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Suppression of the *acuH13* and *acuH31* nonsense mutations in the carnitine/acylcarnitine translocase (*acuH*) gene of *Aspergillus nidulans* by the G265S substitution in the domain 2 of the release factor eRF1

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Abstract

A search for suppressors of the carnitine/acylcarnitine translocase (CACT) deficiency in *Aspergillus nidulans* permitted the identification of the *suaE7* mutation, mapping at a new translational suppressor (*suaE*) gene. The *suaE* gene is essential in *A. nidulans* and encodes the eukaryotic release factor 1 (eRF1). The *suaE7* mutation suppresses two *acuH* alleles (*acuH13* and *acuH31*), both carrying nonsense mutations in the CACT encoding gene that involve the replacement of a CAG (Gln) codon with a premature TAG stop codon. In contrast, the *suaE7* gene does not suppress the *acuH20* amber nonsense mutation involving a TGG → TAG change. The phenotype associated to the *suaE7* mutation strictly resembles that of mutants at the *suaA* and *suaC* genes, two translational suppressor genes previously identified, suggesting that their gene products might functionally interact in translation termination. Sequencing of the *suaE7* gene allowed the identification of a mutation in the domain 2 of the omnipotent class-1 eukaryotic release factor involving the Gly265Ser substitution in the *A. nidulans* eRF1. This mutation creates a structural context unfavourable for normal eRF binding that allows the misreading of stop codons by natural suppressor tRNAs, such as the $tRNAs^{Gln}$. Structural analysis using molecular modelling of *A. nidulans* eRF1 domain 2 bearing the G265S substitution and computer simulation results suggest that this mutation might impair the necessary conformational changes in the eRF1 to optimally recognize the stop codon and simultaneously interact with the peptidyl transferase centre of the 60S ribosomal subunit.

Keywords: *Aspergillus nidulans*; Carnitine/acylcarnitine translocase; *suaE* gene; eRF1; Release factors; Translation termination; Translational suppressors; Natural suppressor tRNAs; Nonsense suppressors; Stop codon translation

1. Introduction

The carnitine/acylcarnitine translocase (CACT) mediates transport of acylcarnitines of different length across the mitochondrial inner membrane for their final oxidation in the mitochondrial matrix (De Lucas et al., 1999; Palmieri, 2004). Deficiency of human CACT results in the most severe disorder of fatty acid β-oxidation and is usually lethal within a few hours or days after birth (Pande et al., 1993; Stanley et al., 1992). The fact that this deficiency is associated with lethality throughout the metazoan lineage accounts for the lack of animal models for the disease in both vertebrates and invertebrates (De Lucas et al., 1999).

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In the filamentous fungus *Aspergillus nidulans*, the CACT is encoded by the *acuH* gene. In contrast with animals, the CACT deficiency in *A. nidulans* is conditionally lethal because, although a functional CACT is required for growth on long-chain fatty acids and C_2 compounds such as acetate, the enzyme is not essential when alternative carbon sources such as carbohydrates or amino acids are utilized (De Lucas et al., 1999). This important metabolic feature, together with the amenability of *A. nidulans* for classical and molecular genetic studies, pinpoints this eukaryotic microorganism as an excellent model to investigate important molecular and biochemical aspects of this inborn error of metabolism. Accordingly, we previously developed a metabolic model that allows the functional characterization of human CACT mutations such that neutral, single residue substitution-causing polymorphisms can be discriminated from potentially pathogenic missense mutations (Perez et al., 2003).

In this work, we utilized the model filamentous fungus *A. nidulans* to identify mutations that suppress the deficiency of *acuH* mutants for growth on acetate and oleate media aiming to discover a putative therapeutic target whose modulation could help in preventing the lethality associated to the CACT deficiency. Although no physiological suppressor of the CACT deficiency was found, we identified and further characterized a suppressor mutation mapping at a new translational suppressor gene in *A. nidulans*, the *suaE* gene, which suppresses two nonsense mutations in the *acuH* gene involving the substitution of a CAG (Gln) codon for a premature TAG amber stop codon.

Cloning and sequencing of the *suaE* gene, as performed in this work, permitted the identification of the *A. nidulans* eRF1. The eukaryotic release factor 1 (eRF1) terminates protein synthesis by recognizing mRNA stop codons at the ribosomal A-site and interacting with the ribosomal peptidyl transferase centre where the peptidyl-tRNA ester bond is hydrolysed so as to release the free polypeptide (Frolova et al., 1999; Ito et al., 2000; Inge-Vechtomov et al., 2003). The eRF1 is composed of three domains. The N-terminal domain (domain 1) is proposed to participate in stop codon recognition (Song et al., 2000; Bertram et al., 2000; Chavatte et al., 2001; Frolova et al., 2002). Domain 2 is responsible for the peptidyl transferase hydrolytic activity (Frolova et al., 1999; Seit-Nebi et al., 2001) and the C-terminal part of eRF1 (domain 3) interacts with eRF3 (eukaryotic release factor 3) (Kisselev et al., 2003).

The phenotype of *suaE7* mutant strains clearly resembles the phenotype of mutants at the *suaA* and *suaC* genes, two translational suppressor genes previously identified in *A. nidulans* (Roberts et al., 1979) and whose unknown gene products presumably interact with eRF1 in the termination of translation. By sequencing the suppressor *suaE7* gene we identified the G265S substitution in the eRF1 domain 2 that must result in limited competitiveness with natural suppressor tRNAs capable of illegitimately translating mRNA stop codons. Molecular modelling was used to build the three-dimensional structures of domain 2 of wild-type and mutant forms of *A. nidulans* eRF1. Our computer simulation results suggest that this missense mutation does not directly affect stop codon recognition or the presumed catalytic domain but likely impairs the necessary conformational changes that must occur in the eRF1 to optimally recognize the stop codon on the mRNA and simultaneously interact with the peptidyl transferase centre located at the 60S ribosomal subunit (Klaholz et al., 2003; Nakamura and Ito, 2003).

2. Materials and methods

2.1. Strains and plasmids

The *A. nidulans* strains used in this work are listed in Table 1. The chromosome-III-specific cosmid library obtained from the Fungal Genetics Stock Center (University of Missouri, Kansas, USA) was employed to clone the *suaE* gene of *A. nidulans*. The *suaE* gene was subcloned using the plasmid pUC19 and established molecular biology techniques (Sambrook et al., 1989). Plasmid pAL3 containing the *Neurospora crassa pyr-4* gene as a fungal selectable marker and the promoter of the *A. nidulans alcA* gene ($alcA^P$) (Waring et al., 1989) was also employed. *Escherichia coli* DH5α (BRL, USA) served as the recipient strain for plasmid transformation.

