

USE OF DEGENERATE PCR TO STUDY THE DYNEIN HEAVY CHAIN GENE FAMILY IN THE PRIMITIVE EUKARYOTE *GIARDIA LAMBLIA*

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Abstract:

Dyneins are a family of motor proteins that serve a variety of motile functions (Karp, 1996). Previous analysis has shown that functional variance in dyneins of different organisms emerged early in the process of evolution (Asai, 1996). Therefore, we chose to study dyneins in the primitive eukaryote, *Giardia lamblia*. In this study, we isolated fragments of dynein heavy chain genes from *Giardia* genomic DNA through degenerate PCR. We then cloned and sequenced these isolated fragments in an effort to determine the number of dynein genes in *Giardia*. Our results thus far have yielded four novel dynein sequences. We plan to compare these findings to those of higher eukaryotes, such as *S. cerevisiae* and *Chlamydomonas*. Also, due to the similarities between axonemal and cytoplasmic dyneins (Karp, 1996), these analyses serve a fundamental role in further studies concerning either class of dynein.

Introduction

Microtubule motors play a critical role in many types of intracellular transport. They are proteins that operate as mechanical motors in conjunction with microtubules. Microtubules are hollow, cylindrical structures, that are formed from tubulin. They are one of the fundamental components of the eukaryotic cytoskeleton and the primary structural element of cilia and flagella. Microtubule motors move unidirectionally along the microtubules while they undergo various conformational changes that make up a mechanical cycle. This mechanical cycle is coupled to a chemical cycle that provides the energy necessary to fuel the movement along the microtubules. There are two types of microtubule motors: kinesins and dyneins. Kinesins are responsible for a wide variety of cytoplasmic movements including: spindle pole separation, vesicle transport, and axonal transport. Most kinesins are plus end-directed microtubule motors when tested in an in vitro motility assay. Motor direction has important consequences because microtubules are organized in polar arrays. For example, since all the microtubules of an axon are orientated with their minus ends facing the cell body and their plus ends facing the synaptic terminals, it was concluded that kinesins were responsible for anterograde axonal transport (reviewed in Karp, 1996). This paper will focus on dynein proteins, of which there are two kinds: cytoplasmic and axonemal. Dynein proteins are minus end-directed microtubule motors, acting in contrast to kinesins. There are two basic roles for cytoplasmic dyneins: they generate a force that aids in the separation of chromosomes during mitosis and they act in minus end-directed microtubule movement of vesicles and organelles through the cytoplasm (reviewed in Karp, 1996). This paper will look specifically at the function of axonemal dynein proteins. Axonemal dynein proteins are the motor proteins that cause the bending of the eukaryotic flagella and cilia (Asai, 1996). This paper describes an effort to classify the number of dynein proteins in the flagella of *Giardia lamblia*.

Cilia and flagella are motile organelles that project from the surface of a variety of eukaryotic cells. They are two versions of the same structure, differing in their patterns of movement and length. Cilia are often found in large numbers on the cell surface and often beat in a coordinated motion. Flagella are longer than cilia and are present in fewer numbers on the cell surface. The force of the flagella pushes an organism forward, while the force of cilia acts to move fluid or specific materials over the cell surface (reviewed in Karp, 1996). The cytoskeleton of both is called the axoneme, which contains an array of microtubule that run longitudinally through the entire organelle (Asai and Brokaw, 1993). The axoneme

consists of nine outer doublet microtubules surrounding a single, central pair of microtubules (figure 1). Each outer doublet consists of one complete microtubule, the A tubule, and one incomplete microtubule, the B tubule. The single central pair of microtubules are surrounded by the central sheath, which is connected to the A tubules via radial spokes. Interdoublet, or nexin, bridges connect the outer microtubules to one another. It should also be noted that the inner and outer dynein arms project from the outer doublets, where the inner arms face the central sheath and the outer arms face towards the boundary of the axoneme. The outer arms contain three globular heads, each which is an ATP-hydrolyzing cross-bridge, that are attached by long tails to a common base. The structure of the inner arms seems to be less conserved, varying with a structure of 2 or 3 globular heads (reviewed in Karp, 1996).

