

University of Massachusetts Medical School

eScholarship@UMMS

---

GSBS Dissertations and Theses

Graduate School of Biomedical Sciences

---

2004-01-22

## ***Chlamydomonas Reinhardtii* ODA5 Encodes an Axonemal Protein Required for Assembly of the Outer Dynein Arm and an Associated Flagellar Adenylate Kinase: A Dissertation**

Maureen Wirschell

*University of Massachusetts Medical School*

### Let us know how access to this document benefits you.

Follow this and additional works at: [https://escholarship.umassmed.edu/gsbs\\_diss](https://escholarship.umassmed.edu/gsbs_diss)



Part of the [Amino Acids, Peptides, and Proteins Commons](#), [Enzymes and Coenzymes Commons](#), and the [Genetic Phenomena Commons](#)

---

#### Repository Citation

Wirschell M. (2004). *Chlamydomonas Reinhardtii* ODA5 Encodes an Axonemal Protein Required for Assembly of the Outer Dynein Arm and an Associated Flagellar Adenylate Kinase: A Dissertation. GSBS Dissertations and Theses. <https://doi.org/10.13028/y6m6-5s14>. Retrieved from [https://escholarship.umassmed.edu/gsbs\\_diss/25](https://escholarship.umassmed.edu/gsbs_diss/25)

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMMS. For more information, please contact [Lisa.Palmer@umassmed.edu](mailto:Lisa.Palmer@umassmed.edu).

*CHLAMYDOMONAS REINHARDTII ODA5* ENCODES AN AXONEMAL PROTEIN  
REQUIRED FOR ASSEMBLY OF THE OUTER DYNEIN ARM AND AN  
ASSOCIATED FLAGELLAR ADENYLATE KINASE.

A Dissertation Presented

By

Maureen Wirschell

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

JANUARY 22, 2004

DEPARTMENT OF CELL BIOLOGY

## COPYRIGHTS

Contents from CHAPTERS II, III, IV, and V of this thesis are copyrighted in 2003 in the following paper submitted for publication in *Molecular Biology of the Cell*:

Maureen Wirschell, Gregory Pazour, Akinori Yoda, Masafumi Hirono, Ritsu Kamiya, and George Witman. (2003). Oda5p, a novel axonemal protein required for assembly of the outer dynein arm and an associated adenylate kinase. *Mol. Biol. Cell.* Submitted.

*CHLAMYDOMONAS REINHARDTII ODA5* ENCODES AN AXONEMAL PROTEIN  
REQUIRED FOR ASSEMBLY OF THE OUTER DYNEIN ARM AND AN  
ASSOCIATED FLAGELLAR ADENYLATE KINASE.

A Dissertation Presented

By

Maureen Wirschell

Approved as to style and content by:

---

Gregory J. Pazour, PhD, Chair of Committee

---

Andrea Pereira, PhD, Member of Committee

---

Dannel McCollum, PhD, Member of Committee

---

William Theurkauf, PhD, Member of Committee

---

Stephen M. King, PhD, External Member of Committee

---

George B. Witman, PhD, Dissertation Mentor

---

Anthony Carruthers, PhD, Dean of the Graduate  
School of Biomedical Sciences

Department of Cell Biology  
January 22, 2004



## ODACKNOWLEDGMENTS

I would like to first thank my advisor, George Witman, PhD, for being an outstanding mentor, scientist and colleague. He provided a work environment that was both productive and supportive. Upon joining the Witman lab, my faith and confidence in science and more importantly, myself, were restored. I have learned that there are still some scientists out there who are professional and have both personal and scientific integrity. I thank George for that.

I thank the members of the Witman lab, both past and present for their kindful instructions and general good humor! Thanks to Yuqing Hou for her scientific discussions and thoughts over the years. You are a very bright student and George is lucky to have you in his lab. To Michael Chapman, one of the newest additions to the Witman lab; thanks for your thoughts and kind words over the last several months. Thanks especially to Bethany Walker for her uplifting and positive personality. It has been a positive influence over the years. You are a “lab-mate” that I miss dearly.

Special thanks to Gregory Pazour for his constant inspiration and guidance over my thesis work and my training. You have been a wonderful advisor in addition to George, and continue to be a thoughtful and supportive advisor to your own students. I could not have picked a finer group of scientists with whom to spend my days.

Thanks are in order to *Chlamydomonas*!! My explorations into the study of outer dynein arm assembly have given me a great opportunity to meet the “*Chlamy* Community”.

My appreciation also extends to James Mobley for his helpful discussions on proteomics and two-dimensional gel electrophoresis. His time and dedication in helping me as a student went above and beyond the call of duty.

Thanks to the Beer and Pizza crew: Kathy, Hector, Angelo, Suz, Richard, Aidan, Emma, Ella and Nora. To my friend Michelle, thank you for all the support you have given me over the years. I will miss you all. Undoubtedly, I will not find better friends anywhere!

Lastly, I would like to thank my husband, Roberto, for his undying support and constant encouragement over these very trying years. You have cheered me on, even when I could not always enjoy what I was doing. I would not have been able to do this without you.

## ABSTRACT

The first type of dynein identified, axonemal dynein (Gibbons and Rowe, 1965), slides adjacent microtubules within the axoneme, generating the force necessary for ciliary and flagellar beating. The outer dynein arm is an important component of the flagellar axoneme, providing up to 60% of the force for flagellar motility. In the absence of the outer arm, cells swim with a slow-jerky motion at about 1/3<sup>rd</sup> the speed of wild-type cells, and the flagellar beat frequency is markedly reduced. Sixteen genes (*ODA1-ODA16*) have been identified that are required for outer arm assembly in *Chlamydomonas reinhardtii*. In addition, *PF13*, *PF22*, and *FLA14* are required for outer dynein arm assembly, but their phenotypes are pleiotropic, suggesting that they affect additional flagellar components. Of the uncloned genes, *ODA5*, *ODA8*, and *ODA10* are of particular interest because they do not encode subunits of the outer arm or the outer dynein arm-docking complex (ODA-DC). Mutant alleles of these genes are unable to complement in temporary dikaryons, suggesting that the gene products interact with each other (Kamiya, 1988). Since the genes encoding all of the known components of the outer dynein arm and the ODA-DC have been characterized, it is of great interest to identify the gene products of these additional, uncloned *ODA* alleles.

The first chapter provides an introduction to the *Chlamydomonas* flagellum, the dyneins in general, the outer dynein arm in particular, and mutations that impinge on the assembly and regulation of this important axonemal structure.

The second chapter addresses the identification and isolation of genomic DNA containing the *ODA5* gene. Utilizing a *NIT1*-tagged *oda5*-insertional mutant, I identified sequences flanking the site of the inserted *NIT1* gene. These sequences were used to isolate wild-type genomic clones spanning the *ODA5* gene. When transformed into the *oda5* mutant, the wild-type clones rescued the mutant phenotype. These results demonstrated the successful isolation of the *ODA5* gene.

The third chapter describes the identification of the *ODA5* gene and its corresponding cDNA. The rescuing genomic fragments were sequenced. Gene modeling was used to predict intron-exon splice sites. Primers to predicted exons were designed and used to obtain the *ODA5* cDNA. The gene structure of Oda5 was analyzed and its predicted amino acid sequence deduced. Secondary structure predictions indicate that Oda5p is likely to contain a series of coiled-coil domains, followed by a poly-glycine sequence and a short, highly charged region. Northern analysis demonstrated that *ODA5* gene expression is upregulated by deflagellation, a hallmark of many flagellar mRNAs.

Data in CHAPTER IV further characterize the Oda5 protein and its association with the axoneme. Oda5p localizes to the flagellum, consistent with the enhancement in mRNA levels in response to deflagellation. Within the flagellum, Oda5p is an axonemal component that is released from the axoneme upon high salt extraction, as are the ODA-DC and the outer dynein arm. However, Oda5p does not associate with this super-complex in the high salt extract as determined by sucrose gradient sedimentation. Oda5p assembles onto the axoneme independently of the outer dynein arm and the ODA-DC, demonstrating it does not require these complexes for localization. Furthermore, Oda5p

assembles onto the axoneme in the *oda8*, but not the *oda10* mutant, demonstrating a role for the Oda10 protein in localization of Oda5p. These data provide the first biochemical evidence for an interaction between Oda5p and Oda10p.

CHAPTER V reveals the discovery of a previously unrecognized phenotype exhibited in both *oda5* and *oda10* mutant strains: a defect in the assembly of a previously unknown flagellar adenylate kinase (AK). The protein levels of this flagellar AK are reduced in *oda5* mutant axonemes, as determined by quantitative mass spectrometry. Direct enzymatic assays confirmed a reduction in AK activity in both *oda5* and *oda10* mutant axonemes, providing a second line of biochemical evidence supporting a complex containing Oda5p and Oda10p. The sequence of the flagellar AK gene and its cDNA were determined.

CHAPTER VI details our efforts to identify the *ODA10* gene. Genomic clones were isolated, which contain sequences at, or near, the *ODA10* locus. Analysis of the genomic clones yielded no insights into the identity of the *ODA10* gene. The inability of these clones to rescue the Oda10- motility phenotype indicates that these clones most likely do not contain an intact *ODA10* gene.

And lastly, CHAPTER VII discusses future experimentation that can be done based on the data provided by the current study.

## TABLE OF CONTENTS

|  |     |
|--|-----|
| Title.....   | i   |
| Copyright.....   | ii  |
| Approval Signatures.....   | iii |
| Acknowledgments.....   | iv  |
| Abstract.....  | vi  |
| Table of Contents.....   | x   |
| List of Tables and Figures.....  | xii |
| <br>   |     |
| CHAPTER I: Introduction.....   | 1   |
| <br>   |     |
| CHAPTER II: Isolation of the <i>ODA5</i> Gene And Rescue of the <i>Oda5-2</i> Insertional<br>Mutant..... | 42  |
| Introduction.....  | 42  |
| Results.....   | 44  |
| Discussion.....  | 49  |
| Materials and Methods.....   | 50  |
| <br>   |     |
| CHAPTER III: Identification of the <i>Chlamydomonas ODA5</i> Gene and Its<br>Corresponding cDNA.....     | 63  |
| Introduction.....  | 63  |

|  |     |     |
|--|-----|-----|
| Results.....   | 65  |     |
| Discussion.....  | 70  |     |
| Materials and Methods.....   | 74  |     |
|  |     |     |
| CHAPTER IV: Analysis of the Oda5 Gene Product and Characterization of the Oda5 |     |     |
| Protein .....  | 87  |     |
| Introduction.....  | 87  |     |
| Results.....   | 89  |     |
| Discussion.....  | 94  |     |
| Materials and Methods.....   | 97  |     |
|  |     |     |
| CHAPTER V: Oda5p and Oda10p Are Required for the Assembly of a Sub-fraction of |     |     |
| Flagellar Adenylate Kinase.....  | 106 |     |
| Introduction.....  | 106 |     |
| Results.....   | 108 |     |
| Discussion.....  | 114 |     |
| Materials and Methods.....   | 118 |     |
|  |     |     |
| CHAPTER VI: The <i>oda10-2</i> Insertional Mutant.....                         |     | 136 |
| Introduction.....  | 136 |     |
| Results.....   | 137 |     |
| Discussion.....  | 147 |     |

Materials and Methods.....150

CHAPTER VII: Future Directions.....163

REFERENCES.....166



**LIST OF TABLES AND FIGURES**

CHAPTER I

|  |    |
|--|----|
| Table I: <i>Chlamydomonas ODA</i> genes.....   | 30 |
| Table II: Mutations affecting outer dynein arm assembly.....                                   | 31 |
| Figure 1: Electron micrographs of a human cilium and a <i>Chlamydomonas</i><br>flagellum.....  | 33 |
| Figure 2: Molecular model for the outer dynein arm and ODA-DC in<br><i>Chlamydomonas</i> ..... | 35 |
| Figure 3: Schematic of the axonemal A-tubule.....  | 37 |
| Figure 4: Schematic of axonemally bound signalling components.....                             | 39 |
| Figure 5: Model of the dynein heavy chain domains.....   | 41 |

CHAPTER II

|  |    |
|--|----|
| Table III: Flagellar beat frequency in temporary <i>oda</i> -mutant dikaryons.....   | 56 |
| Figure 6: Identification of endogenous, genomic DNA sequence at the site of the<br><i>oda5-2</i> mutation.....   | 58 |
| Figure 7: The <i>ODA5</i> gene rescues the <i>Oda5</i> - motility phenotype and the transforming<br>DNA is recovered in the <i>oda5-2</i> rescued strains..... | 60 |
| Figure 8: The <i>oda5-2</i> insertional mutant lacks outer dynein arms and the rescued<br>strain has restored outer dynein arms.....                           | 62 |

## CHAPTER III

|  |    |
|--|----|
| Figure 9: <i>ODA5</i> gene structure and genomic sequence.....               | 78 |
| Figure 10: <i>ODA5</i> intron 3.....   | 80 |
| Figure 11: Sequence of the <i>ODA5</i> cDNA and its product.....             | 82 |
| Figure 12: Phylogenetic tree of Oda5p-related sequences.....                 | 84 |
| Figure 13: <i>ODA5</i> gene expression is upregulated by deflagellation..... | 86 |

## CHAPTER IV

|  |     |
|--|-----|
| Figure 14: Coiled-coil secondary structure of Oda5p.....   | 101 |
| Figure 15: Western blot analysis indicates Oda5p is a salt-extractable, $M_r$ 76,000<br>axonemal protein that sediments at ~5S in sucrose density gradients..... | 103 |
| Figure 16: Oda5p assembles onto the axoneme independently of the outer dynein<br>arm and the ODA-DC.....   | 105 |

## CHAPTER V

|  |     |
|--|-----|
| Table IV: Selected ICAT-labeled peptides identified by ESI/MS/MS.....  | 123 |
| Figure 17: Electron micrographs of Nonidet P-40 and Tergitol-extracted<br>axonemes.....                      | 125 |
| Figure 18: Adenylate kinase activity is reduced in <i>oda5</i> and <i>oda10</i> flagella.....                | 127 |
| Figure 19: The Oda5p-associated adenylate kinase activity is solubilized with Nonidet<br>P-40 detergent..... | 129 |
| Figure 20: Adenylate kinase gene structure, cDNA and deduced protein sequence...                             | 131 |

|  |     |
|--|-----|
| Figure 21: The adenylate kinase gene is tightly linked to the <i>ODA7</i> gene, but does not encode the Oda7 gene product..... | 133 |
| Figure 22: Model for assembly of the Oda5p, Oda8p, and Oda10p complex.....   | 135 |
| CHAPTER VI   |     |
| Table V: Phenotype of meiotic progeny from <i>oda10-2</i> crosses.....   | 154 |
| Figure 23: The V87.2 ( <i>oda10-2</i> ) insertional mutant lacks outer dynein arms.....  | 156 |
| Figure 24: The <i>ODA10</i> genomic clones align with JGI scaffold 1603.....   | 158 |
| Figure 25: The <i>ODA10</i> genomic clones align with JGI scaffold 1307.....   | 160 |
| Figure 26: The deleted sequences are tightly linked to the <i>oda10-2</i> mutation.....  | 162 |

## CHAPTER I

### INTRODUCTION

#### ***Chlamydomonas* is a Model Organism for the Study of the Eukaryotic Flagellum**

*Chlamydomonas*, a unicellular, biflagellate green alga, is an important model organism in the study of flagellar assembly and motility. The strength of *Chlamydomonas* as a model system is due to the wide range of experimental techniques available in this organism. *Chlamydomonas* cells can be grown in large quantities and the flagella isolated for biochemical, pharmacological and ultrastructural analysis of the flagellar apparatus. Electrophoretic analyses of isolated flagella have demonstrated that the flagellar axoneme is composed of >200 individual proteins (Piperno, 1995; Piperno *et al.*, 1977). However, a recent proteomic analysis of the *Chlamydomonas* flagellum suggests this number may be greater than 300 (Agrin *et al.*, 2003). *Chlamydomonas* genetics has proven invaluable in dissecting complex cellular pathways, such as flagellar assembly.

*Chlamydomonas* has well characterized haploid genetics and there exist many mutant strains that exhibit specific defects in flagellar function. Since the absence of flagella is non-lethal, mutations that affect flagellar assembly and motility are readily identified in haploid cells simply by screening cells for altered swimming behaviors. Stable diploids can be produced in *Chlamydomonas*. As a result, complementation in

stable diploids between two mutations with similar phenotypes can be used to test allelism between the pair of mutations. Since diploids cells are capable of mating, mutations in essential genes can also be studied (Harris, 1989). In addition to biochemical and genetic studies, insertional mutagenesis has been instrumental in identifying individual components of the axoneme. This process involves the insertion of transforming plasmid DNA which often (but not always) results in deletion of genomic DNA at the site of insertion. Taken together, these features make *Chlamydomonas* the model organism of choice for studying the structure, function, and assembly of the eukaryotic flagellum. Since the axoneme has been best characterized in *Chlamydomonas*, the details that follow refer to *Chlamydomonas* unless specified otherwise.

### **Flagellar- and Ciliary-Dependent Disease**

While *Chlamydomonas* and humans are separated by millions of years of evolution, human cilia and flagella are remarkably similar to *Chlamydomonas*, both in structure and function (Figure 1A). There are a number of *Chlamydomonas* mutants which exhibit defects in flagellar assembly, structure and motility. The proper assembly of axonemal dyneins into cilia and flagella is critical for ciliary and flagellar motility. Defects resulting from mutations in intraflagellar transport (IFT), dynein assembly and assembly of other axonemal components can lead to diseases such as polycystic kidney disease, retinal degeneration, lateralization defects, and primary ciliary dyskinesia (PCD or immotile cilia syndrome) (Pazour and Rosenbaum, 2002). PCD is a rare autosomal

recessive disorder affecting ~1:10,000-20,000 people. Approximately 50% of PCD patients have *situs inversus* (complete left-right reversal of the internal viscera) due to randomization of the left-right body pattern. PCD with *situs inversus* is referred to as Kartagener syndrome. Symptoms include chronic respiratory and sinus distress caused by reduced mucociliary clearance of the airway. These symptoms result from defects in tracheal ciliary assembly or function, and as a consequence ciliary motility is impaired. Male PCD patients are sterile due to immotile or dysmotile sperm. Female PCD patients are often not completely sterile, but considered “hypofertile” due to dysfunctional oviduct cilia (Afzelius and Mossberg, 1995; Meeks and Bush, 2000).

The molecular mechanisms underlying the assembly and function of *Chlamydomonas* flagella have obvious implications for understanding their counterparts in humans and human disease. Ensuring the proper formation of cilia and flagella is vital, given the importance of these organelles to human health, development, and reproduction.

### **Dynein**

Many types of cellular movements, like flagellar motility, involve molecular motors. Motor proteins hydrolyze nucleotides and use the derived energy to move along cytoskeletal filaments. The first type of dynein identified was axonemal dynein (Gibbons, 1965). It utilizes energy to slide adjacent microtubules within the axoneme to generate the force for flagellar and ciliary movement. Dyneins are usually classified as cytoplasmic dyneins or axonemal dyneins (inner and outer arm dynein). Of the

cytoplasmic dyneins, the major type, Dhc1a (or DHC1), has been implicated in numerous functions, including vesicle transport, localization of the Golgi apparatus, nuclear migration, spindle formation and orientation, centrosome assembly, and chromosome movements (Holzbaur and Vallee, 1994; Purohit *et al.*, 1999; Tynan *et al.*, 2000). The second type of cytoplasmic dynein, Dhc1b/2, is required for retrograde intraflagellar transport (IFT) (Pazour *et al.*, 1999a; Porter *et al.*, 1999), a process required to build and maintain flagellar structures. A null mutation in the *Chlamydomonas DHC1b* gene yields stumpy, short flagella which accumulate electron dense material (Pazour *et al.*, 1999a; Porter *et al.*, 1999). This material has been identified as IFT particles, which are also visible by EM in wild-type flagella as elongated structures tightly associated with the flagellar membrane and the outer doublet microtubules of the axoneme (Kozminski *et al.*, 1993).

The dynein heavy chains (HC) are the key elements of the dynein motor complex and thus have been extensively studied. Through a combination of molecular cloning and PCR-based strategies, a total of twelve different HCs have been biochemically identified in the *Chlamydomonas* flagellum: 3 in the outer dynein arm, 2 from inner arm I1, 6 monomeric inner arm dynein isoforms, and the Dhc1b retrograde motor. (Mitchell and Brown, 1994; Pazour *et al.*, 1999a; Piperno *et al.*, 1990; Porter *et al.*, 1999; Porter *et al.*, 1996; Wilkerson *et al.*, 1994). Two additional HC sequences, identified from RNA derived from cells regenerating their flagella, have not been biochemically characterized, but are believed to comprise part of the heterogeneous inner arm system (Porter *et al.*, 1996).

## The Axonemal Dyneins

### (a) Structure and Assembly

Both cilia and flagella contain a highly organized structure called the axoneme, which is surrounded by the flagellar or ciliary membrane (figure 1A). Typically, the axoneme contains 9 outer doublet microtubules arranged around a central pair of microtubules, called the 9 + 2 formation. The outer doublet microtubules are composed of a complete tubule (A-tubule) fused to an incomplete tubule (the B-tubule). Each microtubule contains protofilaments: 13 in the A-tubule, 10 in the B-tubule, and 13 in each central pair microtubule. A specific type of cilium, called the primary cilium, has a 9 + 0 arrangement of axonemal microtubules and is specifically lacking the internal central pair.

The axoneme contains two main types of axonemal dynein, the inner and outer arm dyneins which are attached to the A-tubule. The arms interact with the B-tubule of the adjacent outer doublet, generating the microtubule sliding that underlies flagellar movement.

The outer and inner arms assemble independently and are located at specific sites within the axonemal superstructure. Inherent to this assembly process is the requirement for unique structural and biochemical properties that ensure the proper targeting of each dynein isoform to its proper site both around and along the length of the axoneme. Outer arm dyneins repeat at 24-nm intervals along the length of the flagellar axoneme (Figure 1B). The outer dynein arm-docking complex (ODA-DC) is thought to



determine the proper binding site for the outer arm structures (Takada and Kamiya, 1994; Wakabayashi *et al.*, 2001). There is only one known species of outer dynein arm, which contains at least 13 polypeptides: 3 heavy chains (HC), 2 intermediate chains (IC), and several light chains (LC1-8) (DiBella and King, 2001) (Figure 2). The outer dynein arm is a well defined structure of the flagellar axoneme. When viewed by EM, the outer arms appear as a crescent-shaped structure with 3 large lobes, each lobe representing one of the three heavy chains.

The inner dynein arms are more heterogeneous in nature. They are organized in a triplet pattern which repeats at 96-nm intervals (Piperno *et al.*, 1990) (Figure 3). The inner arm I1 isoform (subspecies f), containing 2 HCs, is the first component of the triplet and is found along the entire length of the axoneme. I1 is followed by two of the 6 distinct single-headed (i.e. containing one HC) inner arm dynein motors. The single-headed dyneins, termed subspecies a, b, c, d, e, and g, are also known as I2 and I3 collectively (Kagami and Kamiya, 1992; Piperno *et al.*, 1990). Different single headed inner arm isoforms are found in the proximal region of the flagellum as compared to the more distal regions. It is undetermined how the inner arm dyneins are targeted to the axoneme. Presumably, there exist “inner dynein arm-docking complexes (IDA-DCs)” which are required to bind the inner arms to their respective sites along the length of the axoneme (Porter and Sale, 2000). Clearly, the maintenance and regulation of flagellar motility depends on the correct placement of these specific dynein species within the axonemal structure. Hence, the assembly of axonemal dyneins and the mechanisms by which they are targeted, are of considerable interest.

Axonemal dynein, located in both the inner and outer dynein arms, provide the motile force for flagellar motility. The inner dynein arms are responsible for efficient propagation of the flagellar waveform; inner dynein arm mutants (*ida*) have reduced shear amplitude (Brokaw and Kamiya, 1987) and as a result swim with a slow, smooth motion. Since different isoforms of single headed inner dynein arms are targeted to specific regions of the axoneme, this suggests that efficient propagation of waveform requires functionally distinct dynein motors within these different axonemal domains (Piperno and Ramanis, 1991).

The outer dynein arms are required for normal flagellar beat frequency and provide up to 4/5ths of the power for flagellar movement (Brokaw, 1994). Without this structure, flagella beat at a much lower frequency and as a result cells swim with a slow, jerky motion at approximately one-third the speed of wild-type cells. This characteristic swimming phenotype is unique to the outer dynein armless (*oda*) mutants and distinguishes them from other motility mutants.

#### **(b) Photobehavioral Responses and the Axonemal Dyneins**

*Chlamydomonas* cells display two distinct types of behavioral responses to light: phototaxis and photoshock responses, both of which impinge upon the axonemal dyneins. The process of phototaxis, where *Chlamydomonas* cells move towards or away from a light source, involves a change in swimming direction. During phototaxis, the cis and trans-flagella (the cis-flagellum is closest to the eyespot; the trans-flagellum is farther from the eyespot) beat differently in response to small fluctuations in

intraflagellar  $\text{Ca}^{2+}$  concentration in the submicromolar range (Kamiya and Witman, 1984; Ogawa *et al.*, 1995; Omoto *et al.*, 1999; Sakakibara *et al.*, 1993; Takada and Kamiya, 1997). At less than  $10^{-8}$  M  $\text{Ca}^{2+}$ , the trans-flagellum becomes inactive, while at  $10^{-7}$  to  $10^{-6}$  M  $\text{Ca}^{2+}$ , the cis-flagellum is inactive. This effect is seen even in the absence of the outer dynein arms and ODA-DC, suggesting that the machinery responsible for sensing these  $\text{Ca}^{2+}$  changes within the flagellum does not reside in the outer dynein arm/ODA-DC system (Kamiya and Okamoto, 1985). Centrin, an inner arm  $\text{Ca}^{2+}$ -binding light chain, binds two  $\text{Ca}^{2+}$  ions with high affinity ( $K_{\text{Ca}} \sim 1.2 \times 10^{-6}$  M), making centrin a candidate protein for sensing these low level changes in  $\text{Ca}^{2+}$  concentration and transmitting the signal to the inner arm system.

In addition to being required for beat frequency, the outer dynein arm is important in the photoshock, or photophobic response, an avoidance response that occurs upon sudden stimulation with bright light. Dark adapted cells, upon exposure to a sudden burst of light, will stop, transiently swim backwards, and then resume forward swimming. The flagella undergo a shift from an asymmetric waveform to a symmetric waveform upon the transition to backwards swimming.

Microscopic analysis of *oda*-mutant flagella (see Tables I and II for *ODA* gene products and mutations affecting outer arm assembly) demonstrated that the outer dynein arm is required for generating the symmetrical waveform utilized in backward swimming (Kamiya and Okamoto, 1985). Experimental analysis of *oda1* mutant flagella determined the mutant flagella never beat with a symmetrical waveform, indicating that this part of the photoshock response is abolished in the absence of the

outer dynein arm (Kamiya and Okamoto, 1985). When the photoshock response was induced with intense light, the mutant flagella stopped beating and arrested with the flagella in a V-shaped, or "hands-up" position. A different study (Mitchell and Rosenbaum, 1985), determined the photoshock response in *pf28* (i.e. *oda2*, which is defective in the  $\gamma$ HC) was also defective, with cells showing little to no backward swimming in response to photoshock stimuli; however the mutant flagella beat in both the forward and reverse modes, although the reverse mode was aberrant. At low magnification, the mutant cells appear to freeze upon light stimulation, consistent with the analysis of *oda1* mutant flagella. Analysis of *pf28* flagella at higher magnification showed the flagella actually exhibited a partial symmetrical waveform, but remained in the V-configuration during flagellar reversal and had smaller bend amplitudes than wild-type flagella. Although different *oda* mutant alleles were used in these two studies, the slight discrepancy remains unexplained. It is possible that, since the bend amplitudes of *oda*-mutant flagella are greatly diminished during flagellar reversal, the reduced bends were not detected in the *oda1* mutant analysis. Alternatively, different *oda* mutant alleles may exhibit various degrees of defective photoshock response, with some alleles manifesting a "complete" defect, while other alleles exhibit "partial" defects in flagellar reversal. Regardless, in the absence of the outer dynein arm, a productive symmetrical waveform is not achieved, demonstrating that the outer arm is clearly important in this generating this type of flagellar motility.

### (c) Regulation of Dynein Activity

Phosphorylation has been implicated in playing a key role in the regulation of dynein function. Indeed, generation of flagellar motility demands precise temporal and spatial control of dynein function along the length of the axoneme. Evidence suggests that the regulatory elements (phosphatases and kinases) required for this control are built into the axonemal structure (Porter and Sale, 2000) (Figure 4). A regulatory pathway extending from the central pair apparatus, through the radial spokes to the dynein arms, has been structurally and genetically identified through the analysis of suppressor mutations that restore motility to paralyzed flagella (*pf*) mutants that are defective in the central pair or radial spoke structures (Smith and Lefebvre, 1997; Smith and Sale, 1994). This is further supported by biochemical assays showing that microtubule sliding velocity is disrupted in radial spoke mutants and is restored by addition of radial spoke- or dynein-containing fractions derived from radial spoke-containing axonemes (Smith and Sale, 1992). Similar reconstitution experiments demonstrated that inner arm I1 is a critical player in regulation of microtubule sliding (Habermacher and Sale, 1996; Habermacher and Sale, 1997).

Recent studies have revealed the presence of AKAPs (A-kinase anchoring proteins) within the radial spoke system (Gaillard *et al.*, 2001). AKAPs are proteins that “anchor” cAMP-dependent protein kinases (like PKA) and are responsible for targeting these kinases to various cellular locales. One of these AKAPs was determined to be radial spoke protein 3 (Rsp3p) (Gaillard *et al.*, 2001). Rsp3p is located at the base of the radial spoke stalk and would place the kinase in close proximity to inner arm I1 where it would be in a key position to phosphorylate IC138, the only I1 phosphoprotein.

However, inhibitors of PKA fail to completely block phosphorylation of IC138, although these inhibitors restore microtubule sliding activity of radial spokeless axonemes. This suggests additional kinases are involved in IC138 phosphorylation. Intriguingly, additional kinases have been identified within the axonemal superstructure, such as casein kinase 1 and a novel calmodulin-binding kinase identified in the radial spoke (Yang *et al.*, 2000; Yang and Sale, 2000). There is also evidence that calcineurin, a  $\text{Ca}^{2+}$ /calmodulin-dependent type 2b phosphatase, affects I1 function (King and Dutcher, 1997), suggesting that both the cAMP and  $\text{Ca}^{2+}$  signaling pathways are involved in the regulation of flagellar bending.

Dynein arm assembly and the regulation of dynein activity are complex cellular processes. The placement of the arms within the axoneme, and the subsequent control of their activity must occur with extremely high fidelity. Determining the molecular nature of the dynein arms and the mechanisms by which they are assembled and regulated are crucial to understanding how these highly complex structures work.

### **The Heavy Chains (HC)**

The HCs are the force-generating subunits of the dynein motor complex and have been the focus of many studies in both axonemal and cytoplasmic dynein isoforms. All HCs are designed with the same basic architecture (Figure 5). This includes an N-terminal stem domain (~160-kDa) followed by a globular head, which contains the ATPase site(s) (Goodenough and Heuser, 1984; Sale *et al.*, 1985; Witman *et al.*, 1983). A short stalk domain protrudes from the head and contains the MT-binding domain (Gee and

Vallee, 1998; Gee *et al.*, 1997). The stem domain of one HC associates with the stem domain of other HCs and with the IC/LC complex. The HC-HC and HC-IC interacting domains have been mapped to a 150-amino acid region in *Dictyostelium* cytoplasmic dynein (Habura *et al.*, 1999). Similar regions have been identified in *Chlamydomonas* HCs, suggesting that the mechanism of HC-HC and HC-IC interaction is conserved (King, 2000).

The motor domain, the force-producing unit, comprises the C-terminal ~350-kDa of the HCs. It contains the globular head and the MT-binding domain (stalk domain). There are four predicted nucleotide binding sites called P-loop motifs (P1-P4) within the motor domain. These P-loops are contained within larger domains called AAA domains (ATPases associated with cellular activities) of which there are a total of six in the HCs. King (2000) has proposed a model combining the AAA domains and the structure as determined by EM (Figure 5). In this model, each AAA unit comprises one globular subdomain observed by EM, positioning the MT-binding domain and the N-terminal stalk domain on opposite sides of a ring-like structure. The P1 motif within AAA1, GPAGTGKT, is conserved among axonemal and cytoplasmic dyneins and is the site of ATP hydrolysis. The number of P-loops involved in ATP binding is a matter of debate. Studies in sea urchin sperm dynein have suggested that all 4 P-loops are involved in nucleotide binding (Mocz and Gibbons, 1996). However, subsequent experiments using fluorescence anisotropy measurements suggested the presence of only two high-affinity mantATP-binding sites (Mocz *et al.*, 1998) with two additional low affinity binding sites. A two-site model is further supported by studies of the *Chlamydomonas* HCs

(King *et al.*, 1989; Wilkerson *et al.*, 1994). The two site model suggests that two P-loops bind nucleotides with high affinity, yet only one P-loop has hydrolytic activity (P1 in AAA1). The specific roles of the additional P-loops, or AAA domains, within the dynein HCs are unknown, yet they clearly affect dynein motor function. For example, a single base pair substitution in AAA2 of murine left-right dynein (*lrd*) changes a highly conserved glutamic acid to lysine and abolishes dynein activity, resulting in immotile nodal cilia and leading to randomization of left-right asymmetry (Supp *et al.*, 1997).

The MT-binding domain is a projection that emanates from the globular motor domain (Gee *et al.*, 1997). It consists of two regions predicted to form antiparallel coiled-coil segments separated by a ~125-amino acid region that forms the MT-binding site. While the MT-binding activity appears to be a conserved function, the sequence conservation of this domain is not so stringent; only ~25-30% identity between the various dynein isoforms. One interpretation is that this feature reflects differing MT-binding and translocation properties intrinsic to the various dynein isoforms (DiBella and King, 2001).

### **Outer Dynein Arm Components**

The outer dynein arm contains at least 13 known subunits, each providing its own unique contribution to outer arm function. Individual components can play multiple roles in mediating dynein assembly, regulating dynein function, or providing the force-generating power of the dynein motor.



### (a) The Heavy Chains

The outer arm contains three HCs designated  $\alpha$ ,  $\beta$ , and  $\gamma$ .

#### (i) $\alpha$ HC

Several flagellar HCs are phosphorylated, including the  $\alpha$ HC of *Chlamydomonas* outer arm dynein (King and Witman, 1994). There are at least six phosphorylation sites in the  $\alpha$ HC and their locations have been determined by mapping studies (King and Witman, 1994). The current model for HC structure predicts that all six of these phosphorylation sites lie in close proximity to AAA1, where the hydrolytic activity occurs (DiBella and King, 2001; King, 2000). Surprisingly, disruption of the structural gene encoding the  $\alpha$ HC (*oda11*) results in the assembly of an outer arm which lacks only the  $\alpha$ HC and its associated light chain component (LC5), yielding a swimming phenotype that is intermediate between complete outer-armless mutants and wild-type. This feature of the *oda11* mutant is unique and allowed for the precise localization of the  $\alpha$ HC to the outermost lobe of the three-headed outer arm (Sakakibara *et al.*, 1991). The careful analysis of this mutant allowed the dissection of specific contributions of the  $\alpha$ HC in outer arm function. While the  $\alpha$ HC is not required for photoshock, it is required for the *cis*- and *trans*- flagella to beat at different frequencies. Analysis of flagellar beat frequency in *oda11* determined that the two flagella beat at almost identical frequencies, unlike wild-type flagella. This result is consistent with that observed for *oda* mutants that are completely missing the outer dynein arm. Since the  $\alpha$ HC is the only

phosphorylated HC in the outer arm, it is possible that selective phosphorylation of the  $\alpha$ HC in one flagellum mediates the cis-trans frequency imbalance (Sakakibara *et al.*, 1991).

## (ii) $\beta$ HC

In contrast to the  $\alpha$ HC, disruption of the gene encoding the  $\beta$ HC (*oda4*) results in complete loss of the outer dynein arm from the flagellum, indicating that this subunit is critical for the structural integrity and assembly of the outer arm. The *sup-pf-1* mutant is allelic with *oda4* and was identified as an extragenic suppressor of certain paralyzed flagellar (*pf*) mutants which lack the central pair or radial spokes. *sup-pf-1* mutations result in a small deletion within the  $\beta$ HC that restores motility to central-pairless or radial-spokeless mutants. The characterization of this mutation implicated the  $\beta$ HC as a target for regulation of outer arm function by these other axonemal structures.

Another *oda4* allele (*oda4-s7*) results in the production of a truncated  $\beta$ HC, containing only the N-terminal stem and missing the motor domain. This truncation allows assembly of the remaining dynein components, including the  $\beta$ HC N-terminus and its associated LC3. Even though this complex is competent to assemble and is missing only the  $\beta$ HC motor unit, the mutant cells swim at velocities comparable to the null *oda4* allele. Thus, the lack of the beta heavy chain motor impairs outer-arm function more seriously than does the lack of the entire alpha heavy chain, suggesting that the alpha and beta chains play different roles in outer arm function.

### (iii) $\gamma$ HC

Like the  $\beta$ HC, disruption of the  $\gamma$ HC gene (*oda2*) results in complete loss of the outer dynein arm, indicating that this HC is also critical for the structural integrity and assembly of the outer arm. No mutations containing truncated  $\gamma$ HC have been reported. However, our analysis of an insertional allele of *MCD1* (a nuclear gene required for chloroplast gene expression isolated by Clare Simpson and David Stern) determined that the insertional event that removed the *MCD1* gene also removed the 3' end of the  $\gamma$ HC gene. Microscopic analysis of the *mcd1* mutant showed the cells exhibited a classic Oda-swimming phenotype. This mutant expresses low levels of a truncated  $\gamma$ HC, which assembles onto the axoneme. The truncated protein expresses the first ~180-kDa of the  $\gamma$ HC protein. In contrast to the *oda4-s7* allele, *mcd1*-mutant axonemes do not contain the  $\alpha$ HC,  $\beta$ HC, IC1 or IC2, suggesting that this mutation affects assembly of the outer arm (M. Wirschell, unpublished data) in a manner similar to the *oda2* allele. Thus, the N-terminus of the  $\gamma$ HC is not sufficient for stable HC-HC or HC-IC interactions. The reduced levels of axonemally bound, truncated  $\gamma$ HC suggest that it has some intrinsic ability to bind to the axoneme in the absence of the remaining dynein subunits. Indeed, the  $\gamma$ HC alone bundles microtubules *in vitro*, suggesting that it can bind to microtubules via its N-terminus (Sakakibara and Nakayama, 1998).

Complementation tests and linkage analysis revealed that the *sup-pf-2* mutations are alleles of the *ODA2* locus. The *sup-pf-2* mutants are members of a group of dynein

regulatory mutations that restore motility to paralyzed central pair or radial spoke defective strains. The *sup-pf-2* mutations therefore appear to alter the activity of the outer dynein arms by modification of the  $\gamma$ HC. Furthermore, the ATPase activity of the  $\gamma$ HC appears to be activated by sulfhydryl redox state. This enhancement of  $\gamma$ HC enzymatic activity is modulated by the  $\alpha$  and  $\beta$ HCs and their associated thioredoxin LCs (Harrison *et al.*, 2002). Additional regulatory inputs impinge on the  $\gamma$ HC via the LC4  $\text{Ca}^{2+}$ -binding LC (see section on LCs) and LC1 (King, 2000).

#### **(b) The Intermediate Chains (IC)**

The intermediate chains (ICs) are found in dyneins that contain two or more HCs. They are found at the base of the dynein stem domains and are complexed with a series of light chains (LCs). The C-termini of the ICs cloned to date contain conserved WD repeat motifs, a ~40 residue sequence that contains a conserved Try-Asp dipeptide (DiBella and King, 2001; Wilkerson *et al.*, 1995). Exceptions to this rule do exist, such as a unique IC found in *Ciona intestinalis*, human, and sea urchin outer arm dynein (Ogawa *et al.*, 1996; Padma *et al.*, 2001). The N-terminal regions of the ICs are more variable, yet they are clearly essential for IC function (King *et al.*, 1991; Mitchell and Kang, 1993).

*Chlamydomonas* outer arm dynein contains two ICs (Figure 2). Both proteins are required for assembly of the outer dynein arm structure.

#### **(i) IC1**

The N-terminus of IC1 cross-links to  $\alpha$ -tubulin *in situ* (King *et al.*, 1991). These experiments provided the first evidence of the ICs playing a role in cargo binding. Subsequent experiments also have implicated the IC (Karki and Holzbaur, 1995) and LICs (light intermediate chains) of mammalian cytoplasmic dynein (Purohit *et al.*, 1999; Tynan *et al.*, 2000) in cargo binding. However, the interaction of IC1 and  $\alpha$ -tubulin is not sufficient to assemble the outer arm onto the axoneme. A trimeric complex, the ODA-DC, is required to rebind purified outer arm dynein to the axoneme (Takada and Kamiya, 1994). Perhaps the ODA-DC mediates cargo binding in a manner similar to the dynactin complex of mammalian cytoplasmic dynein (Burkhardt *et al.*, 1997).

