Identification of the *fla4* Mutation in *Chlamydomonas reinhardtii*

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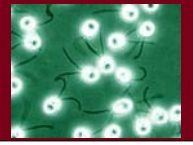
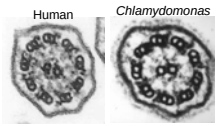


Introduction

•*Chlamydomonas reinhardtii* is a motile unicellular green alga that is commonly used as a model organism to study many aspects of biology. *C. reinhardtii* has a relatively fast generation time, and its vegetative cells are haploid. As a result, flagellar mutants can be easily identified.

•*C. reinhardtii* has 2 flagella located at the anterior end of the cell. Flagellar structure and function is highly conserved throughout many different organisms.

•The basic architecture of a eukaryotic flagellum is 9 doublet microtubules linked to a central pair of singlet microtubules by radial spokes. Motor proteins called dyneins provide the force needed to bend the flagellum. This general design is known as an axoneme, and is present in all motile flagella and cilia (Bray, 2001).



Background of *fla4*

•fla4 is a temperature sensitive mutant strain of *C. reinhardtii* that exhibits normal flagellar function at a permissive temperature of 21ºC, but sheds its flagella at a restrictive temperature of 32º C (Adams *et al.*, 1982).

• Transformation with a large insert bacterial artificial chromosome (BAC 11i21) containing several genes had previously identified a candidate *fla4* gene by phenotypic rescue (Soneral and Porter, unpublished results).

•My goals were to:

- •Identify which one of the genes on the BAC corresponded to FLA4 by subcloning a smaller rescuing insert
- •Sequence the FLA4 gene in wild-type (137C) and mutant (fla4-39B).
- •Identify the mutated nucleotide in *fla4*

Materials & Methods

•Initial sequence was taken from the Joint Genome Institute's (JGI) *Chlamydomonas reinhardtii* genome v. 3.0. When determining primers for RT-PCR, predicted exons were found by BLAST searching for the TPR Repeat genomic sequence against JGI's *Volvox carterii* genome (*V. carteri* is a very similar green alga). Regions of maximum homology were used as predicted exons, and primers were designed to span intron/exon boundaries.

•DNA and RNA isolation, cDNA synthesis, PCR and sequence analysis all followed methods outlined in previous Porter Lab project (Mueller, *et al.,* 2005).

•Sequencing of PCR products was performed by the BioMedical Genomics Center at the University of Minnesota.

•The TPR sequence was cut from BAC 11i21 using restriction enzymes XhoI (on the 5' side) and SacI (on the 3' side). It was ligated into the vector pBluescript and transformed into *E. coli*. Subclones were isolated, linearized, and used to transform fla4-39B cells.

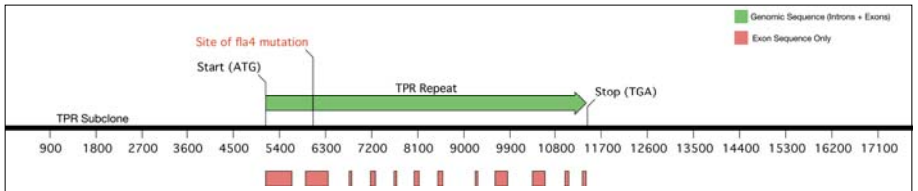


Figure 1: From the original BAC 11i21, TPR Subclone was cut using restriction enzymes XhoI (5') and SacI (3'). fla4-39B cells were transformed with this insert. The fla4 phenotype was rescued by this method. The TPR Repeat gene is labeled with a green bar. The exons were determined by RT-PCR of wild-type (137C) cDNA. Specific information about TPR Repeat's 5' and 3' UTRs have yet to be determined.

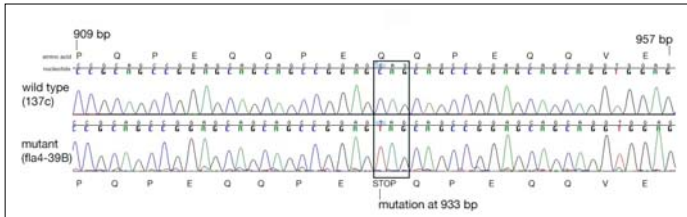


Figure 2:

Sequence results for the region of the genome containing the mutated nucleotide. Base numbers are relative to the start of the gene. In fla4-39B, a cytosine at 933 bp has been replaced by a thymine. This change results in a premature stop codon rather than the glutamine residue seen in 137c.

Figure 3: Phenotypic assay of 137C, fla4-39B, and three rescue strains after 16 hours at 21° and 32°. Flagellated strains appear green. Deflagellation at 32° occurs only in fla4-39B, as is evident by the clear media with green pellet at the bottom of the tube.