Table 1
Aspergillus nidulans strains used in this study

Strain	Genotype	Source or reference
R21	*pabaA1 yA2*	Armitt et al. (1976)
R153	*wA3*; *pyroA4*	Armitt et al. (1976)
G191	*pabaA1 pyrG89*; *uaY9*; *fwA1*	De Lucas et al. (2001)
WH8	*wA3*; *pyroA4*; *acuH8*	De Lucas et al. (1997)
WH13	*wA3*; *pyroA4*; *acuH13*	De Lucas et al. (1997)
WH20	*wA3*; *pyroA4*; *acuH20*	De Lucas et al. (1997)
WH24	*wA3*; *pyroA4*; *acuH24*	De Lucas et al. (1997)
WH31	*wA3*; *pyroA4*; *acuH31*	De Lucas et al. (1997)
YH8	*pabaA1 yA2*; *acuH8*	De Lucas et al. (1997)
YH13	*pabaA1 yA2*; *acuH13*	De Lucas et al. (1997)
YH20	*pabaA1 yA2*; *acuH20*	De Lucas et al. (1997)
YH24	*pabaA1 yA2*; *acuH24*	De Lucas et al. (1997)
YH31	*pabaA1 yA2*; *acuH31*	De Lucas et al. (1997)
PPA14	*biA1*; *ΔacuH::pyr4*$^+$; *argB2*	Perez et al. (2003)
WSol7H	*wA3 suaE7*; *pyroA4*; *acuH13*	This study
YSol7H	*pabaA1 yA2*; *suaE7*; *acuH13*	This study
WSol7	*wA3 suaE7*; *pyroA4*	This study
YSol7	*pabaA1 yA2*; *suaE7*	This study
S7HG	*biA1*; *argB2 suaE7*; *pyroA4*;*acuH13*	This study
G351	*pabaA1*; *alX4 suaA101*; *sB43*; *alcR125*; *fwA1*	Glasgow stock
G724	*pabaA1*; *alX4*; *sB43*; *suaC109*; *fwA1*	Glasgow stock
H9	*pabaA1*; *alX4 suaB111*; *sB43*; *fwA1*	Dr. H. Sealy-Lewis
WG355	*biA1; argB2 galA1*	Perez et al., (2003)
MS2.7	*biA1*;*wA3*;*galA1*;*pyroA4*; *facA303*;*sB3*;*nicB8*;*riboB2*	Dr. M. Grindle

2.2. Aspergillus nidulans media and culture conditions

Aspergillus nidulans was grown in *Aspergillus* complete medium (CM) and *Aspergillus* minimal medium (MM) (Armitt et al., 1976). The following carbon sources were added to the MM for phenotypic analysis of *acuH* mutants: 1% glucose (non-selective conditions), 0.1 M acetate or 6 MM oleate in 1% Tergitol NP40 (Sigma) (selective conditions) (Valenciano et al., 1996). Growth tests of *palB* mutants were carried out in CM containing 0.2 M PO_4HNa_2 (pH 8) (restrictive conditions) and CM containing 0.5 M PO_4H_2Na (pH 5) (non-selective conditions). *A. nidulans* cultures were normally incubated at 37 °C. However, when growth tests of *suaE7, suaA101, suaB111* and *suaC109* strains were performed, the following temperatures were used: 23, 28, 37 and 43 °C. The following nutritional requirements were added as necessary: 1 μg *p*-aminobenzoic acid ml^{-1}, 0.05 μg pyrodoxine ml^{-1}, 0.02 μg biotin ml^{-1}, 200 μg arginine ml^{-1}, 50 μg methionine ml^{-1}, 3 μg panthotenic acid ml^{-1}, 2 μg D-pantolactone ml^{-1}, 2 μg nicotinic acid ml^{-1}, 2.5 μg riboflavin ml^{-1}, 2.44 mg uridine ml^{-1}. *A. nidulans* was transformed by the method of Balance et al. (1983).

2.3. Classical genetic techniques

Standard techniques for genetic manipulation of *A. nidulans* (Pontecorvo et al., 1953; Clutterbuck, 1974) were used to synthesize heterokaryons and diploids for complementation tests, to generate ascospores (meiotic) progeny with various genotypes, and for chromosome assignment by means of the parasexual cycle.

2.4. Molecular genetic techniques

Cosmid and plasmid DNA was isolated by using the Qiagen Plasmid Kit (Qiagen) following the manufacturer's instructions. Genomic DNA was isolated from mycelium grown on 1% glucose MM employing the method described by De Lucas et al. (2001). Southern blotting was carried out by established procedures (Sambrook et al., 1989). DNA sequencing was carried out by using the GeneAmp PCR9700 system on an ABI-PRISM3100 automatic sequencer (Applied Biosystems). The sequence of the *A. nidulans suaE* gene was deciphered by sequencing the plasmids pXB25 and pPS150 using appropriate oligonucleotides. Molecular characterization of the *suaE7* allele was performed by PCR-amplification of genomic DNA from WSol7H strain in four overlapping regions followed by direct sequencing of the PCR-amplified fragments. These PCRs were carried out using the *Pfu* DNA polymerase (Promega) and the following primers: ERFfw1: 5′-TCTCA AGAACCAGGTGGCCAAC-3′, ERFfw2: 5′-TCAAGCC AATCAACACGTCC-3′, ERFf w3: 5′- GTCGCCGGTA TAATTCTTGC-3′, ERFf w4: 5′-CCTC GAATGGCTTG CGGA-3′, ERFrev1: 5′-GA GGCCGCTG AACCGTCAATCT-3′, ERFrev2: 5′- CCTG TTCAGCTCA CTTCC-3′, ERFrev3: 5′-CCTTAACCCTC GCACACT-3′ and ERFrev4: 5′-TCAAGGCTTCGGTGTGAA-3′.

To analyse the essentiality of the *suaE* gene the pALERF1 vector, based on pAL3 (Waring et al., 1989), was constructed. Primers LKPeRF1: 5′-CAGGTACCATACAGCTC-3′ (including a *Kpn*I site) and RBAeRF1: 5′-GGATCCTCAACACCGTAACA-3′(including a *Bam*HI site) were used in a PCR to amplify a 1065-bp fragment from nucleotides −34 to +1032 of the *A. nidulans suaE* gene (Accession no. AF451327). The PCR-amplified fragment lacking the last 135 codons of the *suaE* ORF was cloned into pGEMTeasy giving the vector pGMERF1. The *Kpn*I–*Bam*HI insert of this vector was ligated into the expression vector pAL3, previously digested with the same enzymes, to give pALERF1.

2.5. Molecular modelling and computer simulations

To build the initial model for domain 2 of *A. nidulans* eRF1, the automated comparative protein modelling server SWISS-MODEL (Guex et al., 1999) was employed using as template the X-ray crystal structure of human eRF1 deposited in the Protein Data Bank (http://www.rcsb.org/pdb/) with identification code 1DT9 (Song et al., 2000). For each mutated residue, a manual search for optimal side chain conformations was performed so as to select the rotamer producing the lowest non-bonded energy. A short optimisation run restraining all non-H atoms to their initial coordinates allowed readjustment of covalent bonds and van der Waals contacts without changing the overall conformation of the protein. This was followed by immersion of the molecule in a truncated octahedron of ~8200 TIP3P water molecules with sides extending 8 Å away from any protein atom. Periodic boundary conditions were applied and electrostatic interactions were represented using the smooth particle mesh Ewald method with a grid spacing of ~1 Å. The cutoff distance for the non-bonded interactions was 9 Å. Unrestrained molecular dynamics simulations at 300 K and 1 atm were then run for 10 ns using the SANDER module in AMBER8 (URL: http://amber.scripps.edu/doc8/). All bonds involving hydrogens were restrained to their equilibrium distances using the SHAKE algorithm and an integration step of 2 fs was used throughout. The simulation protocol was essentially as described elsewhere (Marco et al., 2003) and involves a series of progressive energy minimizations followed by slow heating and equilibration periods before data collection. System coordinates were saved every 2 ps after the first 500 ps of equilibration for further analysis. An average structure of the wild-type eRF1 domain 2 was used to model the G265S substitution, and the mutant protein was simulated under the same conditions as before.

3. Results

3.1. Suppression analysis of the carnitine/acylcarnitine deficiency in A. nidulans

In a previous work (De Lucas et al., 1997), we isolated five different mutants in the *A. nidulans acuH* gene. These

acuH mutants were shown to be CACT-deficient and thus unable to grow on acetate or oleate MM (De Lucas et al., 1999) although they grow normally on non-gluconeogenic carbon sources such as carbohydrates. Preliminary characterization of the *acuH* mutant alleles allowed the identification of three nonsense mutations, a missense mutation and a 1 bp-deletion frameshift in the *acuH* coding region. Phenotypic and biochemical analyses demonstrated that all these mutations belong to the loss-of-function class (Perez et al., 2003).