#### **(ii) IC2**

*Chlamydomonas* IC2, in addition to being required for assembly of the outer arm, plays a regulatory role in outer arm dynein activity. Transformation of the *oda6* mutant, containing a defect in the IC2 gene, with a wild-type copy of the IC2 gene resulted in rescue of the outer arm assembly defect and the associated swimming defect. However, mutations in the N-terminus of IC2 allow for assembly of the outer arm, but not rescue of the swimming defect, indicating that the N-terminus of IC2 is critical for outer arm dynein function (Mitchell and Kang, 1993).

#### **(c) The Light Chains (LC)**

Each dynein isoform contains a unique subset of LCs; small subunits which serve a variety of functions. Some LCs are important for structural integrity of the dynein

complex, while others execute regulatory roles in dynein function. Due to the variation in LC composition in different dynein isoforms, it seems reasonable that LC subunit content confers certain functional specificity to each dynein subtype (Tai *et al.*, 1999; Tai *et al.*, 2001). Outer arm dynein contains two main classes of light chains. One class is defined as LCs which interact directly with the HCs. Based on their properties (see below), these LCs are proposed to relay regulatory signals to the HCs. The second class encompasses LCs that interact with the ICs at the base of the dynein HCs in an IC-LC complex. These LCs are essential for assembly of the dynein complex.

#### **(i) Heavy Chain-Associated LCs**

The LC proteins have distinct functional domains. Two LCs in *Chlamydomonas* are capable of  $\text{Ca}^{2+}$  binding: centrin (i.e. caltractin), and LC4. Both proteins are members of the EF-hand family of  $\text{Ca}^{2+}$ -binding proteins and each has four  $\text{Ca}^{2+}$ -binding helix-loop-helix motifs. LC4 is associated with the  $\gamma$ DHC of the outer arm. In LC4, only two of the helix-loop-helix motifs conform strictly to the EF-hand consensus.  $\text{Ca}^{2+}$ -binding assays suggest LC4 binds one  $\text{Ca}^{2+}$  ion at a  $K_{\text{Ca}} \sim 10^{-5}$  M (King and Patel-King, 1995a). The specific function(s) of these  $\text{Ca}^{2+}$ -binding proteins within the flagellum remains undetermined; however LC4 they may be important for the  $\text{Ca}^{2+}$ -dependent photoshock response, which requires the outer dynein arm.

Photoshock is accompanied by a large increase in intraflagellar  $\text{Ca}^{2+}$  concentration from  $10^{-6}$  to  $10^{-4}$  (Bessen *et al.*, 1980). Isolated axonemes beat with a ciliary waveform at  $10^{-6}$  M  $\text{Ca}^{2+}$ , are quiescent at  $10^{-5}$  M  $\text{Ca}^{2+}$ , and beat with a

symmetric waveform at  $10^{-4}$  M  $\text{Ca}^{2+}$ . The  $\text{Ca}^{2+}$ -binding sites of LC4 make this protein an ideal candidate photoshock sensor. Interestingly, the  $\gamma$ HC exhibits a  $\text{Ca}^{2+}$ -dependent microtubule binding activity (Sakato and King, 2000), providing further evidence linking LC4 (the only known  $\text{Ca}^{2+}$ -binding protein in the outer arm) to a  $\text{Ca}^{2+}$ -dependent mechanism for control of outer dynein arm motor function.

The HCs each bind a LC. As stated above, LC4 binds to the  $\gamma$ HC. LC3 binds the  $\beta$ HC within the N-terminal stem domain, while LC5 binds to the  $\alpha$ HC. A recent report indicates that LC3 also binds the  $\gamma$ HC (Harrison *et al.*, 2002). Both LC3 and LC5 contain a thioredoxin redox active site motif (WCGPCK) (Patel-King *et al.*, 1996). In thioredoxin, the dithiol motif converts a vicinal dithiol (two cysteine residues in close opposition) in a different protein to a disulfide bond. The significance of these motifs in the outer arm LCs is unclear. They may allow the outer arm to sense and react to changes in flagellar redox state. A thioredoxin component of sea urchin sperm outer arm dynein has been identified (Ogawa *et al.*, 1996). This protein appears to be a part of a modular IC, containing sequences related to both thioredoxin and nucleoside diphosphate kinase. Homologous ICs from *Ciona intestinalis* and *Homo sapiens* contain the same motifs (Padma *et al.*, 2001). This suggests that sensing redox state is a general phenomenon of flagellar motility and is not *Chlamydomonas* specific.

The  $\gamma$ HC also binds LC1, the largest of the LCs in the outer arm at ~22-kDa. Its binding to the  $\gamma$ HC appears to be hydrophobic in nature and the binding site resides in the  $\gamma$ HC motor domain within AAA1 (Benashski *et al.*, 1999; Pfister *et al.*, 1982). This LC is the first and only LC shown to directly interact with a motor domain. Cross-

linking experiments have demonstrated that LC1 associates with an unidentified axonemal protein of 45-kDA (Benashski *et al.*, 1999).

### **(ii) LCs Associated with the IC-LC Complex**

LC2, LC6, LC7, and LC8 play a structural role in the outer dynein arm and participate in the IC-LC complex at the base of the dynein particle. LC6 and LC8 were first identified in the *Chlamydomonas* outer dynein arm. LC8 has homologues with an extremely high level of sequence conservation, even in organisms that do not contain cilia and flagella (King and Patel-King, 1995b). LC8 is not a dynein specific subunit, as it is found in many cellular complexes including neuronal nitric oxide synthase (Jaffrey and Snyder, 1996; Rodriguez-Crespo *et al.*, 1998; Rodriguez-Crespo *et al.*, 2001), myosin V (Benashski *et al.*, 1997; Benashski and King, 2000; Espindola *et al.*, 2000), and I $\kappa$ B $\alpha$  (Hiscott *et al.*, 1997). Within the flagellum, LC8 is found in both the inner and outer dynein arms, and the radial spokes (Yang *et al.*, 2001), and is required for retrograde IFT (Pazour *et al.*, 1998), suggesting that LC8 is also a component of the Dhc1b motor, although this has not been directly demonstrated. Based on its association in multiple complexes, LC8 presumably functions as an adaptor protein, binding other proteins and promoting the stable assembly of larger protein complexes. In contrast, LC6, which shares 40% homology to LC8, is found in outer arm dynein only. Cross-linking studies indicate LC6 is in close proximity to another outer arm light chain, LC2 (TcTex2) (DiBella *et al.*, 2001). LC2 null mutants are completely unable to assemble the outer arm into the axoneme (Pazour *et al.*, 1999b).



Database analysis and molecular cloning identified the LC7/roadblock (*robl*) class of dynein LCs. The LC7/*robl* family contains many members with multiple isoforms existing in mammalian systems, *Drosophila* and *Chlamydomonas* (Bowman *et al.*, 1999). The *oda15* mutant in *Chlamydomonas* is defective in the gene encoding LC7. *Oda15* mutant flagella do not contain outer dynein arms. Thus, like other LCs in the IC-LC complex, LC7 is required for outer dynein arm assembly in *Chlamydomonas*.

### The ODA-DC

Studies in *Chlamydomonas* have demonstrated that IC1 cross-links to  $\alpha$ -tubulin *in situ* (King *et al.*, 1991). However, IC1 is not sufficient to mediate the targeting and binding of the outer arm to the axoneme. This has been demonstrated using reconstitution assays in which isolated outer arm dynein was recombined with dynein-stripped axonemes. The outer arm was unable to rebind to the axoneme without a 7S complex (the ODA-DC), indicating that IC1 alone is not competent to mediate rebinding (Takada and Kamiya, 1994). These elegant biochemical experiments demonstrated the requirement of the 7S factor in mediating dynein rebinding. EM analysis indicated that this factor correlated with a beak-like projection present on the A-tubule of the outer doublet. The 7S factor was partially purified and determined to contain equimolar amounts of three polypeptides of  $M_r \sim 105$ -kDa (DC1),  $M_r \sim 70$ -kDa (DC2), and  $M_r \sim 25$ -kDa (DC3) (Takada *et al.*, 2002). DC1 (Koutoulis *et al.*, 1997) and DC2 (Takada *et al.*, 2002) are coiled-coil proteins. DC3 contains two consensus EF-hand motifs and two additional non-consensus  $\text{Ca}^{2+}$ -binding motifs (Casey *et al.*, 2003b). *In vitro*  $\text{Ca}^{2+}$ -

binding assays suggest that DC3 binds at least one  $\text{Ca}^{2+}$  ion ( $K_{ca}$  between  $10^{-5}$  and  $10^{-4}$  M), however the significance of the DC3  $\text{Ca}^{2+}$ -binding activity is unknown. The DC3  $\text{Ca}^{2+}$ -binding protein does not appear to be a key player in mediating the  $\text{Ca}^{2+}$ -dependent photoshock response because a modified DC3 gene, which is unable to bind  $\text{Ca}^{2+}$  *in vitro*, exhibits normal photoshock behavior. Mutations in all three of these proteins prevent assembly of the outer dynein arm, confirming their requirement in binding of the outer arm to the axoneme.

Not only is the ODA-DC required for docking the outer arm, but it is postulated to determine the 24-nm repeat of outer arms along the length of the axoneme. The strongest evidence supporting this hypothesis is immunoelectron microscopy showing that DC1 repeats at 24-nm intervals along the length of the outer doublet microtubule (Wakabayashi *et al.*, 2001). DC1 and DC2 are coiled-coil proteins. Coiled-coil domains often participate in protein-protein interactions. Cross-linking and immunoprecipitation experiments imply that DC1 and DC2 are in direct contact with each other (Wakabayashi *et al.*, 2002a; Wakabayashi *et al.*, 2001). A dimer of DC1 and DC2 coiled-coil domains is predicted to form an extended rod-like structure. At 1.5 Å per residue, this structure would have an estimated length of 26.7 nm (Takada *et al.*, 2002). However, rotary shadow EM of recombinant DC1 and DC2 proteins revealed a rod-like structure with a length of ~50-nm (Wakabayashi *et al.*, 2002b). In either case, DC1-DC2 dimers could overlap to form a filament running the length of the axoneme, providing outer arm binding sites every 24-nm.

### Outer Dynein Arm Mutants

Screens for slow swimming strains and cytoplasmic complementation analyses have revealed up to sixteen genes that are required for outer dynein arm assembly, most of which have been cloned and determined to encode components of the dynein motor complex or ODA-DC (Table 1). Mutations in these genes are collectively called *oda* mutants and they fall into several classes. Most fail to assemble an outer arm completely. Additional *oda* mutants have been isolated which have less dramatic effects on outer arm assembly and thus exhibit less pronounced phenotypes (Table II) (DiBella and King, 2001; Sakakibara *et al.*, 1991; Sakakibara *et al.*, 1993).

Four of these genes, *ODA5*, *ODA7*, *ODA8*, and *ODA10*, have yet to be cloned and characterized. Mutant alleles of these genes exhibit the characteristic slow, jerky Oda- swimming phenotype, yet they do not code for proteins of the outer arm proper or the ODA-DC. These proteins may be essential for expression of outer arm genes, assembly of the dynein particle, transport of the outer arm from the cell body to the axoneme, or docking the outer arm within the axoneme (similar to the ODA-DC).

When *Chlamydomonas* gametes of opposite mating type are mixed, fusion between the gametes occurs, resulting in a temporary dikaryon cell containing four flagella and a shared cytoplasm. Fusion of gametes containing mutations at different loci can lead to cytoplasmic complementation which results in assembly of functional components into the four flagella, producing wild-type or near wild-type motility. Certain combinations of known *oda* mutants fail to complement in the dikaryon state (Kamiya, 1988). Based on these analyses, the *oda* mutants fall into three distinct

categories defined by their inability to complement *oda1* (*oda1* and *oda3*), *oda2* (*oda2*, 4, 6, 7, and 9), and *oda5* (*oda5*, 8, and 10). One possible explanation is that each mutant assembles partial “mutant” complexes that are incapable of dissociation and reassembly in the dikaryon cell. Alternatively, the loss of one protein may result in the instability of its binding partner(s), or sequestration of the binding partners such that they are not available for assembly in the dikaryon cell.

The hypothesis of subunit instability is an attractive one and has indeed been shown to be the case for the *oda1* and *oda3* mutants (Takada *et al.*, 2002; Wakabayashi *et al.*, 2001). The inability of *oda5*, 8 and 10 to complement in temporary dikaryons suggests that the products of these genes interact with each other and that in the absence of one of these proteins the entire complex is lost. As mentioned above, these genes do not encode known dynein subunits, yet their phenotypes clearly show that they are required for outer dynein arm assembly.

It seems unlikely that the Oda5, 8, and 10 proteins are functioning in expression of dynein genes. Fowkes and Mitchell investigated the stability and association of outer arm dynein subunits in the cytoplasm of wild-type and individual *oda* mutants (Fowkes and Mitchell, 1998). Their studies demonstrated that the HCs and ICs are pre-assembled in a complex in the cytoplasm of wild-type cells. These experiments detected some reduction in the  $\alpha$ HC (40-60% reduction), the  $\beta$ HC (% reduction not reported), IC1 (60% reduction), and IC2 (40% reduction) protein levels in the *oda5* mutant, suggesting that the HCs and ICs have some level of reduced stability in the absence of the Oda5 protein. Concomitant reductions were not observed in the *oda8* and *oda10* mutant

strains. Fowkes and Mitchell demonstrated that the entire HC-IC complex is present in the cytoplasm of *oda5*, 8, and 10 (and *oda1* and *oda3*). Therefore it is concluded that the Oda5, 8 and 10 proteins are not required for expression of these dynein subunits, nor are they required for the HC-IC association. The nature of observed reduction of these proteins in *oda5* is unknown.

Interestingly, the  $\alpha$ HC was absent in the *oda7* mutant. Immunoprecipitation from *oda7* extracts using a  $\beta$ HC-specific antibody demonstrated the existence of a complex containing the  $\beta$ HC, the  $\gamma$ HC and reduced levels of IC1 and IC2 as compared to wild type. Both ICs are present at normal levels in the cytoplasm of *oda7*; therefore their reduction in the  $\beta$ HC immunoprecipitates from *oda7* lysates indicates a role for this unknown *oda* in the IC-HC interaction. The ICs appear to be important for HC interactions as neither the  $\alpha$ HC nor the  $\gamma$ HC immunoprecipitate with the  $\beta$ HC when the IC dimer is missing (Fowkes and Mitchell, 1998). Identification of the Oda7 protein should reveal new insights into the subunit-subunit interactions within the dynein complex.

An alternative function for the uncloned *odas*, especially *oda5*, 8 and 10 is for the transport of dynein particles into the flagellum. IFT is the process by which the flagellum is built and maintained. Flagellar proteins are constantly turning over and the addition of new components occurs at the flagellar tip, distal to the cell body. It is reasonable to assume that some structural components of the axoneme are assembled in an IFT-dependent manner. Evidence is accumulating to support this hypothesis. The *Chlamydomonas* kinesin-like protein Fla10 (KHP1) is required for transport of a subset

of inner dynein arm components, but not outer dynein arms (Piperno *et al.*, 1996). Quadriflagellate cells carrying a temperature-sensitive mutation in the *FLA10* gene show that assembly of p28 (a LC component of inner arm subtypes a, c and d) into the mutant flagella is abolished at the restrictive temperature, indicating the requirement for anterograde IFT in the assembly of inner arms. In contrast, outer dynein arm transport into the mutant flagella in the quadriflagellate cell was not disrupted at the restrictive temperature, suggesting that outer arm transport is different from inner arm transport. Based on these experiments, it was reasoned that the outer arms may diffuse to their axonemal binding sites or that their transport may require a different motor than Fla10p, although no additional anterograde motors have been identified.

Recently, immunoprecipitations using antibodies to IFT particle components and IFT motors have yielded results indicating that IFT particles associate with acetylated  $\alpha$ -tubulin, radial spoke proteins, and flagellar dyneins, including IC2, LC1, and LC2 of the outer arm and DC1 of the ODA-DC (Qin *et al.*, 2002; Qin *et al.*, 2003). This suggests that outer arms are assembled and/or disassembled in an IFT-dependent manner. How outer arm transport into the flagellum is mediated, and whether any of the uncloned *ODA* genes are involved, will be elucidated as more is learned about IFT and axoneme assembly mechanisms.

#### **At Long-Last--The *ODA5* Gene**

A complete understanding of the role of these unknown *ODA* genes in outer arm assembly will be forthcoming upon identification of their gene products. To that end,

this study begins with the identification of one of these unknown *ODA* genes, *ODA5*, and ends with the discovery of a flagellar adenylate kinase that may function in regulating ATP supply for the outer dynein arm motor. This thesis details the identification of an insertional *oda5* allele, identification of the gene and cDNA sequences for *ODA5*, and characterization of the Oda5 protein (Oda5p).

In this study, *Chlamydomonas* genomic DNA fragments that rescue the Oda5-outer arm assembly defect were isolated. These genomic fragments led to the identification of the *ODA5* gene and its corresponding cDNA. Complete sequence of the gene and cDNA revealed no significantly related homologues, suggesting that *ODA5* is a novel gene. The *ODA5* cDNA predicts a novel coiled-coil protein of ~66-kDa. Northern analysis indicated that *ODA5* expression is upregulated by deflagellation, a hallmark of flagellar mRNA's (Silflow *et al.*, 1982).

This study determined that Oda5p is a *bona fide* axonemal protein, and like the outer dynein arm/ODA-DC, Oda5p is removed from the axoneme by extraction with 0.6 M KCl. However, unlike the outer arm and ODA-DC, the soluble Oda5p sediments as a discrete complex at ~5S. Moreover, Oda5p assembles onto axonemes independently of the outer arm and ODA-DC complexes. Although present in other *oda* mutants, including *oda8*, Oda5p is missing from axonemes of *oda10*. A previous study has shown that mutant alleles of *oda5*, *oda8* and *oda10* do not complement in dikaryons (Kamiya, 1988), suggesting their gene products interact in a complex. The requirement of a functional Oda10p for axonemal localization of Oda5p provides biochemical evidence to support this hypothesis.

To identify proteins dependent on Oda5p for assembly into the axoneme, a quantitative proteomics approach called isotope coded affinity tagging (ICAT) was utilized to analyze the global protein composition of wild-type vs. *oda5* axonemes. ICAT revealed multiple peptides, derived from outer dynein arm or ODA-DC subunits, that are absent or greatly reduced in *oda5* mutant axonemes. ICAT also demonstrated the presence of an adenylate kinase in the flagellum which was reduced by 35-45% in *oda5* axonemes. Analysis of axonemes from a number of *oda* mutants demonstrated that adenylate kinase activity is specifically reduced in *oda5* and *oda10*, providing a second biochemical link between *ODA5* and *ODA10*. The *oda5* and *oda10* Ak-phenotype identified here is a novel finding. This Ak-phenotype is not a universal attribute of outer armless mutants, as it is not present in all *oda* mutants.

Based on previous findings and the data presented in this thesis, we propose that Oda5p is part of a previously unknown axonemal complex, distinct from the ODA-DC, which is required for assembly of the outer dynein arm and an outer arm-associated adenylate kinase. Positioning adenylate kinase in close proximity to the outer arm most likely ensures the efficient recycling of ATP at one of the major sites of ATP hydrolysis in the axoneme.



**Table I.** *Chlamydomonas ODA* genes

| Gene         | Protein           | Reference  |
|--------------|-------------------|--|
| <i>ODA1</i>  | DC2               | (Kamiya, 1988; Takada et al., 2002)                                  |
| <i>ODA2</i>  | $\gamma$ DHC      | (Kamiya, 1988; Mitchell and Rosenbaum, 1985; Wilkerson et al., 1994) |
| <i>ODA3</i>  | DC1               | (Kamiya, 1988; Koutoulis et al., 1997)                               |
| <i>ODA4</i>  | $\beta$ DHC       | (Kamiya, 1988; Mitchell and Brown, 1994; Sakakibara et al., 1993)    |
| <i>ODA5</i>  | this study        | (Kamiya, 1988)   |
| <i>ODA6</i>  | IC2               | (Kamiya, 1988; Mitchell and Kang, 1991)                              |
| <i>ODA7</i>  | ?                 | (Kamiya, 1988)   |
| <i>ODA8</i>  | ?                 | (Kamiya, 1988)   |
| <i>ODA9</i>  | IC1               | (Kamiya, 1988; Wilkerson et al., 1995)                               |
| <i>ODA10</i> | ?                 | (Kamiya, 1988)   |
| <i>ODA11</i> | $\alpha$ DHC      | (Sakakibara et al., 1991)  |
| <i>ODA12</i> | LC2 (Tctex2)      | (Koutoulis et al., 1997; Pazour et al., 1999b)                       |
| <i>ODA13</i> | LC6 (LC8 homolog) | (King and Patel-King, 1995b; Pazour and Witman, 2000)                |
| <i>ODA14</i> | DC3               | (Casey et al., 2003a)  |
| <i>ODA15</i> | LC7               | (Bowman et al., 1999; Pazour and Witman, 2000)                       |
| <i>ODA16</i> | ?                 | (Ahmed and Mitchell, 2003)   |

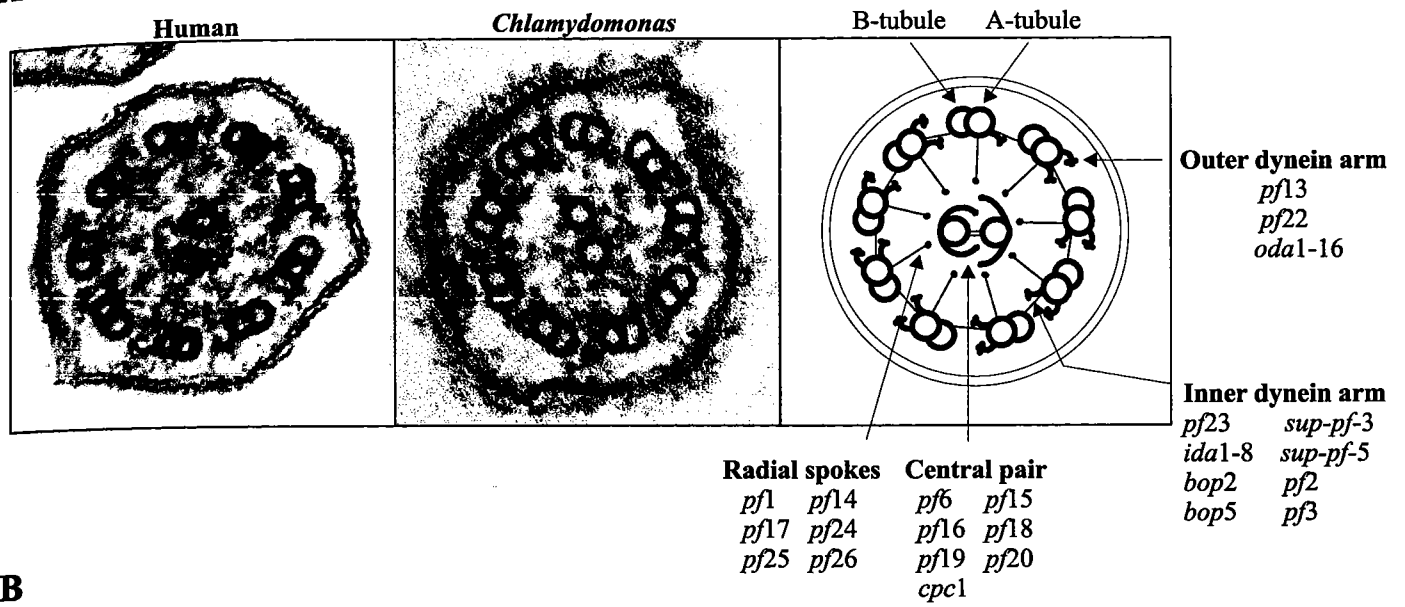
**Table II: Mutations affecting outer dynein arm assembly<sup>1</sup>**

| Gene               | Protein      | Mutant Phenotype   | Reference  |
|--------------------|--------------|--|--|
| <i>ODA1</i>        | DC2          | required for arm assembly  | (Kamiya, 1988; Takada <i>et al.</i> , 2002)                                  |
| <i>ODA2 (PF28)</i> | $\gamma$ DHC | required for arm assembly  | (Kamiya, 1988; Mitchell and Rosenbaum, 1985; Wilkerson <i>et al.</i> , 1994) |
| <i>ODA3</i>        | DC1          | required for arm assembly  | (Kamiya, 1988; Koutoulis <i>et al.</i> , 1997)                               |
| <i>ODA4</i>        | $\beta$ DHC  | required for arm assembly  | (Kamiya, 1988; Mitchell and Brown, 1994; Sakakibara <i>et al.</i> , 1993)    |
| <i>ODA5</i>        | this study   | required for arm assembly  | (Kamiya, 1988)   |
| <i>ODA6</i>        | IC2          | required for arm assembly  | (Kamiya, 1988; Mitchell and Kang, 1991)                                      |
| <i>ODA7</i>        | ?            | required for arm assembly  | (Kamiya, 1988)   |
| <i>ODA8</i>        | ?            | required for arm assembly  | (Kamiya, 1988)   |
| <i>ODA9</i>        | IC1          | required for arm assembly  | (Kamiya, 1988; Wilkerson <i>et al.</i> , 1995)                               |
| <i>ODA10</i>       | ?            | required for arm assembly  | (Kamiya, 1988)   |
| <i>ODA11</i>       | $\alpha$ DHC | not required for arm assembly, except for LC5                        | (Sakakibara <i>et al.</i> , 1991)  |
| <i>ODA12</i>       | LC2 (Tctex2) | required for arm assembly  | (Koutoulis <i>et al.</i> , 1997; Pazour <i>et al.</i> , 1999b)               |
| <i>ODA13</i>       | LC6          | not required for assembly<br>minor swimming defect                   | (King and Patel-King, 1995b; Pazour and Witman, 2000)                        |
| <i>ODA14</i>       | DC3          | partial arm assembly   | (Casey <i>et al.</i> , 2003a; Koutoulis <i>et al.</i> , 1997)                |
| <i>ODA15</i>       | LC7          | required for arm assembly  | (Bowman <i>et al.</i> , 1999; Pazour and Witman, 2000)                       |
| <i>ODA16</i>       | ?            | partial arm assembly   | (Ahmed and Mitchell, 2003)   |
| <i>FLA14</i>       | LC8          | required for arm assembly,<br>short flagella, not outer arm specific | (Pazour <i>et al.</i> , 1998)  |
| <i>PF13</i>        | ?            | required for arm assembly,<br>short flagella                         | (Huang <i>et al.</i> , 1979)   |
| <i>PF22</i>        | ?            | required for arm assembly,<br>short flagella, not outer arm specific | (Huang <i>et al.</i> , 1979)   |

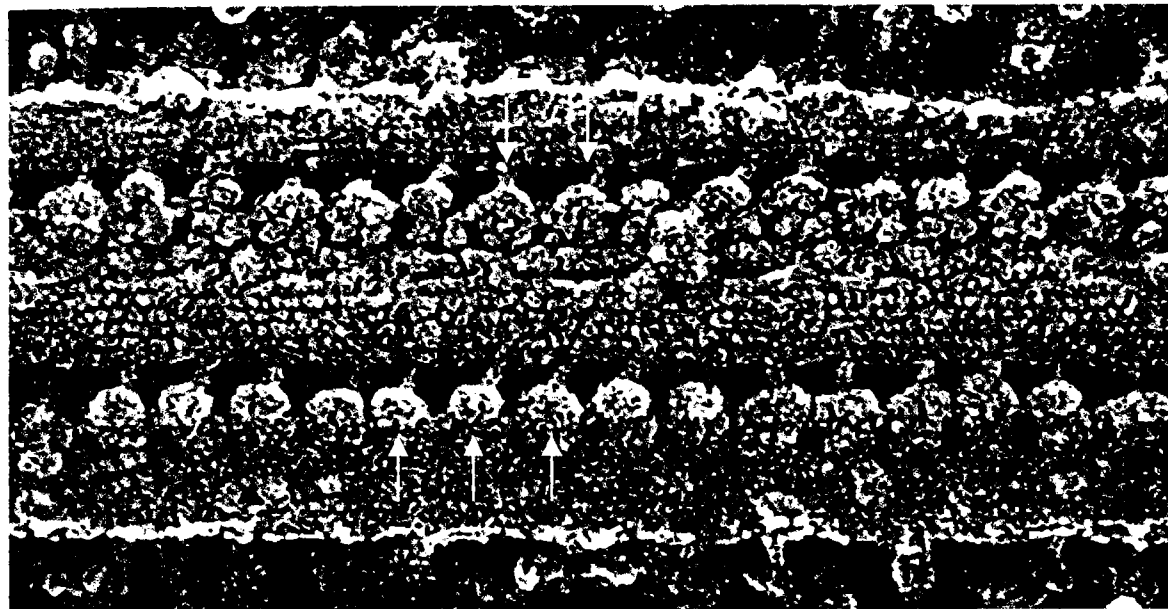
<sup>1</sup>Table II based on Table I in Dibella and King 2001).

**Figure 1: Electron micrographs of a human cilium and a *Chlamydomonas* flagellum.** (A) Cross section EM micrographs through a human cilium(left) and *Chlamydomonas* flagellum (middle) showing the highly conserved axonemal structure (Pennarun *et al.*, 1999 and Witman, unpublished). The schematic (right) labels the structures and shows mutations affecting components of each structure. The axonemal dyneins (outer and inner dynein arms) attach to the A-tubule of the outer doublet microtubule pair. The radial spoke system extends from the outer doublets towards the central pair apparatus, which resides within the center of the axoneme. (B) Longitudinal section of a *Chlamydomonas* axoneme prepared by rapid freeze-deep etch showing the 24-nm periodicity of the outer dynein arms (Hoops and Witman, unpublished).

**A**



**B**



**Figure 1**

**Figure 2: Molecular model for the outer dynein arm and ODA-DC in**

*Chlamydomonas*.  $\alpha$ ,  $\beta$ , and  $\gamma$  denote the three dynein heavy chains; IC1 and IC2 depict the intermediate chains; numbers indicate the light chain subunits (LC1-8). The dynein complex attaches to the A-tubule of the microtubule doublet (gray). This attachment is mediated by a structure termed the Outer Dynein Arm-Docking Complex (ODA-DC), which contains the DC1, DC2, and DC3 subunits.

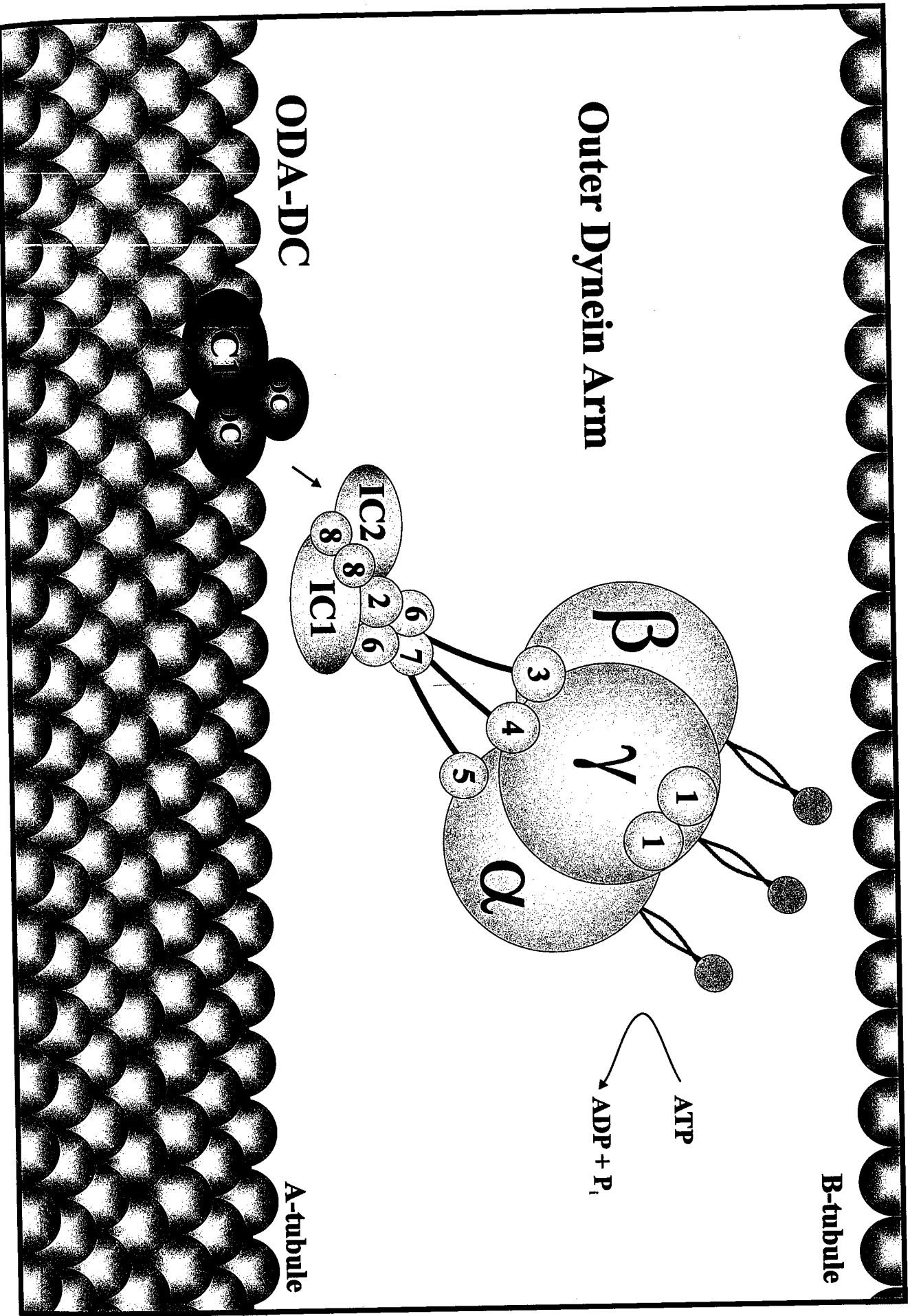
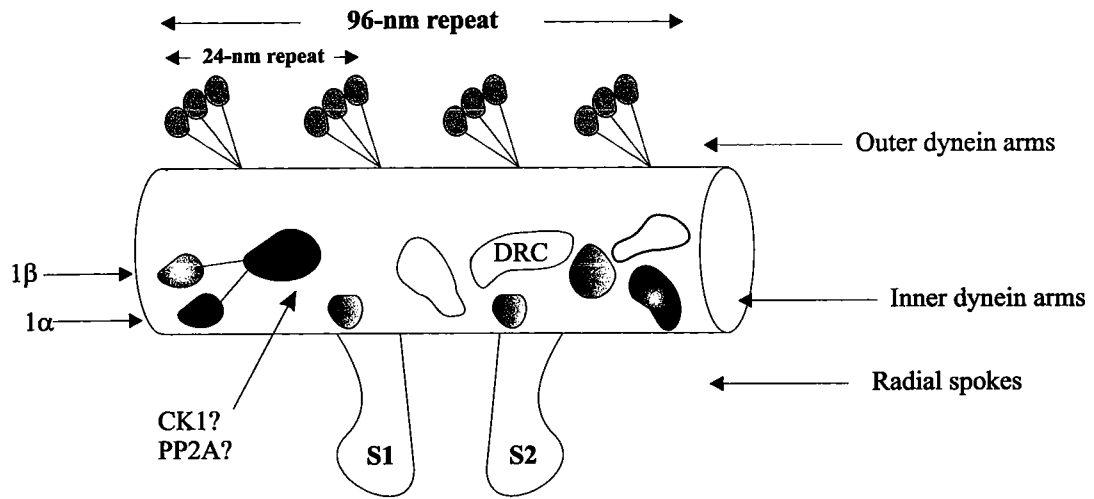


Figure 2

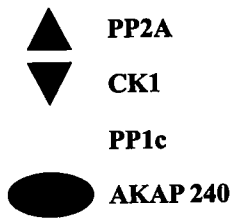
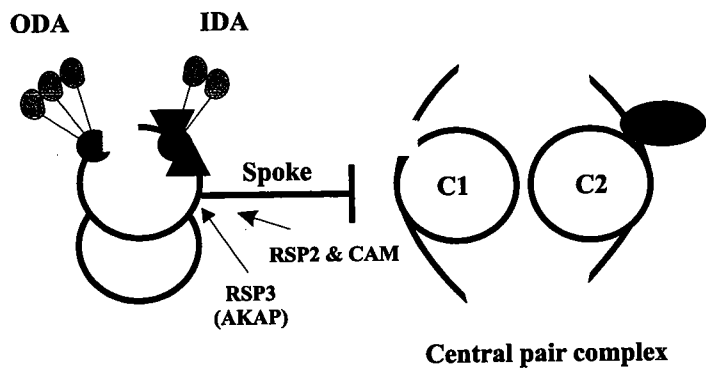
**Figure 3: Schematic of the axonemal A-tubule.** The outer dynein arms repeat every 24-nm, while the inner arm system repeats every 96-nm. Light grey densities depict p28-associated dyneins and the dark grey density depicts a centrin-associated dynein. The putative locations of casein kinase 1 (CK1) and protein phosphatase 2A (PP2A) are marked near I1. The dynein regulatory complex (DRC) is a crescent shaped structure located above the second radial spoke (S2) within the triplet 96-nm inner dynein arm repeat pattern and is thought to regulate dynein activity (modified from Porter and Sale, 2002 *Journal of Cell Biology* 151(5): F37-F42).



**Figure 3**

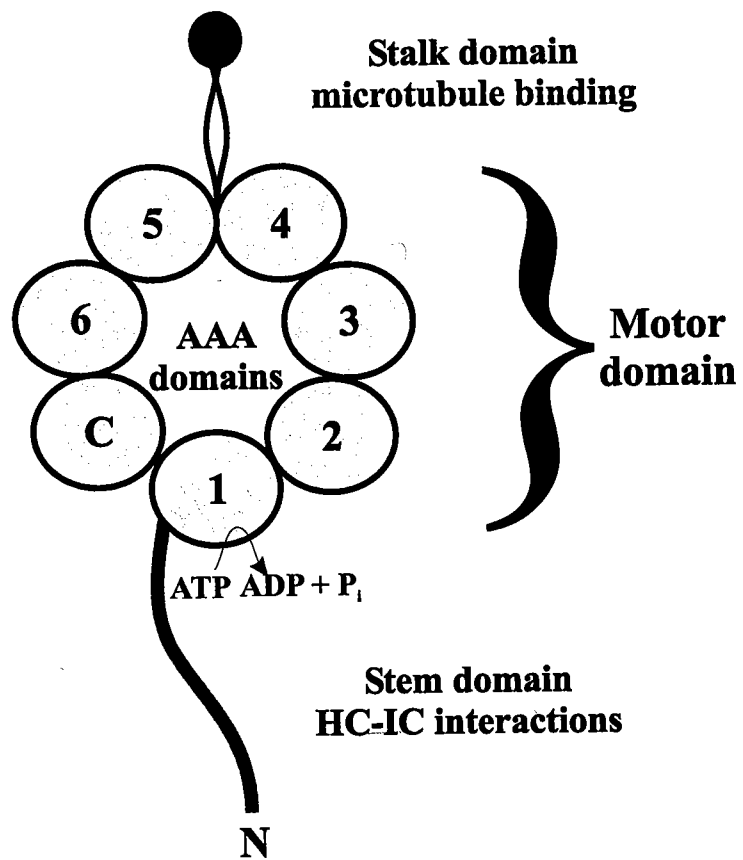


**Figure 4: Schematic of axonemally bound signalling components.** A regulatory pathway extending from the radial spokes to the dynein arms includes components such as casein kinase I (CK1) and protein phosphatase 1c (PP1c) and 2A (PP2A), which have been localized to the central pair and outer dynein arms. Protein kinase A is anchored within the axoneme by axonemal AKAPs (A-kinase anchoring proteins) located in the radial spoke and central pair systems. Calmodulin (CAM) also associates with the radial spokes (from Figure 2A in Porter and Sale, 2002 *Journal of Cell Biology* 151(5): F37-F42).



**Figure 4**

**Figure 5: Model of the dynein heavy chain domains.** The dynein HCs contain three domains. The N-terminal stem domain, the site of HC-IC interactions, is followed by a large globular head which contains the motor domain with its 6 AAA motifs. The P-loop within AAA1 is the site of ATP hydrolysis. The stalk domain protrudes from the globular head and contains the microtubule-binding domain (from Harrison *et al*, *Cell Motility & the Cytoskeleton*. 52: 131-143 2002). N and C represent the N-terminus and C-terminus of the heavy chain respectively.



