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Cloning of opal suppressor tRNA genes of a filamentous fungus reveals two $\mathrm{tRNA}^{\mathrm{Ser}}_{\mathrm{UGA}}$ genes with unexpected structural differences

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The informational suppressors *su4-1* and *su8-1* of *Podospora anserina* were isolated by transformation of *Schizosaccharomyces pombe* UGA mutants. The DNA sequence revealed that they were opal (UGA) suppressor tRNAs. Wild-type alleles were also isolated by hybridization. The DNA sequence showed that they both encode species of $\mathrm{tRNA}^{\mathrm{Ser}}_{\mathrm{UGA}}$. The gene *SU8* has an 18-bp intervening sequence and its primary sequence is very different from that of *SU4*.

Key words: tRNA/opal suppressors/intervening sequence/*Podospora anserina*

Introduction

The filamentous fungus *Podospora anserina* has been used to study the phenomenon of senescence, the fidelity of translation and the ribosome structure-function relationships. An auxotrophic mutant strain (*leu1-1*) has previously been used to recover informational suppressors (Picard-Bennoun *et al.*, 1983). Some of them display the properties expected for tRNA-like nonsense suppressors. They are dominant suppressors which act on specific alleles of many genes. They lie in six loci scattered in the genetic map: *SU4*, *SU8*, *SU10* (Picard-Bennoun *et al.*, 1983), *SU9*, *SU13*, *SU14* (unpublished data). They show a pattern of suppression which indicates that they suppress the same stop codon with various efficiencies. The *su4-1-su8-1* double mutant strain is lethal. This raised the possibility that $su4^+$ and $su8^+$ encode isoaccepting tRNAs recognizing the same codon, and that mutation to suppressor forms depletes the cells of a specific active tRNA. However, these suppressors differ: *su4-1* is a more efficient suppressor than *su8-1* and it blocks sporulation while *su8-1* does not (Picard-Bennoun *et al.*, 1983). The origin of this phenotypical difference was investigated by further genetic analysis of *su4-1* and *su8-1*.

In this paper we present a molecular analysis of the *SU4* and *SU8* genes. The mutant alleles *su4-1*, *su8-1* and their wild-type counterparts were cloned and sequenced. The mutations *su4-1* and *su8-1* act as opal (UGA) suppressors. Both $su4^+$ and $su8^+$ genes encode $\mathrm{tRNA}^{\mathrm{Ser}}_{\mathrm{UGA}}$. Unexpectedly, they have different primary sequences. The *SU8* gene contains an intron. These data are discussed in the light of our present knowledge of eukaryotic tRNA genes.

Results

Selection of the su4-1 and su8-1 opal suppressors in Schizosaccharomyces pombe

The nature of the nonsense mutation suppressed by *su4-1* and *su8-1* was unknown. The opal suppressor hypothesis was first tested as follows. Genomic libraries were prepared from *su4-1* and *su8-1* DNA by ligating *Hin*dIII total digests into the plasmid pHCG31 which carries the *LEU2* gene of *Saccharomyces cerevisiae* and an *ARS* of *S. pombe*. These libraries were used to transform a *S. pombe* strain carrying UGA mutations in the *ADE6* and *GLU1* genes and a non-suppressible mutation in *LEU1* (the *S. pombe LEU1* gene can be complemented by the *S. cerevisiae LEU2* gene). With the *su4-1* library, 26 [Ade^+, Glu^+, Leu^+] transformants were obtained among 20 000 [Leu^+]. With the *su8-1* library, 24 [Ade^+, Glu^+, Leu^+] were obtained among 170 000 [Leu^+] transformants. From these [Ade^+, Glu^+, Leu^+] transformants, recombinant plasmids able to complement the three deficiencies were isolated.

Subcloning and sequence analysis of su4-1 and su8-1

To localize more precisely the active sequence in the inserts of recombinant plasmids, a second step of cloning was made: the *Sau*3A total digest DNA of the inserts ligated into another plasmid (H_2), also able to replicate in *S. pombe*, was used in a transformation of the mutant *S. pombe* strain. For *su8-1* the active fragment was 550 bp in length and its sequence revealed the presence of a tRNA gene. For *su4-1* this *Sau*3A fragment was 1400 bp in length and its partial sequence revealed the presence of a tRNA gene near the *Pvu*II site. The restriction maps of the *Hin*dIII inserts, the localization of the *Sau*3A active fragments and the sequencing strategy are shown in Figure 1. The part of the sequences encoding tRNA are shown in Figure 2A. Each tRNA would read the codon UGA establishing their identity as the opal suppressor genes *su4-1* and *su8-1*. The *SU8* gene contains an 18-bp intron.

