In Aspergillus nidulans the Suppressors suaA and suaC Code for Release Factors eRF1 and eRF3 and suaD Codes for a Glutamine tRNA

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ABSTRACT In Aspergillus nidulans, after extensive mutagenesis, a collection of mutants was obtained and four suppressor loci were identified genetically that could suppress mutations in putative chain termination mutations in different genes. Suppressor mutations in suaB and suaD have a similar restricted spectrum of suppression and suaB111 was previously shown to be an alteration in the anticodon of a gln tRNA. We have shown that like suaB, a suaD suppressor has a mutation in the anticodon of another gln tRNA allowing suppression of UAG mutations. Mutations in suaA and suaC had a broad spectrum of suppression. Four suaA mutations result in alterations in the coding region of the eukaryotic release factor, eRF1, and another suaA mutation has a mutation in the upstream region of eRF1 that prevents splicing of the first intron within the 5′UTR. Epitope tagging of eRF1 in this mutant results in 20% of the level of eRF1 compared to the wild-type. Two mutations in suaC result in alterations in the eukaryotic release factor, eRF3. This is the first description in Aspergillus nidulans of an alteration in eRF3 leading to suppression of chain termination mutations.

KEYWORDS suppression of nonsense mutations in A. nidulans suaD codes for a glutamine tRNA suaA codes for eRF1 suaC codes for eRF3

Isolation of genetic suppressor mutations of chain termination mutations (and anti-suppressors) has proved to be a very powerful tool for identifying components of the translation machinery in a range of model eukaryotic organisms including Saccharomyces cerevisiae, Schizosaccharomyces pombe (Hawthorne and Leupold 1974), Podospora anserina (Coppin-Raynal and Dequard-Chablat 1988), Caenorhabditis elegans (Hodgkin 2005), and Aspergillus nidulans (Martinelli 1994). The early genetic screens made predictions about the possible function of the suppressor genes from phenotypic observations and, much later, these predictions have been confirmed by the molecular data; however, to date this has not been accomplished with Aspergillus nidulans. The mechanism of chain termination is still poorly understood, and it is of interest not only from a fundamental scientific standpoint as it is ubiquitous in all cells, but also from a practical point of view because therapies are being developed to allow read-through of premature chain termination mutations in disease genes (Bidou et al. 2012; Bordeira-Carrico et al. 2012), and vector selection systems are being developed that have a nonsense mutation in the recipient strain and a suppressor mutation in the vector rather than using an antibiotic resistance gene for selection (Oliveira and Mairhofer 2013). In all the organisms where chain termination suppressors have been isolated and characterized at the molecular level, the release factors eRF1 and eRF3 have been found to be essential for polypeptide chain termination. Mutations in either of these proteins can lead to read-through of chain termination mutations (Gagny and Silar 1998; Inge-Vechtomov et al. 2003), but the release factors have other roles in cell organization and cell cycle progression, and characterization of the suppressors from a range of organisms may give important insights into these additional functions (Inge-Vechtomov et al. 2003).

In Aspergillus nidulans, four suppressor loci, suaA, suaB, suaC, and suaD (suasuppressor in Aspergillus) were isolated by co-reversion of mutations in unrelated genes (Roberts et al. 1979). These suppressors were allele-specific and gene-unspecific and were thought to act on
nonsense mutations because of the lack of any residual function in the gene products of the suppressible mutant strains. Subsequent work extended the number of co-suppressible alleles, but the number of new suppressor loci was limited and these have not been characterized further (Al Taho et al. 1984; Martinielli et al. 1984; Sealy-Lewis 1987). The suppressors in the suaB and suaD loci had a restricted pattern of suppression and were semi-dominant to the wild-type suppressor allele, whereas mutations in suaC and suaF had a wider spectrum of suppression and were recessive or semi-dominant depending on the suppressible allele being studied. The suaC and suaF alleles also had a number of pleiotropic alterations, like the omnipotent suppressors in Saccharomyces cerevisiae, that were able to suppress all three classes of chain termination mutation. These included cold sensitivity, slow growth in conditions not requiring the action of the suppressor, poor conidial viability, lowered fertility, and increased sensitivity to aminoglycoside antibiotics (Martinielli 1994). The behavior of the two classes of suppressor led to the hypothesis that the suaB and suaD mutations were acting by alterations in tRNA whereas the suaA and suaC mutations were acting by alterations in ribosomal proteins or release factors. The properties of the suaA and suaC suppressor strains have similarities to the SUP45 and SUP35 omnipotent suppressor strains in Saccharomyces cerevisiae and the sua2 and sua1 suppressors in Podospora anserina that have been shown to code for the release factors eRF1 and eRF3, respectively (Frolova et al. 1990; Stansfield et al. 1995; Gagny and Silar 1998). The suaB111 mutation was identified as a G-to-A alteration in the anticodon CUG of a glutamine tRNA leading to recognition of UAG as a sense codon (Espeso et al. 2005), but the mechanism of action of the other suppressors has remained unidentified. In this article, we identify functions for suaA, suaC (eRF1 and eRF3), and suaD (glutamine tRNA).