**Figure 5**

## CHAPTER II

### ISOLATION OF THE *ODA5* GENE AND RESCUE OF THE *oda5-2* INSERTIONAL MUTANT

#### INTRODUCTION

*Oda5* was originally identified as an outer arm assembly mutant in a screen for slow-swimming phenotypes (Kamiya, 1988). In that study, 10 independent genetic loci were identified and designated *oda1-10*. These 10 loci are distinct from the *pf13* and *pf22* loci, which were previously identified (Huang *et al.*, 1979). These results indicated at least 12 genes are responsible for assembly of the outer dynein arm. Since then, 6 additional *oda* mutations have been identified: *oda11-16* (Table I and II) (Ahmed and Mitchell, 2003; Bowman *et al.*, 1999; Casey *et al.*, 2003a; King and Patel-King, 1995b; Koutoulis *et al.*, 1997; Pazour *et al.*, 1999b; Pazour and Witman, 2000; Sakakibara *et al.*, 1991).

When gametes of opposite mating type fuse, they form quadriflagellate cells (temporary dikaryons) which swim for 2-3 hrs, at which point the flagella resorb. Complementation in temporary dikaryons can be assessed by analyzing the flagellar beat frequency of the resulting quadriflagellate cells ~2 hrs after mating. When the beat frequency of *oda* zygotes was measured, certain combinations of *oda* mutants did not result in rescued motility (Kamiya, 1988). *Oda* mutants fall into one of three classes,

depending on their inability to complement *oda1* (*oda1* or *oda3*), *oda2* (*oda2*, *oda4*, *oda6*, *oda7*, and *oda9*) or *oda5* (*oda5*, *oda8*, and *oda10*). Zygotes within each class fail to undergo dikaryon rescue, although they are clearly competent to rescue when crossed with alternative *oda* mutants (Table III). The significance of this result was not clearly understood at the time. It was speculated that some *oda* mutants produce aberrant outer arm complexes, which are incapable of assembling even when a complete set of outer arm components are present in the dikaryon cell. An alternative hypothesis is that this phenomenon is a consequence of protein instability, such that in the absence of one component of a protein complex, the remaining subunits are unstable and no partial complex is formed. Both are attractive models, because in the quadriflagellate cell the flagella are already formed and complementation does not require the synthesis of new proteins (Luck *et al.*, 1982), suggesting that the interacting proteins must be present and available in the individual *oda* cell in order to rescue to occur.

As stated previously, all of the known components of the outer dynein arm and the ODA-DC have been identified (King, Pazour and Witman, unpublished data). Disruption of most of these genes results in a failure to assemble the outer arm structure. Yet, there are additional *oda* mutants whose gene products have not been determined, namely *oda5*, *oda7*, *oda8*, and *oda10*. These genes are clearly required for assembly of the outer arm, yet their proteins do not comprise known subunits of the outer arm complexes. It is possible that Oda7p encodes a previously unidentified dynein subunit, as it is unable to complement *oda2* ( $\gamma$ DHC), *oda4* ( $\beta$ DHC), and *oda6* (IC2) in dikaryons.

How these genes participate in outer arm assembly can only be understood when the gene products of these loci are known.

The overall goal of this thesis was to identify the gene product of one of these uncloned *oda* mutants. The Oda5, 8, and 10 proteins are of particular interest because they have been hypothesized to interact in a protein complex based on non-complementation in dikaryons. If these gene products truly interact, then identification of one member of this protein complex would be a significant step towards understanding the molecular nature of this complex, and may ultimately lead to the identification of the remaining subunits. The hypotheses of protein complex instability or mutant complexes in these mutants provides the basis for testable models of how these proteins participate in the outer dynein arm assembly pathway.

## RESULTS

### Identification of the *oda5-2* Insertional Mutant

To obtain insertional mutants with defects in motility, strain CC-2454 was transformed with plasmid pMN24 (Fernandez *et al.*, 1989)<sup>2</sup>. This strain was chosen for its ease in transformation as it has the *cw15* mutation, conferring a cell wall-less defect, and the *nit1-305* mutation, a low reverting mutant in the *Chlamydomonas* nitrate reductase gene. *NIT1* is the first enzyme in the pathway for nitrate utilization and is required for cell

---

<sup>2</sup>Insertional mutagenesis was done by our collaborators Akinori Yoda, Masafumi Hirono, and Ritsu Kamiya.

growth when nitrate is the sole nitrogen source. Nit1- mutant cells require a reduced nitrogen source (such as ammonium) for growth. The *NIT1* mRNA is repressed when cells are grown in the presence of ammonium (Fernandez *et al.*, 1989). Plasmid pMN24 contains a 14.5-kb insert bearing a wild-type copy of the *Chlamydomonas NIT1* gene. Insertion of the transforming pMN24 DNA occurs essentially at random, generating disruptions in genes at the site of insertion. Transformants positive for the Nit1+ phenotype were selected and were analyzed by light microscopy for defects in motility. *Oda*- phenotypes were distinguished from other motility mutants by their distinct swimming behavior. *Oda* mutants swim with a slow, jerky motion that is characteristic of loss of the outer dynein arm structure and is clearly distinguishable by light microscopy from motility defects of the inner arm, central pair or radial spoke systems. To determine the nature of the *oda* mutants identified, genetic crosses were performed between these transformants and known *oda* alleles<sup>3</sup>. One transformant, 45B03, was identified as an insertional allele of *oda5*. 45B03 was crossed with the original *oda5-1* mutant (Kamiya, 1988). Tetrads were dissected and no progeny showed wild-type motility (PD:NPD:T = 37:0:0). In addition, temporary dikaryons between *oda5-1* and 45B03 did not undergo any increase in motility during two hours of mating<sup>3</sup>. Thus it is concluded that 45B03 is defective in the *ODA5* gene (allelism determined by Ritsu Kamiya). Loss of the outer arm was confirmed by electron microscopy. To be consistent with standard genetic nomenclature in *Chlamydomonas*, the 45B03 mutation

---

<sup>3</sup>Crosses to determine allelism and temporary dikaryon analysis were done by Ritsu Kamiya.



hereafter will be referred to as *oda5-2*, and the original *oda5* mutant will be designated *oda5-1*.

### Identification of Endogenous DNA Flanking the Site of Insertion

*Chlamydomonas* represents the first photosynthetic organism in which both the chloroplast and nuclear genomes can be transformed (Kindle *et al.*, 1989) and is the only organism in which all three genomes, chloroplast, nuclear and mitochondrial, can be transformed (Lefebvre and Silflow, 1999). Insertional mutagenesis of the nuclear genome has provided a tremendous advantage for cloning genes in *Chlamydomonas* because the inserted DNA can be used to tag the disrupted gene. Usually, one identifies sequence flanking the site of inserted vector sequences. Once flanking sequence is obtained, it can be used to identify wild-type clones overlapping the disrupted gene.

Since no vector sequences stably integrated in the *oda5-2* genome (Figure 6A), DNA flanking the integrated *NIT1* selectable marker was obtained. There are two copies of the *NIT1* gene present in *oda5-2*: the *nit1-305* endogenous gene and the inserted *NIT1* gene. By performing Southern blots with a probe to the 3' end of the *NIT1* gene, we identified a 6-kb restriction fragment length polymorphism (RFLP) present in *oda5-2* (Figure 6B, arrow). The lower bands, detected by the *NIT1* probe, are due to the endogenous *nit1-305* gene present in both the wild-type and *oda5-2* strains. Sequence analysis of the mutant RFLP fragment demonstrated it was a hybrid fragment containing both *NIT1* and unknown endogenous DNA.

To ascertain if the unknown sequences were truly flanking the *NIT1* insertion in *oda5-2*, a small probe was generated to the unknown region and used to probe Southern blots of genomic DNA. The probe, 36.1, recognized an RFLP between wild type and the *oda5-2* mutant (Figure 6C). In wild type, the probe hybridized to an 8-kb *SacII* fragment, while it recognized the 6-kb *SacII* fragment in the *oda5-2* strain. The *oda5-2* strain was backcrossed with a wild-type strain and the progeny analyzed for both motility and the presence of the RFLPs. In all cases, the *NIT1* RFLP and the 36.1 RFLP segregated with the Oda- phenotype, confirming that the *NIT1* insertion and its associated flanking sequence are tightly linked to the *oda5-2* mutation (Figure 6D and E). These results confirmed that the 36.1 probe flanks the site of the *NIT1* insertion that is disrupting the *ODA5* gene.

#### **Isolation of the *ODA5* Gene and Rescue of the *oda5-2* Mutant**

The DNA flanking the integrated selectable marker in the *oda5-2* strain was used as a probe to identify four BACs (18g18, 31d14, 39c13, 27k02) containing that DNA. Restriction mapping indicated these BAC clones had overlapping inserts (data not shown).

To test whether these BAC clones contained the *ODA5* gene, I transformed *oda5-2* with the BACs and screened for rescue of the Oda- phenotype. One BAC clone (18g18) rescued the motility defect in *oda5-2*, suggesting it contained the *ODA5* gene. This BAC contained an insert of ~40-kb. In order to delimit the *ODA5* gene within this BAC, I subcloned smaller fragments from this insert and tested them for rescue. The

smallest rescuing genomic fragment, 50.1, is 6.1-kb in size (Figure 7A) and rescued 22 out of 72 colonies that had been co-transformed with the *ARG7* selectable marker.

Motility assays of wild type, *oda5-2*, and a strain rescued with the 50.1 genomic fragment revealed that both swimming speed and flagellar beat frequency were restored to near wild-type levels in the rescued strain (Figure 7B)<sup>4</sup>. The swimming speed of the rescued strain ( $133 \pm 24 \mu\text{m/s}$ ) was slightly slower than that of wild type ( $172 \pm 13 \mu\text{m/s}$ ), but clearly rescued as compared to the *oda5-2* mutant ( $55 \pm 9 \mu\text{m/s}$ ). The flagellar beat frequency was restored completely (61 Hz for wild type vs. 60 Hz for the rescued strain). To further verify the rescued strains, I performed Southern blot analysis using a probe to the rescuing fragment. In wild type, the probe hybridized to a band of the expected size, whereas in the *oda5-2* strain, the probe did not hybridize to any band, indicating this region is deleted in *oda5-2* (Figure 7C). The probe also hybridized to bands in the rescued strains, demonstrating that the deleted sequences have been restored (Figure 7C). Probes to either end of the 9-kb rescuing fragment (Figure 7A) indicate this entire region is deleted in the mutant (data not shown).

Electron microscopy demonstrated that the outer arms are missing in *oda5-2* and restored in the rescued strains. Figure 8 shows cross sections through the flagella of wild type, the *oda5-2* insertional mutant, and one of the rescued strains. Wild type and material was occasionally observed at the site normally occupied by the outer arm

---

<sup>4</sup>Swimming speed and beat frequency determined by Toshiki Yagi and Ritsu Kamiya.

(Figure 8D). These data demonstrate that the 50.1 rescuing fragment contains the *ODA5* gene.

## DISCUSSION

A 6.1-kb genomic fragment, which rescues the motility defect in *oda5*, was isolated by identifying a mutant *NIT1* RFLP in *oda5-2*. Rescue of the *Oda5*- motility phenotype by restoration of outer dynein arm assembly provides strong evidence that the transforming *Chlamydomonas* genomic DNA contains a wild-type, functional *ODA5* gene. Isolation of this genomic region is a crucial advance towards identifying the *ODA5* gene.

Since the *oda5-2* allele was not tagged by the transforming pUC119 vector sequences, I adapted the standard approach for cloning genes from insertional mutants by isolating RFLPs identifying the inserted copy of the *NIT1* selectable marker gene. The *NIT1* probe identified a 6-kb mutant RFLP in *Sac*II digested genomic DNA from the *oda5-2* strain.

Upon cloning this RFLP fragment, the genomic construct was sequenced and determined to be a chimeric fragment containing both *NIT1* and endogenous sequence adjacent to the *NIT1* insertion. Both the *NIT1* insertion and the endogenous flanking sequence are tightly linked to the *oda5-2* mutation demonstrating that the *NIT1* insertion is disrupting the *ODA5* gene.

The Oda- motility phenotype is directly due to an absence of outer dynein arms. Thus the rescued strains were analyzed by electron microscopy which verified that the outer arms were restored, accounting for the observed rescue in motility. Analysis of both swimming speed and flagellar beat frequency demonstrated that the motility was restored to near wild-type levels. The swimming speed of the rescued strain, while clearly faster than the *oda5-2* strain, is slightly slower than that of wild-type. It is unknown how the swimming speed is somewhat slower than wild type, yet the beat frequency is completely rescued, although a similar phenomenon was observed with *oda14* (Casey *et al.*, 2003a). These results demonstrate that the modified approach to cloning the *ODA5* gene by identifying *NIT1* RFLPs was successful. This modified approach can be adapted to any insertional mutant in which the vector sequences have not stably integrated.

## MATERIALS AND METHODS

### Strains and Culture Conditions

*Chlamydomonas reinhardtii* strains used in this study include: CC-2454 (*cw15*, *nit1-305*, *mt-*), CC-48 (*arg2*, *mt+*), CC124 (*nit1-137*, *nit2-137*, *mt-*), and 137C (*nit1-137*, *nit2-137*, *mt+*), are from the *Chlamydomonas* Genetics Center (Department of Biology, Duke University, Durham, NC). V87.2 (*oda10-2*, *nit1:NIT1*, *NIT2*, *agg1*, *mt-*) (Koutoulis *et al.*, 1997), *oda5-1* (*oda5-1*, *mt+*), 45B03 (*oda5-2*, *cw15*, *mt-*), an insertional allele of *oda5* (provided by R. Kamiya). The *ODA5* allele was confirmed by crossing 45B03

with the *oda5-1* strain (Kamiya, 1988). The insertional mutagenesis experiments to generate the *oda5-2* allele, and all crosses to determine allelism, were performed by our collaborators Akinori Yoda, Masafumi Hirono and Ritsu Kamiya.

*Oda5-2* was crossed to 137C to create strains 88b (*oda5-2, mt-*). 88b was crossed to CC-48 to create strain 112b (*oda5-2, arg2, mt-*). 112b.76, 150, 219, and 220 (*oda5-2: ODA5, arg2, mt-*) were created by transformation of the *ODA5* gene into strain 112b.

*Chlamydomonas* cells were grown in a 14:10 light:dark cycle in the following media: Medium I of Sager and Granick (1953) modified to contain three times the original amount of phosphate (Witman, 1986); R-medium (medium I supplemented with 0.1% sodium acetate); R+Arg (R-medium supplemented with 50 µg/ml arginine); M-N (medium I without nitrogen); TAP (Harris, 1989); TAP+Arg (TAP-medium supplemented with 50 µg/ml arginine); and SGII/NO<sub>3</sub>, (medium II of Sager and Granick [1953] modified to contain 0.003 M KNO<sub>3</sub> as the nitrogen source)

### **Transformations and Insertional Mutagenesis**

All transformations were done using the glass bead method as described previously (Kindle *et al.*, 1989; Koutoulis *et al.*, 1997). The insertional mutant, *oda5-2*, was generated by transforming CC-2454 cells with plasmid pMN24 (Fernandez *et al.*, 1989) containing the *Chlamydomonas NIT1* gene. Transformants positive for *NIT1* were selected on SGII/NO<sub>3</sub> media. Motility mutants were identified by growing positive transformants in liquid culture and screening by light microscopy. Co-transformations

were performed using *ODA5* genomic constructs and p*ARG* 7.8 plasmid (Debuchy *et al.*, 1989); transformants were selected on TAP plates.

### Isolation of the *ODA5* Gene and Rescue of the Oda5- Motility Defect

To obtain sequences flanking the site of the pMN24 insertion in *oda5-2*, genomic Southern blots of wild type and *oda5-2* were screened with a 5-kb probe containing sequences to the 3' end of the *NIT1* gene (Fernandez *et al.*, 1989). A 6-kb RFLP was identified in *SacII*-digested *oda5-2* genomic DNA. 5 to 7-kb genomic fragments from *SacII*-digested *oda5-2* DNA were isolated and subcloned into pSE280 (Invitrogen, Carlsbad, CA). Colony lifts were screened with the *NIT1* probe and one positive colony identified. This plasmid, p36.1, was sequenced and determined to contain both *NIT1* and unknown sequences. A small region of the unknown sequence was amplified by PCR and used to probe Southern blots of genomic DNA from wild type, *oda5-2*, and their meiotic progeny. The 36.1 probe identified a 6-kb *SacII* RFLP that segregated with the Oda5- phenotype.

The 36.1 probe was subsequently used to identify wild-type BAC clones (Clemson University Genome Institute, SC) containing the endogenous sequence. The BAC clones were tested for their ability to rescue the Oda5- phenotype by co-transforming strain 112b with the BAC clones and plasmid p*ARG*7.8 (Debuchy *et al.*, 1989). Transformants were scored for Oda $\pm$  phenotype by light microscopy. The rescuing fragments, 50.1 and 39.2, from one BAC clone were sequenced.

### Isolation and Blotting of Nucleic Acids

*Chlamydomonas* genomic DNA isolations were performed as described previously (Koutoulis *et al.*, 1997). Genomic DNA was separated on 0.8% agarose gels and transferred to Duralon-UV (Stratagene, La Jolla, CA). DNA was cross-linked to the membranes using a Stratalinker at 1200  $\mu$ J (Stratagene). Blots were hybridized in hybridization solution (7% SDS, 1 mM EDTA, 0.25 M sodium monophosphate, pH 7.2) at 65°C overnight, then washed with two changes of 2x SSC, 0.1% SDS (0.3M NaCl, 0.24M sodium citrate, 0.1% SDS), followed by one wash in 0.2x SSC, 0.01% SDS. All washes were done at 65°C for 30 minutes. Hybridization probes were generated by random prime labeling using the Prime-It II kit (Stratagene).

### Colony Lifts

Colony lifts were performed by overlaying bacterial plates with an 80-mm nitrocellulose filter (Gelman Sciences-Pall Corporation, Ann Arbor, MI). Bacterial colonies were allowed to transfer to the nitrocellulose membrane for ~5'. Bacterial cells were lysed, and DNA denatured by a series of washes with 10% SDS, denature buffer (0.5M NaOH, 1.5 M NaCl), renature buffer (1.5 M NaCl, 0.5M Tris), and 2x SSC. Filters were air dried briefly, then crosslinked in a Stratalinker (Stratagene). Filters were incubated in hybridization solution overnight with at least one change of solution, then hybridization probes were added in a fresh aliquot of hybridization solution. Hybridization and washing were performed as described above.



### **Genetic Analysis**

Matings and tetrad analysis were performed according to standard procedures (Dutcher, 1995; Harris, 1989). Meiotic progeny were separated using a glass needle on a micromanipulator (Newport Corp, Irvine, CA). Progeny were scored for motility (Oda +/-) by light microscopy and for Arg +/- by comparing growth on TAP and TAP+Arg plates. Progeny were scored for segregation of the *NIT1/36.1* RFLPs by Southern blot as described above.

### **Motion Analysis**

Swimming speed was determined as described in Kamiya (1988). Briefly, motile cells were imaged by dark-field microscopy and recorded using a CCD camera and VCR. Video images were played on a computer and individual cell positions were tracked as a function of time using Image Tracker PTV software (InterQuest, Osaka, Japan). The swimming velocity of 30 cells was used to determine the average swimming speed for each strain analyzed. Flagellar beat frequency was determined as described previously (Kamiya, 2000). All motion analysis experiments were performed by Toshiki Yagi and Ritsu Kamiya.

### **Electron Microscopy**

Whole cells and isolated axonemes were processed as described previously (Hoops and Witman, 1983). Samples were embedded in a mixture of LX112/Araldite 502 epoxy resin and sectioned at 50-70-nm. All electron microscopy was done at the University of

Massachusetts Medical School Diabetes and Endocrinology Research Center (DERC)

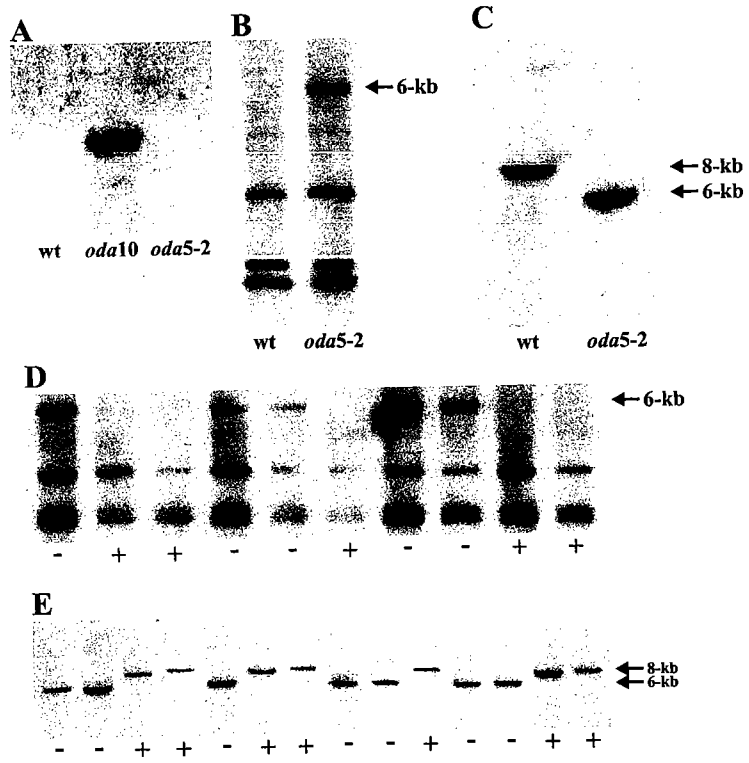
EM Core Facility.

**Table III: Flagellar beat frequency in temporary *oda*-mutant dikaryons<sup>5</sup>**

|              | <i>oda1</i> | <i>oda2</i> | <i>oda3</i> | <i>oda4</i> | <i>oda5</i> | <i>oda6</i> | <i>oda7</i> | <i>oda8</i> | <i>oda9</i> | <i>oda10</i> | <i>pf13a</i> |
|--------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|--------------|--------------|
| <i>oda1</i>  | 24          |             |             |             |             |             |             |             |             |              |              |
| <i>oda2</i>  | 45          | 23          |             |             |             |             |             |             |             |              |              |
| <i>oda3</i>  | 28          | 39          | 23          |             |             |             |             |             |             |              |              |
| <i>oda4</i>  | 45          | 31          | 40          | 23          |             |             |             |             |             |              |              |
| <i>oda5</i>  | 40          | 41          | 40          | 47          | 24          |             |             |             |             |              |              |
| <i>oda6</i>  | 41          | 28          | 40          | 35          | 47          | 24          |             |             |             |              |              |
| <i>oda7</i>  | 43          | 20          | 40          | 29          | 42          | 35          | 25          |             |             |              |              |
| <i>oda8</i>  | 40          | 44          | 44          | 46          | 33          | 43          | 42          | 24          |             |              |              |
| <i>oda9</i>  | 50          | 27          | 49          | 44          | 42          | 26          | 37          | 38          | 24          |              |              |
| <i>oda10</i> | 38          | 42          | 39          | 39          | 24          | 39          | 45          | 26          | 46          | 24           |              |
| <i>pf13a</i> | 38          | 25          | 39          | 26          | 41          | 30          | 29          | 42          | 33          | 36           | --           |
| <i>pf22</i>  | 42          | 26          | 42          | 24          | 40          | 26          | 34          | 41          | 36          | 32           | 19           |

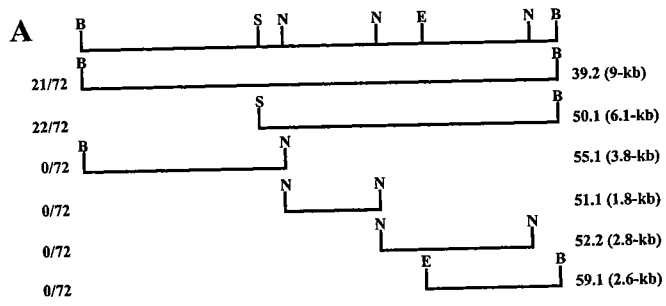
<sup>5</sup>Table III from (Kamiya, 1988).

**Figure 6: Identification of endogenous, genomic DNA sequence at the site of the *oda5-2* mutation.** (A) A Southern blot of genomic DNA isolated from a wild-type strain, an *oda10-2* insertional mutant (Koutoulis *et al.*, 1997) and the *oda5-2* insertional mutant. The blot was probed with pUC119. The *oda5-2* DNA lacks hybridizing bands, indicating the vector sequences were not maintained in the insertion. As expected, wild-type DNA does not contain any hybridizing bands, whereas *oda10*, a pUC-positive insertional mutant, does contain DNA that hybridizes with the vector probe. (B) A Southern blot utilizing a probe to the 3'-end of the *Chlamydomonas NIT1* gene identified a 6-kb genomic *SacII* fragment (arrow) in *oda5-2* but not wild-type DNA. (C) A Southern blot using a probe, 36.1, to endogenous sequence derived from the mutant RFLP fragment identifies the 6-kb *SacII* genomic fragment in *oda5-2*, whereas it hybridizes to an 8-kb genomic fragment in wild-type DNA. (D) and (E) *Oda5-2* was crossed to a wild-type strain and 10 of the resulting progeny were analyzed for motility and presence of the RFLPs identified by *NIT1* (D) and 36.1 (E). Both RFLPs segregate with the Oda- motility phenotype (+ designates wild-type and - designates Oda- phenotype).



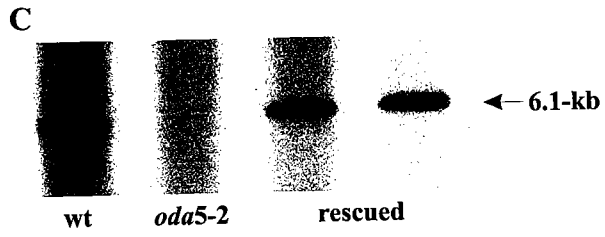
**Figure 6**

**Figure 7: The *ODA5* gene rescues the *Oda5*- motility phenotype and the transforming DNA is recovered in the *oda5-2* rescued strains.** (A) The restriction map for the relevant portion of the rescuing BAC (N, *Nco*I; B, *Bam*H1; E, *Eco*R1; S, *Sal*I). Subclones from the rescuing BAC were tested for their ability to rescue the *Oda5*- motility phenotype. The left column indicates the number of rescued transformants/total number of co-transformants screened. The right column indicates the construct name and the size of the genomic fragment. The smallest rescuing fragment a 6.1-kb *Sal*I-*Bam*H1 fragment (50.1). (B) Motility assays were performed on wild type, *oda5-2*, and one of the rescued strains (*oda5-2* rescued). Both swimming speed and flagellar beat frequency were rescued to near wild-type levels in the rescued strain (n=30 for each strain). (C) Southern blots of *Sal*I/*Bam*H1-digested DNA from wild type, *oda5-2*, and two strains rescued by transformation of *oda5-2* were probed with the transforming DNA. Wild-type DNA contains hybridizing sequences from the *ODA5* region. *Oda5-2* does not contain hybridizing sequences as these regions are deleted. The two rescued strains contain hybridizing sequences, demonstrating that these sequences have been recovered.



**B**

|                       | Swimming speed  | Beat frequency |
|-----------------------|-----------------|----------------|
| wild-type             | 172 ± 13 μm/sec | 61 Hz          |
| <i>oda5-2</i>         | 55 ± 9 μm/sec   | 24 Hz          |
| <i>oda5-2</i> rescued | 133 ± 24 μm/sec | 60 Hz          |



**Figure 7**

**Figure 8: The *oda5-2* insertional mutant lacks outer dynein arms and the rescued strains have restored outer dynein arms.** Electron micrographs of axonemal cross sections from (A) wild type, (B) an *oda5-2* insertional mutant rescued with the 6.1-kb fragment containing the *ODA5* gene (*oda5-2* rescued), and (C and D) the *oda5-2* insertional mutant. Arrows indicate the outer dynein arms in the wild-type and rescued flagella, but the absence of outer arms in the *oda5-2* flagella. In occasional micrographs, some outer arms, or partial arms, were observed in *oda5-2* (arrowhead in D). Scale bar is 100 nm.



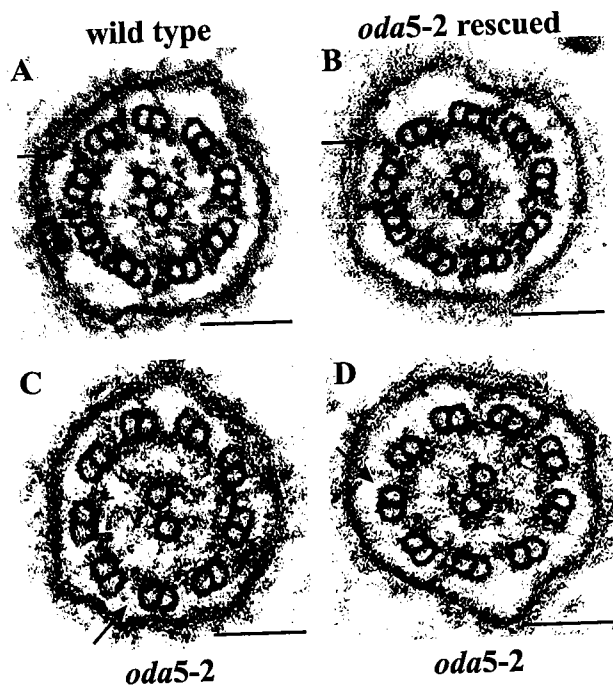


Figure 8

## CHAPTER III

### IDENTIFICATION OF THE *CHLAMYDOMONAS ODA5* GENE AND ITS CORRESPONDING cDNA

#### INTRODUCTION

Estimates of the size of the *Chlamydomonas* genome range from 1.0-1.6 x 10<sup>5</sup>-kb (Harris, 1989). There exists an extensive physical map consisting of 17 linkage groups on which numerous molecular markers have been mapped (Silflow, 1998; Silflow *et al.*, 1995). A noticeable feature of the *Chlamydomonas* genome is its unusually high G/C content, ranging on average between 60-70%. The genome is also highly repetitive, containing repetitive elements in the 3' UTR non-coding regions, large tandem repeats, simple sequence repeats, and telomere repeat sequences. A major genomic walk through the mating-type locus revealed a central domain within that locus that contains major translocations, inversions, deletions and duplications, which most likely lead to suppressed recombination in this region (Ferris and Goodenough, 1994).

*Chlamydomonas* exhibits a codon usage bias, due mainly to its high G/C content. This degree of codon bias may partially explain the low levels of expression observed when foreign genes are introduced into *Chlamydomonas*.

Another common feature of *Chlamydomonas* genes is the presence of multiple small introns present at high frequency. On average, there are four introns per kilobase (kb) of coding sequence with the average intron size being 219-bp (Silflow, 1998). Given the number of introns present in *Chlamydomonas* genes, it has been estimated that the total amount of intron sequence is nearly equivalent to the amount of coding sequence. Introns are an important feature of *Chlamydomonas* genes. They are generally necessary for efficient expression of transgenes. A similar effect is observed in *Volvox carteri*, which also has a high frequency of introns. Most *Chlamydomonas* genes contain introns, although some intronless genes have been reported, including core histone genes (Fabry *et al.*, 1995), and more recently the *oda14* gene (Casey *et al.*, 2003a). The 5' and 3' splice junctions of *Chlamydomonas* introns generally conform to the eukaryotic consensus for these sequences. The 5' and 3' consensus sequences are (C/A)(A/C)G/GTG(A/C)G and (G/A)CAG/(G/A) respectively, with the underlined regions being contained within the intron.

Analysis of many *Chlamydomonas* sequences has generated consensus sequences for the translation start and stop codons. A consensus of (A/C) A (A/C) (A/C) ATG (G/C) was obtained for the context of the start codon; and a consensus sequence of (G/C) TAA (G/A) was obtained for the context of the stop codon. TAA is the predominantly used stop codon in *Chlamydomonas*, although TGA and TAG stop codons are also found.

The 3' UTRs of *Chlamydomonas* genes are often several hundred base pairs in length. A putative polyadenylation consensus sequence, TGTAAG, is found in nearly

90% of *Chlamydomonas* gene sequences analyzed. TGTAG and TGTTA are also used to a lesser extent. This sequence is located ~10-20 bp upstream of the site of polyadenylation. This signal differs from the polyadenylation consensus in other eukaryotes (AATAAA).

Given the sequence complexity of the genome, sequencing of *Chlamydomonas* genes is often quite difficult. Sequence reads tend to be much shorter and primer design is often suboptimal due to high GC content and the highly repetitive nature of the genome. Primer walking to sequence through long genomic regions can be arduous and time consuming. Nonetheless, sequencing of *Chlamydomonas* genomic DNA can be optimized using certain combinations of DNA sequencing mixes (Hills *et al.*, 2002).

This chapter focuses on the identification of the *ODA5* gene within the rescuing genomic fragment and determining the corresponding *ODA5* cDNA. The *ODA5* gene is contained in a 6.1-kb genomic fragment that rescues the *oda5-2* motility and outer arm assembly defect. This fragment is large enough to contain one or more *Chlamydomonas* genes. In order to determine the number of genes present in the 50.1 rescuing fragment, the entire genomic fragment was sequenced. Sequencing of the *ODA5* gene is an important milestone in understanding the nature of how this protein functions in outer dynein arm assembly.

## RESULTS

### The *ODA5* Gene Structure

The rescuing genomic fragment was completely sequenced (Figure 9B). GreenGenie is a gene prediction program designed for predicting *Chlamydomonas* genes (Li *et al.*, 2003). This program was used to predict the number of genes contained within the rescuing fragment and their potential exons (Figure 9A). The results predict a single gene with 10 exons and 9 introns that spans the entire length of the 50.1 rescuing fragment. Note that subclones of 50.1 do not rescue the Oda5- phenotype, indicating that the gene is disrupted in these smaller subclones (Fig. 7A, CHAPTER II). The intron-exon boundaries generally conform to the 5' and 3' splice junction consensus sequences described above. Generally, the intron size was relatively small (171 to 539-bp in size) as is expected in *Chlamydomonas*, except for one large intron of 1433-bp (intron 3). This unusually large intron contains four internal canonical 3' splice sites followed by downstream stop codons (Figure 10). Moreover, the *ODA5* cDNA sequence obtained (described below) confirms the predicted intron-exon boundaries in this region, indicating that intron 3 is an unusually large intron in the *ODA5* gene.

The start codon predicted by GreenGenie does not precisely conform to the consensus sequence for start codons [(A/C) A (A/C) (A/C) ATG (G/C)]. The sequence context of the predicted start codon for *ODA5* is GTCGATGG. An ATG found seven codons upstream conforms somewhat better to the consensus than the predicted start codon. Its sequence context is TCCAATGG. There are two in-frame stop codons upstream of both ATG start codons. It is likely that the first ATG is the site of translation initiation, although it was not predicted by Green Genie. GreenGenie often

has trouble predicting the initial exon in *Chlamydomonas* genes (Susan Dutcher, personal communication).

Similarly, the sequence context of the predicted stop codon in *ODA5* does not precisely conform to the consensus sequence for stop codons. The consensus of (G/C) TAA (G/C) was obtained from analysis of 149 cloned sequences (Silflow, 1998), in which the stop codon TAA was used in 70% of the the genes analyzed. Alternative stop codons TGA and TAG were found in 15% of the sequences studied. The sequence context around the predicted stop codon in *ODA5* is GTGAT. Thus it appears that slightly variant sequences are utilized in the start and stop codons for the *ODA5* gene.

A major effort has been undertaken to sequence the *Chlamydomonas* genome. The first draft of the genome sequence was released in January, 2003, in conjunction with the Joint Genome Institute (JGI) and the Department of Energy (DOE). This draft of the *Chlamydomonas* genome was generated using a whole genome shotgun approach. In addition, EST sequencing projects by both Duke University (North Carolina) and Kazusa DNA Research Institute (Japan) have produced an extensive EST database.

Of interest is that the *ODA5* gene is not found in its entirety in the Joint Genome Institute (JGI) *Chlamydomonas* Genome database. The gene sequence appears to bridge two scaffolds (large contigs of genomic sequence). The 5' end of the *ODA5* gene aligns with scaffold 550, while the 3' end of the *ODA5* gene aligns with scaffold 1102 (Figure 9A). Moreover, the sequence from the ends of the rescuing BAC clone (clone 18g18) align with the same scaffolds that align with the *ODA5* gene sequence, confirming that our analysis is correct. Because most of the *ODA5* gene is not present in the genome

sequence, we were unable to verify the EST and gene predictions to this region using the JGI interface. Nonetheless, our analysis with the GreenGenie program reveals there is only one predicted gene in this region. Our data with *ODA5* has allowed the successful integration of these two scaffolds, which will be reflected in the upcoming release of the new version of the *Chlamydomonas* genome database.

### Cloning the *ODA5* cDNA

PCR primers, designed from the predicted coding regions, were used to amplify the *ODA5* cDNA from wild-type *Chlamydomonas* cDNA libraries (Figure 11). Analysis of the cDNA sequence demonstrated that GreenGenie predicted all of the exons correctly, except the initiating exon. The predicted protein sequence for Oda5 begins with the first in-frame ATG codon (Figure 11).

144-bp downstream from the predicted stop codon is a consensus polyadenylation signal sequence (TGTAAG) which is used in the majority of *Chlamydomonas* genes (Silflow, 1998). The genomic sequence of 50.1 extends an additional 27-bp beyond the polyadenylation sequence to a *Bam*H1 site, which demarcates the 3' end of the rescuing fragment. This reveals 144-bp of 3'UTR up to the polyadenylation sequence. The polyA tail is generally 10 to 20-bp downstream of the polyadenylation signal sequence, thus the 3' *Bam*H1 site of 50.1 leaves just enough sequence to allow for proper polyadenylation of the *ODA5* mRNA.

At position 567-572 of the cDNA sequence is a *Sal*I restriction site. This delineates the 5'-boundary of the 50.1 rescuing fragment. Additional 5'UTR sequences

were identified by PCR and EST database searches. The additional 5'UTR sequence matches genomic sequence 5' to the *Sal*I site. This indicates that the 50.1 rescuing fragment does not contain the entire 5'UTR of *ODA5*, but it does contain the entire coding region and 3' polyadenylation signal. The cDNA sequence spans the entire rescuing fragment and confirms the GreenGenie prediction for all of the exons except the initial exon, which was determined empirically.

### **The Deduced Oda5 protein**

The *ODA5* cDNA predicts a 652-amino acid protein (from the first ATG start codon), with a predicted mass of 66,218.41 Daltons and a pI of 5.41 (Figure 11). BLAST reports do not reveal significantly related sequences, suggesting that *ODA5* is a novel protein. BLAST analysis was performed with the filter on, a default parameter that can filter out low complexity sequence. Filtering the query sequence can eliminate statistically significant, but biologically unrelated hits from the blast output, such as hits against common acidic, basic, or proline-rich regions. In some cases, sequences are masked completely, indicating that the significance of any hits returned against an unfiltered query should be considered suspect. This is indeed the case for *ODA5*.

BLAST reports using an unfiltered *ODA5* query sequence return hits to *Chlamydomonas ODA1* (AAK72125.1), a *Drosophila melanogaster* unknown protein (NP\_650569.1) a 66-kDa axonemal protein from *Ciona intestinalis* (BAB88833.1), a human protein similar to non-muscle myosin heavy chain, type A (XP\_090476.5), a hypothetical protein in *Leishmania major* (CAB55364.1), and a hypothetical protein in human



(BAC04081.1) plus weaker hits to myosin and paramyosin sequences. All of these sequences align within the coiled-coil region of Oda5 (discussed in CHAPTER IV). Phylogenetic analysis demonstrated that these sequences were more closely related to *Chlamydomonas* Oda1 than to Oda5, indicating that they represent potential Oda1 homologs (Figure 12).

### **The *ODA5* mRNA is Upregulated by Deflagellation**

Within the cytoplasm, there are enough flagellar precursors present to build two half-length flagella following flagellar amputation. In order to rebuild two full-length flagella, the cell must turn on synthesis of flagellar mRNAs. As a result, flagellar mRNA levels increase dramatically during flagellar regeneration (Silflow *et al.*, 1982). To determine if this is the case for *ODA5*, Northern blot analysis was performed on RNA isolated from wild-type cells either before deflagellation, or 30-45' post-deflagellation. Using a probe to the antisense strand of the *ODA5* cDNA, I found that the *ODA5* transcript is a ~2.7-kb mRNA that is upregulated during flagellar regeneration (Figure 13). This data suggests that the gene product of *ODA5* is a flagellar protein.

## **DISCUSSION**

This chapter details the identification of the *ODA5* gene within the 50.1 rescuing fragment, and the corresponding *ODA5* cDNA. The *ODA5* gene is contained in a 6.1-kb genomic fragment that rescues the *oda5-2* motility and outer arm assembly defect. In

order to determine the nature of the *ODA5* gene, the entire 6.1-kb rescuing fragment was sequenced. *Chlamydomonas* DNA can often be extremely difficult to sequence. To accomplish sequencing of such a large fragment, a mix of ABI chemistries specifically optimized for difficult templates was used in the sequencing reactions (Hills *et al.*, 2002).