Results obtained in this work (see below) suggested the re-examination of all existing *acuH* mutations in order to confirm the codon change previously reported (Perez et al., 2003). This study confirmed that the published nucleotide changes for all *acuH* mutations were correct (Perez et al., 2003). Nevertheless, it was found that in the *acuH* nonsense mutations the amino acid previously reported (Perez et al., 2003) as replaced by the TAG codon was the preceding to the correct one. Accordingly, in this work it was confirmed that all nonsense mutations in the *acuH* gene: *acuH13*, *acuH20* and *acuH31* (=*acuH253*, Armitt et al., 1976) belong to the amber (UAG) class. Also, it was unequivocally demonstrated that in two of these mutants a premature TAG stop codon replaces a CAG (Gln) codon in the *acuH* gene, while in the *acuH20* strain a TGG (Trp) codon is replaced by the TAG stop codon (Table 2).

Spores (5×10^8 conidia per plate) from the non-leaky strain WH13 carrying the *acuH13* mutation (Tables 1 and 2) were inoculated on selective media (0.1 M acetate MM or 6 mM oleate MM) and incubated for 10 days at 37 °C. Seven revertants growing on acetate medium (Sac strains) and five strains growing on oleate medium (Sol strains) were isolated. These revertants were tested for their ability to use acetate and oleate as the sole carbon source. Only four revertants, 2 Sac and 2 Sol strains, were able to grow on both media indicating that they carry intragenic or extragenic mutations suppressing the effect of the *acuH13* allele. Such strains were crossed with the wild-type strain *A. nidulans* R21 (Table 1) and phenotypic analysis of the progeny confirmed that a strain, named WSol7H, carries an extragenic suppressor gene of the *acuH13* mutation.

This extragenic suppressor gene, named *suaE7* (see below), produces growth retardation and other pleotropic effects at 37 °C (see below) that allowed to distinguish clearly the four different genotypes (*suaE7*; *acuH13*, *suaE*$^+$; *acuH13*, *suaE7*; *acuH*$^+$ and *suaE*$^+$;*acuH*$^+$) of the backcross progeny (Fig. 1), each representing 25% of the meiotic progeny analysed (200 colonies).

In parallel, we utilized an *A. nidulans* Δ*acuH* strain (PPA14) that had been constructed earlier by replacing the entire *acuH* ORF with the *Neurospora crassa pyr-4* gene (Perez et al., 2003). Spores (5×10^8 conidia per plate) from the PPA14 strain were inoculated on 0.1 M acetate MM or 6 mM oleate MM and incubated in the conditions described above. In addition, PPA14 spores were mutagenized with uv-light (LD_{90}) and inoculated on the same media. No revertants able to grow on both acetate and oleate media were isolated suggesting the impossibility to obtain physiological suppressors of the CACT deficiency in *A. nidulans*.

3.2. The WSol7H strain defines a new translational suppressor (suaE) gene in A. nidulans

According to the above statement, it was assumed that the extragenic suppressor represented by the WSol7H revertant belongs to the translational type. Nevertheless, appropriate genetic analyses were performed to confirm the mechanism of suppression. Strain WSol7H was crossed with complementary strains carrying the following *acuH* mutations: Δ*acuH*, *acuH8*, *acuH13*, *acuH20*, *acuH24* and *acuH31* (Table 1). The results obtained indicate that the identified suppressor does not suppress the *acuH8* (missense) mutation, the *acuH24* (frameshift) mutation or the *acuH* deletion. Among the *acuH* nonsense mutations, the *acuH13 and acuH31* alleles were suppressed in a similar manner (Fig. 1) but not the *acuH20* nonsense mutation (Table 2). This result demonstrates that the suppressor gene identified in this work is allele specific and belongs to the translational class. Interestingly, the *acuH13* and *acuH31* mutations involve a CAG → TAG change that causes the replacement of a glutamine codon with the amber stop codon, while the *acuH20* allele consists of a TGG → TAG substitution that results in the replacement of a tryptophan codon with the same stop codon (Table 2). These results show that the isolated suppressor is unable to suppress all amber nonsense mutations in the *acuH* gene, which suggests that its suppression capacity of these *acuH* mutations depends on the codon (amino acid) at which the nonsense mutation occurs.

Table 2
Suppression analysis of *Aspergillus nidulans acuH* mutants by the translational suppressor *suaE7* gene

Strain	Type of mutation	Nucleotide change[a]	Predicted consequence	Suppression by *suaE7*
acuH8	Missense	148 C → T	Pro50Leu	No
acuH13	Amber nonsense	501C → T	Gln134Stop	Yes
acuH20	Amber nonsense	861G → A	Trp254Stop	No
acuH24	Frameshift	430delT	Frameshift after Asp109/Leu110Stop	No
acuH31	Amber nonsense	76C → T	Gln26Stop	Yes
PPA14	Δ*acuH*::*pyr-4*$^+$	del(1-1590)	No CACT	No

[a] Position of nucleotide changes are from the ATG of the *acuH* genomic DNA sequence (De Lucas et al., 1999. EMBL Data Bank Accession No. AJ011563).

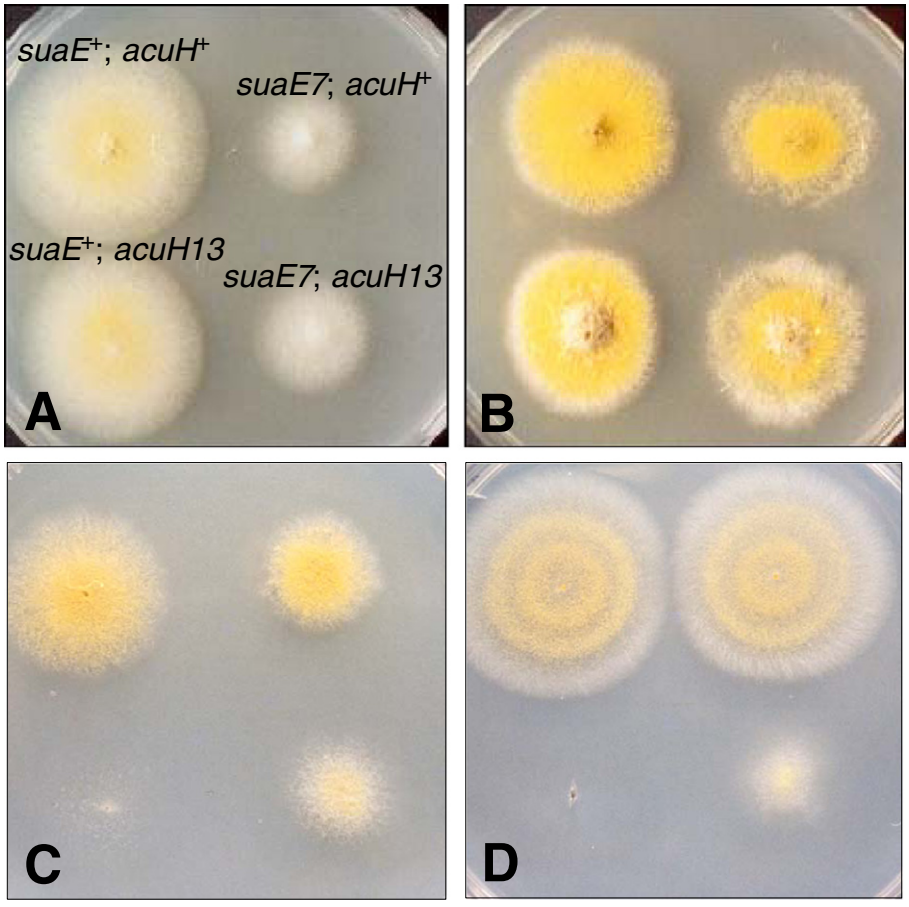

Fig. 1. Growth tests on CM (A), 1% glucose (B), 0.1 M acetate (C) or 6 mM oleate MM (D) of *A. nidulans* strains carrying the four genotypes (*suaE*$^+$; *acuH*$^+$, *suaE7*; *acuH*$^+$, *suaE*$^+$; *acuH13* and *suaE7*; *acuH13*) obtained from a sexual cross between *A. nidulans* R21 wild-type (*suaE*$^+$; *acuH*$^+$) and the revertant WSol7H strain (*suaE7*; *acuH13*). Cultures of MM plates were incubated for 3 days at 37 °C, while the CM plate was incubated for 2 days. In the latter conditions, the growth retardation produced by the *suaE7* gene is much clearer.