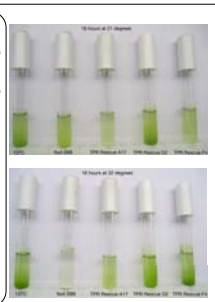
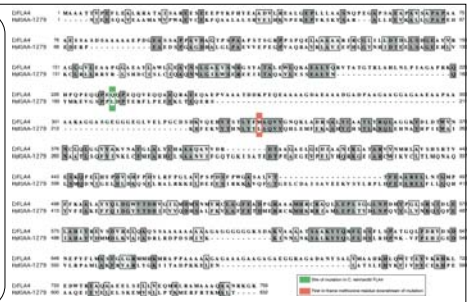


Figure 4: FLA4 peptide sequence, as determined by translation of cDNA, aligned with its human homologue, KIAA-1279. A dark box around corresponding amino acids denotes a match. The lighter box denotes chemically similar residues. Of the 760 amino acids that comprise FLA4, 150 are conserved in KIAA-1279 (19.7%).



Results

Transformation with TPR Subclone rescued fla4 phenotype

•An 18 kb insert (*Figure 1*: TPR Subclone) containing only one complete gene (*TPR Repeat*) was cloned into fla4-39B cells. Of 72 transformants, 3 showed rescue of the mutant phenotype.

•Phenotypic rescue was assayed by suspending duplicate tubes overnight in TAP media at 21°. The next day, half of the tubes were placed in a 32° water bath overnight, while the other half stayed in a 21° incubator. The next morning, flagellated cells give the media a rich green color, indicating swimming cells. Cells that have lost their flagella appear as a green pellet at the bottom of the tube (*Figure 3*).

cDNA sequence revealed valuable information about fla4 gene and protein

Gene Data:

•12 exons in the cDNA (*Figure 1)*

•cDNA sequence (just exon) is 2280 bp (69.9% G+C)

•Genomic sequence (intron and exon) is 6301 bp (67.8% G+C)

Protein Data:

•Molecular Weight: ~78.9 kDa

•Peptide sequence is 760 a.a. residues

•Estimated isoelectric point: 4.70

•Low hydrophobic character

fla4 phenotype is result of nonsense mutation in 2nd exon of TPR Repeat

•By comparing sequence data of PCR samples from wild type (137C) DNA and mutant (fla4-39B), a discrepancy was found at 933 bp, relative to the start of the gene.

•In the second exon of *fla4*, a cytosine nucleotide was replaced by a thymine *(Figure 2).*

•This results in a premature stop codon at 235 aa (of a 760 aa protein) rather than the wild-type glutamine residue.

•This sequence data was retested for accuracy.

Discussion

•Both by sequence analysis and by phenotypic rescue, the *fla4* mutation was shown to be the result of a mutation in a TPR Repeat gene.

•This nonsense mutation could result in several possible products: 1 truncated protein, 2 truncated proteins resulting from another in-frame ATG codon downstream of the mutant stop *(Figure 4*), or no protein at all (i.e. the truncated proteins are degraded or not translated at all).

•By peptide sequence alignment, *FLA4* has several regions of close homology to *KIAA1279* in *Homo sapiens*. *KIAA1279* contains two TPR repeat motifs (Brooks 2005) and is associated with microtubules in neurons (Lyons, *et al.*, 2008).

•Nonsense mutations in *KIAA1279* have been associated with disorders of the nervous system, including mental retardation, facial disfiguration, microcephaly, and Hirschsprung disease. The protein has been shown to be ubiquitous in human tissue (Brooks, *et al.*, 2005).

•In zebrafish, mutations of its *KIAA1279* homologue (*KBP*) have been shown to inhibit outgrowth of axons in nerve cells, resulting in symptoms similar to those seen human mutations (Lyons, *et al.*, 2008).

•This suggests that *FLA4* is an important component for binding microtubules.

•Further study of *FLA4* in *C. reinhardtii* could be used to investigate the role this protein plays in microtubule-binding, a conserved process in which defects may be associated with human neurological disorders.

Future Experiments

•Antibodies specific to the N-terminus and C-terminus will be made to determine which parts of the protein, if any, are being produced in the fla4 strain. These antibodies will also be used to detect expression in the wild-type strain.

•To determine the localization of FLA4, the fla4 mutant will be rescued with a *FLA4*/ GFP (Green Fluorescent Protein) construct. Immunofluorescence microscopy will then be used to detect localization.

•A smaller rescuing subclone would make the gene a bit easier to tag with GFP.

•Further sequence analysis of the 5' and 3' UTRs and the promoter region will help characterize this gene and protein.

References

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