The 50.1 genomic fragment is large enough to contain 1 or more *Chlamydomonas* genes. In order to determine the number of genes present in the 50.1 rescuing fragment, we analyzed the genomic sequence using GreenGenie, a gene prediction program optimized for predicting *Chlamydomonas* genes.

Analysis of the *ODA5* rescuing genomic sequence revealed that only one predicted gene was contained within the 6.1-kb fragment (GreenGenie Gene prediction program). The predicted splice sites generally conform to 5' and 3' consensus sequences.

GreenGenie predicts only one gene within the 50.1 rescuing fragment. PCR primers designed within predicted exons were used to amplify the *ODA5* cDNA. The *ODA5* gene structure is consistent with most *Chlamydomonas* genes, with a few exceptions. (1) Upstream of the predicted start codon is a second in-frame ATG. GreenGenie often fails to accurately predict the initial exon, therefore it is possible that the program did not predict the correct start ATG codon. It is also possible that there are two sites for initiation of translation of the *ODA5* gene, resulting in two Oda5p isoforms. Use of the second ATG (predicted by GreenGenie) would result in a 645 amino acid protein vs. a 652 amino acid protein if the first ATG is utilized. In the majority of

mRNAs, the first AUG downstream from the 5' cap and in frame with the coding sequence is the correct one (Silflow, personal communication). Thus, it is most likely that the first ATG is the actual site of translation initiation, and as such the predicted Oda5 protein sequence begins with the first ATG codon. (2) *ODA5* does not contain a canonical ribosome binding site. Ribosome binding sites, found in the many eukaryotic mRNAs, are a 3-9 bp purine rich sequence (often AGGA or GAGG) located approximately 8-13 nucleotides upstream from the start AUG. Analysis of the *ODA5* sequence does not reveal the presence of such a motif. The *Chlamydomonas ODA1* and *ODA3* sequences also do not contain such a motif located upstream of the initiating codon. It is possible that *Chlamydomonas* utilizes a different initiator sequence than other eukaryotic organisms. (3) The predicted stop codon for *ODA5* is TGA. This stop codon is used in ~15% of *Chlamydomonas* genes analyzed. (4) The *ODA5* mRNA contains a short 3' UTR sequence. A consensus polyadenylation signal sequence, TGTAAG, is present 144 nucleotides downstream from the predicted stop codon. 27-bp beyond that is the 50.1 3' *Bam*H1 site cloning site. This reveals 144-bp of 3'UTR up to the polyadenylation sequence. This is a relatively small 3' UTR for a *Chlamydomonas* gene. The 3' non-coding regions are often several hundred nucleotides in length. The polyA tail is generally added to the mRNA 10-20 bp downstream of the TGTAAG signal sequence. Thus, the 3' *Bam*H1 cloning site of 50.1 leaves just enough sequence to allow for proper polyadenylation of the *ODA5* mRNA. (5) Intron 3 is an unusually large intron for *Chlamydomonas* genes. There are four consensus 3' splice junction sites present within this intron just prior to an extensive region of repetitive C and A/C

content. If any of these four internal splice sites were utilized, an in-frame stop codon would be read, creating a much smaller protein. In spite of the large size of this intron, the cDNA sequence confirms the intron-exon boundaries around intron 3.

At position 567-572 of the cDNA sequence is the 5' *SalI* restriction site, representing the 5'-boundary of the 50.1 rescuing fragment. Additional 5'UTR sequences were identified by PCR and EST database searches. EST clone 1112113G03.y1 (genbank accession # BU654454) aligns with the 5' end of the *ODA5* cDNA sequence. It matches genomic sequence 5' to the 50.1 *SalI* cloning site, indicating that the 50.1 rescuing fragment does not contain the entire 5'UTR of *ODA5*, but it does contain the entire coding region and 3' polyadenylation signal.

Analysis of the larger 9-kb *BamHI* rescuing fragment, 39.2 (Fig 7A), reveals two predicted genes present in the larger fragment. One corresponds to the predicted *ODA5* gene. The second is a small gene, potentially transcribed on the opposite strand, which lies just 5' to the *ODA5* prediction. The *ODA5* prediction obtained using this larger genomic fragment reveals a different initial exon than that predicted using the smaller 50.1 genomic sequence. The initial exon predicted using the larger genomic sequence does not match the cDNA sequence obtained by PCR, indicating that it is not a true exon. This result demonstrates that GreenGenie does not accurately predict the initial exon in *ODA5* when the larger genomic sequence is used for the analysis. The predicted protein encoded by the smaller gene is an unknown protein. BLASTp reveals homology with a hypothetical *Arabidopsis thaliana* protein (Q9LMJ7). The JGI BLAST

server indicates this protein resides on scaffold 550 just upstream of the alignment of the *ODA5* gene sequence.

Within the cytoplasm there are enough flagellar precursors to generate two half-length flagella. In order to rebuild two full length flagella, the cell must turn on synthesis of mRNAs coding for flagellar proteins (Silflow *et al.*, 1982). *ODA5* gene expression is enhanced in response to deflagellation as determined by Northern blot analysis. This feature suggests that the Oda5 protein is a flagellar protein, consistent with its requirement for assembly of the axonemal outer dynein arm.

In conclusion, this chapter details the sequencing and analysis of both the *ODA5* gene and its corresponding cDNA. Identification of the *ODA5* gene is a key step in understanding how this protein functions in outer dynein arm assembly.

## **MATERIALS AND METHODS**

### **Strains and Culture Conditions**

*Chlamydomonas reinhardtii* strains used in this chapter include CC124 (*nit1-137*, *nit2-137*, *mt-*) from the *Chlamydomonas* Genetics Center (Department of Biology, Duke University, Durham, NC) for RNA isolation. *Chlamydomonas* cells were cultured as described in CHAPTER II.

### Isolation and blotting of DNA

Plasmid DNA was isolated according to standard procedures. DNA was separated on 1% TAE agarose gels. Gels were crosslinked in a Stratalinker (Stratagene, La Jolla, CA) at 1200  $\mu$ J for one autocrosslink cycle. DNA was transferred to membrane and Southern blotted as described in CHAPTER II.

### Isolation and blotting of RNA

*Chlamydomonas* RNA was obtained before deflagellation and 30-45 minutes after deflagellation by pH shock (Witman *et al.*, 1972). Total RNA was isolated by LiCl precipitation (Wilkerson *et al.*, 1994) and polyA<sup>+</sup> mRNA selected using Oligo dT cellulose (Ambion, Austin, TX). Polyadenylated RNA was separated on 1% formaldehyde agarose gels and transferred to Immobilon-P using a downward transfer method. RNA was cross-linked to the membranes using a Stratalinker at 1200  $\mu$ J. (Stratagene, La Jolla, CA). A reverse primer (5'-CCGGGTCATGGCGGTGAT-3') to the *ODA5* sequence was used in place of the random primers provided in the Prime-It II random prime labeling kit to generate an antisense *ODA5*-specific probe, which was used to probe Northern blots of isolated mRNA.

### Cloning and Sequencing of the *ODA5* cDNA

The smallest rescuing genomic fragment, 50.1, from BAC clone 18g18 was sequenced. The GreenGenie program predicted potential coding regions within the sequenced 50.1 subclone, and primers (Integrated DNA Technologies, Coralville, IA) within predicted

exons were used to amplify *ODA5* from a cDNA library constructed from polyA<sup>+</sup> mRNA isolated 30' post-deflagellation (Wilkerson *et al.*, 1994) or a gametic cDNA library (gift of William Snell, University of Texas Southwestern Medical Center, Dallas, Texas). cDNA clones were sequenced to confirm intron-exon boundaries. All sequencing was performed by either the Iowa State DNA sequencing facility or the UMMS Nucleic Acid Facility using a combination of ABI chemistries designed for optimized sequencing of difficult templates (Hills *et al.*, 2002).

### Computational Analysis

All sequence assemblies were performed using LaserGene SeqMan (Madison, WI). Primer design was performed using PRIMER3 (Rozen and Skaletsky, 1996, [www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)). To predict regions of coding potential, the genomic sequence was analyzed using the GreenGenie gene prediction program (Li *et al.*, 2003, <http://www.cse.ucsc.edu/%7Edkulp/cgi-bin/greenGenie>). The NCBI BLAST server ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST), Altschul, 1990) was used to search for homologous sequences. ClustalW v. 1.82 (<http://www.ebi.ac.uk/clustalw/>) and NJ Plot (<http://pbil.univ-lyon1.fr/software/njplot.html>) were used for phylogenetic analysis.

**Figure 9: *ODA5* gene structure and genomic sequence.** (A) The upper line illustrates the intron-exon structure of the *ODA5* gene. Rectangles indicate exons and solid lines indicate non-

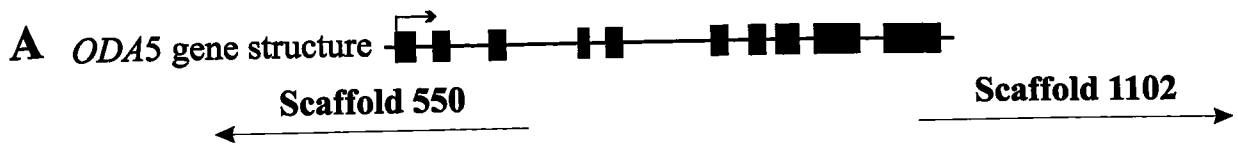
exons were used to amplify *ODA5* from a cDNA library constructed from polyA<sup>+</sup> mRNA isolated 30' post-deflagellation (Wilkerson *et al.*, 1994) or a gametic cDNA library (gift of William Snell, University of Texas Southwestern Medical Center, Dallas, Texas). cDNA clones were sequenced to confirm intron-exon boundaries. All sequencing was performed by either the Iowa State DNA sequencing facility or the UMMS Nucleic Acid Facility using a combination of ABI chemistries designed for optimized sequencing of difficult templates (Hills *et al.*, 2002).

### Computational Analysis

All sequence assemblies were performed using LaserGene SeqMan (Madison, WI). Primer design was performed using PRIMER3 (Rozen and Skaletsky, 1996, [www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)). To predict regions of coding potential, the genomic sequence was analyzed using the GreenGenie gene prediction program (Li *et al.*, 2003, <http://www.cse.ucsc.edu/%7Edkulp/cgi-bin/greenGenie>). The NCBI BLAST server ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST), Altschul, 1990) was used to search for homologous sequences. ClustalW v. 1.82 (<http://www.ebi.ac.uk/clustalw/>) and NJ Plot (<http://pbil.univ-lyon1.fr/software/njplot.html>) were used for phylogenetic analysis.



**Figure 9: *ODA5* gene structure and genomic sequence.** (A) The upper line illustrates the intron-exon structure of the *ODA5* gene. Rectangles indicate exons and solid lines indicate non-coding regions. The initial exon is marked by an arrow indicating the direction of transcription. Below are the JGI scaffolds that align with the ends of the *ODA5* gene. (B) *ODA5* gene sequence: in blue is the EST and PCR derived 5'UTR. Red indicates coding regions. Underlined are two upstream stop codons, and a *Sal1* site (GTCGAC) and a *BamH1* site (GGATCC), which demarcate the 5' and 3' boundaries of the 50.1 rescuing fragment respectively.



**B**

```

1  AGTATGTTGCAGTCGCCCTAGTTTTTCGCAGGACTTAGTGAGTAATGCGCTCAGTTAACTAAACATATGGAGTTTGGCGTCACTGCTCTGAACGCAAAACA 100
101 CTGCGGAGCTGGCAGCTGGCGTCAGGTTTTCGCTGGCGAATGTGTTCAATGCTTCAAAGACCGTGTGTGACAAACTGCAATATTTACGATGCTTAGAGT 200
201 ATATCCGCTCTCACTAGGGCGATGGCATCGCCAACTTGTATGCCAATGCTGCGCGCACCGGAAATGGGTCGATGCATCGTCAAGGAAAGCCGGCGGA 300
301 CCGGATCGTTCGCTGTGCTGCAATCGCATAATGGTACTGAGATGCGGACCATGTCAGCTTTGAGCCAAAGCGCGTTCGCACCGTGGTGACGGATGG 400
401 GCGGGTGGAGACGGGCGGAGAGACTGGGCAAGGTTGCGTTCGCGTGGCGGCTCCAGGTTCCAAGAGAGCAGGGCATGGCGATGGGCAATGCTATTAC 500
501 CGCTTGACATTTCTTAGCTTGACATATTTGATAACCGTAGGGCCCACTGCAAAACCAACTCGAAGGTCGACTGGTATTTGCTTAAAAATCGCTGGCTGT 600
601 ATGGAATGCTCCCGGACAACTTGTACTGTGGCTTACTATTGTAGTCTATCGCGATAAGAACTATAGCGTTTGTGCTTTCCCGGGTTCGCAATTTGGCCAC 700
701 GTATAGGCTCAACTCGCACCTCCAATGGCTCAAACGGCACGTCGATGTGTGACAGAGCTTCAAGGCTCGCATCAAGGAGCTTGAAGCGTTGAAAAAGACT 800
801 GGGCCGATGAGGCGGACGAATCATCGCTCCAGCGTGCACCATCGACCGGCTCAAAGCGGACAAATCAGCGCTCACAACGGACTTGGCCCGCTGG 900
901 CAGCGCGATGACGGCGGTTGCGGCGCTAGTGGCGGGGGGCGCTCGTGGTGTCTGTGAAGGTGAGGGAAGGTTGTGATATTAAGCCCTGGCTACGAAAGT 1000
1001 TTTGGCGGTGACCGTGGCCCGGTTGCCGCGGGCTGTTGGCTCCCGAGCACTGCAAGGACACACCGCAGTATGATATTAAGCCCTGGCTACGAAAGT 1100
1101 TGTGATAGCATCTCGCTCTCGTGTATGCTGTGGAGGTGGTGTGGCACGCGGCTCACGTGTGTTGCTGCCCCACCCGCCCCGGTAGCGTACTCGAATGC 1200
1201 CCTCACCAGTTGACAACCCAGTCAACCGCATCGACGCCACCGGCTGACCTCGCGGCGAGTCAGAGCGGGCGGCAAGCTGACGGAGGTGGCGGACACT 1300
1301 ACAGCAGAAAGGTGGCGGCGTGGGGCAGGCACAGACGCGGGCTGATCTTCAAGGCGGGTGTGGGCGCCGGTGTGGGCGCAAGGCACACGGG 1400
1401 GGTGGAGCTGTAGGAGGCGTGGCGGGCGGCTGCGGTTGGCGGTGACGCGGAGGTGGCTGTGCTGGCTGGCGTACGTTGGCCGGCGGACGGCTG 1500
1501 CTGTTGGCTTGCAGTGGCTGATGGGGTGCACCAGGGCTGGCGGAGCAGCGCTCCACAGTGGAAAGACAGTGTGGGCGTTCGCGGCTTGAACACTTG 1600
1601 AGGGACTTGGGGCGTGTCTGCCCTCACGACAGCGGCGCCAGATTGAGGTAGAGCGGGCGGACGTTGGAGAGCGTGGAGCGGAGGTGGAGCTGGCACAGGCG 1700
1701 CTCTGGCTTCGGCCACCAATGCAATCGCCAGCCAGATTGAGGTAGAGCGGGCGGACGTTGGAGAGCGTGGAGCGGAGGTGGAGCTGGCACAGGCGGAG 1800
1801 GTGCGTGGGTGGGATGGTGTAGATACCAGCCATACTAAACAAACCAATGCAATAGAGCGGAGGAGGAGTGGAAAGAGGCGATGAGCTCTTCG 1900
1901 TGGCAGCGCAATGTTCCCAATTCGAAATGCAACCGGTTGCCAGCCAGGCTCAAACACACGACGCGACGTTCTATCTAGCAATGACAGACTAGG 2000
2001 GCTTGGCGCTGTGGCGGTTGACGGTAGCCGCTTCGAAATTTGCCAGCCAGGCTCAAACACACGACGCGACGTTCTATCTAGCAATGACAGACTAGG 2100
2101 ACACGTTGAATACCCACTAGAAGACATCAAGGAGCGGAGGAGGAGACCAGCCAGGGCGGGACGTTTGCATGTGCAAAAAGGCAAGCGGCAATTAAGTA 2200
2201 GTGAGCGGAAAGGCGCGGAGGCAACAGGAGCGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG 2300
2301 GAGCAAGCAGCATCGGCATGGGCGCCAGCAGGGCCAGGGGAGGGGGTGGTGGGGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG 2400
2401 TGCCAAATCCGGGTCGCCCTCAGCAGGAGAGGGCATCCGGAGCCTGTAGGCGCTAGGAGAGCGGAGCGGACGGGTAATATGGGCACAGGCACACAGAG 2500
2501 GGAGGAGACAACAGCAACCCGAGACACAGGAGAGCTGGCTGGCGGAGAGCTTGGATGTGCAAAAAGGCAAGCGGCAATTAAGTA 2600
2601 CGTAGGCGCACTCAACCCCTAAACCCCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 2700
2701 CACCAGCGCGCCCGCCCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 2800
2801 AAGCACACCCCGCCCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 2900
2901 TCCCCAGAAATCAACCCCTGTATCTCAAGTAAAGGATGGGGTAGGCGTTTACGCGGGTAGCGAATGATGTGGGGAGGTGGAAACCGTGGAGGCGAGGG 3000
3001 CGCGGCTTGGCTGTGGAGCGGTTGAGCATGCTGAGCGTTGGTGAAGGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 3100
3101 ACTTCCACAACGCTCTCACTCCAGTCAACCCCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 3200
3201 GTATAAATATGAAGTAAACCCCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 3300
3301 AGCCATCAAGCGCTTGGAGGTGAGCGCTGGCGGCGGCAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG 3400
3401 TGCCGGACGCTTGGCACATGGGACGCGATATGGCTTACCACCTGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 3500
3501 ACGCGGCGCAGCGGCTTCAACGAGCACATCGCCGCAACCGCGACCTCAAGTCCAGATAGACGCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 3600
3601 GAGGGCGAGCTTGGAGCGGAGGTGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 3700
3701 GCGCAGGTGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 3800
3801 GGGGGTAAATGTGCCCTCAAGTGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 3900
3901 TGAGGACGCGGAGGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 4000
4001 GGGGGCGGGGTTAGGATAAAGGTGACGAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 4100
4101 GCGTTCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 4200
4201 GTCAATGCTTACCAGCAATTCCTCTCACTATCCCTCGCAGGCTCAGCGGCTCGCTTACCAAGTGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGG 4300
4301 GCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 4400
4401 GACGGGGGAGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 4500
4501 GGCCACCCACCCCTCCCTCCCTCGCTCCCTTTCGCGAGCACTCCTCCGTCGTCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 4600
4601 CCTGCTCCCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 4700
4701 CCGAGGCTGCGCTCCCTGCGCGAGGCGCTGCAGCACTACTGGGCGTGGAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 4800
4801 ATCACCCTATGACCCGGGAGCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 4900
4901 GCCGAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 5000
5001 GTCTAATCTGCAGCACTTGCCTGAAAGTCAAAGCGGCGCTCGCATGTGAAGTTACCATCTAAACACCAATGTCGCTTTTCGCACACCCACCCGCA 5100
5101 CCGCAGGCGGAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 5200
5201 CCGCAGGCGGAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 5300
5301 GCGGCTCCCTCGCTCAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 5400
5401 CCGGGCGAGGGCGATGGTGGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 5500
5501 CGTGATGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 5600
5601 GGAGGGGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG 5700
5701 AGGAGGGGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG 5800
5801 GGAGGGGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG 5900
5901 ACTCGTTCCTCGCTTCGTCCTTGCATGTCGCGGCTGACACCAACCGGATGCCCGCTCAGTCTCGGCACCCCTGCCTCTGATGCCCGCT 6000
6001 CCGCTGCGGCTGCGCCCTTCAACGCGGCGTTCGCGCTTACCGTAACTAGTTATGCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 6100
6101 ACCATGTTGGTTCGCAACAGCCTGCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 6200
6201 CCAGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 6300
6301 AGCGCGGGGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG 6400
6401 ACGCGCGCTCCAGTATGAGATCATAGAAGATGAGGATGAGGATGATGAGGAGGATGAGGATGAGGATGAGGATGAGGATGAGGATGAGGATGAGG 6500
6501 CGAGTCTGAAGGCGTATCGGTGGCGTTCACTGTGAGGTTGGATGGGGTTTAAATCGCGGTTGGCGAATGGGTTGGTGGCGTTTGTGCGGCGGCTG 6600
6601 CTGTGGTCTACCGAGCTACTCTTCTGGCTTAGACGGTGTGCGTGGATGATTGGACACTGTAAAGCCTGCTTACAGCGGAGCGCAGGATCC 6693

```

Figure 9

**Figure 10: *ODA5* intron 3.** The consensus sequence for 3' splice sites is (G/A)CAG/(G/A) (Silflow, 1998). This unusually large intron contains four internal canonical 3' splice sites (bold) followed by downstream stop codons (bold and italic). The beginning of exon 4 is in red.

1801 GTGCGTGC GG TGGGGATGGGTTGTAGATACCGCCATACTAAACCAACCCAATGCAATAGAGCGAGGAGAGCGTGGTTCGATGCTTGACAACGGAG 1900  
1901 TGGCACGCGACAATGGTCCCATTCCCGAAATGCACCGAGTTCCCGGGTTCCAGCAAAACATCCCGCAAACCGGTAGAAAGAAGGCGATGAGCTCTTCG 2000  
2001 GTCTTGCGCGTGTGGGCGGTGCTGACGGTAGCCGCTTCGAAATTGCCACCCAGGCTCAAACACACGCGACGCACGTTCTATCTAGCAATGCAGACTAGGC 2100  
2101 ACACGTTGAATACCCACCTAGAAGACATCAAGGGAGCCGAGGAGGAGACCAGCGCCAGGGCGGGACGTTGTCATGTGCAAAAAGGGAAAAGGCAAAACGGT 2200  
2201 GTGAGCGGAAAGGCGCGGAGCGGAAAAGGCAACAGTATTCTATGTGCACAGCCTGGGTGGCAAGAGAGAGGGGAAAAAAGGCAGCGGGCAATTAAGTA 2300  
2301 GAGGCAAGCACGCGATCGGCATGGGGCCGCACGAGGGCCAGGGGAGGGGGTGGTTAGGGGAGAGGCGAGGTTGCGGGCCCGGACGGCTCCCCACAGCCC 2400  
2401 TGCCAATCCGGGTGCCCTCACCCAGGAGAGGGCATCCGGAGCCTGTAGGCGCTAGGGAGAGCGGGACCGGGACGGGGTAATATGGGCAACAGGACACAGAG 2500  
2501 GGAGGAGACAACAGCAACCCGAGACACACGGAGAGCCTGGGTGGCGGAGACGCTGGATGTAGGGCCTGGTAGAAGAGCTTGAATGCCGGGCGCGTGCCT 2600  
2601 CGTAGGGCGCACTCCAACCCCTAAACCCCGGG 2700  
2701 CACCACCCCGGG 2800  
2801 AAGCACACCCACCCCTCATGGGGGAGGGGGGGGGGAAAGGCTACAGGGATATGTTGGCCTGAACATGAGGAGGTGGAGTTTGGCGCTGGAAGGGGGCA 2900  
2901 TCCCCAGAATCAAACCCCTTGTATCTCAAGTAAAGGATGGGGTAGCCGTTACGGGGTACGGAATGATGTGGGGAGGTGGAACCCGTGGAGGCGAGGGC 3000  
3001 CGCGCTTGGCTGTGCGAGCCGGTGCAGCATGCTGAGCGTTGGTGCAAGGGCCGGCCGACGCGCTCAACGCGCGGTGGTCACAGTCCACACCCACC 3100  
3101 ACTTCCACAACGCTCCTCCACCTGCATCACCACCTCCACCACCACCATGTCCACCCTCCACCACCTCCACCACCTCCTCTGCCCGGCTTCT 3200  
3201 GTATAAATATGAAAGTAACCCCGGG

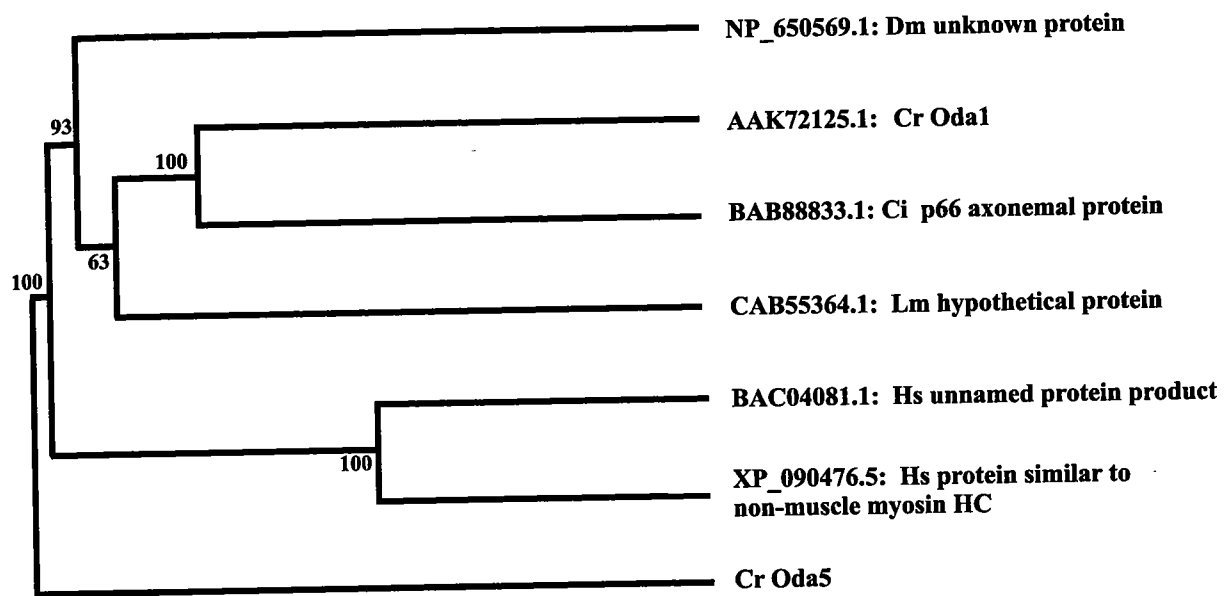
**Figure 10**

**Figure 11: Sequence of the *ODA5* cDNA and its product.** Underlined are two in-frame stop codons (656-658 and 704-706) that are upstream of the predicted start codon; a polyadenylation signal sequence (2828-2832); a *SalI* restriction enzyme site (567-572), which denotes the 5' end of the 50.1 rescuing fragment; and a *BamHI* restriction site (2854-2859), which denotes the 3'-end of the 50.1 rescuing genomic fragment. The sequence from the polyadenylation site to the *BamHI* site is derived from the *ODA5* genomic sequence. Additional 5'UTR sequences were identified by PCR and EST database searches. The additional 5'UTR sequence matches genomic sequence 5' to the *SalI* site. These sequences have been deposited in GenBank/EMBL/DDBJ with accession # AY452532.

1 AGTATGTTGCAGTCGCCCTAGTTTTCGCAGGACTTAGTGAGTAATGCGTCAGTTAACTAACATATGGAGTTGGCGTCACACTGCTCTGAACGCCAAACCA 100  
101 CTGCGGAGCTGGCAGCTGGCGTCAGGTTTTCGCGTGGCGAATGTGTCAATGCTTCAAAGACCGGTGTGTGACAAACTGCAATATTTACGTATGGTCTAGAGT 200  
201 ATATCCGCTCTACTAGGGCGACTGGCATCGCCAATTGTATGCCAACTGCTGCCGGCACCGCGAATGGTTCGATGCATCGTCAAGGAAAGCCGGGCGGA 300  
301 CGGGATCGTTCCCGTTGTCTGCAATACCGATAATGGTACTGAGATGCGGACCATGTCAGCTTTGAGCCAAGCGCGTTCGCACCGTGGGTGACGGGGATGG 400  
401 CGGGGTGGAGACGGGCGGAGAGAGCTGGGCAGGGTTGCGTTTCGCGTGGCGCGTCCAGGGTCCAAGAGAGCAGGGCATGGCGATGGGCAAAATGCTATTAC 500  
501 CGCTTGACATTTCTTAGCTTGGACATATTTGATAACCGTAGGGCCAGTCAAACACCAACTCGAAGGTCGACTGGTATTGCTTGAATAATGCGTGGCTGT 600  
601 ATGGAATGCTCCCGGACAACTTGTACTGTGGCTTACTATTGTAGTCTATCGCGATGAAGCTATAGCGTTTGTGCTTTCGCGGGTTCGCAATTTGGGCAC 700  
701 GTATAGGCTCAACTCGCACCTCCAATGGCCTCAAACGGCAGCTCGATGGTGCACGAGCTTCAGGCTCGCATCAAGGAGCTTGAAGCGTTGAAAAGACTC 800  
M A S N G T S M V H E L Q A R I K E L E A L K K T 25  
1 GGGCCGATGAGGCGGCACGAATCATCGCCTCCAGCGTCCACCATCGACCGGCTCAAAGCGGACAATCAGCGCCTCAACCGGACCTTGGCGCGTTGG 900  
26 R A D E A A R I I A S Q R A T I D R L K A D N Q R L T T D L A A V G 59  
901 CAGCGCGGATGCAGCGCGGTTGCGGCGCTAGTGGCGGCGGGGCGTCCGTCGTTGAGGAGCCACCGGCTGACGCCGGCGCAGTCAAGCGGGCG 1000-  
60 S A D A A A V A A L V A A G A S S V L V K P T G L T P A Q S E R A 92  
1001 GACAAGCTGACGAGGTTGGCGGACCTACAGCAGAAAGATTGAGGTAGAGCGGCGGAGCTGGAGACGCTGGAGCGGGAGGTGGAGCTGGCACAGGGC 1100  
93 D K L T E V A D T Y S R K I E V E R R Q L E T L E R E V E L A Q A 125  
1101 AGGTGCTGGCGATGCGCGGGGAGTGGCGGGGACAGCGGCACACAGGCCATCCATCGCGCCCGCAAAGCCATCAAGGCGCTGGAGACCGGACTGGA 1200  
126 Q V L A M R R D V G G H S A G T Q A I S I A P A K A I K A L E T R L D 159  
1201 TGGCGCGCAGCGGCTTCAACGAGCACATCGCGCCAACCGGACCTCAAGTCCCAGATAGACGCCCGCCCGCGCGCCCGCCATGGAGGCCAT 1300  
160 A A Q R R F N E H I A A N R D L K S Q I D A A G R R A A A M E A M 192  
1301 AGGGCGCAGCTGGAGCGGAGGTTGGCGGGCGTGGCGGGGAGTGGCGGGCGTGCAGGAGGCGGGCGGCGGCGGCGGCGGCGGCGGCGGCTGG 1400  
193 R A Q L E R E V A G L V A A E L A A V Q E A G R V A S E A R H E A L 225  
1401 CGCAGATGGCGGCTTGGCTGTGGCTGAGGACGCGGAGGCGGCGGCGTGGAGTGGAGTGGCGGAGCTGGCGGGCTGCCAGGCGGGCGGACGAGAAGCT 1500  
226 A Q M A A L R V A E D A E A A A F E V E V R E L A A C Q V E L A Q 259  
1501 GCTGCAGGCTCAGCGGCTCGCCTGACCAAGTGGTGGAGGCGGCGGAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 1600  
260 L Q A Q A A R L T K V V E A A E A E A E G G G G A S E P L D L D 292  
1601 AGTGTGGAGCAGGCTTGGCTGGAGGCGGCGGCTCAGCAGTGCAGCGAGCGGCGGCGGCTGCCTCCCTGCCGAGGCGGCTGCAGCACTACCTGGGCC 1700  
293 S V E Q G I G V E A A A Q Q L Q R A A G L P S L P E A L Q H Y L G 325  
1701 TGGAGGCGGCGTCCCTCGAGTGTTCGCGGCTGCAACGAGGCGGCGGAGCGCATCACGCCATGACCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 1800  
326 L E A A S F E L F A A C N E A G S G T Q A I T A M T R E A R E L E A E V R 359  
1801 ACAGATGGAGGCGGAGGCGGTTGGTGGGCGGCGGAGGCGGAGGCGGAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 1900  
360 Q M E A E A V V G R Q H E A I R Q A L G R R D A A L R R A D S V T 392  
1901 GAGCGCGCAGCGCCATCGAGCGCGGCTGGCCATGCTGCCAACGGCATCTCGGTGTGCTGCGGTAGGCTGGGCGGCGGCGGCGGCGGCGGCGGCGG 2000  
393 E R R S A I E R R L A M L R N G I S V C L R R L G A A A A A Q A W 425  
2001 AGGCGGCGCTCCCTCGCTCACAGGCGGCGGCGGAGGCGGAGGCGGAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 2100  
426 E A G A G A P S L T G G G G G G G G G G G G T I L A S R E S G A A E S G A 459  
2101 GGGGGCGGGGCGGGGCGATGGTGGGCGGGCGG 492  
460 G A G A G A M V G G R P P S G S G L Q V A G A P P P H P S V A A C 525  
2201 GCGCACGTGATGGCGGCGTGGCGCTGTTGGAGCAGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 559  
493 A H V M A G L A L L E Q R A S A I I A V Q G T M V G R T N S L P R 525  
2301 CAGCCAGCGGCGGCGGCGGCTGCTGCTCGCTGCTTACCCGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 2400  
526 P A S G R P A S A S L P S P A G T P P R P P S S G G P A A A G T P G 559  
2401 GGCTCAACTTCCACAGCGCAGGAGGCGG 2500  
560 A S T S T A A G G S I I A G V G T R T G A A P G G K A A T A P P 592  
2501 GCTCGGCGGCGGTTGGATCGGGGTTGGGGAAGGGTCTGAGCGCGGGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 625  
593 A A A R V G S G L G K G L S A G P L P S L G T A A S E Y E T I E D 625  
2601 AGGATGAGGATGATGACGAGGATGAGATGCCGTTGACACCGGAGCAGATCCAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG 2700  
626 E D E D D E D E M P L T R E Q I Q A R A Q A S L K A \* 652  
2701 CGAGGTTGGATTGGGGTTTAAATGCGGGTGGCGAATGGGGTGGTGGCGTTGCTGCGGGCGGCTGCTGTTGGTCTACCGAGCCTACTCTTCTGGCTCT 2800  
2801 AGACGGTGTGCGTGGATGATTGGACACTGTAAGCCTGCTTAGACGGCAGCGCAGGATCC 2859

Figure 11

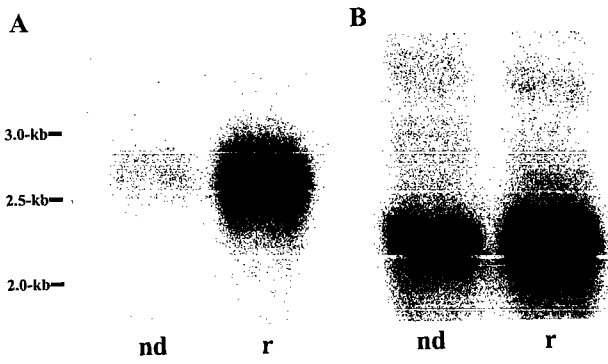
**Figure 12: Phylogenetic tree of Oda5p-related sequences.** Phylogeny indicates that the Oda5-related sequences identified by BLAST using an unfiltered Oda5p query are more closely related to the *Chlamydomonas* Oda1 protein. Cr denotes *Chlamydomonas reinhardtii*; Hs denotes *Homo sapiens*; Ci denotes *Ciona intestinalis*; Lm denotes *Leishmania major*.



**Figure 12**



**Figure 13: *ODA5* gene expression is upregulated by deflagellation.** PolyA<sup>+</sup> mRNA was isolated from wild-type non-deflagellated (nd) cells and cells which were deflagellated and actively regenerating their flagella (r). (A) An *ODA5* cDNA probe was hybridized to northern blots of the isolated mRNA. The probe identifies an induced message at ~2.7-kb, in good agreement with the 2.8-kb size of the *ODA5* cDNA. (B) A cDNA probe to fructose-biphosphate aldolase recognizes a ~2.0-kb mRNA that serves as a loading control; transcription of this gene is not upregulated by deflagellation.



**Figure 13**

## CHAPTER IV

# ANALYSIS OF THE ODA5 GENE PRODUCT AND CHARACTERIZATION OF THE ODA5 PROTEIN

### INTRODUCTION

We have isolated and characterized the previously uncloned *ODA5* gene in *Chlamydomonas reinhardtii*. Sequencing of the *ODA5* cDNA revealed a predicted protein of 652 amino acids with a predicted mass of 66,218.41 Daltons. Analysis of the predicted Oda5 protein (Oda5p) may yield information on functional domains within the protein, providing additional information on the nature of Oda5p and how it functions in outer dynein arm assembly.

The most extensively investigated paradigm for axonemal dynein assembly and targeting is the *Chlamydomonas reinhardtii* outer dynein arm (Pazour and Witman, 2000). Biochemical studies of isolated flagella have revealed detailed information about axonemal dyneins. Both the inner and outer row dyneins can be removed from the axoneme with high salt buffers. The outer dynein arm can be separated into two complexes, sedimenting at 12S and 18S in sucrose gradient sedimentation assays. However, these individual complexes are unable to rebind the axoneme in the absence of a 7S complex called the outer dynein arm-docking complex (ODA-DC). The docking

complex was identified as a factor required to rebind 18S +12S outer arm dynein fractions to axonemes isolated from the *oda1* mutant (Takada and Kamiya, 1994). The 12S fraction contains the  $\gamma$ HC and its associated LCs, while the 18S fraction contains the  $\alpha$  and  $\beta$ HCs with the IC-LC complex.

Takada *et al.* (1992) identified conditions required to keep the entire outer arm intact during its removal from the axoneme and centrifugation steps. These conditions also keep the ODA-DC associated with the intact three headed outer arm. Subsequent experiments determined that the *ODA1* gene encodes the 62-kDa (DC2) component of the ODA-DC (Takada *et al.*, 2002), the 83-kDa (DC1) component is encoded by the *ODA3* gene (Koutoulis *et al.*, 1997), and the 21-kDa (DC3) component is encoded by the *ODA14* gene (Casey *et al.*, 2003a). Mutations in the *oda1*, *oda3* and *oda14* genes prevent assembly of the outer arm, resulting in an Oda- swimming phenotype. Not only is the ODA-DC required for binding of the outer arm, but it has been proposed to determine the 24-nm periodicity of outer arms along the length of the axoneme.

DC1 and DC2 both contain coiled-coil domains which often participate in protein-protein interactions. Cross-linking and immunoprecipitation experiments demonstrate that DC1 and DC2 are in direct contact with each other (Wakabayashi *et al.*, 2002a; Wakabayashi *et al.*, 2001). A dimer of DC1 and DC2 forms a 50-nm rod-like structure (Wakabayashi *et al.*, 2002b) which could overlap along the length of the axoneme, providing outer arm binding sites every 24 nm. Alternatively, the 24-nm periodicity may be determined by additional, unknown factors.

I have isolated and characterized the previously uncloned *ODA5* gene in *Chlamydomonas reinhardtii*. I determined that Oda5 mRNA levels increase during flagellar regeneration, suggesting that the Oda5 protein may localize to the flagellum. Sequencing of the *ODA5* cDNA revealed a predicted protein of 652 amino acids with a predicted mass of 66,218.41 Daltons. Analysis of the predicted Oda5p may yield information on functional domains within the protein, providing additional information on the nature of the Oda5p and how it functions in outer dynein arm assembly.

This chapter details the analysis of the Oda5 protein sequence, the localization of Oda5p and determines its biochemical and assembly attributes. Oda5p is indeed localized to the flagellar axoneme and appears to comprise a novel axonemal complex. In addition to the previously reported complementation data (Kamiya, 1988), I provide biochemical data supporting an interaction between Oda5p and Oda10p.

## RESULTS

### Structure of the Oda5 Protein

*ODA5* was sequenced and the corresponding cDNA identified. The sequence predicts a novel 66-kDa protein containing extensive coiled-coil domains, followed by a non-coiled-coil, highly charged region in the C-terminus (Figure 14). There are five regions containing a high probability of forming coiled-coil structure. The entire region between coils C and D is largely hydrophobic, with 63 out of 126 amino acids being hydrophobic in nature. When the Oda5p sequence is analyzed using the PairCoils

program, regions D and E are predicted to form coiled-coil, but with a lower probability (1.0 and 0.9 for COILS and 0.5 and 0.6 for PairCoils, data not shown). Coiled-coil domains are a common feature of many cytoskeletal proteins and it is likely that Oda5p interacts with itself, or another coiled-coil protein via these regions. Since Oda5p is predicted to interact with Oda10p and/or Oda8p, it is possible that this interaction is mediated via the coiled-coil domains in Oda5p. It will therefore be of considerable interest to identify proteins that interact with Oda5p via these domains.