Since all the *acuH* nonsense mutants belong to the amber (UAG) class, to analyse whether the *suaE7* allele is able to suppress all type of nonsense mutations, we used a different gene, the *palB* gene. Three complete loss-of-function *palB* mutants, each representing a different type (amber, opal or ochre) of nonsense mutation (Herb N. Arst & Miguel A. Penalva personal communication to be detailed elsewhere) were utilized. The *A. nidulans palB* gene encodes a nuclear calpain-like protease (Denison et al., 1995; Nozawa et al., 2003), which acts as a component of the alkaline ambient pH signal transduction pathway (Arst and Penalva, 2003). Mutants at the *palB* locus are easily identified by their inability to grow at alkaline pH (pH 8) while they grow at acidic pH (pH 5). Sexual crosses were established between strain WSol7 (*wA3 suaE7*; *pyroA4*) and the three *palB* mutant strains. Phenotypic analyses of the meiotic progeny (over 200 colonies) from each cross indicated that the *suaE7* gene weakly suppresses all *palB* mutations analysed. Therefore, the *suaE7* mutation has to be considered as a suppressor of all type nonsense mutations.

Further analysis of the phenotype of the WSol7H strain showed intriguing similarities with that caused by mutations in two translational suppressor genes previously identified in *A. nidulans*, the *suaA* and *suaC* genes (Roberts et al., 1979). The *suaA* and *suaC* genes, together with the *suaB* and *suaD* genes, were identified in a search for translational suppressors in *A. nidulans* (Roberts et al., 1979; Martinelli, 1994). Mutations in the *suaA* and *suaC* genes, the first class of translational suppressors, give rise to recessive suppression and cause pleiotropic alterations of morphology. It was proposed that such suppressors map either in a gene coding for a protein involved in translation termination or for an essential ribosomal protein. In contrast, *suaB* and *suaD* mutant alleles, belonging to the second class and postulated to encode suppressor tRNAs, are semi-dominant and do not alter the phenotype in other ways (Roberts et al., 1979; Martinelli, 1994).

Genetic analysis testing for dominance/recessivity in diploids heterozygous for the suppressor *suaE7* gene and homozygous for the *acuH13* mutation showed that the isolated suppressor is recessive. In addition, growth tests of the WSol7H and WSol7 strains, both carrying the *suaE7* allele (Table 1), were performed on CM and 1% glucose MM at different temperatures: 23, 28, 37 and 43 °C. Results obtained demonstrated similar phenotypic alterations to those described for the *suaA* and *suaC* mutants obtained in

the past (Roberts et al., 1979; Martinelli, 1994). Such alterations include very sparse growth at 23 and 43 °C, growth retardation at 28 and 37 °C (Fig. 1) and increased sensitivity to hygromycin. Representative results of growth tests of a *suaE7* strain and available *suaA*, *suaB* and *suaC* mutant strains are shown in Table 3. In addition, we quantified the viability of *suaE7* conidia. The 57% of the conidia formed by the *suaE7* mutants germinated on CM, while the 81% of conidia produced by the R153 (wild-type) strain were viable. Also, we tested the ability of *suaE7* strains to form selfed cleistothecia. The *suaE7* mutants formed only a few cleistothecia which were surrounded by an unusually thick layer of Hülle cells. These cleistothecia contained only a few viable ascospores. These results demonstrate that, as reported for the majority of the *suaA* and *suaC* mutant alleles (Bratt and Martinelli, 1988), the *suaE7* mutation reduces conidial viability and fertility.

Further genetic characterization of the identified suppressor gene involved the use of parasexual genetics. Diploids YSol7::MS2.7 and YSol7H::MS2.7 (Table 1) were obtained by using established procedures. Diploids were treated with benomyl (1 mg/ml) as the haploidizing agent and phenotypic analyses of haploid segregants allowed unequivocally to locate the suppressor gene at chromosome III, as do the *suaA* and *suaB* genes. A sexual cross between strains WSol7H and G351, the latter carrying the *suaA101* allele, was established. Phenotypic analysis of the meiotic progeny analysed (over 200 colonies) demonstrated no linkage between both suppressor mutations. Besides, a second cross was established between strains WSol7H and H9 which carries the *suaB111* allele. Phenotypic analysis of the meiotic progeny (over 200 colonies) demonstrated that both suppressor mutations are unlinked. These results unequivocally indicate the identification of a new translational suppressor gene in *A. nidulans*, which we have named *suaE* based on the nomenclature suggested by Roberts et al., 1979) for the translational suppressors in this model filamentous fungus.

3.3. The suaE7 mutation give rise to the G255S substitution in A. nidulans eRF1

Strain S7HG (*biA1*; *argB2 suaE7*; *pyroA4*;*acuH13*) was derived from a cross between WSol7H and WG355 (Table 1), and used as the recipient strain in transformation experiments to clone the *suaE* gene. To find a positive selection method for cloning the gene, several growth conditions based on the phenotype associated to the *suaE7* gene were tested. It was observed that strain S7HG is unable to grow in 1% glucose MM plus 200 μM hygromycin at 42 °C. Thus, these growth conditions were used to clone the *suaE* gene by transformation. DNA from 20-cosmid pools of the chromosome III-specific library of *A. nidulans* (Brody et al., 1991) was mixed with equimolar amounts of the autonomously replicating plasmid ARp1 (Gems et al., 1991) and used in co-transformation experiments. Following the strategy described elsewhere (De Lucas et al., 1999), a cosmid (L6H06) was identified as capable of restoring all pleiotropic alterations of the S7HG strain associated to the *suaE7* mutation. In addition, the hygromycin-resistant transformants obtained were unable to grow on acetate and oleate media. All these results clearly suggested the identification of a positive cosmid that contains the *suaE* gene and not an unlinked suppressor (see below).

DNA from this cosmid was digested with the following enzymes: *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Xba*I and *Xho*I. Restriction fragments were used in combination with ARp1 DNA to co-transform the strain S7HG. Hygromycin-resistant transformants were obtained for all digestion mixtures except when *Hin*dIII restricts were used. The positive cosmid was digested with *Pst*I and restriction fragments were used in transformation. Only transformation with a 15-kb *Pst*I fragment gave hygromycin-resistant colonies. This fragment was cloned into pUC19 to give pPS150. This large vector, that transformed the S7HG strain to $suaE^{+}$ phenotype at a frequency of 18 transformants per microgram of DNA, was digested with *Xba*I and restricts obtained were used in transformation. A positive 2.5-kb *Xba*I fragment was found. This genomic fragment was cloned into pUC19 to give the plasmid pXB25, which complements the *suaE7* mutation at a similar transformation frequency than pPS150 does. The two strands of the 2.5-kb *Xba*I insert of plasmid pXB25 were sequenced. BLASTN and BLASTX searches unequivocally showed it to contain the ORF except for the last 54 codons of the gene encoding the eRF1 of *A. nidulans* (Han et al., 2005). The remaining 3′ coding region of the *A. nidulans suaE* gene was identified by partial sequencing of the vector pPS150. This vector contains the whole *suaE* gene surrounded by a large genomic region at its 5′ and 3′ ends. The physical map of the *A. nidulans suaE*

Table 3
Growth tests and sensitivity to hygromycin of *A. nidulans* R153 (wild-type) strain and *suaA*, *suaB*, *suaC* and *suaE* mutants

Strain	Growth on CM				Growth on CM + 100 μM hygromycin			
	23 °C	28 °C	37 °C	43 °C	23 °C	28 °C	37 °C	43 °C
R153	4	4	4	3	2	2	3	1
suaA101	1	2	3	2	0	0	2	0
suaB111	4	4	4	3	2	2	2	0
suaC109	0	1	4	3	0	0	1	0
suaE7	1	2	3	1	1	1	2	0

Growth on complete medium (CM) is expressed quantitatively from 0 (no growth) to 4 (maximal growth). Data were scored at the following incubation times: 5 days at 23 °C, 4 days at 28 °C and 3 days at 37 °C and 43 °C. Growth tests on 1% glucose MM were similar to those shown on CM.