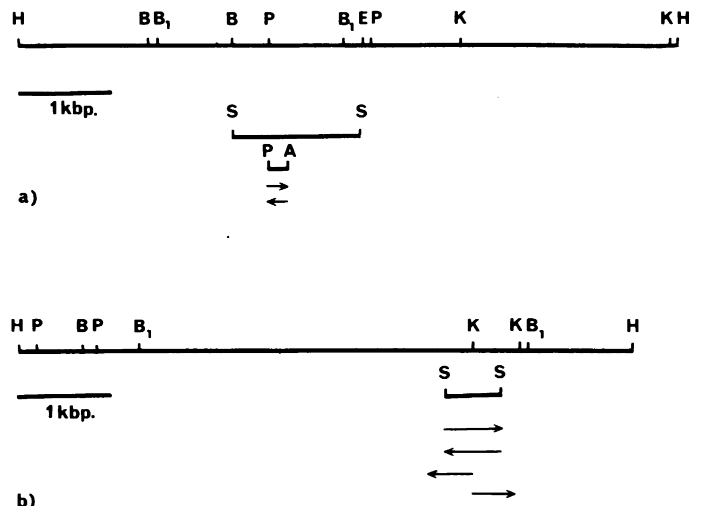

Fig. 1. Restriction maps and the strategy for determining the nucleotide sequence. **(a)** Restriction map of the insert of BH4-21-1. Subcloning localized the suppressor gene *su4-1* in the 200-bp *Pvu*II-*Alu*I fragment indicated under the restriction map. This fragment was sequenced on each strand. **(b)** Restriction map of the insert of BH8-21-1. Subcloning localized the suppressor gene *su8-1* in the 202-bp *Sau*3AI-*Kpn*I fragment indicated under the restriction map. This fragment was sequenced on each strand. Restriction enzyme abbreviations are as follows: B: *Bam*HI, Bl: *Bal*I, E: *Eco*RI, H: *Hin*dIII, K: *Kpn*I, P: *Pvu*II.

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SU8-1 5' TTCTTCTTTCCAAATCAACCAACAGTTAGCATCG
SU4-1 5' ACAACTTTCCACAATTTCCATAATAACAAAAGAA

RRYNNARYGG* GTTCRANNC*

GTCAGCATGGCAGAGTGGTCTAATGCGTTAGACTTCAATATCCATTACATTCAGGTATCTAATTCCTTCGGGAGCGTAGGTTCGAACCCTACTGCTGACG
GGCGCGATGGCAGAGTGGTCTAATGCGTGAGACTTCAA ATCTCATTTCTTCGGAAGCGTCGGTTCAAATCCGGCTCGCGTCG

ATAATCTTTTGCATTTTTCCACACCTACCCATCTC
TTTTTTTGGTATTCTGGGAATTGACAACTCCAATC

Fig. 2. DNA sequence of *su4-1* and *su8-1* genes and flanking regions. Non-coding strands are presented. The underlined portions indicate the extent of the tRNA sequences. *SU8* contains an intervening sequence of 18-bp. The only difference between each suppressor gene and its wild-type allele was a C-G transversion in the anticodon sequence. *: Consensus sequence for the internal control region (R=purine, Y=pyrimidine).