The length of a coiled-coil region can be estimated at  $\sim 1.5\text{\AA}$  per residue (Fraser and MacRae, 1973). If regions A, B, and C form a coiled-coil, then this would form a structure  $\sim 23.55$  nm in length, almost exactly the same length as the outer dynein arm periodicity. It is possible that in conjunction with the ODA-DC, Oda5 participates in determining the correct periodicity of outer arms along the length of the axoneme. However, since Oda5p and the ODA-DC assemble onto the axoneme independently, Oda5p most likely functions in some other aspect of outer arm assembly.

A stretch of 10 glycine residues is found just after the predicted coiled-coil domains end (G<sub>434</sub>-G<sub>444</sub>) (Figure 11). Glycine has typically been called a helix-breaker because it occurs in loops and turns in proteins. Glycine is conformationally more flexible and this flexibility may be required in loops and turns, not helices (Fraser *et al.*, 1967). It is possible this poly-glycine stretch not only breaks the coiled-coil structure, but provides a flexible hinge between the coiled-coil domains and the non-coiled-coil COOH-terminus.

A very acidic region, containing aspartic acid and glutamic acid residues, is found near the COOH-terminus of Oda5p (E<sub>619</sub>-E<sub>634</sub>) (Figure 11). This region may be important for interactions between Oda5p and its binding partners.  $\alpha$ -tubulin,  $\beta$ -tubulin and IC1 of outer arm dynein have highly charged regions. The COOH-terminus of tubulin is exposed along the length of the microtubule and is implicated in binding of microtubule associated proteins (MAPs) (Littauer *et al.*, 1986; Paschal *et al.*, 1989). Oda5 is released from the axoneme by high salt, indicating its association with the axoneme is ionic in nature. It is possible that Oda5 interacts with the axoneme and/or the outer arm through this region, creating a salt-sensitive ionic bond. These features are strikingly reminiscent of the DC1 and DC2 proteins of the ODA-DC, which also have predicted coiled-coil structures, as well as COOH-terminal charged regions.

### **Oda5p is an Axonemal Protein**

In order to facilitate the *in vivo* localization of the Oda5 protein, a polyclonal antibody to an Oda5 fusion protein was generated. In wild-type whole cells, the Oda5p antibody detected a band having a M<sub>r</sub> of 76,000 on SDS-PAGE (Figure 15A). This band was not detected in the null strain, confirming our antibody recognized the correct protein.

Although the antibody cross-reacted with other proteins, it was possible to follow the distribution of Oda5p by comparing fractions from wild type and the *oda5-2* null strain. When cells were deflagellated and cell bodies analyzed, we detected little to no Oda5p in the cell body fraction. Oda5p was highly enriched in isolated whole flagella, demonstrating that Oda5p is a *bona fide* flagellar protein (Figure 15A).

To further localize Oda5p within the flagellum, isolated whole flagella were extracted with Nonidet P-40 detergent followed by 0.6 M KCl (Figure 15B). Oda5p is not present in the detergent-soluble membrane + matrix fraction, but remains associated with the demembrated axoneme, demonstrating that Oda5p is an axonemal component and not a soluble or membrane-associated protein. When the demembrated axonemes were subsequently extracted with 0.6 M KCl, Oda5p was released from the axoneme.

Previous data have shown that the outer arm and the ODA-DC also are released from the axoneme by 0.6 M KCl (Pfister *et al.*, 1982; Takada *et al.*, 1992; Takada *et al.*, 2002). To determine if Oda5p associates with the outer arm or the ODA-DC, the high-salt extract was subjected to sucrose gradient sedimentation, using conditions designed to maintain the outer arm/ODA-DC association (Takada *et al.*, 1992). Western blots of sucrose gradient fractions were probed with antibodies to IC2 and the  $\gamma$ DHC, which confirmed the migration of the outer arm/ODA-DC at the expected position of ~23S (Figure 15C). In contrast, Oda5p migrated at ~5S in these gradients. These data demonstrate that under conditions that remove the outer arm and the ODA-DC as an intact complex, Oda5p is not associated with these two components.

### **Oda5p Assembles Onto the Axoneme Independently of the Outer Dynein Arm and the ODA-DC**

Inasmuch as Oda5p behaved independently of the outer arm and ODA-DC in sucrose gradients, I investigated whether Oda5p can assemble onto the axoneme in the absence of the latter structures. Western blots (Figure 16A) revealed that Oda5p is present in



axonemes of an *oda9* mutant, which is defective in the IC1 gene and fails to assemble an outer arm (Wilkerson *et al.*, 1995), and of *oda1* and *oda3* mutants, which are defective in the DC2 and DC1 components of the docking complex respectively (Koutoulis *et al.*, 1997; Takada *et al.*, 2002) and fail to assemble the ODA-DC and the outer arm. These results show that Oda5p can assemble onto the axoneme independently of the outer arm and the ODA-DC.

#### **Oda5p Assembly Onto the Axoneme is Defective in the *oda10* Mutant**

Because Oda5p, Oda8p and Oda10p have been proposed to interact, I investigated if the localization of Oda5p is disrupted in the *oda8* and *oda10* mutant strains. Western blots revealed that Oda5p does assemble onto *oda8* axonemes. In contrast, Oda5p fails to assemble onto axonemes of the *oda10* mutant (Figure 16A), demonstrating that a functional Oda10 protein is required for proper localization of Oda5p. This provides the first biochemical evidence for an interaction between Oda5p and Oda10p.

#### **The ODA-DC Can Assemble Onto *oda5*, *oda8* and *oda10* Axonemes**

To determine if the ODA-DC can assemble onto the axoneme in the absence of Oda5p, as well as in the absence of *ODA8* and *ODA10* gene products, western blots of isolated axonemes were probed using an antibody to DC2, the 62-kDa component of the ODA-DC (Figure 16B). As expected, axonemes from *oda1* and *oda3*, which do not assemble the ODA-DC, lack DC2, whereas axonemes from *oda9*, which are missing only the outer dynein arm, contain DC2 as do wild-type axonemes. *Oda5*, *oda8* and *oda10*

axonemes also contain DC2. Therefore, DC2 can assemble onto the axoneme in these three mutants, suggesting that the entire ODA-DC can assemble onto axonemes independently of Oda5, Oda8 and Oda10 proteins.

## DISCUSSION

This chapter details the analysis of the Oda5 protein sequence and characterization of the Oda5 protein. Alternative functions have been proposed for the uncloned *odas*, like *oda5*, *oda 7*, *oda 8* and *oda 10* including the intraflagellar transport of the outer arms into the flagellum. IFT is an essential process required for the assembly and maintenance of eukaryotic cilia and flagella. Flagellar proteins are constantly turning over and the addition of new components occurs at the flagellar tip, distal to the cell body (Marshall and Rosenbaum, 2001). Biochemical studies in *Chlamydomonas* indicate that axonemal components interact with the IFT machinery (Qin *et al.*, 2003). Our results do not justify a role for Oda5 (or Oda8 and Oda10) proteins functioning in an IFT-dependent transport of outer dynein arm subunits. IFT proteins are released upon detergent extraction whereas Oda5p is retained on the demembrated axonemes. Rather, our data support a model for an Oda5-complex which functions in the axoneme.

**Oda5p is an Axonemal Protein that Sediments at ~5S.**

Antibodies to Oda5p localize the protein to the axoneme. Oda5p is extracted from the axoneme with high salt, a condition that also removes the outer dynein arm, as well as the ODA-DC (Piperno and Luck, 1979; Takada *et al.*, 1992; Takada *et al.*, 2002). However, Oda5p does not associate with these two structures in the high salt extract, as determined by sucrose gradient sedimentation. One possibility is that Oda5p does associate with these complexes, but the association is disrupted once the complexes are removed from the axoneme. Takada *et al.* (1994) demonstrated that purified outer dynein arm fractions could reassemble onto *oda5* axonemes (Takada and Kamiya, 1994). This is most likely due to the fact that the ODA-DC is present on the *oda5* axonemes, thereby allowing the outer arm constituents to rebind in their correct position.

However, in the absence of Oda5p in *oda5* mutant axonemes, it is not clear how the outer arm can rebind without a functional Oda5p present. One possibility is that the conditions used to purify the 18S +12S outer arm fractions in the studies of Takada *et al.* (1994) actually preserved an association of the Oda5p with the outer arm. The conditions used in the current study were designed to maintain the association of the ODA-DC with the outer arm (Takada *et al.*, 1992; Takada *et al.*, 2002), however, there may be subtle differences between the two isolations that have not been identified. The outer arm is sensitive to hydrostatic forces, and dissociates if these forces are too high. It is possible that the same is true for the interaction of Oda5p with the outer arm. While

---

<sup>6</sup>In Takada *et al.*, (1992), the centrifugation conditions were 180,000 x g for 4.5 hrs in a Hitachi RPS 55T-2 rotor. These conditions maintain the outer arm-ODA-DC as a ~23S complex. This complex is also kept intact using a SW50.1 rotor at 182,788 x g (39,000 rpm) for 7 hr (Takada *et al.*, 2002). Our centrifugation conditions used a SW55ti rotor at 39,000 rpm (184,832 x g) for 4.5 hrs.

our centrifugation conditions maintained the outer arm-ODA-DC as an intact complex, the slightly higher g-forces<sup>6</sup> used in our centrifugation conditions may be strong enough to release of Oda5p from the outer arm. A second possibility is that the outer dynein arm requires modification, which facilitates assembly *in vivo*. The purified outer arms used in the reconstitution experiments (Takada and Kamiya, 1994; Takada *et al.*, 1992) were derived from wild-type axonemes, in which the outer arm had already assembled and functioned within the axoneme. Any such modification would already have been made to the purified dynein fractions used for reconstitution. It is possible that *in vitro* outer dynein arm assembly is a different situation than assembly *in vivo*.

Nonetheless, the sedimentation of Oda5p in the sucrose gradients indicates that Oda5p is not a previously unidentified component of the outer arm, or the ODA-DC. Oda5p co-sediments with BSA at ~5S in sucrose gradients. BSA is a 66-kDa protein that sediments at ~5S in its monomeric form (reported range is 4.3S-5S) (Budnick and Fitzgerald, 2003; Denning *et al.*, 2003; Steinberg and Schliwa, 1996). The 66-kDa Oda5p could also be sedimenting as a monomer in the gradients. Alternatively, since Oda5 is predicted to contain extensive coiled-coil secondary structure, it is possible that Oda5 is complexed with its binding partners, forming an extended structure, similar to the extended rod-like structure formed by the DC1 and DC2 components of the ~7S ODA-DC.

**Oda5 Assembles Onto the Axoneme in Many, But Not All *oda* Mutants.**

To further study the localization Oda5p, we investigated the assembly of Oda5p onto the axoneme in a number of known *oda* mutants. Oda5p assembles onto the axoneme in *oda* strains that are defective in either outer dynein arm or ODA-DC components. Oda5p assembles onto *oda8* axonemes, however it fails to assemble onto axonemes from *oda10*. This demonstrates that Oda5p can assemble onto the axoneme independently of both the outer dynein arm and the ODA-DC and does not require these complexes for localization. The finding that Oda5p behaves differently than the outer arm and ODA-DC further supports the hypothesis that Oda5 participates in a different complex.

#### **Oda5p Interacts with Oda10p**

The inability of *oda5*, *oda8* and *oda10* mutants to complement in temporary dikaryons suggested that the Oda5, Oda8, and Oda10 proteins interact in a complex (Kamiya, 1988). Our finding that Oda5p is missing from axonemes of the *oda10* mutant demonstrates that a functional Oda10p is required for the localization of Oda5p, and provides the first biochemical evidence for an interaction between these two proteins. In contrast, Oda5p was not missing from axonemes of the *oda8* mutant. It is possible that Oda8p is associated with Oda5p and Oda10p, but is not required for binding of Oda5p to the axoneme.

#### **MATERIALS AND METHODS**

### Strains and Culture Conditions

*Chlamydomonas reinhardtii* strains used in this study include: CC-2229 (*oda1*, *mt+*), CC-2233 (*oda3-1*, *nit1*, *nit2*, *AC17*, *mt-*), and 137C (*nit1-137*, *nit2-137*, *mt+*) are from the *Chlamydomonas* Genetics Center (Department of Biology, Duke University, Durham, NC). *oda8+* (*oda8*, *mt+*), *oda10+* (*oda10-1*, *mt+*) (Kamiya, 1988), *oda9-V5* (*oda9*, *mt+*) (Wilkerson *et al.*, 1995), and *oda5-2* (*oda5-2*, *cw15*, *nit1-305:NIT1*, *mt-*). Strain 112b.221.4 (*oda5-2*: HA-*ODA5*, *arg2*, *mt-*) was created by transformation of an HA-tagged *ODA5* gene into strain 112b (*oda5-2*, *arg2-*, *mt-*). *Chlamydomonas* cells were grown as described in CHAPTER II.

### Isolation of Flagella and Flagellar Fractionations

Flagella were isolated by the method of Witman (1986) and extracted with 1% Nonidet P-40 (NP-40) (Calbiochem, La Jolla, CA) in HMDEKP (30 mM Hepes pH 7.4, 5 mM MgSO<sub>4</sub>, 1 mM DTT, 0.5 mM EGTA, 25 mM KCl, and 1 mM PMSF) as indicated. Demembrated axonemes were subsequently extracted with 0.6 M KCl in HMDEKP buffer. High-salt extracts were fractionated on 5-20% sucrose gradients under conditions that maintain the association of the outer arm and the ODA-DC (Takada *et al.*, 2002). A mixture of bovine thyroglobulin, catalase, and bovine serum albumin (Sigma) was run in a parallel gradient for S-value determination.

### Polyclonal Antibody Production

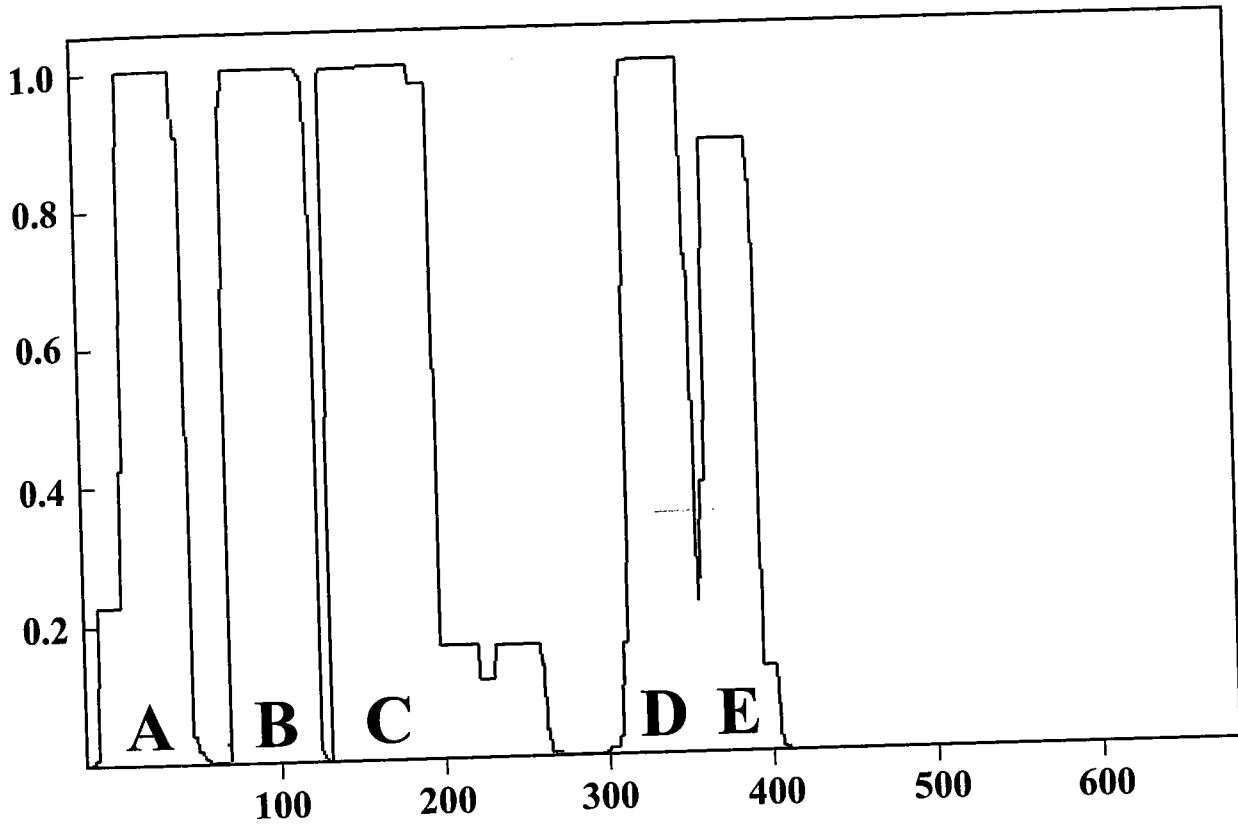
A cDNA encoding an NH<sub>2</sub>-terminal fragment of Oda5p was subcloned into the pMAL vector (Invitrogen) to create a construct containing the first 154 amino acids of Oda5p fused to the maltose binding protein (MBP). This fusion protein was bacterially expressed, purified and used to immunize rabbits for polyclonal antibody production (Invitrogen). Affinity purification was performed using an Oda5-GST fusion protein containing the same NH<sub>2</sub>-terminal fragment of Oda5.

### **Western Blots and Immunoprecipitations**

SDS-PAGE and western blots were performed according to standard methods. Protein extracts from intact cells were prepared by centrifuging cells and mixing the cell pellets with sample buffer (10mM Tris pH 8.0, 32 mM dithiothreitol, 1 mM EDTA, 10% sucrose, and 1% SDS). Samples were heated for 10 minutes and sheared with a 22g needle. Axonemal fractions were prepared as described above and dissolved in sample buffer. Proteins were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Cambridge, MA). Oda5p was localized using the anti-Oda5p polyclonal antibody diluted 1:1000 in 5% horse serum in 1x TBST (TBS plus 0.5% tween-20). IC2, the  $\gamma$ DHC, and DC2 were detected using monoclonal antibodies 1869A, 12 $\gamma$ B (King *et al.*, 1985), and an anti-DC62 polyclonal antibody (Wakabayashi *et al.*, 2001) respectively. HRP-conjugated secondary antibodies (Pierce, Rockford, IL and Sigma) were used at 1:2000.

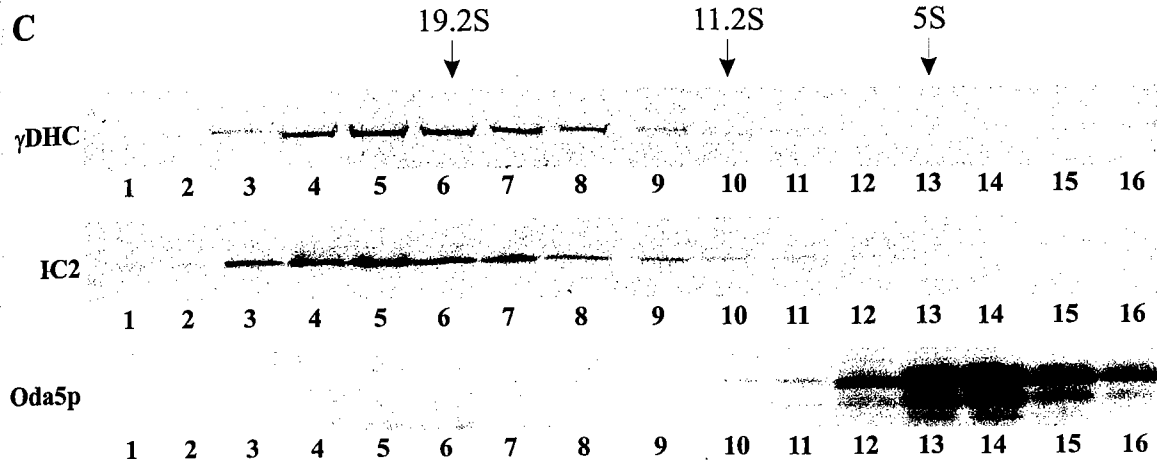
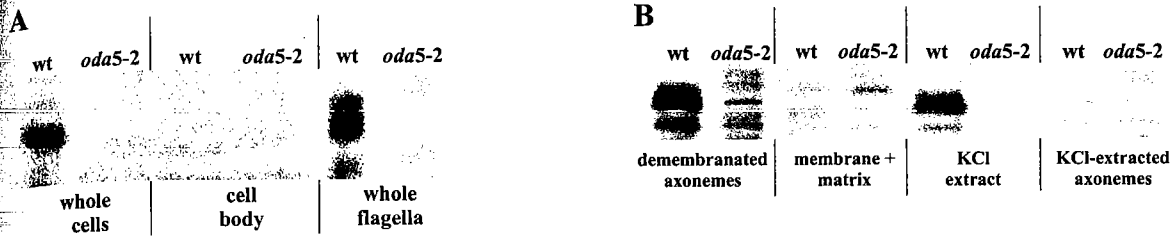
**Figure 14: Coiled-coil secondary structure of Oda5p.** Graphical representation of the predicted coiled-coil regions in the Oda5 protein as determined using the COILS program (MTIDK matrix, with a 2.5 weighting of hydrophobic positions a and d, and a window size of 28) (Lupas *et al.*, 1991). The x-axis is amino-acid number; the y-axis is the probability that the sequence will form coiled-coil secondary structure.





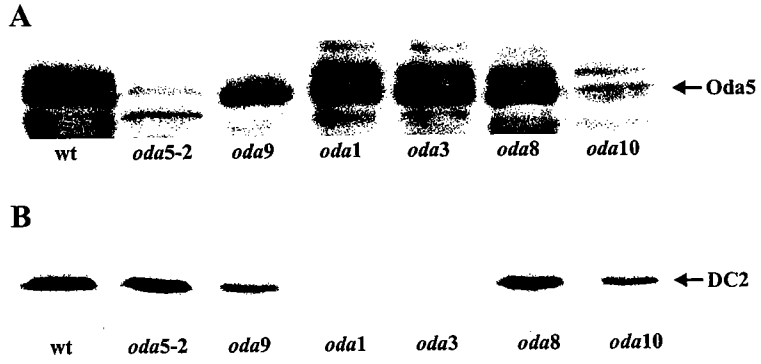
**Figure 14**

**Figure 15: Western blot analysis indicates Oda5p is a salt-extractable,  $M_r$  76,000 axonemal protein that sediments at  $\sim 5S$  in sucrose density gradients.** (A) The Oda5 antibody recognizes a  $M_r$  76,000 band in wild-type whole cells that is absent from the *oda5-2* whole cells, confirming the antibody recognizes the correct protein. This band is not detected in cell bodies lacking flagella (middle panel), yet it is readily detected in whole flagella (right panel). (B) Oda5p remains associated with the axoneme after Nonidet P-40 detergent extraction (demembrated axonemes) and is not detected in the Nonidet P-40 detergent-soluble membrane + matrix fraction (membrane + matrix). Extraction of demembrated axonemes with 0.6 M KCl releases Oda5p into the KCl extract; none is detected in the KCl-extracted axonemes. (C) Sucrose gradient fractions were probed with antibodies to outer dynein arm components IC2 and  $\gamma$ -DHC, and with the Oda5p-antibody. The outer dynein arm/ODA-DC complex sediments at  $\sim 23S$  as expected; however, Oda5p sediments at  $\sim 5S$ .



**Figure 15**

**Figure 16: Oda5p assembles onto the axoneme independently of the outer dynein arm and the ODA-DC.** (A) Western blot of axonemes isolated from wild type (wt) and *oda5-2*, *oda9*, *oda1*, *oda3*, *oda8*, and *oda10*, and probed with the Oda5p-antibody. Oda5p is detected on axonemes from wild type and all the *oda* mutants except *oda5-2* and *oda10*. (B) A western blot of axonemes from the same strains was probed with an antibody to the DC2 component of the ODA-DC. DC2 assembles onto axonemes in all strains except *oda1* and *oda3*, which are defective in DC2 and DC1, respectively.



**Figure 16**

## CHAPTER V

Oda5p AND Oda10p ARE REQUIRED FOR THE ASSEMBLY OF A SUB-FRACTION OF FLAGELLAR ADENYLATE KINASE

## INTRODUCTION

Kamiya (1988) identified three complementation groups of *oda* mutants. Possible explanations for these results may be that (1) partial complexes are generated in each *oda* mutant cell and these "mutant" complexes are incapable of reassembly in the dikaryon; (2) loss of one protein may result in sequestration of binding partner(s) such that they are unaccessible for assembly in the dikaryon cell; or (3) the loss of one protein may result in the instability of its binding partner(s). For the *oda2* complementation group, Fowkes and Mitchell (1998) provide the first evidence that lack of complementation among *oda* mutants results from the prevention of complex preassembly in the cytoplasm. However, there are also mutants in this group which exhibit subunit instability. Expression of both intermediate chains is disrupted in the *oda6* and *oda9* mutants, which contain defects in the structural genes for IC2 and IC1 respectively. Therefore, their association is necessary for their stable expression in the cytoplasm.

The *ODA7* gene product is unknown; therefore, its expression could not be directly assessed in these experiments. However, the *oda7* mutation results in instability of the  $\alpha$ -DHC (*ODA11*), and prevents pre-assembly of the ICs with the remaining  $\beta$ - and  $\gamma$ -DHCs. Thus, a combination of subunit instability and complex preassembly defects may contribute to lack of complementation in the *oda2* complementation group. The model of subunit instability is further supported by the *oda1* complementation group. Oda1p and Oda3p interact in the ODA-DC. Expression of these proteins is compromised in both *oda1* and *oda3* mutants (Wakabayashi *et al.*, 2001), demonstrating that their association is necessary for their stable presence in the cytoplasm.

The non-complementation of *oda5*, *oda8*, and *oda10* has suggested that the products of these genes interact with each other (Kamiya, 1988), such that in the absence of one component of the complex, the remaining subunits are unstable. If this scenario is correct, then additional proteins will be absent in the *oda5* mutant flagella. The identification of these missing proteins will be an important step towards identifying Oda5p-interacting proteins.

This chapter details the use of isotope coded affinity tagging (ICAT) to identify Oda5p-associated proteins. ICAT is a quantitative mass spectrometry-based method for comparing the global protein expression profile, or "proteome", of two different samples. This approach revealed that the absence of Oda5p (and Oda10p) is correlated with a reduction in the level of a previously uncharacterized flagellar adenylate kinase (AK). The results suggest that Oda5p is part of a complex that includes the flagellar AK. Therefore, the Oda5p-containing complex is not only essential for outer arm

assembly, but probably anchors AK in close proximity to the arm, ensuring that both high-energy phosphate bonds of ATP can be efficiently utilized at the axonemes major site of power production.

## RESULTS

### Oda5-associated Proteins

In an effort to identify proteins dependent upon Oda5p for axonemal localization, I used the recently developed ICAT technique (Han *et al.*, 2001) to compare proteins present in wild-type vs. *oda5* flagellar fractions. ICAT allows one to determine the relative amounts of individual proteins in the two fractions being compared.

Subassemblies of the outer dynein arm are known to remain in the cytoplasm of mutants unable to assemble the arm due to loss of an outer arm protein (Fowkes and Mitchell, 1998), and preassembled axonemal complexes are present in the flagellar matrix, even in non-regenerating flagella (Qin *et al.*, 2002; Qin *et al.*, 2003). To best identify Oda5-interacting proteins, it was necessary to perform the ICAT on as comprehensive a set of flagellar proteins as possible. Therefore, for the ICAT experiments, it was important to remove the cell bodies by isolating the flagella, and selectively remove the flagellar matrix fraction, which might contain Oda5p-interacting proteins that are stable but unable to attach to the axoneme in the *oda5* mutant, while leaving as many non-matrix proteins as possible. To do this, isolated flagella from wild-type and *oda5-1* cells were treated with the non-ionic surfactant Tergitol Type NP-40,



which disrupts but does not completely dissolve the flagellar membrane (Figure 17), releasing the soluble matrix<sup>7</sup>. The axonemes and residual membrane were then collected by centrifugation, leaving the matrix in the supernatant. The resulting pellets were then washed, solubilized in SDS and urea, and labeled with the isotopically light (wild type) and isotopically heavy (*oda5*) forms of the ICAT reagent, which specifically labels cysteines. The labeled samples were combined, digested with trypsin, fractionated by cation exchange chromatography, and the labeled peptides purified by affinity chromatography. The purified, labeled peptides were analyzed by electrospray tandem mass spectrometry (ESI-MS/MS). The ratio of the isotopically heavy (*oda5-1*) to isotopically light (wild type) peptide in each peptide pair was determined from the signal intensities of the peaks as the pair eluted into the mass spectrometer. Peptide sequences were identified from the MS/MS spectra by searching the *Chlamydomonas* 20021010 EST database, or the BLAST non-redundant database with *Chlamydomonas* specified as the organism. Peptide and EST sequences were then used to search the *Chlamydomonas* genome database.

Although the ICAT analysis identified only some of the known axonemal proteins, the results for these were as expected (Table IV). For example, peptides representing the  $\alpha$ ,  $\beta$  and  $\gamma$  heavy chains of the outer dynein arm were present in *oda5-1* flagella in amounts ranging from 0 to 0.24 times their wild-type levels. In contrast, peptides representing proteins of the ODA-DC (DC1), inner dynein arm (p28), and

---

<sup>7</sup>Tergitol membrane solubility properties were discovered and characterized by Nathan Agrin and Maureen Wirschell.

central-microtubule-pair-complex (PF6) were not reduced in *oda5-1* compared to wild-type. One notable exception is a peptide from the p28 LC, which was reported with a ratio of 1.95, while a second peptide from the same protein had a ratio of 1.0-- the expected ratio for this protein. This reflects a low-level error rate by the ProICAT software, which is not 100% accurate in determining the peak signal intensities of the MS spectra (Derek Smith, UVic Genome BC Proteomic Centre, personal communication). In spite of the occasional inaccuracy in quantitation, these results validate the ICAT approach for the quantitative comparison of flagellar fractions.

A peptide representing a previously unidentified WD-repeat protein was present in *oda5-1* at about two-thirds its amount in wild type (Table IV); this protein (Green Genie 492.9) is represented in the EST database (20021010.5906.1).

Moreover, four other peptides, all derived from the same predicted gene, were present in *oda5-1* in amounts ranging from 0.54 to 0.64 their wild-type levels. These peptides represent an adenylate kinase (AK) protein. The identification of four independent peptides from the same protein, all with similar quantitation ratios, lends confidence to the ICAT result, which indicates that the levels of this AK are reduced in *oda5-1* mutant flagella.

### **Flagellar AK Activity is Reduced in *oda5***

AK activity has previously been observed within the *Chlamydomonas* flagellum (Watanabe and Flavin, 1976). To confirm the apparent reduction of this enzyme in

*oda5-1* flagella, I directly assayed AK enzymatic activity in Tergitol-extracted axonemes from wild-type, *oda1*, *oda3*, *oda5-1*, *oda8*, *oda9*, *oda10-2* and rescued strains.

Compared to its level in wild type, AK activity was reduced by ~30-50% in *oda5-1* and *oda10-2* but not in *oda1*, *oda3*, *oda8* or *oda9* (Figure 18). Corresponding reductions in activity also were observed in *oda5-2* and *oda10-1* (data not shown). A strain rescued for the Oda- motility defect with an HA-tagged *ODA5* gene construct had wild-type levels of AK activity, demonstrating that it also is rescued for the Ak- defect. Therefore, the HA-epitope does not interfere with the function of Oda5 in outer dynein arm or AK assembly. The ~35-50% reduction in AK activity in *oda5* axonemes correlates well with the 35-45% reduction in AK protein levels in *oda5-1* as determined by ICAT. The similar reduction observed in *oda10* provides additional evidence for a biochemical connection between Oda5p, Oda10p and AK. The loss of AK activity is not a general consequence of the failure to assemble the outer arm or ODA-DC, as AK activity is normal in *oda1*, *oda3*, and *oda9* mutants lacking these structures.

Interestingly, I found that if wild-type flagella were extracted with Nonidet P-40 rather than Tergitol, the fraction of AK activity that is deficient in *oda5-1* mutants is soluble. When wild-type and *oda5* flagella were extracted with Tergitol, the AK activity in the resulting pellet was 35% less in *oda5* than in wild type, whereas the AK activity in the soluble fraction was equivalent in wild type and *oda5* (Figure 19). However, when flagella were demembranated with Nonidet P-40, the amount of AK activity remaining in the axonemes was equivalent in wild type vs. *oda5*, but now the amount of AK activity in the soluble fraction was reduced ~40% in *oda5* as compared to wild type

(Figure 19). The most likely explanation for these results is that the AK is associated with the Oda5p complex via a Nonidet-sensitive bond. Alternatively, since Nonidet, but not Tergitol, completely removes the flagellar membrane (Figure 19), the Oda5p-associated AK may be a membrane-associated protein.

The overall AK activity is diminished in the Nonidet P-40 flagellar fractions, as compared to Tergitol-flagellar fractions. Since Nonidet is a stronger detergent, as evidenced by its ability to solubilize the flagellar membrane, it is possible that it depresses the activity of the AK enzyme. Nevertheless, the level of reduction detected in *oda5* vs. wild-type flagellar fractions obtained with Nonidet P-40 is consistent with that observed for flagellar fractions obtained with Tergitol.

### **The Flagellar AK Gene**

The flagellar AK gene is split between two overlapping scaffolds in the current *Chlamydomonas* genomic database (Figure 20A) and is predicted to encode an AK with a mass of ~27-kDa and homology to AKs in other organisms. The gene contains three exons. The cDNA sequence was derived from EST sequences and PCR-amplified cDNA clones (Figure 20B). The gene utilizes consensus splice sites for the intron-exon junctions, except the exon2-intron2 junction. It also uses slightly variant start and stop sequences; the sequence context around the start codon is ACAGATGG, and the stop codon is GTGAG, using the TGA stop codon rather than the more commonly used TAA (see CHAPTER III for the consensus sequences for splice junctions, and start and stop sequence context).

The NH<sub>2</sub>-terminus is unique and may be important for the localization of this AK isoform to the flagellum. The third exon contains the homology to AK enzymes.

AKs have two highly conserved motifs, the AK signature domain and the ATP-binding domain or P-loop (Figure 20C). The AK signature domain constitutes part of the catalytic cleft and contains conserved arginine and aspartic acid residues ([LIVMFYWCA]-[LIVMFYW](2)-D-G-[FYI]-P-R-X(3)-[NQ]). The P-loops of AKs deviate from the usual P-loop consensus sequence ([AG]-X<sub>4</sub>-G-K-[ST]) in that the last position is occupied by a glycine instead of a serine or threonine (Prosite PDOC00017). The flagellar AK contains the conserved aspartic acid and arginine in the signature motif (FLIDGFPRALDQ) and the conserved glycine substitution within the P-loop domain (GGPGSGKG); all of which are encoded by the third exon, as are the four ICAT-identified peptides (Figure 20C).

#### **The Adenylate Kinase Gene is Tightly Linked to the *ODA7* locus, But Does Not Encode the *Oda7* Gene Product**

Physical mapping of the AK gene revealed that it resides on Linkage Group I near the known markers *ARG7*, *PBT302*, *GBP1* and *RB47* (Nancy Haas, personal communication) (Figure 21A). Interestingly, both the *ODA7* and *ODA8* genes map to the same region of Linkage Group I (Kathir *et al.*, 2003). To determine if the AK gene is near the *ODA7* or *ODA8* genes, I screened Southern blots of meiotic progeny derived from a cross between these uncloned *oda* mutants and CC-2290; a strain that is interfertile, yet shows a high degree of polymorphism with standard laboratory strains of

*Chlamydomonas reinhardtii*<sup>8</sup>. The AK probe detected an RFLP that segregated with the Oda7-motility phenotype, but not the Oda8-motility phenotype, indicating that the probe is tightly linked to the *ODA7* gene (Figure 21B). To investigate the possibility that the *ODA7* gene and the *AK* gene are one and the same, I assessed the AK activity in Tergitol-extracted *oda7* flagella. AK activity was normal in *oda7* mutant flagella (Figure 21C), suggesting that the flagellar AK does not encode the Oda7 gene product.

## DISCUSSION

### A Flagellar AK Associates with the Oda5p/Oda10p Complex

ICAT analysis revealed that a flagellar AK was reduced 35-45% in *oda5-1* compared to wild type. AK catalyzes the reversible reaction:  $2 \text{ ADP} \leftrightarrow \text{ATP} + \text{AMP}$ . Direct enzymatic assays confirmed that flagellar AK activity was reduced in *oda5*, and indicated that the AK activity was similarly reduced in *oda10* but not in the other *oda* mutants. Therefore, a portion of the newly identified AK is dependent upon Oda5p and Oda10p for its incorporation into the flagellar structure. Because the flagellar AK is not reduced in other mutants that lack the outer arms or the outer arms together with the ODA-DC, it is not simply a subunit of either of these structures. Rather, it appears to be specifically associated with the Oda5p/Oda10p complex. AK activity is not reduced in *oda8*, consistent with our finding that Oda5p is assembled onto *oda8* axonemes. It is

---

<sup>8</sup>Crosses to CC-2290 and Southern blotting of DNA derived from the progeny of these crosses were done by Gregory Pazour.

possible that Oda8p functions only in binding of the outer arm, while Oda5p and Oda10p function in assembly of both the outer arm and adenylate kinase.

### **The Flagellar AK**

The Oda5-associated flagellar AK is solubilized from the wild-type flagellum by treatment with Nonidet P-40 but not Tergitol. Because the AK is not predicted to contain transmembrane domains, and because its presence in the flagellum is dependent upon Oda5p, which is an axonemal protein, it is probable that the AK also is an axonemal component, and that it is associated with the axoneme via interactions that survive the Tergitol treatment but are disrupted by Nonidet P-40.

AK activity previously has been reported in cilia and flagella from several organisms (Nakamura *et al.*, 1999; Noguchi *et al.*, 2001; Schoff *et al.*, 1989; Watanabe and Flavin, 1976), although neither the specific AK nor its location in the flagellum were determined. The apparent association of the AK with Oda5p and Oda10p in the current study, and the fact that the latter are required for outer dynein arm assembly, suggests that the AK is held in close proximity to the outer arm by the Oda5p/Oda10p complex. I propose that the outer dynein arm -- one of the major ATP-hydrolyzing structures of the axoneme -- is intimately associated with an ATP-regenerating system in order to achieve efficient conversion of ADP to ATP and AMP, thus ensuring that both high-energy phosphate bonds of ATP are readily accessible to this important force-generating machine.

That the flagellar AK is reduced but not completely absent in the *oda5* and *oda10* mutants indicates that the AK is located at additional sites within the axoneme. One likely site for the remaining flagellar AK is the inner dynein arm system. Such a localization would place the AK at two major sites of ATP utilization in the axoneme.

The AK gene was mapped to Linkage Group I, near the *ODA7* locus. Direct enzymatic assays of *oda7*-mutant flagella demonstrate that AK activity is normal in *oda7*; thus the flagellar AK is not the Oda7 gene product. Although tight linkage between AK and *ODA8* was not found, *ODA8* also resides on linkage group I. The fortuitous location of the AK gene may prove useful for ultimately identifying these uncloned *ODA* genes.

### **A Model for the Oda5p, Oda8p, and Oda10p Complex in Outer Arm and AK Assembly**

Based on the non-complementation data and our biochemical data, we propose that Oda5p, 8p, and 10p interact in a protein complex. A model for the assembly of this complex is shown in Figure 22. In this model, Oda5p, Oda8p, and Oda10p interact in a complex that is targeted to the axoneme, where it functions in outer dynein arm and AK assembly. If either the *ODA5* or *ODA10* genes are disrupted, then the remaining subunits are unstable and no partial complex is formed, yielding the Ak- and Oda- phenotypes. If the *ODA8* gene is disrupted, then Oda5p (and potentially Oda10p) forms a stable, partial complex that is targeted to the axoneme. This study demonstrates that Oda5p is assembled onto the axoneme in the absence of Oda8p. It remains an open



question as to whether Oda10p also localizes to the axoneme in *oda8* mutants. In any case, I propose that a partial complex containing Oda5p, and possibly Oda10p, is sufficient for assembly of the outer arm associated-AK, but not for assembly of the outer dynein arm itself, yielding only the Oda- phenotype in *oda8* mutants.

The inability of *oda5*, *oda8* and *oda10* mutants to complement in temporary dikaryons can be explained by this model. Dikaryons between *oda5* and *oda10* do not complement because the entire complex is missing in both strains. Dikaryons between *oda8* and either *oda5* or *oda10* do not complement because both strains would be missing the Oda8 protein. The dikaryon rescue only looks at the increase in beat frequency of the resulting quadriflagellate cell. Therefore, the complementation data only addresses the Oda- phenotype, not the Ak- phenotype. If the flagellar AK activity could be measured in the dikaryon cell, one would predict that the Ak- phenotype would not be complemented in an *oda5* and *oda10* dikaryon. However, dikaryons between *oda8* and *oda5*, or *oda8* and *oda10*, would be expected to complement for the Ak- phenotype, while not complementing the Oda- phenotype.

The current findings will facilitate studies to identify the gene products of the *ODA8* and *ODA10* genes. These studies will provide further insight into the nature of this complex, as well as providing additional insights into the assembly mechanisms of the outer dynein arm and its associated adenylate kinase.