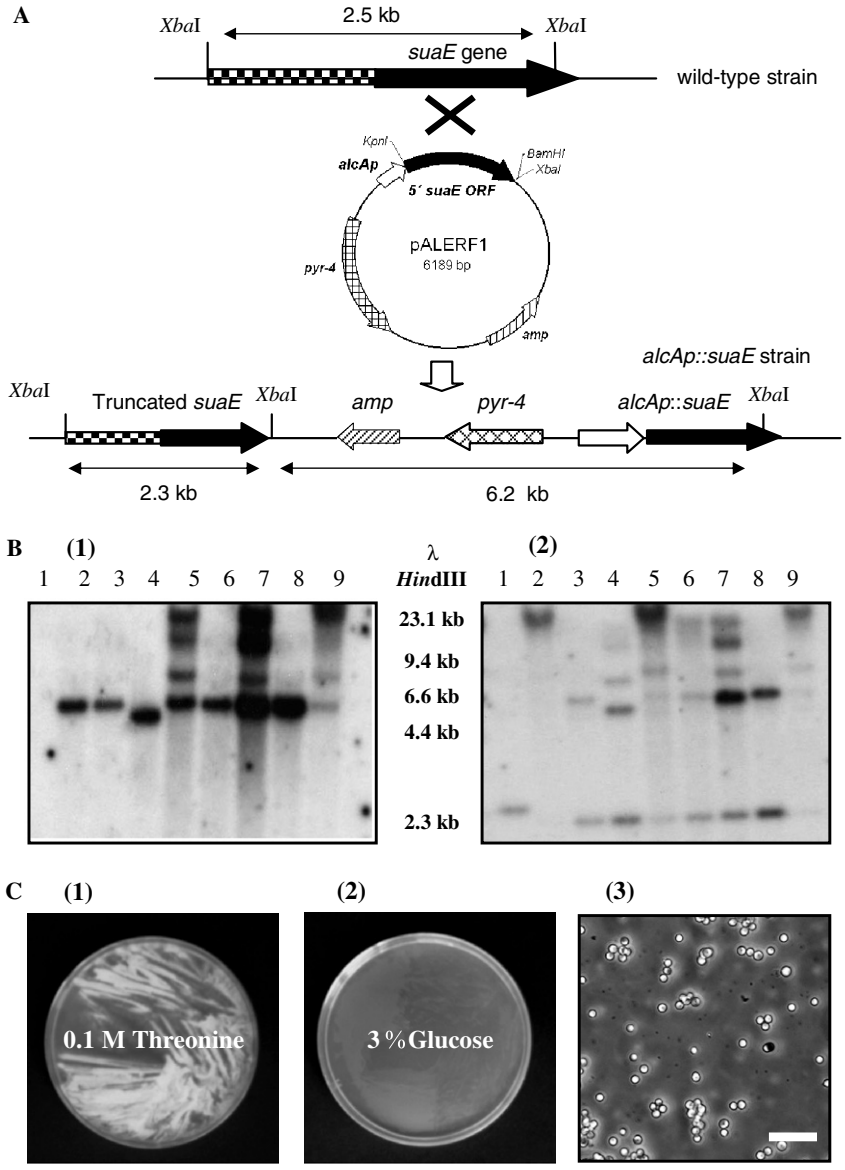

Fig. 2. Identification by Southern blotting and characteristic phenotype of *A. nidulans* transformants expressing the *suaE* gene under control of the *alcA* promoter. (A) Schematic representation of the integration of a single copy of plasmid pALERF1 at the genomic *suaE* locus leading to the replacement of the *suaE* promoter by the $alcA^P$. Chromosomal and plasmid *Xba*I sites are indicated. Sizes of fragments hybridizing with the *pyr-4* or the *suaE* probe in the wild-type strain and correct transformants are shown. (B) Southern blot analysis of genomic DNA from the recipient strain G191 ($pyrG^-$) and $pyr\text{-}4^+$ strains obtained by transformation with plasmid pALERF1. (B.1) Genomic DNA from strain G191 (lane 1) and $pyr\text{-}4^+$ transformants (lanes 2–9) digested with *Xba*I and hybridized with a 1.2-kb *Hin*dIII internal fragment of the *N. crassa pyr-4* gene. (B.2) Genomic DNA from strain G191 (lane 1) and $pyr\text{-}4^+$ transformants (lanes 2–9) hybridized with a 1065-bp *Kpn*I–*Bam*HI region containing the *suaE* ORF except the last 135 codons. Transformants shown in lane 3 and 8 express the *suaE* gene under control of the *A. nidulans* $alcA^P$. (C) Essentiality analysis of the *suaE* gene in *A. nidulans*. (C.1) Phenotypic analysis showed normal growth of $alcA^P$-*suaE* transformants in inducing conditions (0.1 M threonine MM) at 37 °C. (C.2) In repressing conditions (3% glucose MM) these transformants were unable to grow. (C.3) Microscopic analysis demonstrated that eRF1-depleted spores are unable to germinate. These spores started to burst after 20 h incubation on selective medium at 37 °C. Bar represent 20 μm.

gene (Han et al., 2005), its complete genomic sequence (Accession No. AF451327) (URL: www.broad.mit.edu/annotation/fungi/aspergillus/) and the deduced eRF1 protein sequence (Accession No. AAM46702) are all available.

In addition, we analysed the essentiality of the *suaE* gene by putting this gene under control of the promoter of the *A. nidulans alcA* gene ($alcA^P$). As described in Section 2, we constructed the plasmid pALERF1 that contains a transcriptional fusion of the $alcA^P$ to the *suaE* ORF truncated at its 3′ end (Fig. 2). This plasmid was used to transform the *A. nidulans* G191 strain (Table 1) and transformants were selected on 0.1 M glycerol MM to permit the growth of $pyrG^+$ strains in which the *suaE* gene was expressed under control of the *A. nidulans* $alcA^P$. Southern blot analysis allowed the identification of several $pyrG^+$ transformants with a hybridization pattern consistent with the replacement of the *suaE* promoter by the $alcA^P$. Phenotypic analysis of two of these transformants in 3% glucose MM or in

YEPD (repressing conditions) (Romero et al., 2003) showed that eRF1-depleted spores are unable to germinate (Fig. 2). However, these transformants grew as the wild-type strain when incubated in 0.1 M threonine MM (inducing conditions). Microscopic analysis showed that in repressing media these conidia swelled until they almost double their size. Nevertheless, these conidia did not show any other germination feature. Consequently, germ tube emergence was never observed. Finally, these spores started to burst after 20 h incubation at 37 °C. These results demonstrate that the *suaE* gene is an essential gene in *A. nidulans*.