**MATERIALS AND METHODS**

*A. nidulans* strains, media, growth conditions, and manipulations

Aspergillus media and growth conditions were as described by Cove (1966). The scoring of the suppressible alleles in alX, sb and niaD, are described by Sealy-Lewis (1987). Genetic techniques were as described by Clutterbuck (1974). The strains used are listed in Supporting Information, Table S1. Gene symbols are as previously described (Clutterbuck 1993, 1997) and the characterized sequence changes of suppressible alleles are listed in Table 1.

**Molecular techniques**

Standard molecular techniques are described by Sambrook et al. (1989). *Aspergillus* genomic DNA was prepared as described by Jones and Sealy-Lewis (1989). DNA was extracted from the strains [H3, H103, H44, H44(27), H44(23), H44(32), H7, H7 rev16] and PCR primers (Table S2) were designed to amplify overlapping sections of the entire coding region of the genes. The same primers were used for sequencing the PCR products. The coding region of both genes was sequenced on both strands. Where a change was identified compared with the sequence in the database, the wild-type was sequenced in that region to confirm the change. RT-PCR was performed using the GE Healthcare Illustra Ready-to-Go RT-PCR beads. The PCR products for eRF1 and the tubulin controls were standardly run on 1.3% TAE agarose gels or 5% TBE polyacrylamide gels. Crystal structure prediction of eRF1 and eRF3 was achieved through EsyPred3D Web server (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/ esypred/) and Swiss-model (http://swissmodel.expasy.org/).

**Transformation experiments, protein analyses, and cell imaging**

Transformation of *Aspergillus* was performed as described by Tilburn et al. (2005). Strains expressing GFP C-terminally tagged fusions of wild-type or mutant SuaA protein were generated as described by Yang et al. (2004). Transformation DNA cassettes were obtained by fusion PCR procedures of three fragments comprising, in the following order, the 5′-UTR/suaACDS, gfp/riboBAf and 3′-UTR region of suaA, as described in Figure 1. Strains MAD1427 and H1885 were used to generate strains expressing SuaA-GFP, and H1885 and H1884 were used to express SuaA105-GFP and SuaA23-GFP proteins, respectively.

The suaA23 mutation was re-introduced into the suppressible strain H44 by transformation. The fragment containing the suaA23 allele was PCR-amplified using gDNA from H44(23) as the template and primer pairs suaA1 and suaA2. Transformants were positively

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**Table 1 Alleles with known chain termination sequence changes**