#### **A WD-Repeat Protein Also Is Reduced in *oda5* Flagella**

ICAT analysis also identified one peptide, from a predicted WD-repeat protein, that was reduced in *oda5-1* flagella by about the same amount as the AK. It is tempting to speculate that this protein may be associated with the AK. The protein has homologues in other flagellated organisms, including *Homo sapiens*, *Mus musculus*, *Macaca fascicularis*, *Anopheles gambiae*, and *Drosophila melanogaster*. Further characterization of the protein will be required to clarify its relationship, if any, to the flagellar AK.

#### **Utility of ICAT for Comparing Proteins in Flagella of Different Mutants**

Our ICAT analysis correctly reported the relative levels, in wild-type vs. *oda5* flagella, of peptides that were identified as being derived from known axonemal proteins, as well as those from the previously undescribed flagellar AK. The main shortcoming of the ICAT approach was its inability to identify a more comprehensive set of flagellar proteins, as evidenced by its failure to identify peptides from a greater percentage of known axonemal proteins. This was, most likely, due to the long duty cycle (~14 s) of the mass spectrometer used, which prevented many peptides from being selected for fragmentation for MS/MS analysis. This problem should be greatly alleviated by newly available instruments, which have much faster data acquisition rates and thus can analyze a much larger number of peptides from a sample of the same complexity. With the use of such instruments, ICAT and similar quantitative proteomic approaches (Aebersold and Mann, 2003) are likely to become very valuable for identifying specific proteins whose levels are altered in mutant vs. wild-type flagella.

## MATERIALS AND METHODS

### Strains and Culture Conditions

*Chlamydomonas reinhardtii* strains used in this study include: CC-2290 (*mt-*) and 137C (*nit1-137, nit2-137, mt+*) are from the *Chlamydomonas* Genetics Center (Department of Biology, Duke University, Durham, NC). Additional strains include: *oda1+* (*oda1, mt+*), *oda3+* (*oda3, mt+*), *oda5+*, (*oda5-1, mt+*), *oda7+* (*oda7, mt+*), *oda8+* (*oda8, mt+*), *oda9+* (*oda9, mt+*), *oda10+* (*oda10-1, mt+*) (Kamiya, 1988), V87.2 (*oda10-2, nit1:NIT1, NIT2, agg1, mt-*) (Koutoulis *et al.*, 1997), *oda5-2* (*oda5-2, cw15, nit1-305:NIT1, mt-*), and 112b.221.4 (*oda5-2: HA-ODA5, arg2, mt-*). *Chlamydomonas* cells were grown as described in CHAPTER II.

### Construction of an HA-tagged *ODA5* Gene

Plasmid p125.5 was created by ligating a 2.6-kb *EcoR1-BamH1* fragment containing the terminal 2/5<sup>th</sup> of the *ODA5* gene into the pBS-SK+ vector (Stratagene). p125.5 was digested with *HincII*, which cuts in the SK+ vector, 5' to the *EcoR1* cloning site, and at position 5901 in the *ODA5* gene. This produces a 2.3-kb fragment and a 3.1-kb fragment which includes the SK+ vector plus the 3' end of the *ODA5* gene. A 136-bp *Sma1* fragment was isolated from p3xHA (Silflow *et al.*, 2001) and ligated into the 3.1-kb *HincII* fragment from p125.5, creating plasmid p127.3. The 2.3-kb *HincII* fragment from p125.5 was ligated into the *Stu1* site of p127.3, creating plasmid p128.8. Lastly,

plasmid p50.1 was digested with *Sal1-EcoR1* and plasmid p128.2 was digested with *EcoR1-BamH1*. The 3.5-kb *Sal1-EcoR1* fragment from p50.1 was ligated into the pUC18 vector, along with the 2.6-kb *EcoR1-BamH1* fragment from p128.8. This creates construct p130.2, which contains the *ODA5* gene with the triple HA-epitope tag located 15 amino acids upstream of the *ODA5* stop codon. All constructs were sequenced and verified by restriction mapping to determine proper orientation and reading frame.

#### **Isolation of Flagella and Flagellar Fractionations**

Flagella were isolated by the method of Witman (1986) and extracted with 1% Nonidet P-40 (Calbiochem, La Jolla, CA) or 1% Tergitol Type NP-40 (Sigma, St. Louis, MO) in HMDEK (30 mM Hepes pH 7.4, 5 mM MgSO<sub>4</sub>, 1 mM DTT, 0.5 mM EGTA, 25 mM KCl) plus 1 mM PMSF as indicated.

#### **Isotope Coded Affinity Tagging (ICAT)**

ICAT was performed at the University of Victoria GenomeBC Proteomics Centre. Briefly, pellets of Tergitol-treated flagella from wild type and *oda5-1* were resuspended in 6 M urea, 0.1% SDS and then labeled using the ICAT reagent kit (Applied Biosystems Inc., Foster City, CA). The samples were combined, digested with trypsin, and the resulting peptide mixture fractionated into 4 or 10 fractions. The peptides were then affinity purified using a streptavidin column. The results are the pooled analyses of both the 4- and 10-fraction experiments. Analysis was performed on an Applied

Biosystems/MDS QStar hybrid LC/MS/MS quadrupole TOF system and quantitation performed using Applied Biosystems ProICAT software.

### **Adenylate Kinase Assays**

AK activity was determined by the method of Watanabe and Flavin (1976) with slight modifications. AK was assayed by coupling the formation of ATP from ADP to NADP<sup>+</sup> reduction in the presence of hexokinase and glucose-6-phosphate dehydrogenase. The reaction mixture consisted of 55 mM Tris pH 7.9, 40 mM glucose, 2 mM MgCl<sub>2</sub>, 1 mM ADP, 0.18 mM NADP<sup>+</sup>, 1 unit each of hexokinase and glucose-6-phosphate dehydrogenase, and 1 mM sodium(meta)vanadate to inhibit dynein ATPases. The reaction mixture was preincubated for 10 minutes to consume any ATP contaminating the ADP. Flagellar fractions were added to the reaction and the adenylate kinase activity measured by monitoring the change in absorbance at 340 nm that accompanied the production of NADPH. Data points were collected every 30 seconds for 10 minutes. Assays were performed in triplicate on three independently isolated flagellar samples.

### **Cloning of Flagellar AK cDNA**

GreenGenie (Li *et al.*, 2003) was used to analyze the flagellar AK genomic structure. Primers (Integrated DNA Technologies, Coralville, IA) to predicted exons were used to amplify the AK cDNA from a gt10 cDNA library constructed from mRNA isolated from synchronously grown cells harvested one hour after the beginning of the dark period (Pazour and Witman, unpublished data). Comparison of the cDNA sequence vs. the

genomic sequence from the *Chlamydomonas* genome database (Joint Genome Institute, Walnut Creek, CA) verified the intron-exon boundaries and untranslated regions.

### **Genetic Analysis**

Matings and tetrad analysis between CC-2290 and *oda7* and *oda8* were performed according to standard procedures (Dutcher, 1995; Harris, 1989) by Greg Pazour.

### **Isolation and Blotting of DNA**

DNA from meiotic progeny was isolated and Southern blots of *Pst*I-digested genomic DNA were probed with the AK cDNA as described in CHAPTER II.

**Table IV:** Selected ICAT-labeled peptides identified by ESI/MS/MS

| Peptide   | Ratio <i>oda5</i> /WT | Protein                          | JGI Scaffold |
|---|-----------------------|----------------------------------|--------------|
| <i>Peptides from known outer dynein arm proteins absent or greatly reduced in oda5-1:</i> |                       |                                  |              |
| CGYSVANGR   | 0.05                  | $\alpha$ -DHC                    | 820          |
| ELEICK  | 0.00                  | $\alpha$ -DHC                    | 820          |
| SQAVDASEYEALCR  | 0.14                  | $\alpha$ -DHC                    | 820          |
| TECYR   | 0.24                  | $\alpha$ -DHC                    | 820          |
| AVDAWCAQVAATSDEK  | 0.18                  | $\beta$ -DHC                     | 209          |
| CPVYTTEAR   | 0.012                 | $\beta$ -DHC                     | 209          |
| DFYGDCMK  | 0.09                  | $\gamma$ -DHC                    | 406          |
| <i>Peptides from known axonemal proteins not reduced in oda5-1:</i>                       |                       |                                  |              |
| CEAIEK  | 1.95                  | Inner dynein arm p28 light chain | 809          |
| ETGICPIR  | 1.00                  | Inner dynein arm p28 light chain | 809          |
| SVCIGAEQGLR   | 1.33                  | DC1                              | 768          |
| GGSCAFYESEQLR   | 1.15                  | PF6                              | 751          |
| <i>Other peptides reduced in oda5-1:</i>  |                       |                                  |              |
| TVLFFDCPEEEMEK  | 0.61                  | Adenylate kinase                 | 31           |
| ALDQAEQFESSIMPCK  | 0.57                  | Adenylate kinase                 | 655          |
| CHVISAVAAPDDVYGK  | 0.55                  | Adenylate kinase                 | 655          |
| CEALMK  | 0.65                  | Adenylate kinase                 | 31           |
| VQALDFSCDER   | 0.64                  | WD-repeat protein                | 492          |

**Figure 17: Electron micrographs of Nonidet P-40 and Tergitol-extracted axonemes.** Tergitol-extracted axonemes contain co-sedimenting flagellar membranes (left panel, arrows), while Nonidet-extracted axonemes are virtually free of flagellar membranes (right panel). Scale bar is 300 nm.





Tergitol



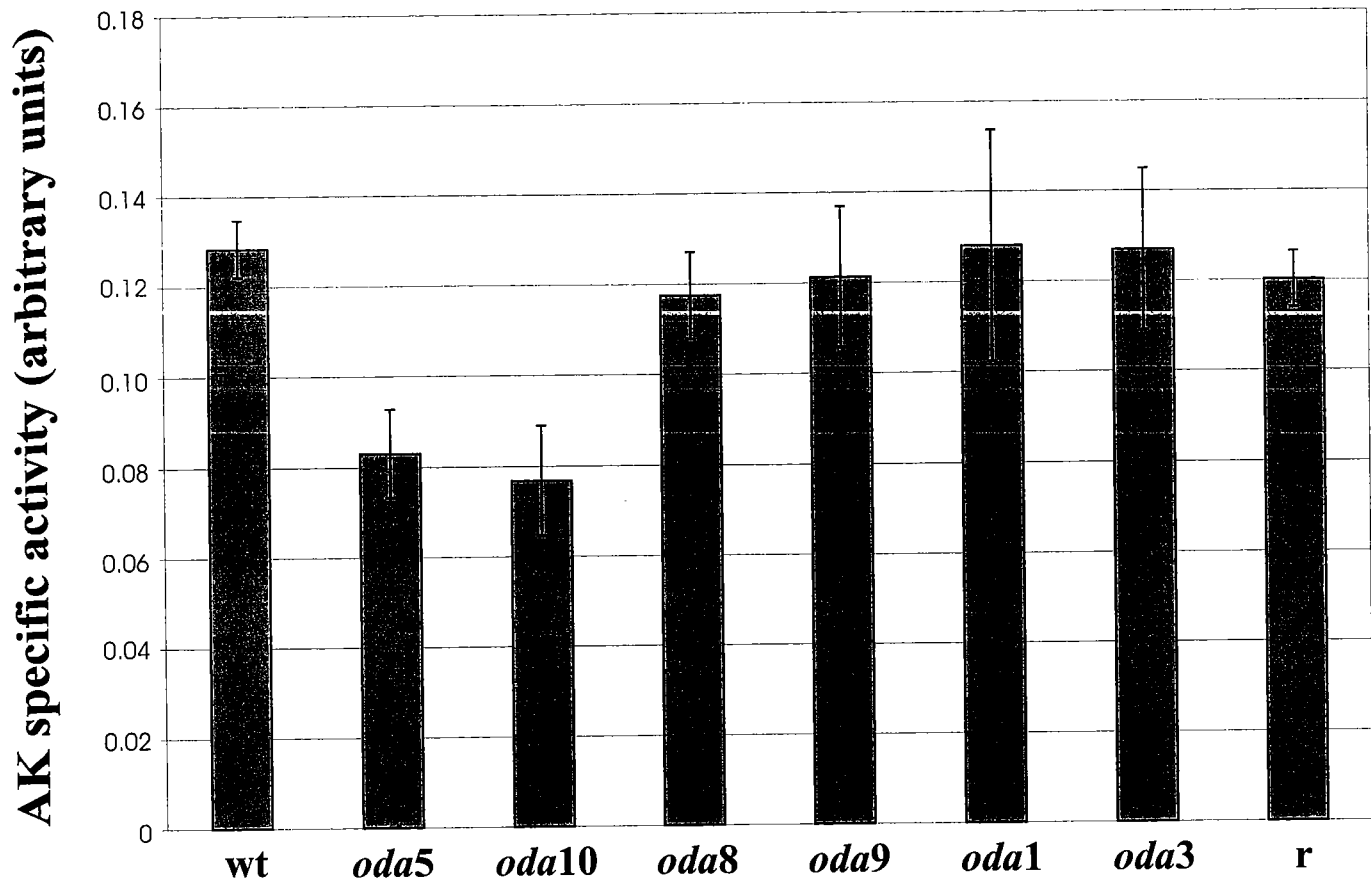
Nonidet

**Figure 17**

**Figure 18: Adenylate kinase activity is reduced in *oda5* and *oda10* flagella.**

Adenylate kinase activity was measured in Tergitol-treated axonemes from wild type, *oda1*, *oda3*, *oda5*, *oda8*, *oda9*, *oda10*, and an HA-tagged *ODA5* rescued strain (r).

Adenylate kinase activity is specifically reduced in *oda5* and *oda10* mutant axonemes, but restored to the wild-type level in the strain transformed with an HA-tagged *ODA5* gene construct. The histogram shows the AK specific activity (in arbitrary units) for each strain. Error bars represent the standard deviation calculated from three independent measurements.



**Figure 18**

**Figure 19: The Oda5p-associated adenylate kinase activity is solubilized with Nonidet P-40 detergent.** When flagella are treated with Tergitol, the Oda5p-associated AK activity remains in the pellet. However, when flagella are treated with Nonidet P-40, the Oda5p-associated AK activity is released into the supernatant. The histogram shows the AK specific activity (in arbitrary units) of each fraction. Error bars represent the standard deviation calculated from three independent measurements.

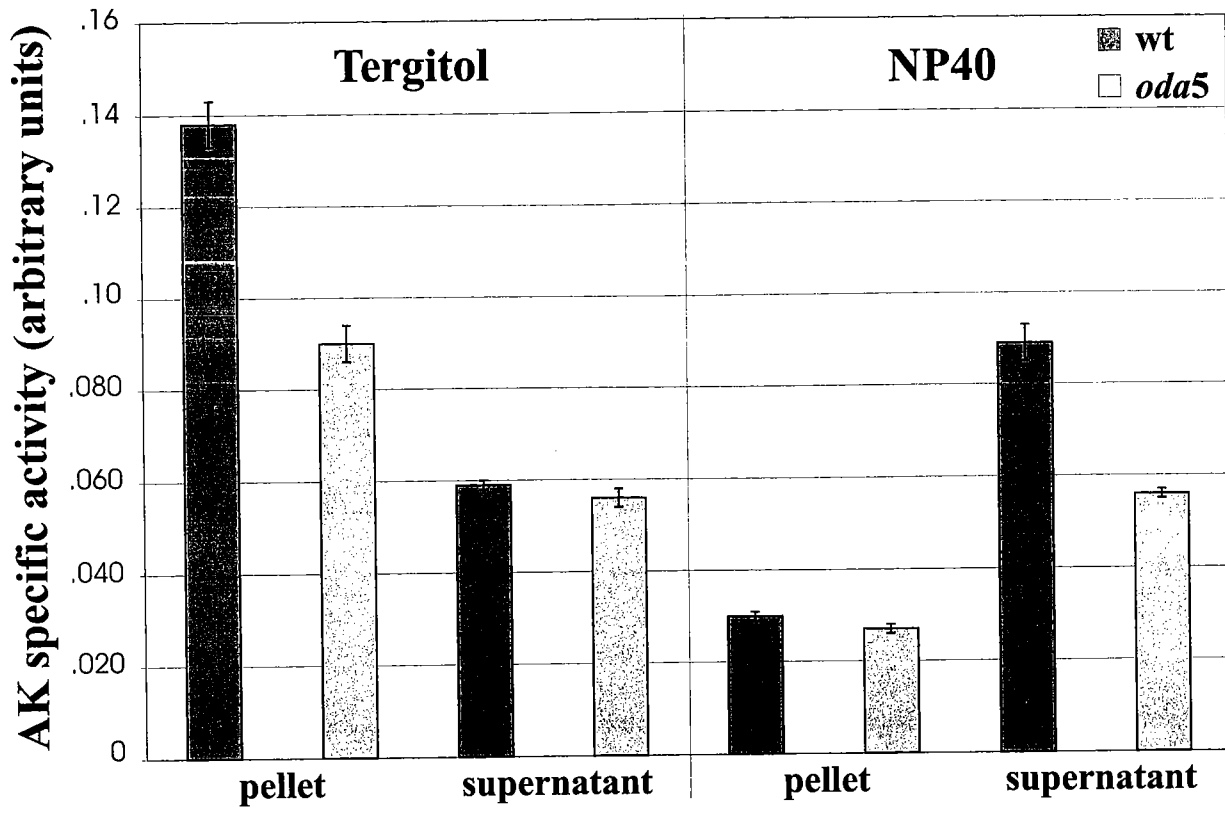


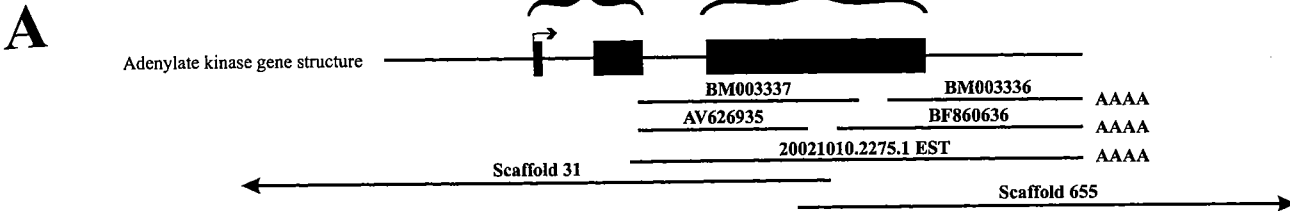
Figure 19

**Figure 20: Adenylate kinase gene structure, cDNA and deduced protein sequence.**

(A) Intron-exon structure of the AK gene. Black boxes indicate the three exons; solid lines indicate the non-coding regions; arrow indicates the direction of transcription. The gene is split between two overlapping scaffolds (Scaffolds 655 and 31). EST sequences (20021010.2275.1 Unigene consensus, BM003337, BM003336, BF 860636, and AV626935) and PCR-amplified cDNA clones verified the AK gene structure. The first two exons are unique, while the third exon contains sequences with homology to AKs.

(B) The AK cDNA sequence and deduced protein sequence. Underlined are an upstream, in-frame stop codon, TAG; a consensus polyadenylation signal sequence, TGTA, located 521-bp downstream of the stop codon; and the peptides identified by ICAT. The shaded regions denote the AK P-loop domain (amino acid 68-75) and the AK signature motif (amino acid 141-152). These sequences have been reported in GenBank/EMBL/DDBJ with accession # AY452531. (C) The P-loop and the AK signature motif are highly conserved among AKs from diverse organisms.

# unique AK homology



**B**

```

1 TGGTGTGCTGACCTTGTCTTGTCTAGCAACTCTGTATTGTGCTTTGACAAGCGGCTGTAACGCAAACCTTGAACCTTGACC 80
81 GCATAGAGCCCAAACGACTTCGCGCACGCGTGCCTCCACGGCCGCTAGGGCCATCTGGACAGCCCAAGCACAGATGGG 160
1 CTGTGGAGCTAGCAAAACGGAGCAGCCGAGCAAGCCGGAGCCAGCTGTGCCTAAAGAGCCATCATCTCCTGCGATGGGCG 240
3 CGASKTEQPSKPEPAVVPKEPSSPAMG 28
241 GAAAACCCAGCAAGCCTCAGCAGCAGCGCCGAGCGCCAGCGCAAACAGAGGAGATTGCGCTGCGCCCGTGCCTGGC 320
29 GKPSKPKPQQQAAPSAPAQTEEIALPPVPG 55
321 AGCCTACCCGCGATGCCAGATCGTGTTCGTGCTCGGCGCCCGCGCAGCGCAAGGGCAGCAATGCGACAAGATTAA 400
56 SLPADAQIVFVLLGGPGSGKGTQCDKIK 82
401 GGCCGACTATGAGTGCCTGCACCTCAGCGCCGGCAGCTGCTGCGCGCTGAGGTGAAGAGCGGCAGTGAGGTGCGCCTGA 480
83 ADYECVHLSAGDLLRAEVKSGSEVGL 108
481 AGTGGAGGCGCTGATGAAGGAGGGCAAGCTCGTCCGGTGGTGTACGCTCAACCTGCTGAAGCGGGACATGAGCC 560
109 KCEALMKEGKLVPAVATLNLKRDMA 135
561 AGCGGTGGCAAGTTCTTCCTCATCGACGGCTTTCGCGCGCGCTGGACCAGGCGGAGCAGTTTGAAGCAGCATTATGCC 640
136 SGGKFLFLIDGEPRLALDIOAEQFESSIMP 162
641 GTGCAAGACGGTGTCTTCTCGACTGCCCGGAGGAGAGATGAGAAGCGCCTGCTGAAGCGGGGAGACCAGCGCG 720
163 CKTVLFFEDCFEEMEKRLKRGETS 188
721 GCAGCGACGACAACCGGACACCATTCGCAAGCGCTTCCGACCTTCTGGATCAGTCCGCTGCCGTAAGGACCACTAC 800
189 RSDDNADTIRKRFRTFLDQSLPVKDH 215
801 CTTACGAGGGCAAGTGCCACGTGATCAGCGCTGTGGCGCTCCGACGATGTGTACGGCAAGGTGAAGGTGGCCTTGA 880
216 L T Q G K C H V I S A V A A P D D V Y G K V K V A L E 242
881 GGGCTGAGCGTGGTGAAGAAGTGAGCGGTTCTTGAGAATTGTGACCAGTCCGACTGAGGGTGAAGTATTTCCGGAACAC 960
243 G L S V V K K * 249
961 ATGGATTGTACTGTGTGAAAGCGTTTATGTATTGTGTTGGGTGCCGTGAGACGGTGTACTGCAGCACGCTTTCGTGATGA 1040
1041 CTACGCGGTATGACTGCGTGACGCTTGAACCTGGCAGCAGCCCGGCATCACGCATATCAGTAACTTGACGTTCCGACCAGC 1120
1121 ATGGAGGTCACGGGCGCCTAATAGCAGGACTTGCTTGTATCAAAAAGGGCAATGGGGTACCGCCAACATCCGGCACTAG 1200
1201 TGCCCATTCATGTTAATTGTTTGTGAGGCTAGGCGAAGACTGCATGAGTTCCTCAGGATGCATGCAGACACATTAAGCTG 1280
1281 ATGGTATTGCAGTGTGAGAATGCAGACGACTCGGAGCGCTGATGAGCGGAGCGCAGAAATGGTGCATGCACCTCTGCTCA 1360
1361 GCAACAGCCCCCGTACTGGCTGACGTCTAGCAAAGCTCGGGAAGCCATGCCAGTGCACAAAATGTAAGTGATCAGT 1440
1441 TGTGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1476
    
```

**C**

AK signature consensus:  
[LIVMFYWCA] - [LIVMFYW] 2-D-G- [FYI] -P-R-X3- [NQ]

P-loop consensus: [A/G]-X<sub>1</sub>-G-K-G

|                                      |              |          |
|--------------------------------------|--------------|----------|
| <i>Chlamydomonas reinhardtii</i>     | FLIDGFPRALDQ | GGPGSGKG |
| <i>Caenorhabditis elegans</i>        | FLIDGYPREVAQ | GGPGSGKG |
| Chicken-AK1                          | FLIDGYPREVKQ | GGPGSGKG |
| Rabbit-AK1                           | FLIDGYPRQVQQ | GGPGSGKG |
| Mus Musculus-AK1                     | FLIDGYPREVKQ | GGPGSGKG |
| Bovine-AK1                           | FLIDGYPRQVQQ | GGPGSGKG |
| Pig-AK1                              | FLIDGYPREVKQ | GGPGSGKG |
| Rat-AK1                              | FLIDGYPREVKQ | GGPGSGKG |
| <i>Homo sapiens</i>                  | FLIDGYPREVQQ | GGPGSGKG |
| Trypanosome                          | YVIDGFPRKMDQ | GGPGSGKG |
| Fern-AK1                             | FLIDGFPRNEEN | GGPGSGKG |
| Mushroom-AK                          | FLIDGFPRKMDQ | GGPGAGKG |
| <i>Oryza sativa japonica</i>         | VLIDGFPRCEEN | GGPGSGKG |
| <i>Cyprinus carpio</i>               | YLIDGYPREVKQ | GGPGSGKG |
| <i>Clonorchis sinensis</i>           | FLIDGYPHELEQ | GGPGSGKG |
| <i>Prunus armeniaca</i>              | FLIDGFPRSEEN | GGPGSGKG |
| <i>Dictyostelium discoideum</i>      | FLVDGFPRNEEN | GGPGSGKG |
| <i>Arabidopsis thaliana</i>          | FLIDGFPRNEEN | GGPGSGKG |
| <i>Oryza sativa</i>                  | FLIDGFPRNEEN | GGPGSGKG |
| Ura6 <i>Saccharomyces cerevisiae</i> | FLIDGFPRKMDQ | GGPGAGKG |
| <i>Neurospora crassa</i>             | FLIDGFPRKMDQ | GGPGAGKG |
| <i>Schistosoma japonicum</i>         | FLIDGYPRELDQ | GGPGSGKG |

Figure 20

**Figure 21: The adenylate kinase gene is tightly linked to the *ODA7* gene, but does not encode the *Oda7* gene product.** (A) The AK gene maps to linkage group I, near known markers *ARG7*, *PBT302*, *GBP1*, and *RB47*. Both the *ODA7* and *ODA8* genes reside on the same linkage group near these same molecular markers. (B) An adenylate kinase cDNA was used to probe Southern blots of progeny derived from crosses between *oda7* vs. CC-2290, or *oda8* vs. CC-2290, a strain that is interfertile, yet shows a high degree of polymorphism with standard laboratory strains of *C. reinhardtii*. The probe detected an RFLP (arrows) which segregated with the *Oda7*-motility phenotype, but not with the *Oda8*-phenotype. The upper band indicates the wild-type (CC-2290) fragment, while the lower band indicates the mutant fragment. (C) Direct enzymatic assays of *oda7* mutant flagella indicated that adenylate kinase activity is not abrogated in *oda7*. The histogram shows the AK specific activity (in arbitrary units) for each strain. Error bars represent the standard deviation calculated from three independent measurements.



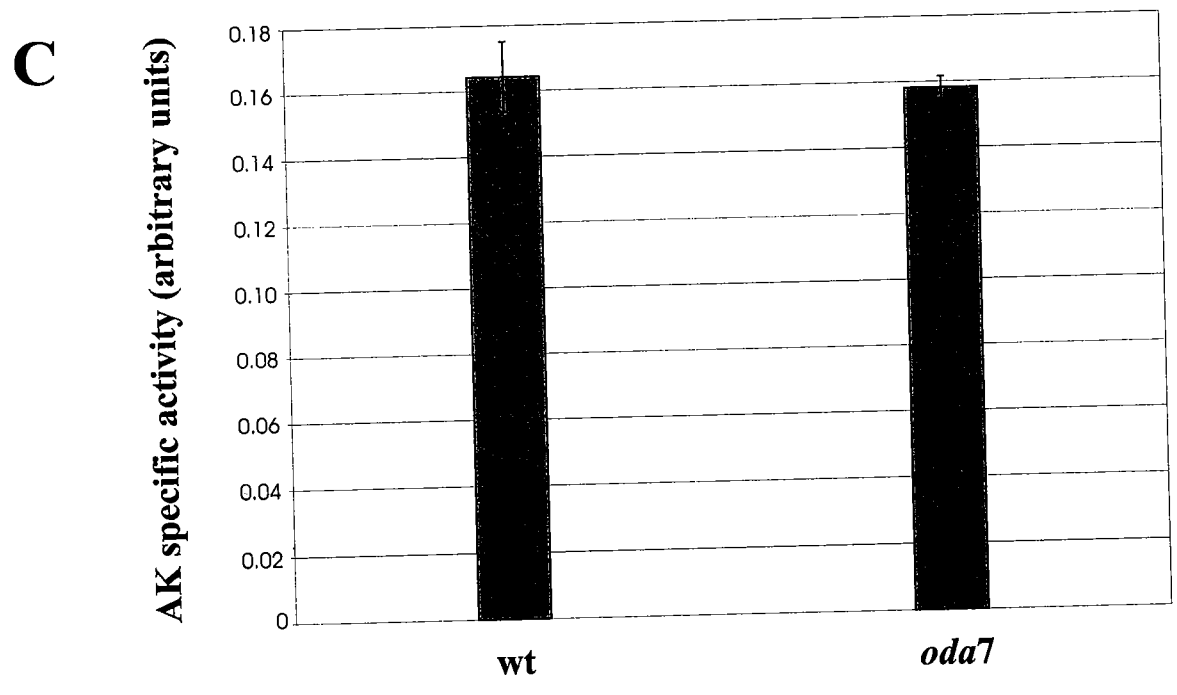
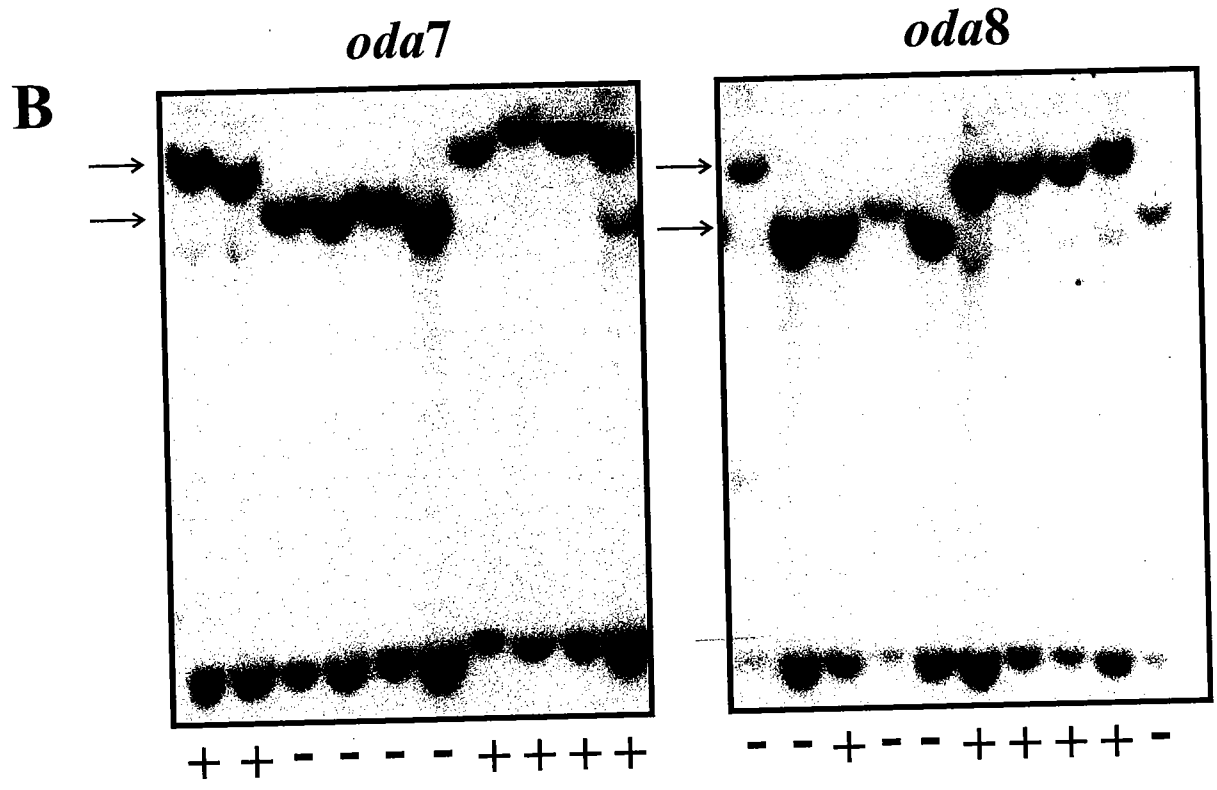
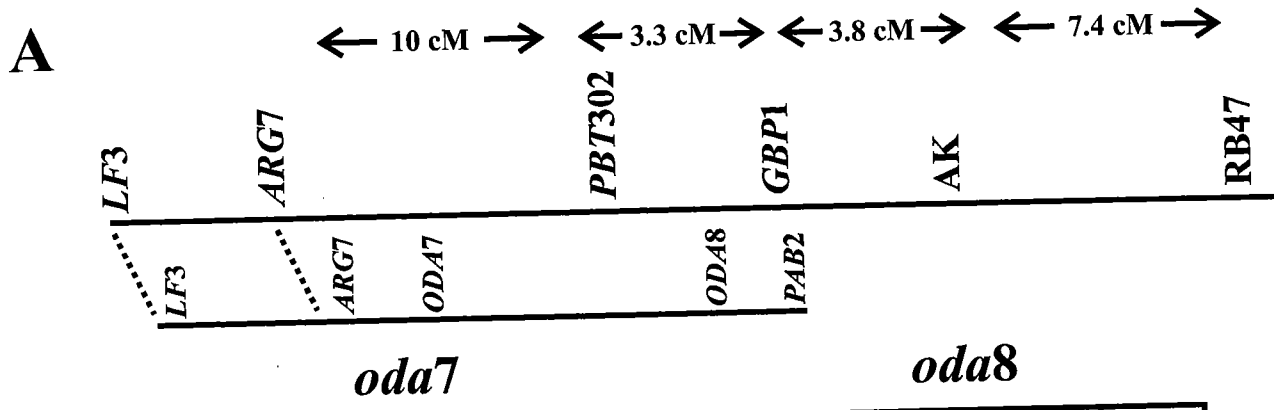
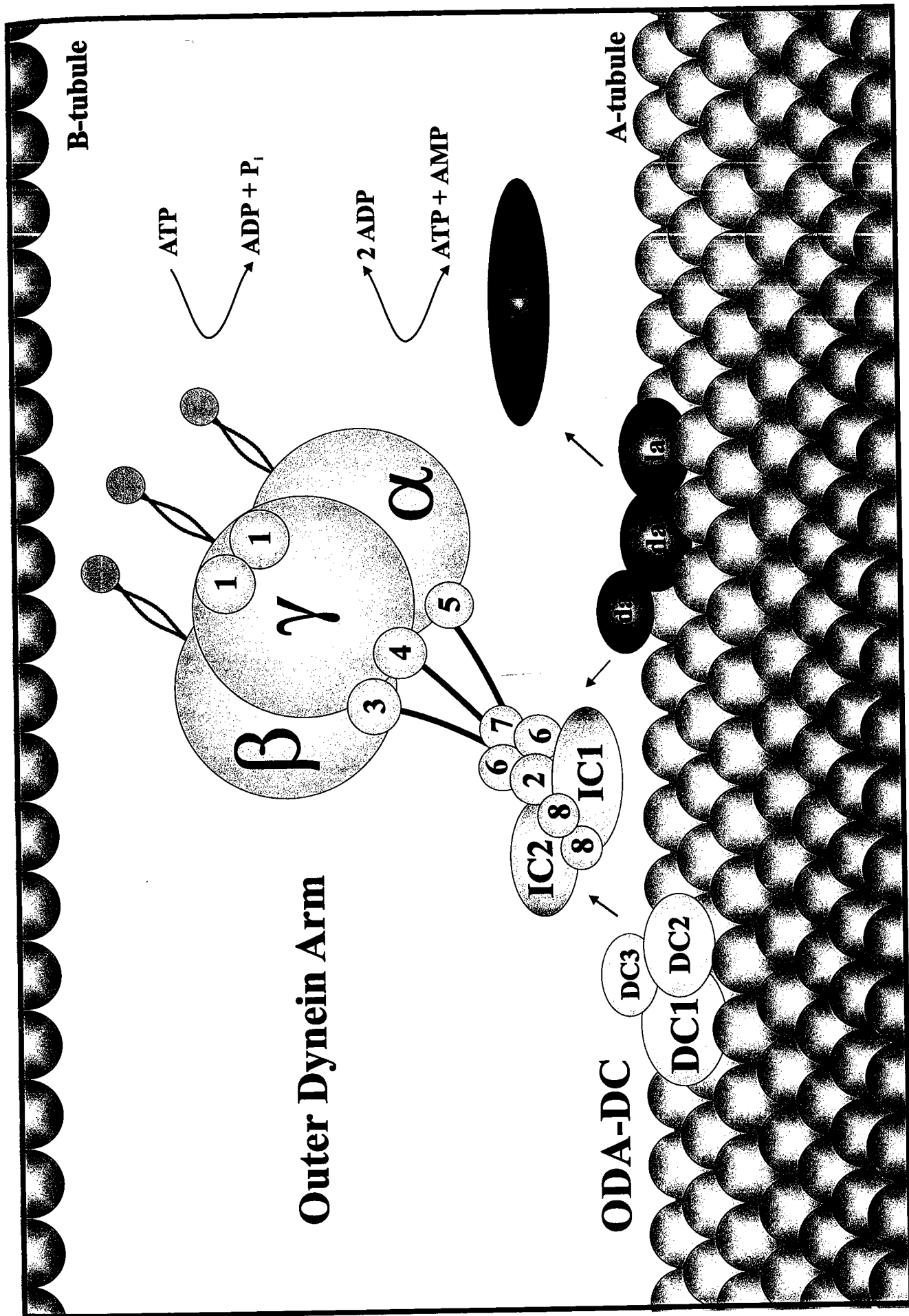


Figure 21

**Figure 22: Model for assembly of the Oda5p, Oda8p, and Oda10p complex.** In wild-type cells, the Oda5, 8 and 10 proteins assemble into a complex which localizes to the axoneme where it functions in assembly of the outer dynein arm and its associated adenylate kinase. If either the *ODA5* or *ODA10* genes are disrupted, then the remaining subunits are unstable and no partial complex is formed. In this case, neither outer arm assembly nor adenylate kinase assembly occurs. If the *ODA8* gene is disrupted, then Oda5p (and possibly Oda10p) are stable, forming a partial complex, which is targeted to the axoneme. Assembly of the partial complex is sufficient for targeting of adenylate kinase, yet no outer arm is assembled in *oda8* as the entire complex is not complete.



**Figure 22**

## CHAPTER VI

### THE *oda10-2* INSERTIONAL MUTANT

#### INTRODUCTION

*Oda10* was originally identified as an outer arm assembly mutant in the same screen that identified *oda5-1* (Kamiya, 1988). This study reported 10 new, independent genetic loci required for outer dynein arm assembly that were designated *oda1-oda 10*.

To obtain motility mutants by insertional mutagenesis, Koutoulis *et al.* (1997) transformed *nit1* mutant strains of *Chlamydomonas* (g1 or 1330.1) with plasmid pGP505 (Pazour *et al.*, 1995) containing a wild-type *NIT1* gene in the pUC119 vector (Fernandez *et al.*, 1989). Selected mutants exhibited a wide range of motility defects, including slow-jerky swimmers (potential *oda* mutants), slow-smooth swimmers (potential *ida* mutants), aflagellate cells, uniflagellate cells, paralyzed flagella, and long flagella phenotypes. Insertional mutagenesis in *Chlamydomonas* usually results in deletions or rearrangements at the site of insertion. These genetic alterations can be detected by RFLP analysis using appropriate probes.

In two independent insertional mutagenesis experiments, Koutoulis *et al.* (1997) examined 9,881 transformants and found 39 that manifested the *Oda-* phenotype. Southern blotting using probes to the previously cloned outer dynein arm heavy and

intermediate chains demonstrated that 12 of the latter were defective in *ODA4* and 15 were defective in *ODA9*. The remaining 12 *oda*-like mutants did not exhibit RFLPs to the known outer dynein arm heavy and intermediate chain probes, suggesting that they were defective in uncloned genes that are necessary for outer dynein arm assembly or function. Electron microscopy revealed that five of these unknown *oda*-like mutants were indeed missing the outer dynein arm structure; these were classified as "complete" *oda* mutants. Seven others showed partial loss of the outer arm and a few cell lines contained intact outer arm structures, although they manifested the Oda- swimming phenotype.