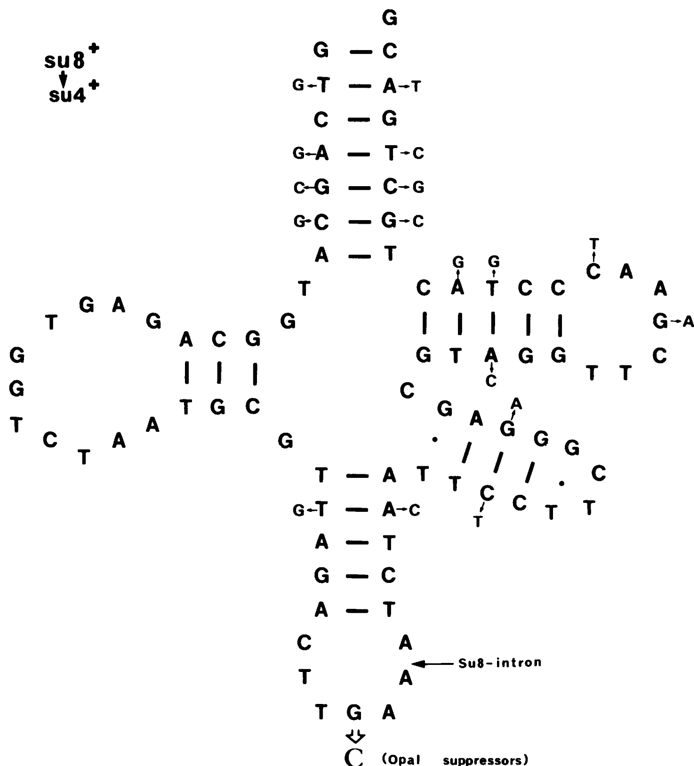

Fig. 3. *su4* and *su8* genes in the cloverleaf conformation. The arrows indicate the variations between *su8* and *su4*.

Each of the suppressor genes shares general features with all eukaryotic elongator tRNA genes: (i) internal control regions follow the consensus sequences (Figure 2) (Traboni *et al.*, 1982); (ii) the coding sequence is followed by a run of Ts in the non-coding strand that can serve as a transcription terminator signal; (iii) *SU8* contains an intervening sequence which may be located one nucleotide away from the 3′ end of the anticodon. As shown in Figure 3, *SU4* and *SU8* may be drawn as potential tRNA structures which are in agreement with general rules for eukaryotic elongator tRNAs (Grosjean *et al.*, 1982). The length of the extra arm is consistent with genes encoding tRNA^{Ser} or tRNA^{Leu}, since only leucine and serine tRNAs have been reported to have V-loops larger than five nucleotides (Sprinzl and Gauss, 1984).

Cloning and DNA sequences of su4⁺ and su8⁺

Using the *su4-1 Hin*dIII-*Hin*dIII insert as a probe, a clone of the $su4^+$ allele was isolated in the *su8-1* library. Similarly, a clone of the $su8^+$ allele was isolated in the *su4-1* library with the *su8-1 Hin*dIII-*Hin*dIII insert as a probe. These alleles were also sequenced. The results confirmed those obtained with *su4-1* and *su8-1*, except that both anticodons were found to be UGA, establishing that both wild-type genes specified a $\mathrm{tRNA}^{Ser}_{UGA}$.

Table I. Number of differences between the coding sequences of *P. anserina*, *S. cerevisiae* and *S. pombe* $\mathrm{tRNA}^{Ser}_{UGA}$ genes

	P. anserina *SU8**	*S. cerevisiae* *SUQ5*	*S. pombe* *SUP3**
su4	17	24	23
*su8**	–	26	19
SUQ5	–	–	12

The absence of a nucleotide is counted as a difference. Intron sequences are not included in the comparison.
*: tRNA which contains an intron.

Discussion

The genetic analysis of the mutations *su4-1* and *su8-1* in *P. anserina* has shown that they have the usual characteristics of tRNA-like nonsense suppressors (Picard-Bennoun *et al.*, 1983). These mutations exhibit the same pattern of suppression but the level of suppression of *su4-1* is greater than that of *su8-1* (unpublished results), and the *su4-1* strains are defective in the process of sporulation. The lethality of the double mutant *su4-1-su8-1* suggests that such a strain is depleted of a specific active tRNA and no other tRNA is able to decode the codon usually read by the product of *SU4* and *SU8*. The *su4-1* and *su8-1* suppressors and the wild-type alleles were isolated and sequenced. The two suppressor genes encode tRNAs reading the opal codon. The wild-type genes encode the $\mathrm{tRNA}^{Ser}_{UGA}$. The gene *SU8* has an 18-bp intervening sequence and its primary sequence is very different from that of *SU4*.

