHABLAT and COPPIN-RAYNAL 1984), arguing for a similar deficiency in all the antisuppressor mutants. This defect, as for *AS7* mutants, is clearly due to increased accuracy because the phenotype can be reverted by paromomycin and by suppressor mutations since it was shown that both approaches decrease error level in *P. anserina* wild-type and antisuppressor strains *in vivo* (COPPIN-RAYNAL 1981) and *in vitro* (PICARD-BENNOUN 1981; COPPIN-RAYNAL 1982). A possible explanation to account for the discrepancies between the various mutants is that there might be a threshold level in the alteration of translation before seeing any ascospore

formation defect. Some mutants may not reach this threshold and be fertile.

Several models can be proposed to account for an effect of translation error in ascospore formation. First, as we proposed for Crippled Growth (SILAR *et al.* 1999), expression of a gene required during the sexual cycle may necessitate a translation error. Second, because accuracy is maintained through activated consumption of energy (KURLAND 1992) and because sexual reproduction takes place in *P. anserina* when nutrients are exhausted, it is possible that energy is lacking to complete this last step in the development of the sexual cycle. Finally, as changes in accuracy are often associated with changes in ribosome processivity (KURLAND 1992), it is possible that large proteins required for ascospore formation are not produced in sufficient amount in the mutants.

To differentiate between these models, we first attempted to correlate accuracy and ascospore formation defect. This was possible with the *AS4* mutants because they display a wide range of alterations both at the translational and fertility levels. Strikingly, antisuppression efficiency, as measured with readthrough promoted by two types of suppressors, is very variable depending on the system used to test it. Even though some trends can be discerned (*AS4-29* acts efficiently whereas *AS4-44* acts moderately on all types of suppressors; *AS4-30* and *AS4-11* efficiently antagonize the effects of tRNA suppressors, but not that of the omnipotent suppressor; and *AS4-27* and *AS4-43* have the opposite outcome), different orders between the various *AS4* antisuppressors are obtained even when measuring the same kind of effect (*e.g.*, either antagonism of a deficient release factor or insertion of a tRNA suppressor). Compare, for example, the effect of *AS4-29* and *AS4-43* on readthrough associated with *su1-1* (Table 1). This suggests that, even for the same mechanism, the *AS4* mutations have a codon-specific effect. Accordingly, analysis of two EF1A antisuppressor mutants of *Escherichia coli* yielded the same results (TAPIO and ISAKSSON 1990). This prevents us from ascertaining a classification based on this criterion in order to relate it to the severity of the ascospore formation defect. However, at least in the *AS4* mutants, we noted a relationship between resistance to paromomycin and ascospore impairment (the more resistant, the more affected in ascospore formation; see Table 1 and first section of RESULTS). Moreover, *AS7* mutants that display a strong ascospore formation defect are also highly resistant to paromomycin (COPPIN-RAYNAL and LE COZE 1982). Because the paromomycin resistance level is probably an indicator of global accuracy level, this result points toward one of the global hypotheses.

We also tried to identify the cellular mechanisms impaired during ascospore formation. Indeed, the first hypothesis suggests a specific effect during ascus formation whereas the second and the third ones should pro-

mote a more global disturbance. At least two distinct phenomena impair the ascospore formation in the *AS4* mutants: defects in spindle plus nuclear localization and nuclear death after high condensation of the chromatin. The observed mislocalization of both spindles and nuclei could be related to the described interaction of eEF1A with the cytoskeleton (DURSO and CYR 1994). However, a recent article shows that this interaction is conditional and domain dependent, thus questioning the relevance of the *in vitro* data (MOORE and CYR 2000). Interestingly, the spindle localization defect associated with the *AS4* mutations is not due to faulty interactions with microtubules *per se*, but is due to an indirect effect through translation modification since normal ascospore formation is restored when translation accuracy is decreased. These data would therefore again point toward the global hypotheses. Confirming this is the fact that in the ascospores, no special localization of the eEF1A-GFP fusion was detected. During our search of suppressors of the fertility defect we isolated two unlinked mutations that do not seem to be involved in accuracy control. Isolation of the corresponding genes will definitely shed some light on the involved processes.

Unexpectedly, some antisuppressor mutations previously described as lethal (PICARD-BENNOUN 1976) are in fact viable but present a very altered growth pattern associated with a lack of perithecia production. Analysis of *AS4-33* showed that, at least in this mutant, fertility is not dependent on error level. Therefore, eEF1A exerts another control on sexual reproduction independently from accuracy monitoring.

Unlike ascospore formation, longevity is clearly not controlled by accuracy in the *AS4* mutants. This confirms previous data that suggested the same conclusion (BELCOUR *et al.* 1991; SILAR *et al.* 1997, 2000b). How can *AS4* mutations affect life span? The answer to this question is not straightforward, especially because many mechanisms contribute to life span definition in *P. anserina* (ROSSIGNOL and SILAR 1996). Strikingly, the increased life span of the *AS4* antisuppressors is a recessive phenotype whereas the increased life span of *AS4* suppressor mutations is dominant (SILAR *et al.* 2000b). It is therefore possible that eEF1A controls longevity through several mechanisms. We are currently testing this hypothesis by associating various eEF1A alleles.

The GFP tagging of eEF1A confirms the previously reported cellular localization (GANGWANI *et al.* 1998 and references therein). eEF1A is mostly located in the cytoplasm, but can transiently accumulate within the nucleus. In yeast and mammals (GANGWANI *et al.* 1998), the nuclear localization is correlated with cellular proliferation. In these organisms, it was shown that eEF1A binds the ZPR1 protein that relocalizes to the nucleus when growth is stimulated. Disruption of the interactions between the two proteins results in the accumulation of cells in the G2/M phase of the cell cycle (GANGWANI *et al.* 1998). Although we previously were able to

observe such a correlation between cell cycle and nuclear localization of a GFP-tagged ribosomal protein (LALUCQUE and SILAR 2000), we could not detect any correlation between growth status and nuclear localization of the eEF1A-GFP protein. In addition to a nuclear localization, we have also observed eEF1A foci in the cytoplasm. Similar cytoplasmic accumulation has been seen during *Xenopus* oogenesis (VIEL *et al.* 1990). We have no indication of the possible role of this particular localization. Note that, like MOORE and CYR (2000), we did not observe any obvious association with the cytoskeleton, questioning the *in vivo* relevance of an interaction between eEF1A and the cytoskeleton in normal conditions.

Finally, there is no evident correlation between fertility and longevity in *P. anserina*. The case of the double mutant strain *AS4-30 su3-8* is quite striking because it is almost immortal, but has a near wild-type fertility and reproduces as fast as wild type. Apart from the *Rgs31* mutant strain (CONTAMINE and PICARD 1998), all immortal mutants previously analyzed are female sterile (TUDZYNSKI and ESSER 1979; DUFOUR *et al.* 2000) or show a delayed reproduction due in part to slow growth (case of *PaTOM70-1*, JAMET-VIERNY *et al.* 1997b; V. CONTAMINE, personal communication).

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