Molecular characterization of the *suaE7* mutation was performed by PCR amplification of the *suaE7* allele from the revertant WSol7H strain as stated in Section 2. The 906G > A transition in the *suaE* ORF was identified. This mutation results in the G265S substitution in the eRF1 of *A. nidulans*. In addition, we sequenced the *suaE* gene from several strains obtained in this work that carry the *suaE7* allele or the wild-type $suaE^{+}$ gene (Table 1). The results obtained unequivocally correlate the presence of the 906G > A transition at the *suaE* gene with the pleiotropic growth defects and the suppression of the *acuH13* and *acuH31* mutations (Fig. 1).

It has been proposed that the omnipotent eRF1 and aRF1 (archaea class-1 release factor) have originated from an archaeal-like version in the common ancestor of eukayotes and archaea (Inagaki and Ford, 2000). In contrast, the two bacterial class-1 release factors (RF1 and RF2) do not posses any obvious sequence homology with eRF1 or aRF1 (Nakumura and Ito, 2003). An alignment of eRF1 sequences from representative eukaryotes including the fungi *A. nidulans* and *Saccharomyces cerevisiae*, the ciliate *Tetrahymena thermophila* and the mammal *Homo sapiens*, together with the aRF1 sequences from the archaeas *Methanosarcina mazei* and *Pyrococcus abysii*, is shown in Fig. 3. It can be seen that a significant level of sequence identity exists among domains 1 (residues 1–141 using the numbering of human eRF1) of eRF1s and aRF1s. The domain 1 characteristic motif NIKS involved in recognition of all stop codons is present in both aRF1s and eRF1s except for that from *Tetrahymena thermophila*, a ciliate with a nonstandard genetic code that utilizes only UGA as stop (Lozupone et al., 2001; Kisselev et al., 2003). Also, a significant level of sequence identity is found among domains 2 (residues 145–275 of human eRF1) of eRF1s and aRF1s. As can be seen, the characteristic GGQ tripeptide in domain 2 that is required for peptidyl-tRNA hydrolysis is also conserved in both eukaryotic and archaeal RFs. In contrast, a very low level of sequence identity exists among domains 3 (residues 276–437 of human eRF1) of both types of class-1 RFs. Interestingly, the glycine 265 residue of domain 2 of the *A. nidulans* eRF1 (G253 in the human eRF1) is present in all of these sequences, which are representative of both *Eukarya* and *Archaea*. The conservation of this glycine in all eukaryotic and archaeal sequences analysed (not shown) clearly suggests a relevant role of this amino acid in eRF1 structure and/or function.

3.4. Structural consequences of the G265S substitution in domain 2 of *A. nidulans* eRF1

The overall architecture of domain 2 of *A. nidulans eRF1* is very similar to that of human eRF1, as expected from the high degree of sequence identity (Figs. 3 and 4). Likewise, Gly265, which is positionally equivalent to Gly263 in human eRF1, is located close to the N-terminus of the kinked α-helix that connects domains 2 and 3. Our modelling and simulation results for domain 2 of both wild-type and G265S mutant fungal eRF1 suggest that no gross conformational changes take place in this domain as a consequence of this mutation (Fig. 4). The evolution of the root-mean-square deviation from the initial structure (Fig. 5) shows that most of the fluctuation is due to the high mobility of the loop embedding the GGQ motif, as expected, whereas the α-helix remains very stable in both cases. Nonetheless, we note that the flexibility of this helix, which has been suggested to participate in the hinge motions involving both protein domains, is likely to be affected by the good hydrogen bonds that the hydroxyl group in the side chain of the mutated Ser265 can establish with the backbone of adjacent protein residues that make up the preceding loop.

4. Discussion

A search for suppressors of the CACT deficiency in the model fungus *A. nidulans* has suggested the impossibility of isolating physiological suppressor genes for this genetic and metabolic disease, the most severe disorder of fatty acid β-oxidation in humans. Nevertheless, this suppressor analysis has allowed the identification and further genetic and structural characterization of a novel mutation (*suaE7*) in the omnipotent class-1 release factor of eukaryotes (eRF1) that suppresses two of the three existing amber nonsense mutations in the *A. nidulans* CACT encoding (*acuH*) gene.

Translation termination in eukaryotes is controlled by the release factor complex, a heterodimer formed by association of eRF1 and eRF3. This complex recognizes a stop codon located in the ribosomal A-site and triggers the release of the nascent peptide (see Inge-Vechtomov et al., 2003, for a review). Genetic and biochemical studies in *S. cerevisiae,* in which the essential *SUP45* and *SUP35* genes encode, respectively, the eRF1 and eRF3 proteins, have demonstrated that eRF1 acts as the key factor in the termination of translation while the eRF3 has a stimulating role (Bertram et al., 2001; Inge-Vechtomov et al., 2003).

Resolution of the three-dimensional structure of human eRF1 by X-ray crystallography has revealed the molecular mechanism of eRF1 activity (Song et al., 2000). The overall shape and dimensions of eRF1 resemble those of a tRNA molecule with domains 1, 2, and 3 of eRF1 corresponding to the anticodon loop, aminoacyl acceptor stem and T stem of a tRNA molecule, respectively (Fig. 3). Domain 1, containing the highly conserved motif NIKS, is proposed to participate in stop codon recognition (Song et al., 2000; Bertram et al., 2000; Chavatte et al., 2001; Frolova et al.,