Allelism was tested by complementation for the five complete *oda* mutants containing defects in unknown genes. Stable diploids were generated by crossing the unknown *oda* mutants to previously reported mutants whose defective gene products are unknown: *oda1*, *oda3*, *oda5*, *oda7*, *oda8*, *oda10*, *pf13* and *pf22*. This study determined that cell lines V40 and F60 did not complement the *oda3* mutant, whereas cell line V87.2 failed to complement *oda10*, indicating they are new alleles at these loci.

By using one of the *oda3* insertional mutants, Koutoulis *et al.* (1997) succeeded in cloning the *ODA3* gene and characterizing the *ODA3* gene product, which encodes the DC1 component of the ODA-DC. The same strategy was applied to the cloning of the *ODA10* gene, yet *ODA10* has proved to be more difficult and its successful cloning has not been achieved.

## RESULTS

### Characterization of V87.2, an Insertional Allele of *oda10*

V87.2 was generated by insertional mutagenesis of wild-type strain g1 with plasmid pGP505 (Koutoulis *et al.*, 1997). To determine if V87.2 was an allele of a previously known *oda* mutant, genetic crosses were performed between V87.2 and the known *oda* mutants, as well as *pf13* and *pf22*, which also fail to assemble the outer arms. Stable diploids were selected and complementation assessed by qualitatively analyzing the motility by light microscopy. If a stable diploid cell exhibited wild-type motility, it was considered positive for complementation, indicating the two parental *oda* alleles contained mutations in different genes. If a stable diploid cell exhibited the Oda- swimming phenotype, it was considered non-complementing, indicating the two parental *oda* alleles contained mutations in the same gene. Only stable diploids between V87.2 and *oda10* exhibited the Oda- phenotype (Koutoulis *et al.*, 1997). Furthermore, meiotic progenies obtained from a cross between V87.2 and *oda10-1* were analyzed by light microscopy for motility. All meiotic progeny exhibited the Oda- motility phenotype<sup>9</sup> (Table V).

Electron microscopy demonstrated that the outer dynein arms are completely missing in V87.2 axonemes, consistent with the Oda- motility phenotype exhibited by this strain (Figure 23). These results demonstrate that V87.2 is an insertional allele of *oda10*, which exhibits defects in outer dynein arm assembly. The original *oda10*

---

<sup>9</sup>V87.2 allelism determined by Anthony Koutoulis (Koutoulis *et al.*, 1997).

mutation (Kamiya, 1988) is hereafter referred to as *oda10-1*, and V87.2 is hereafter referred to as *oda10-2*.

Molecular mapping demonstrated that both *ODA10* and LC7, an outer dynein arm light chain, map to Linkage Group VIII (Kathir *et al.*, 2003; Silflow *et al.*, 1995). To rule out the possibility that *ODA10* encodes the LC7 dynein light chain, we generated stable diploids between *oda10-2* and *oda15*, which is defective in the LC7 gene<sup>10</sup>. All stable diploid cells exhibited wild-type motility, indicating that *oda10* is not defective in the gene encoding LC7.

#### **Isolation of Endogenous Sequence Flanking the *oda10-2* Mutation**

To determine if *oda10-2* is a tagged allele, the mutant was mated to a wild-type strain and meiotic progeny analyzed for motility and for segregation of the Nit1+ phenotype as well as for presence of pUC119 vector sequences in the genome. In all cases, the Oda10- motility phenotype segregated with the ability to grow on nitrate as the sole nitrogen source (Nit+ phenotype) (Anthony Koutoulis, unpublished data), indicating the *oda10-2* allele is tagged by the inserted *NIT1* selectable marker. The *oda10-2* mutation is also tagged with the inserted pUC119 vector sequences (see Figure 6A, CHAPTER II). Furthermore, the Oda10- phenotype segregated in a 2:2 manner, consistent with it being caused by a single insertional event that disrupted the *ODA10* gene (Table V). The presence of pUC119 vector in the *oda10-2* genome provides a convenient means for

---

<sup>10</sup>strains *oda15* strains provided by Dr. Gregory Pazour.

cloning sequence flanking the insertion that is tightly linked to the *oda10-2* mutation. To obtain DNA that is located next to the integration site, a  $\lambda$ -phage genomic library was generated from the *oda10-2* cell line and phage clones hybridizing with a pUC119 probe were selected<sup>11</sup>.

By probing wild-type and *oda10-2* genomic DNA with fragments from the puc-positive  $\lambda$ -phage, a 1.5-kb genomic fragment (2.1) was identified that gave an RFLP on Southern blots of genomic DNA digested with *PvuII*. The mutant RFLP fragment is ~1.7-kb, while the 2.1-probe hybridizes to a 2- to 3-kb fragment in wild-type DNA<sup>11</sup> (Anthony Koutoulis, unpublished data). The 2.1 genomic fragment was used to screen wild-type cosmid and  $\lambda$ -phage genomic libraries. Four cosmid clones (cos22, cos31, cos32, and cos33) and 22  $\lambda$ -phage genomic clones were selected for further characterization<sup>11</sup>.

To determine if any of the positive genomic clones contained the *ODA10* gene, they were co-transformed into the *oda10-2* mutant along with the pARG7.8 plasmid (Auchincloss *et al.*, 1999; Debuchy *et al.*, 1989). No wild-type swimmers were observed with any of the clones tested, indicating that an intact *ODA10* gene was not present in these clones<sup>12</sup>.

---

<sup>11</sup>V87.2 library construction, identification of the 2.1 probe and genomic clones, and segregation of the mutant RFLP were done by Anthony Koutoulis.

<sup>12</sup>transformations were done by Anthony Koutoulis, Bethany Dickert and Maureen Wirschell. Crosses between *oda10-2* and CC-1820 were done by Maureen Wirschell.



### Identification of Additional Probes to the *oda10* Genomic Region

As none of the cosmid or  $\lambda$ -phage isolates contained a functional copy of the *ODA10* gene, the 2.1-probe was used to screen a *Chlamydomonas* cDNA library. One cDNA clone, pcODA10.1<sup>13</sup>, gave an RFLP on Southern blots of wild-type and *oda10-2* genomic DNA (Figure 24A), as did the 2.1-genomic probe (not shown). Since pcODA10.1 was easier to propagate than 2.1, this cDNA was used as a probe to screen a *Chlamydomonas* genomic BAC library. Although the hybridization signal intensity was very low, positively hybridizing BAC clones were obtained from InCyte Genomics (now Clemson University Genome Institute). Southern blotting of the BAC DNA determined the BAC clones to be false positives and these BACs were not pursued further (data not shown).

In an effort to further understand the genomic region that is tightly linked to the *oda10-2* mutation, one cosmid clone, cos22, was selected for further characterization. This clone positively hybridized with the pcODA10.1 cDNA probe on Southern blots, whereas cosmid clones cos31, cos32 and cos33 either did not hybridize, or hybridized very weakly (Bethany Dickert, data not shown). Because cos22 was extremely difficult to propagate in bacteria, smaller subclones from cos22 were generated and used to characterize the *oda10* region further. Two fragments from cos22, NN4 and N6, were chosen for further study. Both of these genomic fragments are deleted in the *oda10-2*

---

<sup>13</sup>Identification of the pcODA10.1 cDNA clone was done by Anthony Koutoulis.

mutant (Figure 24B and C).

Southern blotting of meiotic progenies from a cross between *oda10-2* and wild type demonstrated that the deleted sequences segregated with the Oda- phenotype, indicating that the deleted region is tightly linked to the *oda10-2* mutation (Figure 26).

### Identification of Additional BAC Clones and $\lambda$ -Phage Clones

The two independent genomic fragments, NN4 and N6, that map within the *oda10-2* deletion, were used to screen the *Chlamydomonas* BAC library in an effort to identify additional genomic clones covering the *ODA10* region. Six BAC clones were identified (BACs 08L13, 04c12, 28a07, 38h12, 28a20, 24L14); however none of the six clones hybridized with both probes. Southern blotting verified that none of the BACs selected contained sequences that hybridized with both deletion probes. These results suggest that the *ODA10* genomic region is not intact in this BAC library.

Nonetheless, DNA from these BAC clones was transformed into the *oda10-2* cell line to test for rescue of the Oda10- phenotype. Again, no wild-type swimming transformants were obtained, demonstrating that these BAC clones do not contain an intact *ODA10* gene.

The two deletion probes were subsequently used to screen the  $\lambda$ -phage clones that had previously been isolated using the original 2.1 probe. Six phage clones out of 22 hybridized with both deletion probes. Five hybridized with only one deletion probe

(NN4) and 9 hybridized with the other (N6). Two phage clones did not hybridize with either deletion probe and were omitted from further study.

Eight  $\lambda$ -phage isolates were selected for further analysis; the six double positive clones and two additional clones; each positive for one of the deletion probes. One of the double positive clones did not reproduce and was only positive for one probe in subsequent screens. The remaining seven phage clones reproduced their original hybridization patterns and were isolated to purity by three successive rounds of screening. Phage DNA was isolated and used to transform the *oda10-2* mutant. No positive transformants were obtained, demonstrating that the phage isolates do not contain an intact *ODA10* gene.

### **Restriction Mapping and Sequencing of the *ODA10* Genomic Region**

Since none of the genomic clones (cosmid, BAC, or  $\lambda$ -phage) rescued the *Oda10*-phenotype, we attempted to characterize the *ODA10* genomic region further by restriction mapping and sequencing. Subclones from two of the  $\lambda$ -phage clones, and from cos22 were generated and used along with pcODA10.1 for sequencing and Southern blotting of wild-type genomic DNA. The Southern blotting generated a restriction map of the *ODA10* region (not shown). Sequencing of the constructs yielded no insights as to the identity of the *ODA10* gene. Blast searching of the GenBank *Chlamydomonas* EST database identified EST clones that overlapped with portions of the genomic sequence and searching the JGI *Chlamydomonas* Genome database (version 2) revealed that Scaffold 235 contained sequences in the *ODA10* region (Figure 24).

One of the  $\lambda$ -phage subclones, 24.8, is a ~9-kb *Not*I genomic fragment. One end of this fragment (24.8R) matches ESTs and gene model C\_2350003, representing ATP/ADP transporters (Figure 24). Using a probe to the EST sequences, we found the mRNA is not induced by deflagellation, contrary to what is expected for the *ODA10* mRNA (not shown). The EST probe hybridizes to bands of the same size on Southern blots of wild type and *oda10-2* genomic DNA, suggesting this region of the genome is not compromised in the *oda10-2* insertional mutant (Figure 24F). PCR analysis of genomic DNA confirms these sequences are not deleted in the *oda10-2* mutant (Figure 24E).

The opposite end of 24.8 (24.8F) matched EST sequences and gene model C\_2350002 representing a glutathione peroxidase-like protein. Probes to this end of 24.8 demonstrated that this region is deleted in the *oda10-2* insertional mutant (Figure 24D). The mRNA transcript is not enhanced by deflagellation, an expected feature of the *ODA10* mRNA (not shown).

Moreover, EST sequence AW707899 encoding the glutathione peroxidase-like protein, is found in this region. However, the other end of this EST clone, AW707900 matches sequence encoding a putative 40S ribosomal protein in scaffold 13 (C\_130042), suggesting that either genomic sequences are missing from both our 24.8 construct and the JGI scaffold, or that this EST clone contains two different cDNA sequences (Figure 24G).

The 24.8 plasmid was remarkably difficult to propagate in bacteria. In many cases, as the plasmid replicated, the insert would be deleted, or rearranged. In some

instances, sequence analysis revealed that vector sequences had recombined within the ~9-kb insert. The recombination observed with 24.8 was a common feature to many of the isolated genomic clones. As a consequence, the entire ~9-kb *Not1* insert in 24.8 was not sequenced to completion. To date, the sequence compiled consists of a 3.513-kb contig at the end encoding the putative ATP/ADP transporter, and a 3.880-kb contig at the end that lies within the *oda10-2* deletion. A ~1.6-kb internal region remains unsequenced. However, Scaffold 235 contains only a 485-bp gap between the sequenced regions of 24.8. Therefore, I conclude that the 24.8 construct contains additional non-*Chlamydomonas* genomic DNA sequences.

When compared to version 2 of the JGI *Chlamydomonas* genome database, a small gap is observed in the 24.8 sequence. This discrepancy indicates that the genomic sequence obtained from the  $\lambda$ -phage clones, the JGI sequence, or both are incomplete.

Sequence from constructs overlapping the deletion (NN4 and N6) and from the region flanking the deletion in *oda10-2* (pcODA10.1) were analyzed against the JGI *Chlamydomonas* genome database (Figure 24). Both regions align with scaffold 235, within contigs 1, 2, 3, and 4. There are four gene predictions within this region: C\_2350008, C2350011, C\_2350001, and C\_2350007 whose predicted sequences encode novel proteins (C\_2350008, C\_2350001, C\_2350007) or a cysteine proteinase protein (C\_2350011). The C\_2350007 gene model predicts a 56 amino acid protein that resides within the C\_2350001 gene model. It is possible this is an erroneous prediction. No ESTs are found within these two predictions to verify the gene models.

The pcODA10.1 cDNA overlaps with the C\_2350011 gene model that encodes the predicted proteinase. Northern blot analysis of wild-type mRNA did not detect an upregulated mRNA (not shown), suggesting that the pcODA10.1 cDNA does not encode *ODA10*. Comparison of the pcODA10.1 sequence and the genomic sequence compiled from the  $\lambda$ -phage subclones with the JGI genomic sequence indicates that there is a 1.196-kb gap between pcODA10.1 and the  $\lambda$ -phage subclones. Within this region, there is a 736-bp gap in the JGI scaffold, indicating this region is not sequenced in the genome database.

Once again, comparison of the JGI scaffold sequence to the sequence compiled from the  $\lambda$ -phage and cosmid subclones demonstrated the presence of small deletions, two of which overlap with gaps in the JGI scaffold sequence, indicating that our genomic constructs are incomplete. The genomic constructs within this region also demonstrated difficulties with bacterial propagation, although not quite as severe as that observed with 24.8.

The remaining sequence of Scaffold 235 contains five gene models, C\_2350009, C\_2350010, C\_2350004, C\_2350005, and C\_2350006. Further inspection of the gene models by BLAST demonstrates that C\_2350010 does not match any significantly related sequences in the databases, indicating this gene model predicts a novel protein. C\_2350004 contains a BAH domain which is associated with DNA methylation, replication, and transcriptional regulation and blastp reveals a low level of homology within the N-terminus to DNA methyltransferases. C\_2350005 has a small region

containing homology to GATA Zinc-finger binding proteins and C\_2350006 has homology to D-alanine-D-alanine ligase and related ATP-grasp enzymes from bacteria.

BLAST results indicate that a small region of C\_2350009 matches *Homo sapiens* and *Mus musculus* predicted proteins. More importantly, the proteomics analysis of the *Chlamydomonas* flagellum identified six peptides (DINEFLTNELK, TAEAAALT SGYDDTAR, LQDQGYQAAESEEVLGALDGR, QTEEELAR, SLTTSALESK, YQDHGDLRQVDVVR) from this predicted protein, which was extracted with 0.6M KCl (Agrin *et al.*, 2003), an expected feature for an Oda5-interacting protein. To determine if this gene is disrupted in the *oda10-2* genome, I performed real-time PCR on DNA derived from wild type and *oda10-2*, using primers located within and adjacent to the gene model (Figure 25A). No defect was detected in this gene, suggesting this region of the genome is not disrupted in *oda10-2* (Figure 25B).

### **Physical Mapping of *ODA10* Using Probes to the *ODA10* Genomic Region**

Physical mapping using RFLP markers has generated a molecular map of the *Chlamydomonas* genome (Kathir *et al.*, 2003). The molecular map is based on a set of 136 random progeny derived from a cross between a standard laboratory strain of *C. reinhardtii* (21gr) and a field isolate S1-C5 (Kathir *et al.*, 2003). The molecular markers consisted of random genomic clones, specific genes or cDNA contributed by individual researchers, and genomic, cDNA, or expressed sequence tag (EST) sequences obtained from the Genbank database.

In order to understand more about the *ODA10* genomic region, RFLP analysis was used to map the *ODA10* probes identified in this study. The *ODA10* gene has been mapped to Linkage Group VIII ([http://www.biology.duke.edu/chlamy\\_genome/nuclear\\_maps.html](http://www.biology.duke.edu/chlamy_genome/nuclear_maps.html)). The orientation of a region on Linkage Group VIII containing *ODA10* remained undetermined. Utilizing two probes to *ODA10* genomic region (corresponding to the NN4 and N6 constructs), Kathir and colleagues (University of Minnesota, St Paul, MN) found that one of our probes (N6) mapped to a set of markers on Linkage Group VIII. The second probe (NN4) was found to be repetitive on their Southern blots and was not useful for mapping purposes. The pcODA10.1 cDNA was at first found to map to Linkage Group XII/XIII, yet upon further characterization, did not reproduce the same hybridization patterns. The mapping of pcoda10.1 was not pursued further. Nevertheless, the successful mapping of one probe to Linkage Group VIII provided further evidence that the *ODA10* constructs that were identified are derived from the correct region of the genome.

## DISCUSSION

V87.2 was identified in a screen for slow-swimming *oda* mutants and represents an insertional allele of the uncloned *ODA10* gene. Mutations in this gene prevent outer dynein arm assembly, thus *ODA10* is an essential component in the outer arm assembly pathway.



Fowkes and Mitchell (1998) speculated that the gene products of *ODA5*, *ODA8*, and *ODA10* may encode uncharacterized outer dynein arm light chains. Our findings with *ODA5* suggest this is not the case. Rather, these three proteins appear to interact in a complex that is distinct from the outer dynein arm. Prior to our identification of the *ODA5* gene, nothing was known about the proteins encoded by these genes.

### The *ODA10* Gene

Since *oda10-2* is a tagged allele, we were able to use the inserted vector sequences to clone endogenous DNA that is tightly linked to the *oda10-2* mutation. This sequence was used to obtain additional genomic fragments that cover a large portion of the *oda10* locus.

The mutation that causes the *Oda10-2* phenotype is a large deletion that most likely removes the *ODA10* gene. We isolated wild-type genomic sequences that span the deletion, including DNA on either side of the deletion. However, when compared to the JGI genome database, small gaps present in the sequence obtained from our genomic clones were observed. These discrepancies indicate that the genomic sequence obtained from our clones is compromised. Given the observed recombination events and difficulties with the bacterial propagation of our constructs, it is also possible that the JGI scaffold sequence is incomplete due to similar difficulties. In spite of the large genomic region covered by our clones, we were unable to rescue the *oda10-2* mutant, further verifying that our genomic clones did not contain an intact *ODA10* gene.

Taken together, the lack of rescue, the difficulties in propagating the various genomic clones, and the incomplete genomic sequences, suggest that the *ODA10* genomic region is a particularly difficult region of the *Chlamydomonas* genome to propagate in bacteria and as a result is not completely represented in most genomic libraries. Nonetheless, the probes to the *ODA10* genomic region segregated with the Oda10- motility phenotype, indicating that they are tightly linked to the *oda10-2* mutation. One possibility is that a second insertional event occurred in the *oda10-2* strain. This second insertional event may have disrupted the *ODA10* gene that is very tightly linked to the genomic region we have cloned, thus our probes segregate with the Oda10-motility phenotype. As the *Chlamydomonas* Genome sequence is updated, the sequences obtained by our efforts may prove useful in ultimately determining the identity of the *ODA10* gene.

#### **A Reverse Genetic Approach to Identifying Oda10p**

Since a forward genetic approach to identifying *ODA10* was unsuccessful, a reverse genetic approach may be more fruitful in identifying this elusive protein. The sequencing of the *Chlamydomonas* genome has made it possible to take a large-scale mass spectrometry-based approach to the identification of flagellar proteins (Agrin *et al.*, 2003). This major undertaking has identified many of the previously known flagellar proteins and may prove to be a useful method for identifying unknown flagellar proteins.

Six peptides from the C\_2350009 gene model were identified in a proteomics analysis of the *Chlamydomonas flagellum* (Agrin *et al.*, 2003). Similar to Oda5p, the NH<sub>2</sub>-terminal ~1/2 of the C\_2350009 predicted protein contains a high probability of forming coiled-coil structure, followed by a C-terminal non-coiled-coil region. Within this gene model there is a small gap in the JGI Scaffold sequence, indicating that this region is not sequenced completely. The missing genomic sequence may alter the gene prediction and the predicted protein sequence, which contains the peptides identified in the proteomics analysis.

Real-time PCR suggests this gene is not compromised in the *oda10-2* genome. Nonetheless, this protein may be the most likely Oda10p candidate and I am currently pursuing the possibility that this gene contains a small mutation that is not detected by real-time PCR.

Our attempts to identify Oda5-associated proteins by ICAT did not reveal any peptides residing in the *ODA10* genomic region. Thus, no clues to the identity of the Oda10 protein were forthcoming from these experiments. Nonetheless, the Oda10 protein may be revealed upon further identification of Oda5p-associated proteins.

## **MATERIALS AND METHODS**

### **Strains and Culture Conditions**

*Chlamydomonas reinhardtii* strains used in this study include: CC124 (*nit1-137, nit2-137, mt-*), CC-2247 (*oda10-1, mt-*), CC-1030 (*pf13*), CC-1382 (*pf22*), CC-2264 (*ida1,*

*mt+*), and CC-1820 (*arg7-2, mt-*) all from the *Chlamydomonas* Genetics Center (Department of Biology, Duke University, Durham, NC). V87.2 (*oda10-2, mt+*) was identified as an insertional allele of *oda10* (Koutoulis *et al.*, 1997). *g1* (*agg1, nit1, NIT2, mt+*) was used for transformation of pGP505. 3167.2 (*oda15, mt-*), H8 (*arg7, mt-*), and 3184.1 (*oda15, mt+*) are from Gregory Pazour<sup>10</sup>.

V87.2 was crossed to CC124 to create strains 4a (*oda10-2, mt+*), 4b (*ODA10*), 4c (*ODA10*) and 4d (*oda10-2*), and to strain H8 to create 2913.1 (*oda10-2, arg7*) and 2907.2 (*oda10-2, arg7*). CC-2247 was crossed to *g1* to create 2976.2 (*oda10-1, nit1, NIT2*), and to CC-2264 to create 2855.2 (*oda10-1, ida1*). 4a was crossed to CC-1820 creating 51b (*oda10-2, mt-, arg 7-2*) which was used for transformation purposes. 63b (*oda15, arg2, mt+*) and 68d (*oda15, arg2, mt+*) were generated by crossing 3167.2 to CC-48 and 3184.1 to CC-1826 respectively. 63b and 68d were crossed to 51b to generate stable diploids SD210 (*oda15/ODA15; oda10/ODA10*), SD211 (*oda15/ODA15; oda10/ODA10*), and SD215 (*oda15/ODA15, oda10/ODA10*). *Chlamydomonas* cells were cultured as described in CHAPTER II.

### **Transformations and Insertional Mutagenesis**

All transformations were done using the glass bead method (Kindle, 1990) as described (Koutoulis *et al.*, 1997). Co-transformations were performed using *ODA10* genomic constructs and pARG 7.8 plasmid (Auchincloss *et al.*, 1999; Sizova *et al.*, 1995) and transformants selected on TAP plates without arginine. Strain V87.2 was generated by the same method (Koutoulis *et al.*, 1997). Briefly, *g1* cells was transformed with plasmid pGP505 (Pazour *et al.*, 1995) containing the *Chlamydomonas NIT1* gene.

Transformants positive for *NIT1* were selected on SGII/NO<sub>3</sub> media. Motility mutants were identified by growing positive transformants in liquid culture and screening by light microscopy. The *ODA10* allele was confirmed by crossing V87.2 with the *oda10-1* strain (Kamiya, 1988).

#### **Isolation of the Endogenous Sequence Flanking the *oda10-2* Mutation.**

To determine if *oda10-2* is a tagged insertional allele, Southern blots of genomic DNA from wild type and *oda10-2* were probed with pUC119. A positive hybridizing band was detected in *oda10-2* genomic DNA, demonstrating that the inserting vector sequences from pGP505 stably integrated into the *oda10-2* mutant.

A genomic  $\lambda$ -phage library was constructed using genomic DNA from the *oda10-2* strain as described (Koutoulis *et al.*, 1997). To obtain sequences flanking the site of pGP505 insertion, phage clones containing pUC119 were isolated from the above library. These phage clones were digested with a variety of restriction enzymes, and Southern blots probed with pGP505 to identify genomic fragments that did not contain pUC119 or *NIT1*. One fragment 2.1, which does not contain the sequences from the transforming vector, detected an RFLP between wild type and *oda10-2*, indicating it is flanking the site of the pGP505 insertion<sup>11</sup>. This genomic fragment was used to identify a cDNA clone (pcODA10.1), which also detects the mutant RFLP<sup>13</sup>. This RFLP segregates with the Oda10- motility phenotype, indicating it is tightly linked to the *oda10-2* mutation<sup>11</sup> (Anthony Koutoulis, unpublished data).

### **Identification of Genomic Clones Residing At or Near the *ODA10* Locus**

These probes were used to identify wild-type cosmid, BAC, and  $\lambda$ -phage genomic clones that spanned the deletion in *oda10-2*. Rescue of *oda10* was tested by co-transforming strains 51b (Maureen Wirschell), 2796.2, 2907.2, 2908.1 and 2913.1 (Anthony Koutoulis and Bethany Dickert) with the genomic clones and a selectable marker. Strain 2855.2 is non-motile and was transformed without selection, then screened for the presence of motile cells (Bethany Dickert). Transformation was performed using the glass bead method as described above. Transformants were scored for the Oda $\pm$  phenotype by light microscopy.

### **Isolation and Blotting of Nucleic Acids**

*Chlamydomonas* nucleic acids were isolated; Southern and Northern blots were processed as described in CHAPTER II.

### **Real-time PCR**

Using primers designed within and adjacent to the C\_2350009 gene prediction, wild type and *oda10-2* genomic DNA were amplified using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA). Quantitation of the PCR amplification was performed using a DNA engine Opticon continuous fluorescence detection system (MJ Research, Waltham, MA). PCR was performed for 35 cycles, with a plate read after each cycle and a melting curve was generated every 0.2°C, between 30°C and 98°C.

**Colony Lifts**

Colony lifts were performed as described in CHAPTER II.

**Genetic Analysis**

Matings, tetrad analysis, and generation of stable diploids were performed according to standard procedures (Dutcher, 1995; Harris, 1989). Progeny were scored for segregation of pUC119 and *NIT1* by Southern blot as described in CHAPTER II.

**Electron Microscopy**

Cells were processed for electron microscopy as described in CHAPTER II.

**Table V: Phenotype of meiotic progeny from *oda10-2* crosses**

| Cross                                | Motility <sup>14</sup> |    |
|--------------------------------------|------------------------|----|
|                                      | +                      | -  |
| <sup>15</sup> CC124 x V87.2          | 23                     | 19 |
| <sup>16</sup> <i>oda10-2</i> x V87.2 | 0                      | 65 |

<sup>14</sup> + refers to wild-type motility; - refers to Oda- motility.

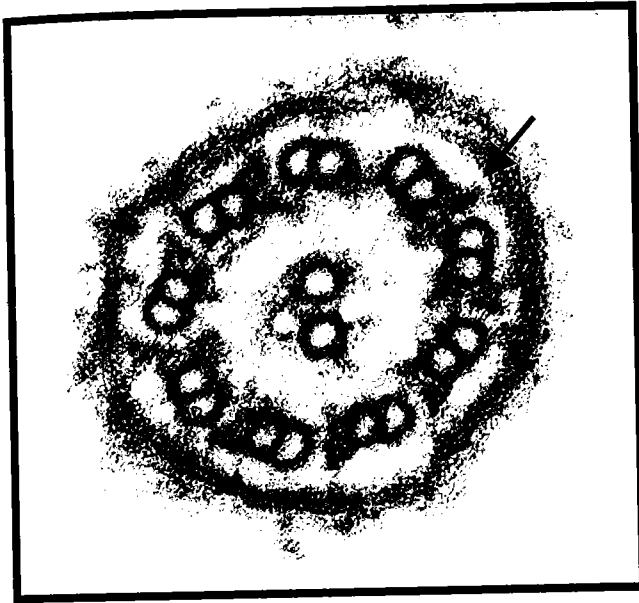
<sup>15</sup> The Oda- phenotype segregated in approximately a 2:2 manner (23:19) in the backcross to the wild-type strain CC124.

<sup>16</sup> All progeny had the Oda- phenotype when V87.2 was crossed to the original *oda10-1* allele, indicating that V87.2 is an insertional allele of *oda10*.

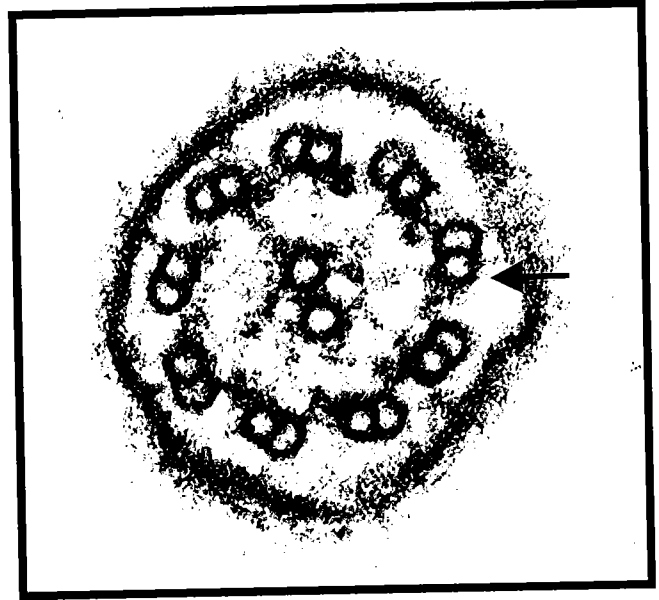


**Figure 23: The V87.2 (*oda10-2*) insertional mutant lacks outer dynein arms.**

Electron micrographs of axonemal cross sections from wild-type and V87.2 (*oda10-2*) insertional mutant. Arrows indicate the outer dynein arms in the wild-type flagella, but the absence of outer arms in the mutant flagellum.



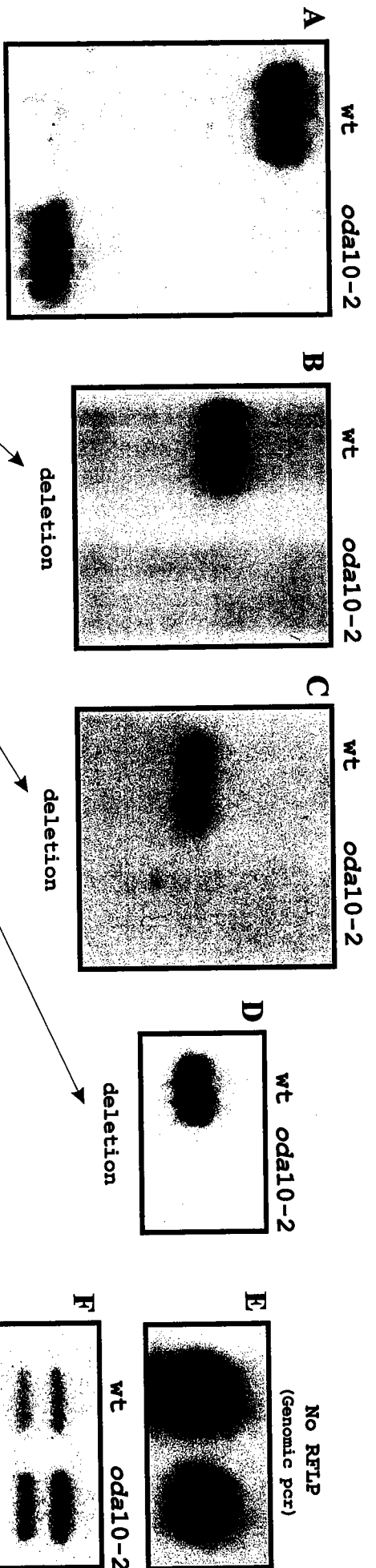
wild type



*oda10-2*

Figure 23

**Figure 24: The *ODA10* genomic clones align with JGI scaffold 235 in version 2 of the *Chlamydomonas* Genome database.** The JGI scaffold is represented as a black bar with the red-shaded boxes indicating gaps in the JGI sequence. The JGI gene models are depicted in blue and EST sequences are shown in light purple. Above the scaffold are the constructs (green bars) isolated from the *ODA10* region and how they compare to the JGI scaffold. Note the presence of small gaps in our constructs as compared to the JGI sequence. These constructs contain DNA that is flanking the insertion in *oda10-2* (A, E, and F) or contain DNA that is deleted in *oda10-2* (B, C, and D).



C\_2350008: Novel protein

C\_2350011: (G9V3U6) 26/29-kDa proteinase (*Drosophila*)

C\_2350001: Novel protein

C\_2350007: Novel protein

C\_2350002: Glutathione peroxidase-like protein

C\_2350003: Plastidic ATP/ADP Transporter (Citrus hybrid cultivar)

C\_2350009: Similar to Human and mouse predicted proteins

C\_2350010: Novel protein

C\_2350004: DNA methyltransferases

C\_2350005: GATA Zinc-finger binding proteins

C\_2350006: D-alanine-D-alanine ligase AND ATP grasp enzymes

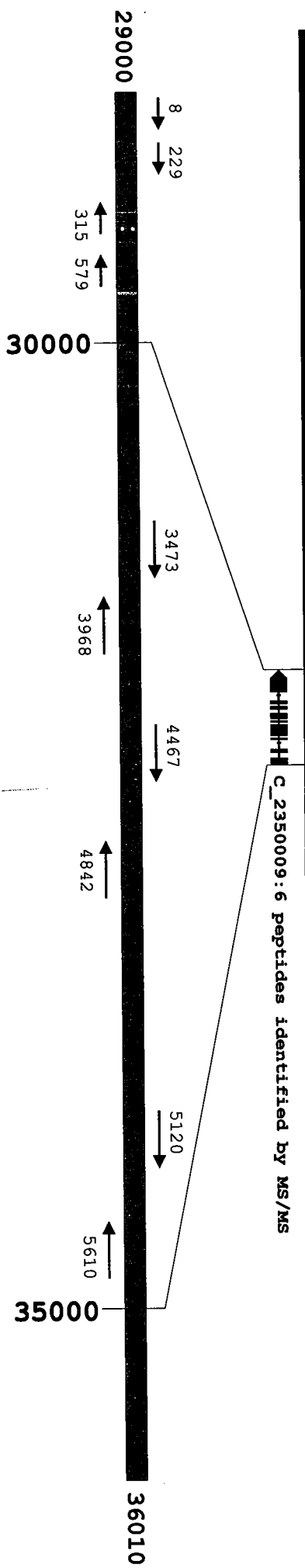
Includes AW707899 (AW707900, the other end of AW707899, hits scaffold 13)

Figure 24

**Figure 25: Gene C-2350009 is not defective in the *oda10-2* strain.**

Six peptides predicted by gene model C\_2350009 were identified in the *Chlamydomonas* flagellar high-salt extract (Agrin *et al.*, 2003). (A) The JGI scaffold is shown along with the *ODA10* genomic constructs. The region encompassing the C\_2350009 gene model is expanded. Arrows illustrate the orientation of primers designed within and adjacent to this gene relative to the scaffold. Below are the primer sequences. (B) Graphs of the fluorescence intensity (x-axis) vs. PCR cycle number (y-axis) are shown for each primer pair. Real-time PCR did not detect any defects in *oda10-2* DNA as determined by the ability of all primer sets to amplify product from both wild-type and *oda10-2* genomic DNA.

pcODA10.1 NNA  
 N6 24.8 F 24.8 R

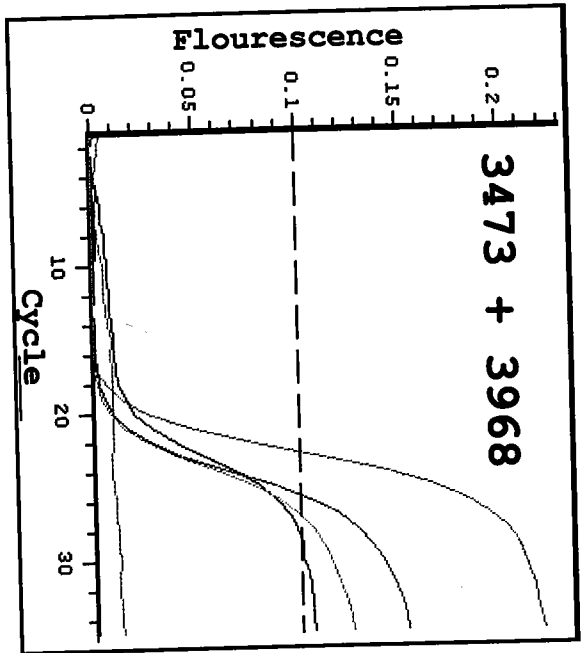
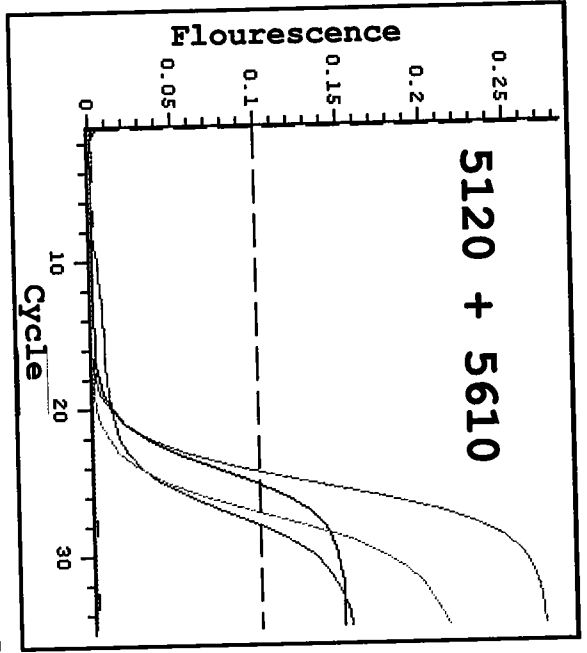
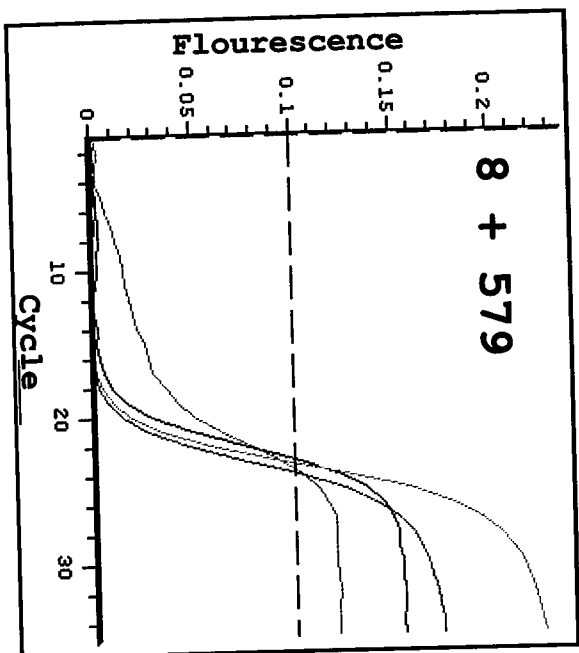
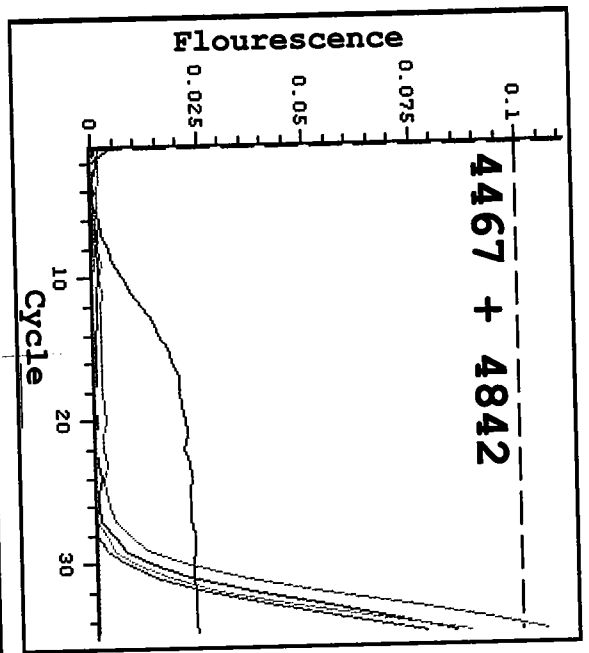
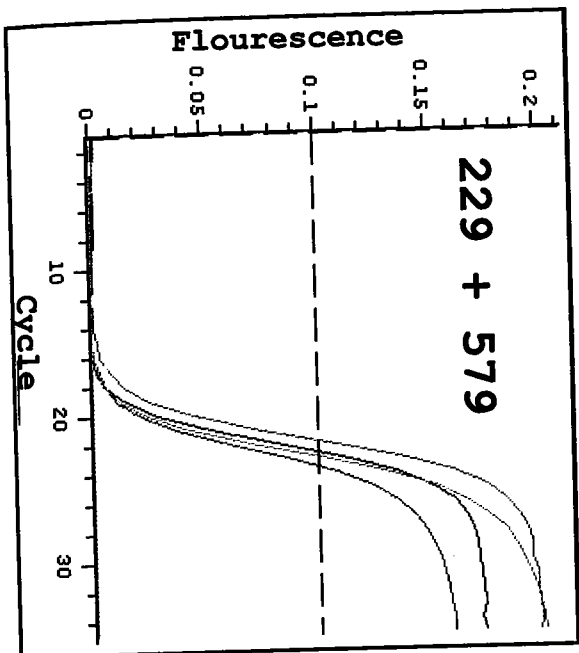
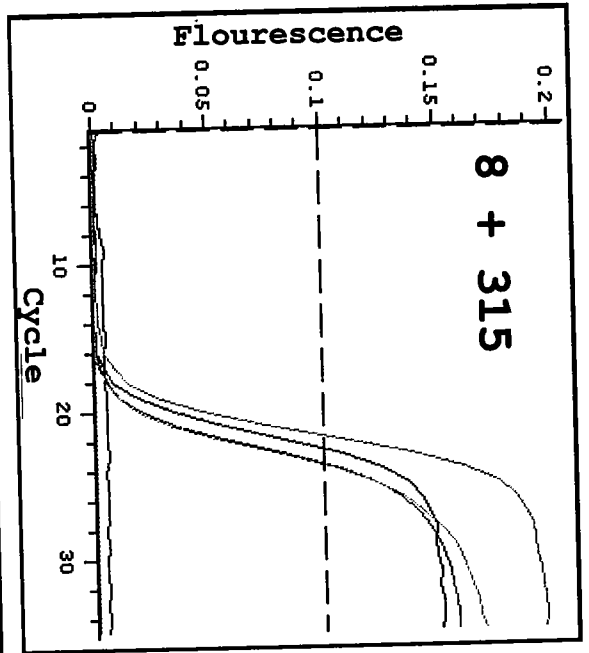


|      |                       |
|------|-----------------------|
| 8    | CGAAATTAAGTGAGGCCAAA  |
| 229  | GAAGAGCTGGGCAAGCTAGA  |
| 315  | CATCACAACCGAGCTCACAC  |
| 579  | AGGTCCGACAGCTTCTCAAG  |
| 3473 | TCCTCTTGGTGGTCACTCTCC |
| 3968 | GGGGTTAGCAGTCATTGAA   |
| 4467 | CACGTCATCACTCCCTTTT   |
| 4842 | GGTGGTGGCGCTTATGTAT   |
| 5120 | AGGTTGAGCTTCTGCCGCTC  |
| 5610 | CAACTGCCCGGCTAATGAACT |

Figure 25A

\_\_\_\_ No DNA; -DMSO  
\_\_\_\_ Wild type; -DMSO  
\_\_\_\_ oda10-2; -DMSO

\_\_\_\_ No DNA; +DMSO  
\_\_\_\_ Wild type; +DMSO  
\_\_\_\_ oda10-2; +DMSO



**Figure 25B**

**Figure 26: The deleted sequences are tightly linked to the *oda10-2* mutation.** The *oda10-2* insertional mutant was crossed to a wild-type strain and tetrads dissected. Southern analysis of the offspring (4a-4d), using a probe to the N6 construct (Figures 24C and 25A), demonstrated that the deletion segregates with the Oda motility defect (+ designates wild-type motility; - designates *oda* motility). The offspring shown are the meiotic progeny of one complete tetrad. A total of 20 progeny (complete tetrads and random progeny) were analyzed.