<table>
<thead>
<tr>
<th>Mutant Allele</th>
<th>Nucleotide Change</th>
<th>Amino Acid Affected</th>
<th>Amino Acid Change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>alX4a</td>
<td>CAG to TAG</td>
<td>Gln 409</td>
<td>Gln to TAG</td>
<td>This study</td>
</tr>
<tr>
<td>alcR125b</td>
<td>TGG to TAG</td>
<td>Trp 149</td>
<td>Trp to TAG</td>
<td>Dr. B. Felenbok, personal communication</td>
</tr>
<tr>
<td>areA600</td>
<td>TCG to TAG</td>
<td>Ser 646</td>
<td>Ser to TAG</td>
<td>Kula et al. (1990) and Langdon et al. (1995)</td>
</tr>
<tr>
<td>areA601</td>
<td>AAA to TAA</td>
<td>Lys 206</td>
<td>Lys to TAA</td>
<td>Prof. H. N. Arst, personal communication</td>
</tr>
<tr>
<td>alaD67</td>
<td>UGG to UGA</td>
<td>Trp 131</td>
<td>Trp to TGA</td>
<td>Flippeli et al. (2001)</td>
</tr>
<tr>
<td>acuH13</td>
<td>CAG to TAG</td>
<td>Gln 134</td>
<td>Gln to TAA</td>
<td>Martinez et al. (2007)</td>
</tr>
<tr>
<td>acuH31</td>
<td>CAG to TAG</td>
<td>Gln 26</td>
<td>Gln to TAA</td>
<td>Martinez et al. (2007)</td>
</tr>
<tr>
<td>acuH20</td>
<td>TGG to TAG</td>
<td>Trp 254</td>
<td>Trp to TGA</td>
<td>Martinez et al. (2007)</td>
</tr>
<tr>
<td>ngA1</td>
<td>TTA to TGA</td>
<td>Leu 269</td>
<td>Leu to TGA</td>
<td>Han et al. (2005)</td>
</tr>
<tr>
<td>palC143</td>
<td>TTA to TGA</td>
<td>Leu 223</td>
<td>Leu to TGA</td>
<td>Tilburn et al. (2005)</td>
</tr>
<tr>
<td>palF15</td>
<td>TTA to TGA</td>
<td>Leu 189</td>
<td>Leu to TGA</td>
<td>Herranz et al. (2005)</td>
</tr>
<tr>
<td>palB7</td>
<td>GGA to TGA</td>
<td>Gly 791</td>
<td>Gly to TGA</td>
<td>Peñas et al. (2007)</td>
</tr>
<tr>
<td>palB513</td>
<td>TTA to TGA</td>
<td>Leu 552</td>
<td>Leu to TGA</td>
<td>Peñas et al. (2007)</td>
</tr>
<tr>
<td>brlA23</td>
<td>GAG to TAG</td>
<td>Gin 317</td>
<td>Gin to TAA</td>
<td>Griffith et al. (1999)</td>
</tr>
<tr>
<td>brlA17</td>
<td>GAA to TAA</td>
<td>Glu 118</td>
<td>Glu to TAA</td>
<td>Griffith et al. (1999)</td>
</tr>
<tr>
<td>brlA19</td>
<td>TAC to TAA</td>
<td>Tyr 395</td>
<td>Tyr to TAA</td>
<td>Griffith et al. (1999)</td>
</tr>
<tr>
<td>brlA4</td>
<td>CAA to TAA</td>
<td>Gin 334</td>
<td>Gin to TAA</td>
<td>Griffith et al. (1999)</td>
</tr>
</tbody>
</table>

a alX4 is the gene for allantoinase, AN4603, C1218T.
b alcR125b codes for the transcription factor AN8978, G506A to stop. Although all the mutants listed have chain termination mutations within the coding region, not all the mutants have been shown to be suppressible (see text).
Figure 1 Generation of recombinant strains expressing GFP-tagged versions of SuaA. The cartoon (A) denotes the eRF1 locus (AN8853 on chromosome 1) indicating the position of the introns. The arrows show the positions of the single base changes for suaA105 (in the splice site for the upstream ORF) and suaA23 (in the coding region of the third exon). The cartoon below shows the DNA cassettes generated by fusion PCR used in transformation (constructs were either wild-type or contained the suaA23 or suaA105 mutation) together with the primer pairs that were used (green arrows). The cartoons in (B) show the strains transformed and the resultant status of the gene after recombination into the positions of the single base changes for suaA105 (in the splice site for the upstream ORF) and suaA23 (in the coding region of the third exon). The arrows show the positions of the single base changes for suaA105 (in the splice site for the upstream ORF) and suaA23 (in the coding region of the third exon).

