

Demonstration that the *Neurospora crassa* mutation *un-4* is a single nucleotide change in the *tim16* gene encoding a subunit of the the mitochondrial inner membrane translocase

Aric Wiest, Michael Plamann, and Kevin McCluskey

Fungal Genetics Stock Center, School of Biological Sciences, University of Missouri-Kansas City

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The *Neurospora crassa* temperature sensitive mutation known as *un-4* has been shown by a map-based complementation approach to be a single nucleotide change in the open reading frame of the mitochondrial inner membrane translocase subunit *tim16* (NCU05515).

Many mutations in *Neurospora crassa* are only known by a morphological or other visible phenotype. For many of these, the actual open reading frame responsible remains unknown. Among these are several temperature-sensitive lethal mutations known as *unknown* (Inoue and Ishikawa, 1970; Ishikawa and Perkins, 1983). As part of our continuing effort to define the gene defect associated with these otherwise anonymous temperature sensitive mutations, we have identified *un-4* as a missense mutation in the *tim16* (translocase of the inner mitochondrial membrane) gene. We used a complementation approach to identify an open reading frame that conferred the ability to grow at 37°C on the *un-4* strain FGSC 2172. Since Schmidhauser et al. (1999) reported that *un-4* was on a cosmid with *lys-5*, we attempted to complement the *un-4* lesion using cosmid G13:G8 from the Orbach Sachs pMOcosX library (Orbach and Sachs, 1991; Vollmer and Yanofsky, 1986). While this cosmid was reported to complement *un-4* (Schmidhauser et al., 1999), it did not do so in our hands (Table 1). Indeed, in assembly 7 of the *Neurospora* genome (Galagan et al., 2003), this cosmid maps to linkage group IV on contig 43, while *un-4* and *lys-5* are on linkage group VI. Based on the location of *lys-5* (NCU05526) on contig 22, we chose several cosmids from contig 22 and found that two did restore the ability to grow at 37°C to the *un-4* strain FGSC 2172 (Table 1). Three genes were identified as possible candidates based on the overlapping regions of complementing cosmids. These three candidate genes were amplified with PCR and tested for their ability to complement the *un-4* mutation (Table 2). This approach allowed the identification of NCU05515.3 as the likely open reading frame that is mutated in the *un-4* strain.

DNA Sample	Hygromycin resistant colonies at Room Temperature	Colonies at 37°C
pMOcosX G13G8	>350	0
pMOcosX X17C7	>100	0
pMOcosX X9D6	>100	70
pLorist6Xh ^a 108A6	>20	0
pLorist6Xh 16C3	>50	0
pLorist6Xh 36D9	50	>100
No DNA	0	0

Table 1. Identification of cosmids that complement *un-4*. All cosmids tested carry the hygromycin resistance cassette.

^a (Kelkar et al., 2001)

DNA sequence obtained directly from PCR amplified genomic DNA from strain 2172 showed a single C to T transition at position 293 of the coding sequence of NCU05515.3. This results in a serine to phenylalanine change in the polypeptide at position 98. The serine at position 98 is conserved among most fungi but in some higher eukaryotes, this position is occupied by a threonine (Figure 1). This region is part of a J-like domain and is thought to interact with *Tim14* via hydrogen bonds (Mokranjac et al., 2006).

Transforming DNA	Amplified fragment size	Presumptive Function	# colonies at 37°C	# colonies at 37°C
NCU05514.3	2327	Hypothetical	0	0
NCU05515.3	935	Mitochondrial import inner membrane (tim16)	21	25
NCU05516.3	3032	similar to Golgi membrane domain	0	0
pLorist6xh36D9	-	(transformation control)	>100	>100
No DNA			0	0

Table 2. Identification of PCR products that complement *un-4*. Data from two transformation experiments are shown.

<i>Neurospora crassa</i>	KGGSFYLSKVVRAERLE
<i>Chatomium globosum</i>	KGGSFYLSKILRARERIE
<i>Neosartorya fischeri</i>	KGGSFYLSKILRARERIE
<i>Aspergillus clavatus</i>	KGGSFYLSKILRARERIE
<i>Aspergillus fumigatus</i>	KGGSFYLSKILRARERIE
<i>Aspergillus nidulans</i>	KGGSFYLSKILRARERIE
<i>Aspergillus terreus</i>	KGGSFYLSKILRARERIE
<i>Giberella zeae</i>	KGGSFYLSKIVRAKERFE
<i>Aspergillus oryzae</i>	KGGSFYLSKILRARERIE
<i>Magnaporthe grisea</i>	KGGSFYLSKILRARERLE
<i>Coccidioides immitis</i>	KGGSFYLSKILRARERIE
<i>Phaeosphaeria nodorum</i>	KGGSFYLSKILRARERIE
<i>Ashbya gossypi</i>	KGGSFYLSKIYRAAERLK
<i>Maconellicoccus hirsutus</i>	KGGSFYLSKVVRAKERLD
<i>Caenorhabditis elegans</i>	KGGTLVLSKVVRAKERID
<i>Caenorhabditis briggsae</i>	KGGTFYLSKVYRAKERID
<i>Ostreococcus tauri</i>	RSGETFYLSKVFRARERLM

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Figure 1. Alignment of amino acid sequence from the J-like region of Tim16 among fungi and select eukaryotes. Position 98 is indicated by the carat (^) underneath. *M. hirsutus* is *Maconellicoccus hirsutus*, also known as the hibiscus mealybug. *Caenorhabditis elegans* and *C. briggsae* are both nematodes. *Ostreococcus tauri* is a unicellular green alga.

The demonstration that *un-4* defines the *tim16* gene adds value to strains carrying this mutation. The ability to study the interactions of subunits of the mitochondrial protein import motor will be enhanced by the ability to use a temperature-sensitive mutation to control the action of the motor.

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