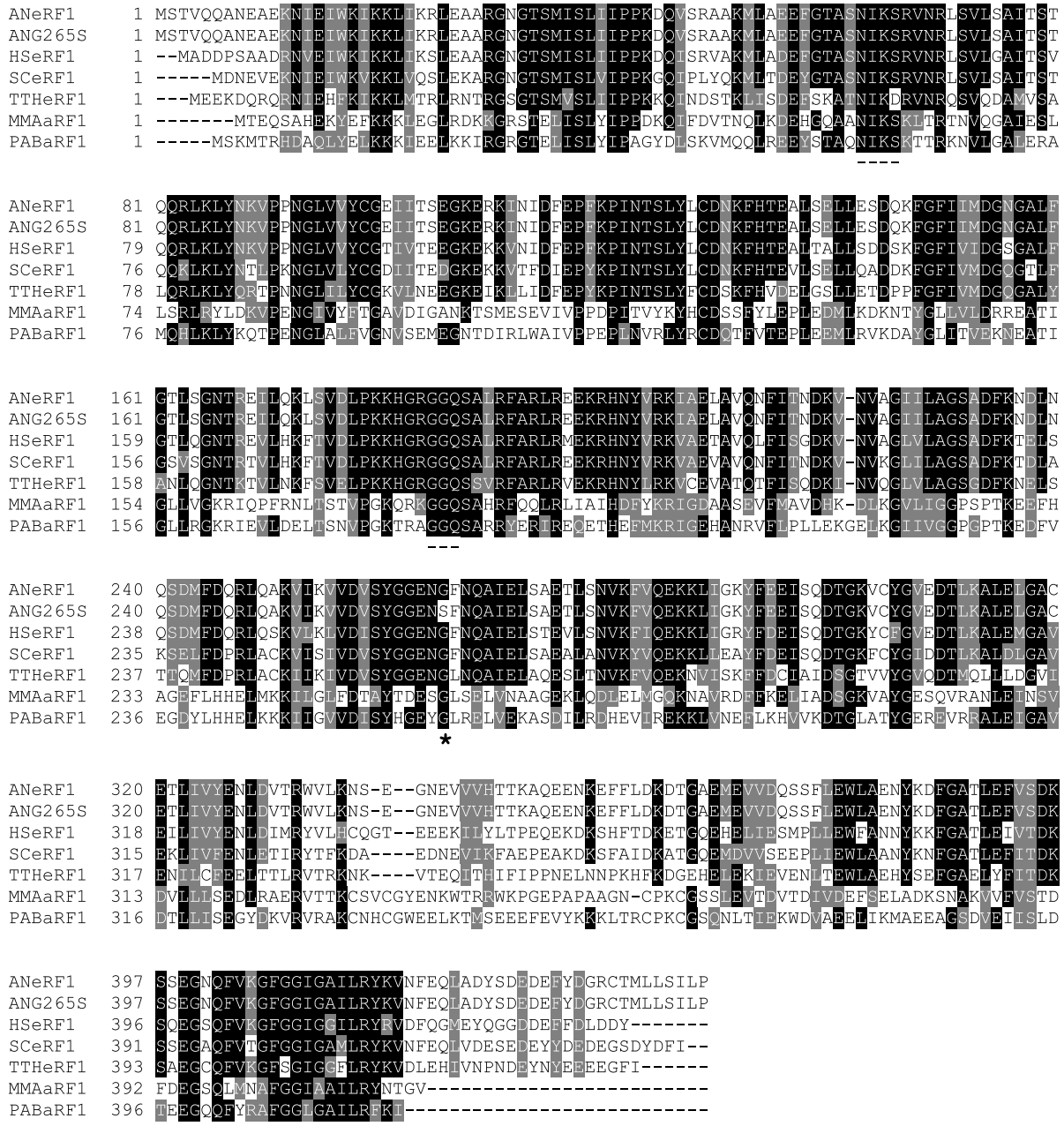

Fig. 3. Alignment using the program CLUSTAL W (1.8) of the amino acid sequence of the eRF1 from the fungi *Aspergillus nidulans* wild-type (lane 1) and *suaE7* mutant strain (lane 2) , *Saccharomyces cerevisiae* (lane 4), the ciliate *Tetrahymena thermophila* (lane 5) the mammal *Homo sapiens* (lane 3) with the aRF1 sequence from two representative archaeas: *Methanosarcina mazei* (lane 6) and *Pyrococcus abyssi* (lane 7). Identical amino acids in at least five of the sequences appear black shaded in the box-shade representation. Similar amino acids in at least five of the sequences are shaded gray. The characteristic motifs of domain 1 (NIKS) and domain 2 (GGQ) of eRF1 and aRF1 are underlined. The G265S amino acid substitution resulting from the *A. nidulans suaE* mutation is marked by an asterisk. Notice that the *A. nidulans* glycine 265 is fully conserved in these representative sequences from *Eukarya* and *Archaea*.

2002). Domain 2, which is responsible for the peptidyl transferase hydrolytic activity, includes a GGQ motif that has been highly conserved throughout evolution (Seit-Nebi et al., 2001). Domain 3 interacts with the eRF3 (eukaryotic release factor 3), a class-2 translational termination factor that displays ribosome-dependent GTPase activity and is involved in the recycling of class-1 factor (Kisselev et al., 2003). Domains 1 and 2 are connected by a hinge region comprising residues 1–7 and 142–144 whereas domain 3 connects with domain 2 via a kinked helix that has been compared to a spring breaker (Ma and Nussinov, 2004). In the crystal structure of human eRF1 ("open" conformation), the separation between NIKS and GGQ motifs is about 100 Å whereas the distance between the ribosomal decoding and peptidyl transferase centre, as inferred from cryoelectron microscopy studies, is around 70 Å, which is precisely the distance separating a tRNA anticodon and the CCA acceptor stem (Klaholz et al., 2003; Nakamura and Ito, 2003). This means that to fit the ribosome binding pocket eRF1 must adopt a "closed" conformation to bring

these motifs closer in a process that has been suggested to depend on solvation and the protonation state of histidine residues (Ma and Nussinov, 2004).

The G265S substitution in the *A. nidulans* eRF1 (equivalent to G263S in the human eRF1) reported in this work is, to the best of our knowledge, the first mutation identified in a fully conserved residue of the eRF1/aRF1 domain 2 that does not affect the GGQ context. Therefore, it is unlikely that this mutation affects directly the eRF1 catalytic domain represented by the GGQ motif. In addition, it must be noted that the structural change brought about by the G265S mutation in domain 2 is relatively subtle (Fig. 4), as one would expect in a protein that cannot be significantly altered without seriously compromising its normal essential function. The structural variability during molecular dynamics simulations in aqueous solution was also found to be rather low and of similar magnitude for both wild-type and mutant proteins (Fig. 5), with most of the variation arising from the very flexible loop region, as expected. For this reason, we tend to think that the different efficiencies of the two eRF1 proteins to act as release factors are likely dependent on differences in the conformational properties of the spring helix connecting domain 2 to domain 3. By stabilizing the N-terminus of the helix, we hypothesize that this mutation might be preventing some of the functionally relevant conformational changes that are needed for ribosome binding and/or for triggering the ribosomal peptidyl transferase activity. Gaining further inside into this possibility through computer simulation would require additional modelling of the whole protein docked into the ribosome binding pocket but this is beyond the scope of the present work.

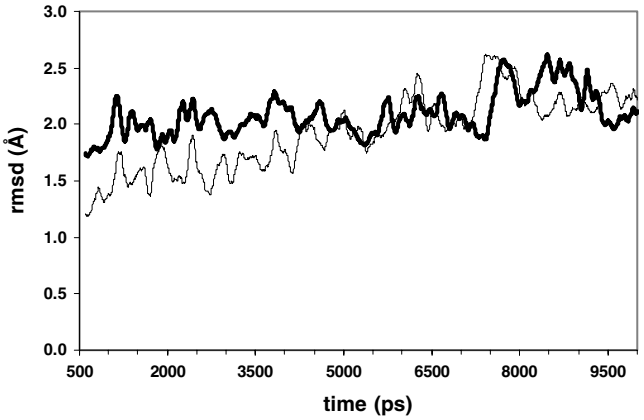

Fig. 5. Time evolution of the root-mean-square deviations (rmsd) of the coordinates of backbone atoms with respect to the initial structure for the whole of domain 2 in both wild-type (thick line) and G265S mutant (thin line) eRF1 structures.

Although the process of peptide chain termination is normally effective, stop codons are not always recognized efficiently. This fact provides a regulatory mechanism of gene expression that is extensively utilized by positive-sense ssRNA viruses (Beier and Grimm, 2001). The misreading of termination codons is achieved by a variety of naturally occurring suppressor tRNAs that seem to be present in all organisms. Such natural nonsense suppressor tRNAs contain structural and nucleoside modifications that permit not only reading their cognate sense codons but also illegitimate nonsense translation (Beier and Grimm, 2001; Bertram et al., 2001). The natural cytoplasmic UAG/UAA suppressor tRNAs generally found in eukaryotes are