The functional differences between *su4-1* and *su8-1* may be related to the structural differences between *SU4* and *SU8*. The pleiotropic effects of *su4-1* may be correlated either to a higher expression of the gene *SU4*, or to the wobble possibilities of the *su4-1* or *su8-1* tRNA which theoretically could read *Opal* (UGA) as well as *Try* (UGG) codons. In the first hypothesis the levels of the two mature tRNA are different either because of the transcription rate or because the processing of the pre-tRNA is a limiting or regulated step. In the second hypothesis, we propose that the U at position 34 is modified (U*) in *su8-1* tRNA to prevent wobble, as was demonstrated in yeast for the $\mathrm{tRNA}^{Glu}_{U*UC}$ (Sekiya *et al.*, 1969; Kobayashi *et al.*, 1974), whereas *su4-1* tRNA has no such nucleotide modification restricting the wobble possibilities and inserts the serine amino acid at the *Opal* and *Try* codons. The latter substitution may be harmful to a particular process

like sporulation. The presence of an intervening sequence within *SU8* pre-tRNA might account for this difference in modification (Johnson and Abelson, 1983). A similar conclusion has been reached concerning *sup10* and its isoacceptors in *S. pombe* (Sumner-Smith *et al.*, 1984).

As *su4*$^+$ and *su8*$^+$ encode isocoder tRNAs (recognizing the same codon) their sequence differences are somewhat unusual. The number of differences between the primary sequence of *SU4* and *SU8* is similar to the number of differences between two $tRNA^{Ser}$ belonging to different species (Table I), whereas as a rule such isoacceptors are very similar within a species. In *S. cerevisiae*, the three $tRNA^{Ser}_{AGA}$ genes sequenced out of 11 are identical (Page and Hall, 1981), as are the three $tRNA^{Ser}_{UGA}$ (Broach *et al.*, 1981). In *S. pombe*, the sequence of the two $tRNA^{Ser}_{UGA}$ genes are similar except at the point corresponding to the tip of the extra arm (Amstutz *et al.*, 1985); two $tRNA^{Arg}_{ACG}$ genes differ only by one nucleotide in position 37 (Gamulin *et al.*, 1983). The first case of low homology of isocoders in eukaryotes was reported recently for a $tRNA^{Gly}_{CCC}$ gene (Shortridge *et al.*, 1985) which has only 69% homology with a human placenta $tRNA^{Gly}_{CCC}$ (Gupta *et al.*, 1980). In *S. pombe*, *sup-8* and *sup-10*, which have significantly different primary structures, as proved by hybridization, have been proposed to be isoacceptors decoding UUA (Leu) (Sumner-Smith *et al.*, 1984). This might be a situation reminiscent of *SU4* and *SU8* in *P. anserina*.

Intergenic conversion has been postulated as a mechanism for maintaining homology amongst members of a multigenic family (Klein and Petes, 1981), and indeed interchromosomal gene conversion of tRNA loci has been demonstrated in *S. pombe* (Munz *et al.*, 1982; Amstutz *et al.*, 1985). Thus, it is possible that the acquisition of an intron by one member of the ancestral family of $tRNA^{Ser}_{UGA}$ genes (Cedergren *et al.*, 1981) would have released this gene from the correction mechanism of gene conversion and allowed a relatively high degree of heterology to be maintained. In *P. anserina* the coding sequence differences observed between *su4-1* and *su8-1* may be correlated with the presence of an intervening sequence within *SU8*. Extending this hypothesis, our supposition is that a major difference between *sup-8* and *sup-10* of *S. pombe*, if both have the anticodon UAA, would be the absence of an intron in *sup-10*.

The lethality of the *su4-1-su8-1* double mutant strain suggests that this strain is depleted of the $tRNA^{Ser}_{UGA}$ and that no other tRNA is able to decode the UCA codon. However, more than two such tRNA genes might exist. In particular, we cannot exclude a poorly expressed member of the $tRNA^{Ser}_{UGA}$ gene family (as might be *su9*, *su10*, *su13* or *su14*) which would be unable to alleviate the lethality of the *su4-1-su8-1* double mutant. If *P. anserina*, like *S. cerevisiae* and *S. pombe* (Sprinzl and Gauss, 1984), contains a major $tRNA^{Ser}$ species with the modified anticodon IGA which is theoretically capable of translating the codons UCU, UCC and UCA, we can conclude that this major $tRNA^{Ser}$ does not translate the codon UCA. This restriction of the original wobble hypothesis (Crick, 1966) was already inferred in *S. pombe* (Munz *et al.*, 1981) and *S. cerevisiae* (Guthrie and Abelson, 1982).

Finally, the cloned tRNA suppressors were used in DNA transfection experiments with *P. anserina* (Brygoo and Debuchy, 1985).