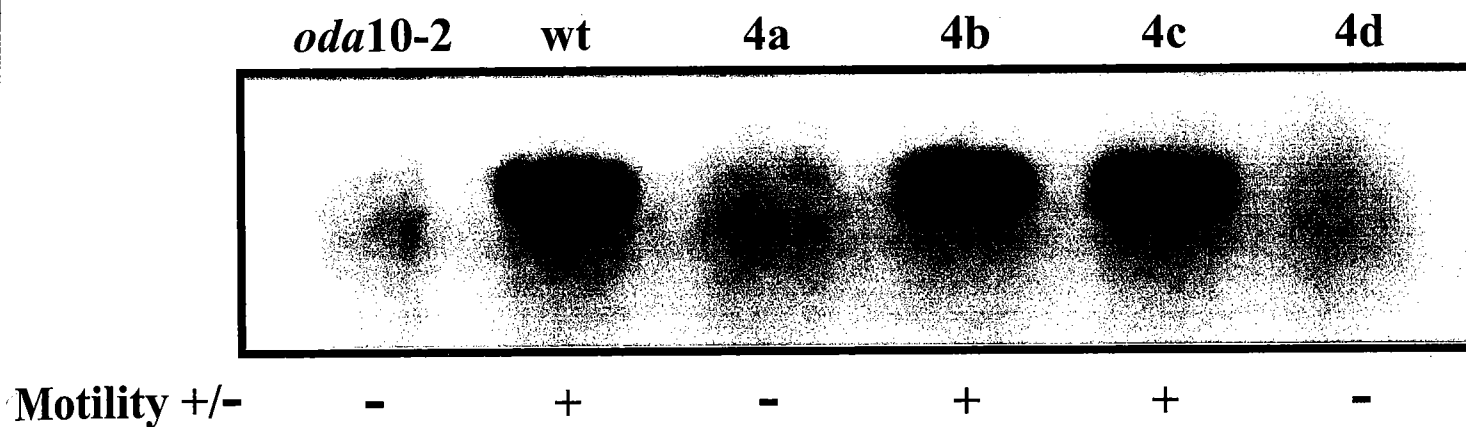


Figure 26

## CHAPTER VII

### FUTURE DIRECTIONS

The cloning of the *ODA5* gene has led to the identification of the founding member of a novel complex that is necessary for the assembly of the outer dynein arm. This complex is comprised of at least the Oda5 and Oda10 proteins, but most likely includes the flagellar adenylate kinase identified here and the Oda8 protein as well. The use of the HA-tagged Oda5 protein may prove useful for identifying proteins that interact with Oda5. HA-Oda5 is specifically immuno-precipitated with an HA-specific antibody. While one-dimensional SDS-PAGE analysis of the immune complexes did not yield conclusive results, analysis of the immune complexes by two-dimensional gel electrophoresis may prove more useful. Cross-linking of flagellar fractions may also yield insights into Oda5-interacting proteins. Since the HA antibody is more specific than the Oda5-polyclonal antibody, these experiments would be performed in the HA-*ODA5* rescued strain.

Oda5 participates in a novel complex which assembles independently of the outer arm and ODA-DC, yet it is required for assembly of the outer dynein arm. How this complex mediates arm assembly remains unclear. Cross-linking studies may lend themselves well to identifying components of the axoneme, the outer arm, or the ODA-DC that interact with the Oda5 complex.

During the course of this thesis work, it was discovered that Oda5 and Oda10 are also required for the targeting of a flagellar adenylate kinase that is intimately associated with the outer dynein arm. This discovery identifies a new machinery, likely to involve ATP production, associated with dynein structure and raises a number of interesting avenues of exploration. How is this adenylate kinase interacting with the Oda5 complex? Is the interaction direct or indirect? The interaction between Oda5 and the AK is sensitive to Nonidet P-40. Can the Nonidet P-40 sensitivity be exploited to further characterize this AK? What proteins interact with the AK? What is the phenotype of cells defective in this AK gene? Addressing these fundamental questions regarding the adenylate kinase can provide additional insight into flagellar motility and possibly establishes a new principal for targeting of adenylate kinase.

To begin to address these questions, an antibody to the AK protein will need to be generated. This antibody will have to be highly specific as there are likely to be AKs present in the cytosol as well as additional AKs in the *Chlamydomonas* flagellum (Agrin *et al.*, 2003). Indeed, a central pair associated protein, Cpc1p, appears to have an adenylate kinase domain (D.R. Mitchell, personal communication). Since the Oda5-associated AK has a unique N-terminus, a peptide antibody designed to this region may provide the specificity required to detect this particular AK. In lieu of a specific antibody, the AK assay will still be a powerful tool for analyzing AK complexes. Watanabe and Flavin (1976) determined that the *Chlamydomonas* flagellum contained two peaks of AK activity, one at 4.3S and a second peak at 2S. Using the *oda5* or *oda10* mutant strains for comparison, the AK assay could be used to analyze sucrose gradient

or column fractions of detergent-soluble flagellar components. Identification of fractions that contain AK activity in wild type but lack AK activity in the mutants may provide valuable information on putative AK complexes. These fractions could be compared to wild type by silver staining of 1D or 2D gels to see if there are specific proteins that are missing or reduced in the mutant fractions.

Furthermore, since the AK gene maps to linkage group I, in the vicinity of both *ODA7* and *ODA8*, this gene may prove useful for the cloning of both of these uncloned *ODA* genes. This AK is also found at additional axonemal sites other than the outer arm. The location of these sites remains undetermined, although we speculate that the inner dynein arm system is a likely candidate. The AK assay can be readily employed to analyze inner dynein arm (*ida*) mutant flagella to ascertain if any of the known *ida* alleles manifest an Ak- phenotype.

To better understand the effects of this AK on dynein function, a mutant cell line defective in the AK gene can be generated. Currently, the Witman lab is using a quantitative PCR based screen for analyzing our collection of motility mutants. Using PCR primers to the AK gene, I found no candidate mutants that are defective in this gene. This collection contains mutants with a variety of defects ranging from slow-smooth swimmers, slow-jerky swimmers, aflagellate cells, unflagellate cells, and cells with paralyzed and long flagellar phenotypes. It is possible that the phenotype of an AK knockout is either lethal, or is simply not represented in the phenotypes contained within this collection. In the absence of an insertional mutant, a targeted knockdown of AK can be attempted through RNAi. Analysis of the RNAi knockdown phenotype will

further characterize the role this AK serves in flagellar motility and may also aid in identifying other axonemal structures that associate with this AK.

## REFERENCES

- Afzelius, B.A., and B. Mossberg. 1995. Immotile-Cilia Syndrome (Primary Ciliary Dyskinesia), Including Kartagener Syndrome. *In* The Metabolic and Molecular Bases of Inherited Disease. Vol. III. C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, editors. McGraw-Hill, Inc., New York. 3943-3954.
- Agrin, N., J.D. Leszyk, K.M. Green, J.E. Evans, G.J. Pazour, and G.B. Witman. 2003. Exploring the *Chlamydomonas* Flagellar Proteome. *Mol Biol Cell*. 14.
- Ahmed, N.A., and D.R. Mitchell. 2003. *oda16-1* contains a gene that rescues slow-swimming *Chlamydomonas*. *Mol Biol Cell*. 14:in press (abstract).
- Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J Mol Biol*. 215:403-410.
- Auchincloss, A.H., A.I. Loroch, and J.D. Rochaix. 1999. The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: cloning of the cDNA and its characterization as a selectable shuttle marker. *Mol Gen Genet*. 261:21-30.
- Benashski, S.E., A. Harrison, R.S. Patel-King, and S.M. King. 1997. Dimerization of the highly conserved light chain shared by dynein and myosin V. *J Biol Chem*. 272:20929-20935.

- Benashski, S.E., and S.M. King. 2000. Investigation of protein-protein interactions within flagellar dynein using homobifunctional and zero-length crosslinking reagents. *Methods*. 22:365-371.
- Benashski, S.E., R.S. Patel-King, and S.M. King. 1999. Light chain 1 from the *Chlamydomonas* outer dynein arm is a leucine-rich repeat protein associated with the motor domain of the gamma heavy chain. *Biochemistry*. 38:7253-7264.
- Bessen, M., R.B. Fay, and G.B. Witman. 1980. Calcium control of waveform in isolated flagellar axonemes of *Chlamydomonas*. *J Cell Biol*. 86:446-455.
- Bowman, A.B., R.S. Patel-King, S.E. Benashski, J.M. McCaffery, L.S.B. Goldstein, and S.M. King. 1999. *Drosophila roadblock* and *Chlamydomonas* LC7: A Conserved Family of Dynein-associated Proteins Involved in Axonal Transport, Flagellar Motility, and Mitosis. *J. Cell Biol*. 146:165-180.
- Brokaw, C.J. 1994. Control of flagellar bending: a new agenda based on dynein diversity. *Cell Motil Cytoskeleton*. 28:199-204.
- Brokaw, C.J., and R. Kamiya. 1987. Bending patterns of *Chlamydomonas* flagella: IV. Mutants with defects in inner and outer dynein arms indicate differences in dynein arm function. *Cell Motil Cytoskeleton*. 8:68-75.

- Budnick, M.O., and R.S. Fitzgerald. 2003. Improved manufacturing techniques enable bovine serum albumin to shed its low-tech image. *IVD Technology*.
- Burkhardt, J.K., C.J. Echeverri, T. Nilsson, and R.B. Vallee. 1997. Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *J Cell Biol.* 139:469-484.
- Casey, D.M., K. Inaba, G.J. Pazour, S. Takada, K. Wakabayashi, C.G. Wilkerson, R. Kamiya, and G.B. Witman. 2003a. DC3, the 21-kDa subunit of the outer dynein arm-docking complex (ODA-DC), is a novel EF-hand protein important for assembly of both the outer arm and the ODA-DC. *Mol Biol Cell.* 14:3650-3663.
- Casey, D.M., T. Yagi, R. Kamiya, and G.B. Witman. 2003b. DC3, the Smallest Subunit of the *Chlamydomonas* Flagellar Outer Dynein Arm-docking Complex, Is a Redox-sensitive Calcium-binding Protein. *J Biol Chem.* 278:42652-42659.
- Debuchy, R., S. Purton, and J.D. Rochaix. 1989. The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the *ARG7* locus. *Embo J.* 8:2803-2809.



- Denning, D.P., S.S. Patel, V. Uversky, A.L. Fink, and M. Rexach. 2003. Disorder in the nuclear pore complex: The FG repeat regions of nucleoporins are natively unfolded. *PNAS*. 100:2450-2455.
- DiBella, L.M., S.E. Benashski, H.W. Tedford, A. Harrison, R.S. Patel-King, and S.M. King. 2001. The Tctex1/Tctex2 class of dynein light chains. Dimerization, differential expression, and interaction with the LC8 protein family. *J Biol Chem*. 276:14366-14373.
- DiBella, L.M., and S.M. King. 2001. Dynein motors of the *Chlamydomonas* flagellum. *Int Rev Cytol*. 210:227-268.
- Dutcher, S.K. 1995. Mating and tetrad analysis in *Chlamydomonas reinhardtii*. *Methods Cell Biol*. 47:531-540.
- Espindola, F.S., D.M. Suter, L.B. Partata, T. Cao, J.S. Wolenski, R.E. Cheney, S.M. King, and M.S. Mooseker. 2000. The light chain composition of chicken brain myosin-Va: calmodulin, myosin-II essential light chains, and 8-kDa dynein light chain/PIN. *Cell Motil Cytoskeleton*. 47:269-281.
- Fabry, S., K. Muller, A. Lindauer, P.B. Park, T. Cornelius, and R. Schmitt. 1995. The organization structure and regulatory elements of *Chlamydomonas* histone genes reveal features linking plant and animal genes. *Curr Genet*. 28:333-345.

- Fernandez, E., R. Schnell, L.P. Ranum, S.C. Hussey, C.D. Silflow, and P.A. Lefebvre. 1989. Isolation and characterization of the nitrate reductase structural gene of *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci U S A*. 86:6449-6453.
- Ferris, P.J., and U.W. Goodenough. 1994. The mating-type locus of *Chlamydomonas reinhardtii* contains highly rearranged DNA sequences. *Cell*. 76:1135-1145.
- Fowkes, M.E., and D.R. Mitchell. 1998. The role of preassembled cytoplasmic complexes in assembly of flagellar dynein subunits. *Mol Biol Cell*. 9:2337-2347.
- Fraser, R.D., B.S. Harrap, T.P. Macrae, F.H. Stewart, and E. Suzuki. 1967. Effect of glycyl residues on the stability of the alpha-helix. *Biopolymers*. 5:251-257.
- Fraser, R.D.B., and T.P. MacRae. 1973. Conformation in Fibrous Proteins and Related Synthetic Polypeptides. Academic Press, Inc, New York. 682 pp.
- Gaillard, A.R., D.R. Diener, J.L. Rosenbaum, and W.S. Sale. 2001. Flagellar radial spoke protein 3 is an A-kinase anchoring protein (AKAP). *J Cell Biol*. 153:443-448.
- Gee, M., and R. Vallee. 1998. The role of the dynein stalk in cytoplasmic and flagellar motility. *Eur Biophys J*. 27:466-473.

- Gee, M.A., J.E. Heuser, and R.B. Vallee. 1997. An extended microtubule-binding structure within the dynein motor domain. *Nature*. 390:636-639.
- Gibbons, I.R. 1965. Chemical dissection of cilia. *Arch Biol (Liege)*. 76:317-352.
- Goodenough, U., and J. Heuser. 1984. Structural comparison of purified dynein proteins with in situ dynein arms. *J Mol Biol*. 180:1083-1118.
- Habermacher, G., and W.S. Sale. 1996. Regulation of flagellar dynein by an axonemal type-1 phosphatase in *Chlamydomonas*. *J Cell Sci*. 109 ( Pt 7):1899-1907.
- Habermacher, G., and W.S. Sale. 1997. Regulation of flagellar dynein by phosphorylation of a 138-kD inner arm dynein intermediate chain. *J Cell Biol*. 136:167-176.
- Habura, A., I. Tikhonenko, R.L. Chisholm, and M.P. Koonce. 1999. Interaction mapping of a dynein heavy chain. Identification of dimerization and intermediate-chain binding domains. *J Biol Chem*. 274:15447-15453.
- Han, D.K., J. Eng, H. Zhou, and R. Aebersold. 2001. Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. *Nat Biotechnol*. 19:946-951.

- Harris, E.H. 1989. The *Chlamydomonas* Sourcebook: A Comprehensive Guide to Biology and Laboratory Use. Academic Press, San Diego. 780 pp.
- Harrison, A., M. Sakato, H.W. Tedford, S.E. Benashski, R.S. Patel-King, and S.M. King. 2002. Redox-based control of the gamma heavy chain ATPase from *Chlamydomonas* outer arm dynein. *Cell Motil Cytoskeleton*. 52:131-143.
- Hills, H.G., M. Blomberg-Wirschell, and B. Rajeshkumar. 2002. DNA sequencing of difficult templates. Approaches to using ABI BigDye with dGTP and Amersham chemistries and altered cycling conditions. *In* Association of Biomedical Resource Facilities.
- Hiscott, J., P. Beauparlant, P. Crepieux, C. DeLuca, H. Kwon, R. Lin, and L. Petropoulos. 1997. Cellular and viral protein interactions regulating I kappa B alpha activity during human retrovirus infection. *J Leukoc Biol*. 62:82-92.
- Holzbaur, E.L., and R.B. Vallee. 1994. DYNEINS: molecular structure and cellular function. *Annu Rev Cell Biol*. 10:339-372.
- Hoops, H.J., and G.B. Witman. 1983. Outer doublet heterogeneity reveals structural polarity related to beat direction in *Chlamydomonas* flagella. *J Cell Biol*. 97:902-908.

- Huang, B., G. Piperno, and D.J. Luck. 1979. Paralyzed flagella mutants of *Chlamydomonas reinhardtii*. Defective for axonemal doublet microtubule arms. *J Biol Chem.* 254:3091-3099.
- Jaffrey, S.R., and S.H. Snyder. 1996. PIN: an associated protein inhibitor of neuronal nitric oxide synthase. *Science.* 274:774-777.
- Kagami, O., and R. Kamiya. 1992. Translocation and rotation of microtubules caused by multiple species of *Chlamydomonas* inner-arm dynein. *J Cell Sci.* 103:653-664.
- Kamiya, R. 1988. Mutations at twelve independent loci result in absence of outer dynein arms in *Chlamydomonas reinhardtii*. *J Cell Biol.* 107:2253-2258.
- Kamiya, R. 2000. Analysis of cell vibration for assessing axonemal motility in *Chlamydomonas*. *Methods.* 22:383-387.
- Kamiya, R., and M. Okamoto. 1985. A mutant of *Chlamydomonas reinhardtii* that lacks the flagellar outer dynein arm but can swim. *J Cell Sci.* 74:181-191.
- Kamiya, R., and G.B. Witman. 1984. Submicromolar levels of calcium control the balance of beating between the two flagella in demembrated models of *Chlamydomonas*. *J Cell Biol.* 98:97-107.

- Karki, S., and E.L.F. Holzbaur. 1995. Affinity Chromatography Demonstrates a Direct Binding between Cytoplasmic Dynein and the Dynactin Complex. *J. Biol. Chem.* 270:28806-28811.
- Kathir, P., M. LaVoie, W.J. Brazelton, N.A. Haas, P.A. Lefebvre, and C.D. Silflow. 2003. Molecular Map of the *Chlamydomonas reinhardtii* Nuclear Genome. *Eukaryot Cell.* 2:362-379.
- Kindle, K.L. 1990. High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci U S A.* 87:1228-1232.
- Kindle, K.L., R.A. Schnell, E. Fernandez, and P.A. Lefebvre. 1989. Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. *J Cell Biol.* 109:2589-2601.
- King, S.J., and S.K. Dutcher. 1997. Phosphoregulation of an inner dynein arm complex in *Chlamydomonas reinhardtii* is altered in phototactic mutant strains. *J Cell Biol.* 136:177-191.
- King, S.M. 2000. AAA domains and organization of the dynein motor unit. *J Cell Sci.* 113 ( Pt 14):2521-2526.

- King, S.M., B.E. Haley, and G.B. Witman. 1989. Structure of the alpha and beta heavy chains of the outer arm dynein from *Chlamydomonas* flagella. Nucleotide binding sites. *J Biol Chem.* 264:10210-10218.
- King, S.M., T. Otter, and G.B. Witman. 1985. Characterization of monoclonal antibodies against *Chlamydomonas* flagellar dyneins by high-resolution protein blotting. *Proc Natl Acad Sci U S A.* 82:4717-4721.
- King, S.M., and R.S. Patel-King. 1995a. Identification of a Ca(2+)-binding light chain within *Chlamydomonas* outer arm dynein. *J Cell Sci.* 108 ( Pt 12):3757-3764.
- King, S.M., and R.S. Patel-King. 1995b. The M(r) = 8,000 and 11,000 outer arm dynein light chains from *Chlamydomonas* flagella have cytoplasmic homologues. *J Biol Chem.* 270:11445-11452.
- King, S.M., C.G. Wilkerson, and G.B. Witman. 1991. The Mr 78,000 intermediate chain of *Chlamydomonas* outer arm dynein interacts with alpha-tubulin *in situ*. *J Biol Chem.* 266:8401-8407.
- King, S.M., and G.B. Witman. 1994. Multiple sites of phosphorylation within the alpha heavy chain of *Chlamydomonas* outer arm dynein. *J Biol Chem.* 269:5452-5457.

- Koutoulis, A., G.J. Pazour, C.G. Wilkerson, K. Inaba, H. Sheng, S. Takada, and G.B. Witman. 1997. The *Chlamydomonas reinhardtii* ODA3 gene encodes a protein of the outer dynein arm docking complex. *J Cell Biol.* 137:1069-1080.
- Kozminski, K.G., K.A. Johnson, P. Forscher, and J.L. Rosenbaum. 1993. A motility in the eukaryotic flagellum unrelated to flagellar beating. *Proc Natl Acad Sci U S A.* 90:5519-5523.
- Lefebvre, P.A., and C.D. Silflow. 1999. *Chlamydomonas*: the cell and its genomes. *Genetics.* 151:9-14.
- Li, J.B., S. Lin, H. Jia, H. Wu, B.A. Roe, D. Kulp, G.D. Stormo, and S.K. Dutcher. 2003. Analysis of *Chlamydomonas reinhardtii* genome structure using large-scale sequencing of regions on linkage groups I and III. *J Eukaryot Microbiol.* 50:145-155.
- Littauer, U.Z., D. Giveon, M. Thierauf, I. Ginzburg, and H. Ponstingl. 1986. Common and distinct tubulin binding sites for microtubule-associated proteins. *Proc Natl Acad Sci U S A.* 83:7162-7166.
- Luck, D.J., B. Huang, and G. Piperno. 1982. Genetic and biochemical analysis of the eukaryotic flagellum. *Symp Soc Exp Biol.* 35:399-419.



- Marshall, W.F., and J.L. Rosenbaum. 2001. Intraflagellar transport balances continuous turnover of outer doublet microtubules: implications for flagellar length control. *J Cell Biol.* 155:405-414.
- Meeks, M., and A. Bush. 2000. Primary ciliary dyskinesia (PCD). *Pediatr Pulmonol.* 29:307-316.
- Mitchell, D.R., and K.S. Brown. 1994. Sequence analysis of the *Chlamydomonas* alpha and beta dynein heavy chain genes. *J Cell Sci.* 107 ( Pt 3):635-644.
- Mitchell, D.R., and Y. Kang. 1991. Identification of *oda6* as a *Chlamydomonas* dynein mutant by rescue with the wild-type gene. *J Cell Biol.* 113:835-842.
- Mitchell, D.R., and Y. Kang. 1993. Reversion analysis of dynein intermediate chain function. *J Cell Sci.* 105 ( Pt 4):1069-1078.
- Mitchell, D.R., and J.L. Rosenbaum. 1985. A motile *Chlamydomonas* flagellar mutant that lacks outer dynein arms. *J Cell Biol.* 100:1228-1234.
- Mocz, G., and I.R. Gibbons. 1996. Phase partition analysis of nucleotide binding to axonemal dynein. *Biochemistry.* 35:9204-9211.

- Mocz, G., M.K. Helms, D.M. Jameson, and I.R. Gibbons. 1998. Probing the nucleotide binding sites of axonemal dynein with the fluorescent nucleotide analogue 2'(3')-O-(-N-Methylantraniloyl)-adenosine 5'-triphosphate. *Biochemistry*. 37:9862-9869.
- Nakamura, K., K. Iitsuka, and T. Fujii. 1999. Adenylate kinase is tightly bound to axonemes of *Tetrahymena* cilia. *Comp Biochem Physiol B Biochem Mol Biol*. 124:195-199.
- Noguchi, M., T. Sawada, and T. Akazawa. 2001. ATP-regenerating system in the cilia of *Paramecium caudatum*. *J Exp Biol*. 204:1063-1071.
- Ogawa, K., R. Kamiya, C.G. Wilkerson, and G.B. Witman. 1995. Interspecies conservation of outer arm dynein intermediate chain sequences defines two intermediate chain subclasses. *Mol Biol Cell*. 6:685-696.
- Ogawa, K., H. Takai, A. Ogiwara, E. Yokota, T. Shimizu, K. Inaba, and H. Mohri. 1996. Is outer arm dynein intermediate chain 1 multifunctional? *Mol Biol Cell*. 7:1895-1907.
- Omoto, C.K., I.R. Gibbons, R. Kamiya, C. Shingyoji, K. Takahashi, and G.B. Witman. 1999. Rotation of the central pair microtubules in eukaryotic flagella. *Mol Biol Cell*. 10:1-4.

- Padma, P., A. Hozumi, K. Ogawa, and K. Inaba. 2001. Molecular cloning and characterization of a thioredoxin/nucleoside diphosphate kinase related dynein intermediate chain from the ascidian, *Ciona intestinalis*. *Gene*. 275:177-183.
- Paschal, B.M., R.A. Obar, and R.B. Vallee. 1989. Interaction of brain cytoplasmic dynein and MAP2 with a common sequence at the C terminus of tubulin. *Nature*. 342:569-572.
- Patel-King, R.S., S.E. Benashki, A. Harrison, and S.M. King. 1996. Two functional thioredoxins containing redox-sensitive vicinal dithiols from the *Chlamydomonas* outer dynein arm. *J Biol Chem*. 271:6283-6291.
- Pazour, G.J., B.L. Dickert, and G.B. Witman. 1999a. The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagellar assembly. *J Cell Biol*. 144:473-481.
- Pazour, G.J., A. Koutoulis, S.E. Benashski, B.L. Dickert, H. Sheng, R.S. Patel-King, S.M. King, and G.B. Witman. 1999b. LC2, the *Chlamydomonas* homologue of the t complex-encoded protein Tctex2, is essential for outer dynein arm assembly. *Mol Biol Cell*. 10:3507-3520.
- Pazour, G.J., and J.L. Rosenbaum. 2002. Intraflagellar transport and cilia-dependent diseases. *Trends Cell Biol*. 12:551-555.

- Pazour, G.J., O.A. Sineschekov, and G.B. Witman. 1995. Mutational analysis of the phototransduction pathway of *Chlamydomonas reinhardtii*. *J Cell Biol.* 131:427-440.
- Pazour, G.J., C.G. Wilkerson, and G.B. Witman. 1998. A dynein light chain is essential for the retrograde particle movement of intraflagellar transport (IFT). *J Cell Biol.* 141:979-992.
- Pazour, G.J., and G.B. Witman. 2000. Forward and reverse genetic analysis of microtubule motors in *Chlamydomonas*. *Methods.* 22:285-298.
- Pennarun, G., E. Escudier, C. Chapelin, A.M. Bridoux, V. Cacheux, G. Roger, A. Clement, M. Goossens, S. Anselem, and B. Duriez. 1999. Loss-of-function mutations in a human gene related to *Chlamydomonas reinhardtii* dynein IC78 result in primary ciliary dyskinesia. *Am J Hum Genet.* 65:1508-1519.
- Pfister, K.K., R.B. Fay, and G.B. Witman. 1982. Purification and polypeptide composition of dynein ATPases from *Chlamydomonas* flagella. *Cell Motil.* 2:525-547.
- Piperno, G. 1995. Two-dimensional separation of axonemal proteins. *Methods Cell Biol.* 47:107-112.

- Piperno, G., B. Huang, and D.J. Luck. 1977. Two-dimensional analysis of flagellar proteins from wild-type and paralyzed mutants of *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci U S A*. 74:1600-1604.
- Piperno, G., and D.J. Luck. 1979. Axonemal adenosine triphosphatases from flagella of *Chlamydomonas reinhardtii*. Purification of two dyneins. *J Biol Chem*. 254:3084-3090.
- Piperno, G., K. Mead, and S. Henderson. 1996. Inner dynein arms but not outer dynein arms require the activity of kinesin homologue protein KHP1(FLA10) to reach the distal part of flagella in *Chlamydomonas*. *J Cell Biol*. 133:371-379.
- Piperno, G., and Z. Ramanis. 1991. The proximal portion of *Chlamydomonas* flagella contains a distinct set of inner dynein arms. *J Cell Biol*. 112:701-709.
- Piperno, G., Z. Ramanis, E.F. Smith, and W.S. Sale. 1990. Three distinct inner dynein arms in *Chlamydomonas* flagella: molecular composition and location in the axoneme. *J Cell Biol*. 110:379-389.
- Porter, M.E., R. Bower, J.A. Knott, P. Byrd, and W. Dentler. 1999. Cytoplasmic dynein heavy chain 1b is required for flagellar assembly in *Chlamydomonas*. *Mol Biol Cell*. 10:693-712.

Porter, M.E., J.A. Knott, S.H. Myster, and S.J. Farlow. 1996. The dynein gene family in *Chlamydomonas reinhardtii*. *Genetics*. 144:569-585.

Porter, M.E., and W.S. Sale. 2000. The 9 + 2 axoneme anchors multiple inner arm dyneins and a network of kinases and phosphatases that control motility. *J Cell Biol*. 151:F37-42.

Purohit, A., S.H. Tynan, R. Vallee, and S.J. Doxsey. 1999. Direct interaction of pericentrin with cytoplasmic dynein light intermediate chain contributes to mitotic spindle organization. *J Cell Biol*. 147:481-492.

Qin, H., D.R. Diener, S. Geimer, X. Liu, H. Tran, D.G. Cole, and J. Rosenbaum. 2002. Evidence of IFT complexes association with motors and cargoes. *In* The Tenth International Conference on the Cell and Molecular Biology of *Chlamydomonas*, Vancouver, B.C.

Qin, H., D.R. Diener, S. Geimer, X. Liu, H. Tran, D.G. Cole, and J. Rosenbaum. 2003. Intraflagellar transport particles associate with and transport axonemal proteins required for flagellar assembly and maintenance. *Mol Biol Cell*. 14:in press (abstract).

- Rodriguez-Crespo, I., W. Straub, F. Gavilanes, and P.R. Ortiz de Montellano. 1998. Binding of dynein light chain (PIN) to neuronal nitric oxide synthase in the absence of inhibition. *Arch Biochem Biophys.* 359:297-304.
- Rodriguez-Crespo, I., B. Yelamos, F. Roncal, J.P. Albar, P.R. Ortiz de Montellano, and F. Gavilanes. 2001. Identification of novel cellular proteins that bind to the LC8 dynein light chain using a pepscan technique. *FEBS Lett.* 503:135-141.
- Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol.* 132:365-386.
- Sager, R., and S. Granick. 1953. Nutritional studies with *Chlamydomonas reinhardtii*. *Ann N Y Acad Sci.* 466:18-30.
- Sakakibara, H., D.R. Mitchell, and R. Kamiya. 1991. A *Chlamydomonas* outer arm dynein mutant missing the alpha heavy chain. *J Cell Biol.* 113:615-622.
- Sakakibara, H., and H. Nakayama. 1998. Translocation of microtubules caused by the alphabeta, beta and gamma outer arm dynein subparticles of *Chlamydomonas*. *J Cell Sci.* 111 ( Pt 9):1155-1164.

- Sakakibara, H., S. Takada, S.M. King, G.B. Witman, and R. Kamiya. 1993. A *Chlamydomonas* outer arm dynein mutant with a truncated beta heavy chain. *J Cell Biol.* 122:653-661.
- Sakato, M., and S.M. King. 2000.  $Ca^{2+}$  modulates the microtubule-binding activity of  $\beta\gamma$  and  $\gamma$  heavy chain subparticles of *Chlamydomonas* outer arm dynein. In American Society for Cell Biology. Vol. 11. Mol Biol Cell, San Francisco. 194a.
- Sale, W.S., U.W. Goodenough, and J.E. Heuser. 1985. The substructure of isolated and in situ outer dynein arms of sea urchin sperm flagella. *J Cell Biol.* 101:1400-1412.
- Schoff, P.K., J. Cheetham, and H.A. Lardy. 1989. Adenylate kinase activity in ejaculated bovine sperm flagella. *J Biol Chem.* 264:6086-6091.
- Silflow, C.D. 1998. Organization of the nuclear genome. In *The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas*. J.D. Rochaix, M. Goldschmidt-Clermont, and S. Merchant, editors. Kluwer Academic Publishers, The Netherlands. 25-40.
- Silflow, C.D., P. Kathir, and P.A. Lefebvre. 1995. Molecular mapping of genes for flagellar proteins in *Chlamydomonas*. *Methods Cell Biol.* 47:525-530.



- Silflow, C.D., M. LaVoie, L.W. Tam, S. Tousey, M. Sanders, W. Wu, M. Borodovsky, and P.A. Lefebvre. 2001. The Vfl1 Protein in *Chlamydomonas* localizes in a rotationally asymmetric pattern at the distal ends of the basal bodies. *J Cell Biol.* 153:63-74.
- Silflow, C.D., P.A. Lefebvre, T.W. McKeithan, J.A. Schloss, L.R. Keller, and J.L. Rosenbaum. 1982. Expression of flagellar protein genes during flagellar regeneration in *Chlamydomonas*. *Cold Spring Harb Symp Quant Biol.* 46 Pt 1:157-169.
- Sizova, I.A., T.V. Lapina, and A.V. Kozlov. 1995. [Analysis of a chromosomal DNA fragment isolated from a hybrid autonomous plasmid of *Chlamydomonas reinhardtii*]. *Genetika.* 31:324-332.
- Smith, E.F., and P.A. Lefebvre. 1997. The role of the central apparatus components in flagellar microtubule assembly and microtubule assembly. *Cell.Motil.Cytoskel.* 38:1-8.
- Smith, E.F., and W.S. Sale. 1992. Regulation of dynein-driven microtubule sliding by the radial spokes in flagella. *Science.* 257:1557-1559.
- Smith, E.F., and W.S. Sale. 1994. Mechanisms of flagellar movement: functional interactions between dynein arms and the radial spoke-central pair apparatus

complex. *In* Microtubules. C.W. Lloyd, editor. John Wiley & Sons, Inc, New York. 381-392.

Steinberg, G., and M. Schliwa. 1996. Characterization of the Biophysical and Motility Properties of Kinesin from the Fungus *Neurospora crassa*. *J. Biol. Chem.* 271:7516-7521.

Supp, D.M., D.P. Witte, S.S. Potter, and M. Brueckner. 1997. Mutation of an axonemal dynein affects left-right asymmetry in inversus viscerum mice. *Nature*. 389:963-966.

Tai, A.W., J.Z. Chuang, C. Bode, U. Wolfrum, and C.H. Sung. 1999. Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1. *Cell*. 97:877-887.

Tai, A.W., J.Z. Chuang, and C.H. Sung. 2001. Cytoplasmic dynein regulation by subunit heterogeneity and its role in apical transport. *J Cell Biol.* 153:1499-1509.

Takada, S., and R. Kamiya. 1994. Functional reconstitution of *Chlamydomonas* outer dynein arms from alpha-beta and gamma subunits: requirement of a third factor. *J Cell Biol.* 126:737-745.

- Takada, S., and R. Kamiya. 1997. Beat frequency difference between the two flagella of *Chlamydomonas* depends on the attachment site of outer dynein arms on the outer-doublet microtubules. *Cell Motil Cytoskeleton*. 36:68-75.
- Takada, S., H. Sakakibara, and R. Kamiya. 1992. Three-headed outer arm dynein from *Chlamydomonas* that can functionally combine with outer-arm-missing axonemes. *J Biochem (Tokyo)*. 111:758-762.
- Takada, S., C.G. Wilkerson, K. Wakabayashi, R. Kamiya, and G.B. Witman. 2002. The outer dynein arm-docking complex: composition and characterization of a subunit (oda1) necessary for outer arm assembly. *Mol Biol Cell*. 13:1015-1029.
- Tynan, S.H., A. Purohit, S.J. Doxsey, and R.B. Vallee. 2000. Light intermediate chain 1 defines a functional subfraction of cytoplasmic dynein which binds to pericentrin. *J Biol Chem*. 275:32763-32768.
- Wakabayashi, K., S.M. King, and R. Kamiya. 2002a. Analysis of proteins interacting with the outer dynein arm docking complex (ODA-DC) from *Chlamydomonas* by chemical crosslinking. *Mol Cell Biol*. 13, supplement:184a.
- Wakabayashi, K., S. Takada, M. Hayashi, J. Usukura, G.B. Witman, and R. Kamiya. 2002b. Structure and property of the outer-dynein-arm docking complex (ODA-

DC) studied using recombinant proteins. *In* The Tenth International conference on the Cell & Molecular Biology of *Chlamydomonas.*, Vancouver, Canada. 167.

Wakabayashi, K., S. Takada, G.B. Witman, and R. Kamiya. 2001. Transport and arrangement of the outer-dynein-arm docking complex in the flagella of *Chlamydomonas* mutants that lack outer dynein arms. *Cell Motil Cytoskeleton.* 48:277-286.

Watanabe, T., and M. Flavin. 1976. Nucleotide-metabolizing enzymes in *Chlamydomonas* flagella. *J Biol Chem.* 251:182-192.

Wilkerson, C.G., S.M. King, A. Koutoulis, G.J. Pazour, and G.B. Witman. 1995. The 78,000 M(r) intermediate chain of *Chlamydomonas* outer arm dynein is a WD-repeat protein required for arm assembly. *J Cell Biol.* 129:169-178.

Wilkerson, C.G., S.M. King, and G.B. Witman. 1994. Molecular analysis of the gamma heavy chain of *Chlamydomonas* flagellar outer-arm dynein. *J Cell Sci.* 107 ( Pt 3):497-506.

Witman, G.B. 1986. Isolation of *Chlamydomonas* flagella and flagellar axonemes. *Methods in Enzymology.* 134:280-290.

- Witman, G.B., K. Carlson, J. Berliner, and J.L. Rosenbaum. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. *J Cell Biol.* 54:507-539.
- Witman, G.B., K.A. Johnson, K.K. Pfister, and J.S. Wall. 1983. Fine structure and molecular weight of the outer arm dyneins of *Chlamydomonas*. *J. Submicrosc. Cytol.* 15:193-197.
- Yang, P., R. Bodiwala, and W.S. Sale. 2000. Structural interaction of calmodulin, dynein light chain LC8 and radial spoke protein 2 in the flagellar radial spoke. *Mol Biol Cell.* 11:538a.
- Yang, P., D.R. Diener, J.L. Rosenbaum, and W.S. Sale. 2001. Localization of calmodulin and dynein light chain LC8 in flagellar radial spokes. *J Cell Biol.* 153:1315-1326.
- Yang, P., and W.S. Sale. 2000. Casein kinase I is anchored on axonemal doublet microtubules and regulates flagellar dynein phosphorylation and activity. *J Biol Chem.* 275:18905-18912.