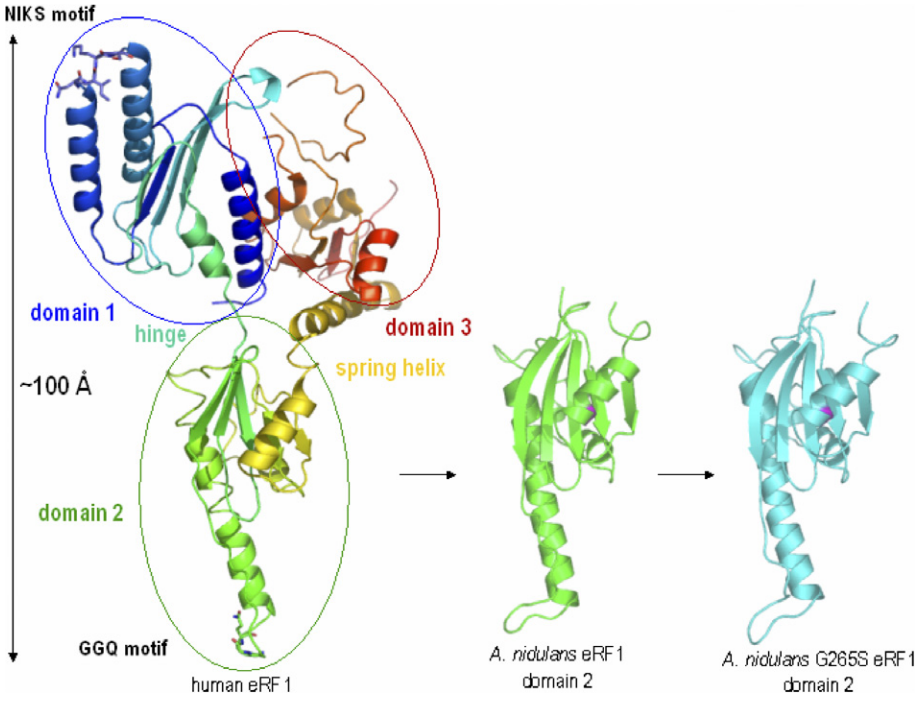

Fig. 4. Ribbon representation (colour coded from the *N*-terminus [blue] to the *C*-terminus [red], with intermediate values ramping smoothly) and domain structure of the crystallographically solved human eRF1 structure (left) that was used as template to model both wild-type (middle) and G265S (right) domain 2 of *A. nidulans* eRF1. The position of the suppressor mutation in the spring helix is highlighted in magenta.

$tRNA^{Tyr}$, $tRNA^{Gln}$ and $tRNA^{Leu}$ while the cytoplasmic $tRNA^{Trp}$, $tRNA^{Arg}$ and $tRNA^{Cys}$ act as UGA suppressors (Beier and Grimm, 2001).

In natural conditions, these cryptic tRNAs are non-competitive with translation termination, as the release factor complex specifically binds to the nonsense codons. However, mutations in the eRF1 or eRF3 that hamper normal eRF binding and/or activity decrease the competitiveness of these factors against these natural suppressor tRNAs. As a consequence of this illegitimate nonsense translation, a fraction of newly synthesized proteins are abnormally enlarged and thus become non-functional, which may substantially decrease cell viability and growth.

In *Saccharomyces cerevisiae*, the pleiotropic effects associated to missense mutations in the eRF1 or eRF3 include temperature sensitivity, osmotic sensitivity (Inge-Vechtomov and Andrianova, 1970) increased sensitivity to paromomycin (Mironova et al., 1982) and retardation of cell-cycle completion (Inge-Vechtomov et al., 2003; Valouev et al., 2004). In *A. nidulans*, two different missense mutations in the eRF1 have been reported so far, the *suaE7* mutation provoking the G265S substitution (characterized in this work) and the *snpA6* mutation causing the E117K substitution (Han et al., 2005). The *snpA6* mutation suppresses the *npgA1* mutation, a mutation in a 4′ phosphopantetheinyl transferase encoding gene (Keszenman-Pereyra et al., 2003) that results in a colourless colony. Phenotypic analyses have shown that temperature sensitivity and growth retardation at 37 °C are characteristic features associated to both existing eRF1 missense mutations in *A. nidulans*. In addition, a deeper phenotypic analysis performed in this work, showed that the *suaE7* mutation produces lowered fertility, poor conidial viability and increased sensitivity to hygromycin. Similar pleotropic alterations to those of the *suaE7* mutants were reported for mutations in the *A. nidulans suaA* and *suaC* genes (Roberts et al., 1979; Martinelli, 1994), two translational suppressor genes whose gene products are still unknown. Phenotype comparison among *suaE*, *suaA* and *suaC* mutants clearly suggests that the *suaA* and *suaC* genes participate in the termination of translation with eRF3 and eRF1/eRF3-interacting proteins (poly(A)binding protein or UPF proteins) (Inge-Vechtomov et al., 2003) which thus become possible candidates for their respective gene products.

Molecular characterization of all existing *acuH* mutations and their suppression analyses have demonstrated that the translational suppressor *suaE7* gene is allele-specific being able to suppress nonsense mutations but not frame-shift or missense mutations in the *acuH* gene. Besides, results obtained from the suppression analyses of opal, amber and ochre nonsense mutations in the *palB* gene have indicated that the suppressor *suaE7* mutation is also gene-unspecific and able to suppress the three types (amber, opal and amber) of nonsense mutations.

It was found that the two *acuH* alleles suppressed by the *suaE7* gene, *acuH13* and *acuH31*, carry nonsense mutations involving a Gln → TAG stop codon change. These substitutions occur at residue 134 of the *A. nidulans* CACT in the *acuH13* allele and at amino acid 26 in the *acuH31* allele. Curiously, the remaining *acuH* nonsense mutation, *acuH20,* resulting in a Trp → amber stop codon substitution at residue 254 of AcuH is not suppressed by *suaE7*. Taken together, these results strongly suggest that the *suaE7* suppression of the *acuH13* and *acuH31* alleles involves the participation of the cytoplasmic UAG/UAA suppressor $tRNAs^{Gln}$. In accord with this explanation, it was shown, firstly, that these $tRNAs^{Gln}$ containing a CUG or an UmUG anticodon exist in all prokaryotes and eukaryotes (Sprinzl et al., 1998; Beier and Grimm, 2001) and secondly, that glutamine is the most common amino acid encoded in the read-through of UAG (Harrell et al., 2002). Competition at a premature UAG codon between one of these two $tRNAs^{Gln}$ isoacceptors and the eRF1 carrying the G265S mutation would give a chance to the correct insertion of a glutamine residue at this position within the CACT protein sequence instead of terminating polypeptide chain elongation. Consequently, a fraction of wild-type AcuH molecules should be synthesized in cells carrying both the *suaE7* and *acuH13* or *acuH31* mutations thus leading to partial restoration of growth on selective medium such as acetate or oleate MM (Fig. 1). In contrast, the lack of suppression of the *acuH20* mutation by the *suaE7* gene indicates that no natural UAG/UAA suppressor tRNAs allow the insertion of tryptophan or another amino acid that can functionally replace Trp254 in the *A. nidulans* CACT. Interestingly, this tryptophan residue is fully conserved in the CACTs sequence through evolution (De Lucas et al., 1999; Perez et al., 2003).

Sequence context surrounding the termination codon appears to play a relevant role in determining the efficiency of translation termination in prokaryotes and eukaryotes. Accordingly, it has been proposed that most naturally occurring termination codons lie within a context that promotes efficient translation termination. In contrast, premature translation termination codons introduced by mutation would not be subject to such selective pressure. Several sequences consisting of 1–6 nucleotides upstream and/or downstream of the termination codons have been proposed to increase or decreased the translational read-through efficiency in eukaryotes (Brown et al., 1990; Bonetti et al., 1995; Beier and Grimm, 2001; Harrell et al., 2002). However, no definitive mechanistic model has emerged for the role of these nucleotides, being especially difficult to correlate the codon context with the efficiency of the UAG read-through (Tork et al., 2004; Cridge et al., 2006). In addition, more complex signals like stem-loop or pseudoknot structures have also been correlated with the increase of the read-through efficiency (Beier and Grimm, 2001). Taking into account this knowledge, we performed an analysis of sequences surrounding the premature TAG stop codon of the *acuH13*, *acuH20* and *acuH31* mutant alleles. This study failed in identifying any codon context that could predict differences in the UAG read-through efficiency among these mutations. This fact, suggests that

the codon context surrounding the *acuH20* mutation may not be responsible for the lack of suppression of the *acuH20* mutant allele.

During the preparation of this manuscript Han et al. (2005) reported the identification of the *A. nidulans snpA* gene encoding eRF1 as a temperature-sensitive suppressor of the *npgA1* mutation. We here propose, as the most appropriate, the denomination of *suaE* (=*snpA*) gene for the *A. nidulans* translational suppressor gene encoding the eRF1, based on the nomenclature suggested by Roberts et al. (1979) for the translational suppressors in this model filamentous fungus.

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