Table 2 Sequence changes in suppressors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Product</th>
<th>Nucleotide Change</th>
<th>Gene Product Alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>suaB111a</td>
<td>gln tRNA AN9659</td>
<td>contig 161 (CTG to CTA)</td>
<td>Anticodon change recognizes UAG</td>
</tr>
<tr>
<td>suaD103</td>
<td>gln tRNA AN9669</td>
<td>contig 168 (CTG to CTA)</td>
<td>Anticodon change</td>
</tr>
<tr>
<td>suaD108</td>
<td>gln tRNA AN9669</td>
<td>contig 168 (CTG to CTA)</td>
<td>Anticodon change recognizes UAG</td>
</tr>
<tr>
<td>suaA105</td>
<td>eRF1 AN8853</td>
<td>−209 G to A</td>
<td>Alteration in upstream region</td>
</tr>
<tr>
<td>suaA101</td>
<td>eRF1 AN8853</td>
<td>(TCC to TAC) C104A</td>
<td>S35Y, N113K</td>
</tr>
<tr>
<td>suaA23</td>
<td>eRF1 AN8853</td>
<td>(AAC to AAG) C452G</td>
<td></td>
</tr>
<tr>
<td>suaA32</td>
<td>eRF1 AN8853</td>
<td>(AAA to AAC) A656C</td>
<td></td>
</tr>
<tr>
<td>suaA27</td>
<td>eRF1 AN8853</td>
<td>(ATT to TCT) A978T.T979C</td>
<td></td>
</tr>
<tr>
<td>suaC109</td>
<td>eRF3 AN2080</td>
<td>(TAT to AAT) T1186A</td>
<td>Y396N</td>
</tr>
<tr>
<td>suaC109</td>
<td>eRF3 AN2080</td>
<td>(TAT to AAT) T1186A</td>
<td>Y396N, N415H</td>
</tr>
<tr>
<td>suaC300</td>
<td>eRF1 AN8853</td>
<td>(AAC to CAC) A1243C</td>
<td>E117K</td>
</tr>
<tr>
<td>snpA6b</td>
<td>eRF1 AN8853</td>
<td>(GAG to AAAG) G462A</td>
<td>G265S</td>
</tr>
<tr>
<td>suaE7c</td>
<td>ANIA_O8853</td>
<td>(GGT TOAGT) G906A</td>
<td></td>
</tr>
</tbody>
</table>

aEspeso et al. (2005).

bHan et al. (2005).

cMartinez et al. (2007).

dANIA_O8853 is the prefix given to the suaE7 mutation (aspergillusgenome.org).
restore the wild-type amino acid to allantoinase (Alx), but for the other two mutated proteins there would be a substitution of glutamine for tryptophan in AlxR and glutamine for serine in AreA600, which are presumably non-functional. There are 174 tRNAs annotated (Iriarte et al. 2012), and of these seven are glutamine tRNAs. There are two glutamine tRNAs that recognize the codon CAA and five that recognize the preferred codon CAG (Lloyd and Sharp 1991; Iriarte et al. 2012). The suppressor tRNAs that have been selected independently (suaB111, suaD103, and suaD108) are all in tRNAs that recognize CAG codons.

**suaA codes for the release factor eRF1**

The suaA mutations were closely linked to each other and were shown not to recombine in large numbers of progeny, making it likely that they were all mutations within the same gene (Sealy-Lewis 1987). suaA mapped on chromosome III and was closely linked to phenA in the region of eRF1. suaA101, suaA27, suaA32, and suaA23 all showed nucleotide changes in the coding region of eRF1 (ANIA 8853 version 5) (Table 2, Figure 1) compared to the wild-type sequence and the likelihood of there being a mutation in suaA in independent isolates if this is not the gene responsible for the phenotype must be very small. To confirm that a suaA mutation is responsible for the suppression, strain H44 (pabaA1; alx4; alcR125; niaD500) was transformed with a fragment containing suaA23 and allantoin-utilizing transformants were selected. The transformants were also suppressed for the alcR125 and niaD500 mutations showing suppressed growth on ethanol medium and nitrate medium, respectively (Figure 2).

Viable gene replacements of wild-type suaA with suaA+ tagged with GFP (in strain H1888: yA2 pantoB100; riboB2; alx4; sB43; aldA67) were obtained that expressed the SuaA-GFP fusion showing that the GFP tag does not interfere with the function of eRF1 in chain termination. SuaA-GFP showed a preferential cytoplasmic localization where its activity is expected for translation termination (Figure 3A). Interestingly, nuclei were not fluorescent, suggesting the presence of a nuclear-export system acting on SuaA to ensure exclusion of the translational machinery from the nucleoplasm in this fungus.

To investigate further the suaA23 suppressor function, a construct containing the 5'UTR and suaA23 coding region fused to the gfp/riboB8 cassette was used to transform strain H1888: yA2 pantoB100

![Figure 2](image) **Figure 2** Growth testing of strains on 1% glucose minimal medium with 5 mM ammonium tartrate (left) or 0.6 mM allantoin as nitrogen source (right). The strains are control strains (only relevant genotype shown). 1. Wild-type; alx4 suaA+. 2. alx4 suaA105. 3. alx4 suaA27. 4. alx4 suaA32. 5. alx4 suaA23. Individual transformants: 4, 5, 6, 7, 8 of H44 (alx4 containing strain transformed with a fragment containing the suaA23 mutation; see Materials and Methods).