Materials and methods

Bacterial strains, cloning vehicles, media and general methods

Escherichia coli strain BJ5183 was used for the construction of libraries and the recovery of plasmids. The plasmids pHCG31, H_2, pBR328 and recombinant plasmids were grown in *E. coli* strain HB101. *E. coli* was grown in L broth. General methods for transformation, growth of plasmids and recovery of DNA were as described (Maniatis *et al.*, 1982).

S. pombe strain, cloning vehicles, media and general methods

The genotype of the *S. pombe* strain PT611 was *ade6-704* (UGA), *glu1-57* (UGA), *leu1-32*, h$^+$. The vector used was pHCG31 carrying an autonomously replicating sequence of *S. pombe*, the gene *LEU2* of *S. cerevisiae* and a part of pBR322 containing the replication origin and the β-lactamase gene. Alternatively, the vector H_2 was used, carrying a mitochondrial autonomously replicating sequence of *P. anserina*, the gene *LEU2* of *S. cerevisiae* and a part of pBR328 including the replication origin, the β-lactamase and chloramphenicol acetyl transferase genes. *S. pombe* was grown in the minimal medium YNB (yeast nitrogen base without amino acid and ammonium sulfate, Difco) supplemented as required with adenine (75 mg/l), leucine (50 mg/l) and glutamic acid (4.1 g/l) or ammonium sulfate as specified by the manufacturer. The transformation procedure was based on that described previously (Beach *et al.*, 1982) and is identical until the first wash with 20 mM sodium citrate phosphate pH 5.6. The cells were resuspended in 20 mM sodium citrate phosphate pH 5.6, 40 mM EDTA and incubated for 30 min at 30°C. After centrifugation, the cells were washed with 1.2 M sorbitol, 50 mM sodium citrate phosphate pH 5.6 and, after recentrifugation, resuspended in 5 ml of the same buffer. 100 μl of a 5 mg/ml zymolyase 60 000 (Seikogaku Kogyo Co. Ltd.) solution in 50% glycerol were added to the suspension and incubated for 30 min at 37°C. Then 250 μl of cytohelicase (Industrie Biologique Française) were added and the cells incubated until the optical density of an aliquot of the suspension in water was half of the O.D. of the same dilution in the buffered sorbitol. Cells were then centrifuged, washed in 1.2 M sorbitol, 50 mM sodium citrate phosphate pH 5.6 and resuspended in 1.2 M sorbitol, 50 mM sodium citrate phosphate pH 5.6, 5 mg/ml yeast extract, 5 mg/ml glucose. After an incubation of 1 h at 30°C, the cells were washed three times as described (Beach *et al.*, 1982) and transformation continued in the same way, except that the final incubation before plating was omitted. Recovery of plasmids in *E. coli* was as described (Beach *et al.*, 1982).

P. anserina strains, nomenclature, media and general methods

For the *P. anserina* strains the typography rules are as follows: the allelic forms of a gene are scripted in small letters and the generic name of the gene is scripted in capital letters. The mutant strains used in these studies were *su4-1* and *su8-1* (Picard-Bennoun *et al.*, 1983). The solid and liquid media for growth of *P. anserina* were as described (Rizet and Engelman, 1949; Esser, 1974). For total DNA preparation, mycelium was grown in M1 supplemented with 2.5 mg/ml yeast extract for 3 days at 28°C. The mycelium from five Roux flasks was harvested, lyophilized, ground and then suspended in 20 ml, 50 mM Tris-HCl, pH 9, 50 mM EDTA, 2% sarcosyl. The suspension was mixed with 14 ml of phenol pH 9:chloroform: isoamyl alcohol (10:10:0.4), incubated at room temperature for 10 min and centrifuged for 15 min at 10 000 *g* in glass tubes. The preceding step was repeated with the supernatant and 6 ml of the organic mixture. After a second centrifugation the supernatant was dialyzed in 10 mM Tris-HCl pH 7.5, 1 mM EDTA and then 1.26 g CsCl added per ml. Ultracentrifugation is performed for 60 h at 70 000 *g*. The viscous fractions containing DNA were recovered.

Miscellaneous procedures

Restriction and DNA modification enzymes were used as specified by the manufacturers. DNA sequence analysis was by the dideoxy method (Sanger *et al.*, 1977). General molecular methods were all as previously described (Maniatis *et al.*, 1982).

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