![Figure 3](image) **Figure 3** Localization and expression levels of SuaA/eRF1-GFP fusion. (A) Fluorescence images of cells of a transformant expressing SuaA-GFP fusion (H1888 transformed with the suaA::gfp construct; see Figure 1, MAD4903). (B) Western blot showing levels of SuaA-GFP fusion in total protein extracts of two transformants of suaA105::gfp (transformant 1 is MAD4904) compared with a suaA+::gfp transformant (MAD4903). Graph shows relative intensity of SuaA-GFP detection bands compared to actin levels.
TASNIKS and YxCxxxF are part of the region that recognizes the stop codons (Bertram et al. 2000; Frolova et al. 2002; Bulygin et al. 2011). Domain 2 contains a conserved GGQ motif that plays a role in the interaction of eRF1 with the peptidyl transferase center in the ribosome and triggers peptidyl transferase center (Frolova et al. 1999; Seet-Nebi et al. 2001) and is involved in stimulation of GTPase activity in eRF3 (Cheng et al. 2009). The C-terminal domain 3 involves in binding eRF3, which has a ribosome-dependent GTPase activity: the hydrolysis of GTP is necessary for a conformational change in the eRF1 and eRF3 complex that allows for the efficient release of the polypeptide (Alkalaeva et al. 2006; Fan-Minogue et al. 2008). Recent studies of S. cerevisiae suggest that the conformation of eRF1 might be different for recognition of UAA/UAG and UGA, with C124 and an associated epitope being important for UGA decoding (Fan-Minogue et al. 2008; Merritt et al. 2010).

suaA23 involves a single amino acid change within the conserved YxCxxxF region ([N131I], YLCDNKF–YLCDIKF in the β-sheet domain 1), whereas suaA105 involves two amino acid changes, an S35Y change and a change in the β-sheet region of domain 1 (N113K); both these suppressor strains have very similar specificities with regard to the suppressible alleles alX4 (UAG), areA600 (UAA), and areA601 (UAA) (Sealy-Lewis 1987) (Figure 5 and Figure 6). They both suppress the null phenotype for alX4 and areA600 but not for areA601. The conserved NIKS region is involved in recognition of the first U of the codon. Recent studies using perfluorophenyl analogs of UAA and UAAA with human eRF1 have implicated the YxCxxxF motif as binding to purines in the second and third positions as well as a novel site 26-AAAR-28. Position 28 is conserved as either arginine or lysine in a number of species and the positive side chains of these amino acids would interact with the negatively charged phosphates 3’ of adenosines in the third and fourth positions of the tetrapeptide (Fan-Minogue et al. 2008; Bulygin et al. 2011). Analogous studies using similar derivatives of guanines have shown that in addition to association with the YxCxxxF motif, the Thr-32 in the conserved 31-GTx-33 motif is the major target for cross-linking (Bulygin et al. 2010). Molecular modeling suggests that recognition of the UAG/ UAA and UGA in stop codons is associated with a different conformation of eRF1 (Fan-Minogue et al. 2008). suaA23 has a change within the conserved YxCxxxF region and can suppress the UAG mutations within alX4 and areA600 but is still able to terminate at the UAA mutation. An interpretation of these results is that the change in the YxCxxxF motif is able to discriminate between the UAG and UAA codon. In the case of suaA101, where there are two changes in the protein, it is not possible to deduce whether both of the changes are necessary for the phenotype, but the change S35Y produces a change in the conserved residue that was implicated as being important in human eRF1 for binding guanines (31-GTx-33 human numbering) and 33- GTS-35 in A. nidulans (Kryuchkova et al. 2013). The S35 residue has been changed and the suaA101 strain is unable to terminate at the UAG codons within alX4 and areA600, but it is able to terminate at the UAA codon. The alterations in eRF1 that lead to suppression reduce the efficiency of termination, but the protein must retain some function because there is only one copy of the termination factors eRF1 and eRF3 in A. nidulans, and termination must still be able to occur at normal stop codons. The context of the stop codon is very important. Where stop codons occur through mutation within a coding sequence that are not in a preferred context, these mutations will be more subject to read-through by the altered release factors. Suppression at these codons is dependent on the natural suppressor activity of normal tRNAs; which tRNA will be inserted is presumably a competition between these naturally occurring tRNAs and the release factors (Beier and Grimm 2001).

suaA32 has a change in domain 2 (K181N) in the region of the GGQ domain that plays a role in the interaction of eRF1 with the peptidyl transferase center (Frolova et al. 2000) and is also close to the region implicated in the stimulation of the GTPase activity of eRF3 (Cheng et al. 2009). suaA27 has a change in domain 3 in the β-sheet (I289S) (Figure 5 and Figure 6). In contrast to suaA101-containing and suaA23-containing strains, suaA32 and suaA27 suppress alX4, areA600, and areA601 mutations, thus having specificity for both UAG and UAA mutations. The suaA32 and suaA27 mutations are less likely to affect the discrimination between the three stop codons but would be more likely to generally reduce the efficiency of chain termination, and so they are able to suppress both the UAG and UAA mutations. The mutation in suaA27 is in a conserved amino acid very close to the start of domain 3 that interacts with eRF3. The hydrophobic amino acids in Schizosaccharomyces pombe eRF1: Phe288, Ile291, Tyr298, Phe300, and Phe405 have been implicated in eRF3 interaction (amino acids within A. nidulans -5). From work in S. cerevisiae, it has been found that the structural changes within eRF1 can result in alterations in the level of eRF1, but the structural change itself can alter the read-through observed (Hatin et al. 2009; Merritt et al. 2010). We cannot distinguish between these alternatives in this study. It is evident, however, that mutations within eRF1 can lead to suppression of both UAG and UAA mutations. suaA23, suaA105, suaA101, and suaA32 suppressor strains were crossed to the UGA containing aldA67 mutant strain (Table 1) and there was no suppression of the aldA mutant phenotype at 37°C, but at 25°C some suppression was observed in the suaA23 aldA67 (but not suaA101 aldA67), suaA32 aldA67, and suaA105 aldA67 double mutant, showing that eRF1 acts on UGA as well as UAG and UAA mutations.
There were two previous publications in which suppressor mutations in eRF1 were described in *A. nidulans*. In the first article, Han et al. (2005) described a mutation in the pantothenyl transferase, *ngA1*, that resulted in a lack of pigmentation in the conidia and the hyphae that could be suppressed by a conditional mutation in a second gene, *snpA6*, at 37° and 42°, but not at 25°. The *snpA6* suppressor was shown to involve an E117K mutation in domain 1 eRF1 (Figure 5) of a UGA stop codon in *ngA1*. Martinez et al. (2007) described a suppressor, *supE7*, of two UAG mutations in the acetyl carnitine transferase carrier protein (*acuH13* and *acuH31* but not *acuH20*) that involved a change in domain 2 of eRF1 (G265S). It was proposed that this amino acid change might affect the conformational properties of the hinge region between domains 2 and 3, interfering with ribosome binding or peptidyl transferase activity (Figure 5). *supE7* also suppressed UAA and UGA mutations in pal− strains (Martinez et al. 2007). The *supE7* suppressor mapped on chromosome III and had very similar properties to the *suaA* and *suaC* suppressors, but it was found by a cross with *suaA101* to be unlinked to *suaA101*. Because the four *suaA* mutations have sequence changes in eRF1, all these mutations are in eRF1 like *supE7* (it seems likely that the cross data were incorrect but it has not been possible to repeat this cross because the *supE7* strain is no longer viable) and the *suaA* mutations can suppress all three classes of chain termination codons. We propose that *suaA* and *snpA6* are reclassified as *suaA* mutations, because the *suaA* mutations were the first to be described and *suaA* is located on the genetic map. The only alteration that was found in the *suaA105* strain was in the region upstream of the coding region of the genes at −209 with respect to the start codon. This coincides with an alteration in the 5’ slice site, GT to AT, in the upstream ORF. We predicted that this would lead to a failure of splicing of the intron in the mutant that could affect the initiation of translation leading to lower levels of expression of eRF1. In RT-PCR of the wild-type and mutant RNA with a forward primer that split the first intron and a reverse primer that was after the second intron, only a PCR product was seen for the wild-type amplification, and sequencing of this product confirmed the position of the three introns as annotated. A PCR amplification where the forward primer was within the first intron and the reverse primer split the second intron in the coding sequence produced a product for the *suaA105* strain, but not for the wild-type strain, and sequencing of the PCR product confirmed the presence of an unspliced 5’ intron but correct splicing of the first two introns in the coding sequence (data not shown). Epitope (-GFP) tagging of the wild-type and mutant proteins resulted in reduced expression in the *suaA105* strain (Figure 3B). As described for other constructs, a genomic fragment containing *suaA105* mutation was fused to the gfp/riboB<sup>+</sup> cassette and was used to transform strain H1884 (Table S1) to prototrophy on riboflavin medium. A feasible explanation for the effect of altered mRNA processing due to *suaA105* mutation is at the protein levels, because a 20% reduction is observed in the mutant background compared to wild-type. Lower levels of expression of eRF1 could lead to suppression by the natural tRNAs that are able to translate chain termination codons as sense, because they would be able to compete more efficiently for translation of the termination codons. However, the *suaA105::gfp* transformants did not show suppression of *algX* (Figure 4), suggesting an interference of GFP in the functionality of this low-expressed but wild-type SuaA tagged protein.

**suaC codes for eRF3**

The *suaC109* suppressor–containing strain is cold-sensitive (fails to grow at 25°). *suaC* maps on linkage group VII, shows linkage to *choA* (data not shown), and has a similar broad spectrum of suppression to the *suaA* suppressor strains. *suaC109* suppresses the UAG mutations in *algX*, *alcB125*, and *areC600*, as well as the UAA mutation in *areA601* (Sealy-Lewis 1987) and *brlA17*, but not the UAA mutations
in brlA19 or brlA4 or the UAG mutation in brlA24 (Griffith et al. 1999). suaC109 strains were crossed with UGA containing pal strains palC143, palF15, and palB7, and palB513 (mutations that affect pH regulation) in an attempt to establish whether suaC109 could suppress UGA mutations, but the crosses were infertile. The location of suaC on the genetic map was in the region of eRF3, and so DNA extracted from the suaC109 strain was sequenced and a single change T1186A (Y396N) was found in the sequence predicted to encode eRF3, AN2080. The sequence as annotated has one intron and encodes a protein of 708 amino acids. The eRF3 comprises two domains: the N and M regions (amino acids 1–253 in S. cerevisiae) and the C region (amino acids 254–685) that contain a GTPase fold (amino acids 254–479) and also interact with eRF1 (Kong et al. 2004). Crystallography studies between the eRF1 from H. sapiens and S. pombe and the C-terminus of the eRF3 from S. pombe, which lacks the GTPase domain, has shown that there is an interaction between the C-terminus of eRF3 and the C-terminal domain, but there is additional evidence from small-angle X-ray scattering analysis that an interaction between domain 2 (R192 eRF1 H. sapiens) of eRF1 is required for stimulation of the GTP-binding and hydrolysis activities of eRF3 (Cheng et al. 2009).

There is considerable variability between eRF3s in the N and M domains in different eukaryotes, but the C-terminal domain is highly conserved. The A. nidulans eRF3 shows no similarity with the ERF3 from H. sapiens in the first 269 amino acids, but thereafter it has 53% identity. S. cerevisiae has no identity over the first 53 amino acids but has 48% identity thereafter, and S. pombe shows similarity over 99% of the protein with 49% identity. In S. cerevisiae, the N-terminal portion of the protein has been shown to be important for the cytoplasmically inherited prion determinant (PSI+), which when aggregated impairs termination and acts as a suppressor of nonsense mutations (Ter-Avanesyan et al. 1994). The N-terminal region contains four tandem repeats of the sequence, PQGGYQQYN, similar to mammalian prion repeats (Stansfeld et al. 1998).
Tuite 1994; Lindquist 1997). This sequence is missing from the sequence in S. pombe and A. nidulans, but S. pombe contains a region that is rich in repeats of APST (Ito et al. 1998) that, again, is not a feature of the A. nidulans sequence. Examination of the A. nidulans eRF3 for repeated elements using REPRO (http://www.ibi.vu.nl/programs/reprowww/) did not reveal any obvious repeats, but there was a preponderance of Glu, Ala, and Tyr amino acids between amino acids 53 to 156 in the N-terminus that would produce a very

**Figure 7** Multiple alignment of ERF3. Alignments using ClustalW are shown for A. nidulans XP_659684.1 (locus tag AN2080), S. cerevisiae AFD29160.1, S. pombe NP_588225, and H. sapiens ERF3A NP-002085.2. There are two genes in mammals that code for eRF3, eRF3A, and eRF3B, and they differ in their N-termini. We have used eRF3A for the alignment because the expression levels of this gene have been shown to control the formation of the termination complex (Chauvin et al. 2005). The explanation of conservations underneath the alignment is explained in Figure 6. Amino acid changes in the suppressor strains are indicated above the alignment with the amino acid that has changed shaded. There are repeated sequences reported for both S. cerevisiae and S. pombe, and these are underlined in the respective sequences (see text for references).
hydrophilic flexible region. In the meiotic analysis of many nonsense suppressor mutations in *A. nidulans*, there has been never any evidence of cytoplasmic inheritance as exhibited by the PSI² strain in *S. cerevisiae* and, therefore, no evidence of prion formation (H. M. Sealy-Lewis, unpublished data).

The alteration in eRF3 in *suaC109* was in the C-terminus and has been located using the crystal structure of *S. pombe* for modeling (Table 2, Figure 7 and Figure 8). We also sequenced a mutant strain that was selected as a revertant of the cold-sensitive phenotype of *suaC109* (Figure 7 and Figure 8). We also sequenced a mutant strain that was selected as a revertant of the cold-sensitive phenotype of *suaC109*. Figure 7 and Figure 8). We also sequenced a mutant strain that was selected as a revertant of the cold-sensitive phenotype of *suaC109*. Figure 7 and Figure 8). We also sequenced a mutant strain that was selected as a revertant of the cold-sensitive phenotype of *suaC109*.

The revertant of the *suaC109* phenotype has been renamed *suaC500*. Both changes are thus in the C-terminus (Table 2, Figure 7 and Figure 8). The revertant still had suppressor activity but was cold-insensitive. The cold sensitivity was thus a property of the altered eRF3 and not a consequence of a read-through product in an unrelated gene. Both *suaA* and *suaC* mutations result in a number of pleiotropic changes, which could result from read-through of proteins; in addition, it has been suggested for both eRF1 and eRF3 that they might have a translation-independent role, because eRF1 and the myosin-light chain have been shown to interact in *S. cerevisiae* and mutations in eRF1 can suppress defects in cytokinesis (Valouev et al. 2004). The N-terminal region of eRF3 in *S. cerevisiae* has also been shown to interact with other proteins such as Ltt1p (a protein of unknown function) and Slap1, which is involved in cytokoskeletal assembly; this suggests a translation-independent role for eRF3 (Bailleul et al. 1999; Urakov et al. 2001).

The reason for the cold sensitivity in the *suaC109* strain is unknown, but it could involve a failure to assemble with eRF1, GTP, or the ribosome at lower temperatures, as has been described for ribosome assembly in *E. coli* (Guthrie et al. 1969). This could be reversed by further changes within the eRF3 molecule. Also, in *S. cerevisiae* it has been shown that the GTPase domain of eRF3 interacts with Upf1p, which is involved in nonsense-mediated decay, and any changes that interfere with their interaction could also lead to nonsense suppression (Amrani et al. 2006; Ivanov et al. 2008). Genome-wide interaction studies have identified a large number of interactions between eRF3 and eRF1 and other proteins in *S. cerevisiae* (von der Haar and Tuitt 2007), and there may be other proteins that interact with the GTPase domain of eRF3.

**CONCLUSION**

The suppressor mutations in eRF1 are found in all three domains of the protein but in eRF3 they are confined to the conserved C-terminal domain, which is similar to the findings in *S. cerevisiae* (Merritt et al. 2010). The *suaA* and *suaC* suppressor strains were characterized by the fact that each mutant has a distinct but unique phenotype, and it is clear that the diverse interactions of eRF1 and eRF3 can lead to these properties. With regard to the *suaA* suppressors, they can result in temperature-sensitive phenotypes for some suppressed proteins but not others [e.g., *suaA27* and *areA600* or *suaA101* and *amdSl005* result in a phenotype where growth is stronger at 25°C than 37°C (Sealy-Lewis 1987)]. This suggests that the same amino acid is not inserted at the stop codon for the different suppressor alleles and implies that the different eRF3 mutants compete differently with the natural suppressor tRNAs in the cell. The molecular details of how the release factors interact both with each other and with other factors on the ribosome should yield further insights into the process.

**ACKNOWLEDGMENTS**

We thank Nadia Brooker, Wen Chen, and Sasha Demetriou, who contributed to this work as part of undergraduate projects, and Professor Herb Arst for the provision of the *pol4* strains. E.A.E. thanks the Spanish Ministerio de Economia y Competitividad for support through grant BFU2012-33142. L.M. holds an FPI fellowship associated with grants BFU2009-08701 and to BFU2012-33142.

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