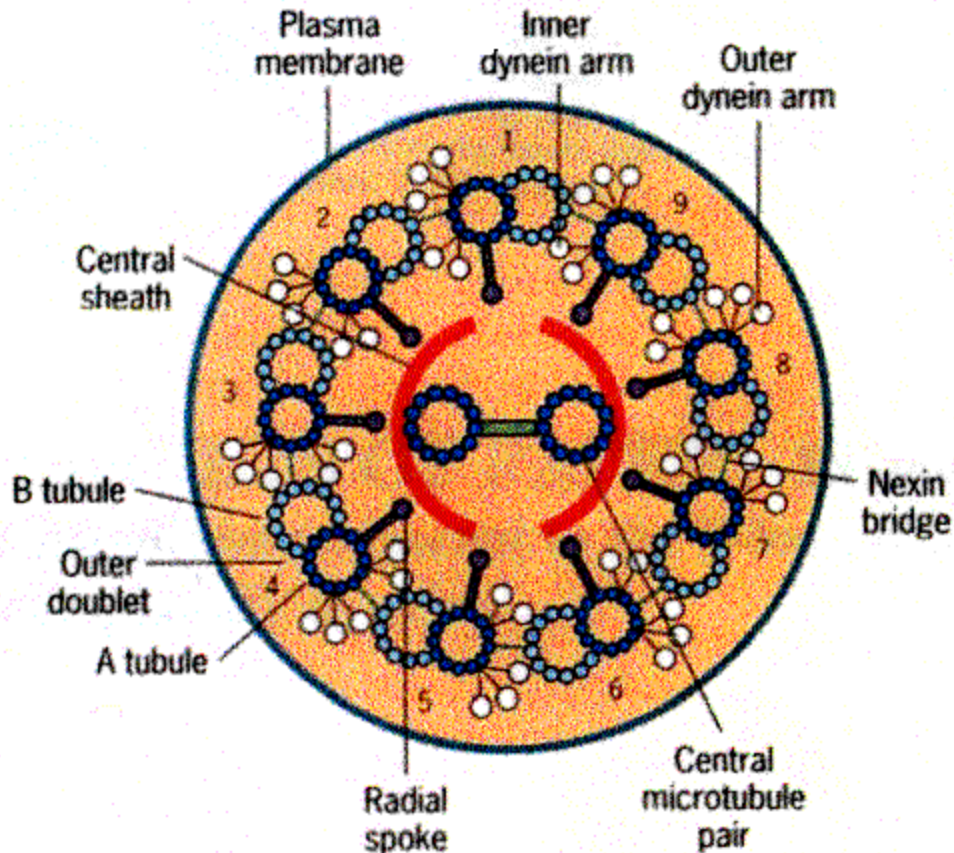


Figure 1: The structure of a ciliary or flagellar axoneme (Karp, 1996).

The axoneme contains the motor activity for the beating activity of the cilia and flagella. Dynein is the sole ATPase that is responsible for the hydrolysis of ATP for the axoneme. It has been found that the outer arms regulate the beat frequency, while the inner arms control the waveforms of the cilia/flagella (Piperno, 1990). One specific study looked into whether the oscillation of the flagella is a property of the dynein arms themselves, or whether oscillation requires an intact axoneme. This experiment led to the surprising conclusion that only a few, and possibly only one, dynein arms could produce strong oscillations (Shingyoji et al., 1998).

The motility, or oscillation, of the cilia and flagella occurs via the sliding microtubules mechanism. The dynein arms act as swinging cross-bridges that generate the forces required for ciliary or

flagellar movement (reviewed in Asai and Brokaw, 1993). Cilium bending occurs when the doublets on one side of the bend slide beyond those on the other side. This bends are initiated repeatedly and propagate along the length of the axoneme and produce relative motion of the cell body and the surrounding fluids (Asai and Brokaw, 1993). The dynein bend can be divided into three basic steps repeated in a cycle: head attachment, tilting (which leads to force generation), and dissociation (Shingyoji et al., 1998). Resistance is needed to cause the bend; otherwise the doublets would simply slide past each other and provide no motion. The radial spokes, the interdoublet links, or both, provide this resistance (Asai, 1996).

While both the outer and inner dynein arms are responsible for the bending of cilia and flagella, the outer arm is probably the most extensively studied. These studies have described outer arm dynein complex that is characterized by its number of light, intermediate, and heavy chains. Dyneins contain two or three heavy chains, that contain the ATPase activity mentioned above, up to four intermediate chains, and several light chains (reviewed in Asai and Brokaw, 1993). According to Asai and Brokaw, one can deduce a lot of information about the functional domains of dynein just from the primary structure of its heavy chain (Asai and Brokaw, 1993). They describe three different domains of the heavy chain that relate to the three major aspects of dynein function. First, there is a microtubule binding. Second, there is a site cargo binding. Third, there is the globular head, which contains the activity required for Mg^{2+} -ATP hydrolysis. This domain is characterized by a highly conserved P-loop which is located near the middle of the sequence (Asai and Brokaw, 1993). The fact that this region contains some absolutely conserved sequences was utilized in the design of the degenerate oligonucleotide primers that were used for polymerase chain reaction (PCR) in the Asai and Criswell experiment as well as in this study (Asai and Criswell, 1995).

Many studies of dyneins, up until this time, have focused on *Saccharomyces cerevisiae* (yeast), *Chlamydomonas*, or *Tetrahymena* as very simple model organisms for higher eukaryotes such as *Homo sapiens*. However, phylogenetic distance trees show that yeast are actually relatively close to humans in terms of eukaryotic differentiation (Sogin, 1991). This study is focusing on a *Giardia lamblia* in an attempt to determine the number of dynein proteins in an organism from the earliest beginnings of the eukaryotic lineage. *Giardia* was specifically chosen because it is the simplest eukaryotic organism that still has flagella. As of 1996, there were 12 different dynein heavy chain sequences available, from various organisms and cell types. There exists a wide evolutionary separation among the sources of these dyneins, yet these sequences are still very similar (Asai, 1996). The experiments described in this paper have the goal of comparing *Giardia*'s dynein proteins with the 12 complete sequences in a search for either sequence homology or differences.

In conclusion, this purpose of this experiment is to determine the number of different dynein proteins present in the primitive eukaryotic organism *Giardia*. Degenerate PCR has been used to amplify the P-loop encoding fragments in an effort to determine the number of dynein proteins encoded in the genome.

Materials and Methods

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to amplify the region of *Giardia*'s dynein heavy chain gene that encodes the conserved catalytic P-loop. Two previously constructed primers, A2 and S2, were used because they flanked the region of interest (figure 2). The 5' ends of the primers were created with poly Gs or poly Cs, which helps protect the ends during amplification. PCR was first performed using *Saccharomyces cerevisiae* and *Tetrahymena* genomic DNA as control templates, in an effort to perfect the PCR program before using it on *Giardia*. Once it was working well, PCR was performed on *Giardia* genomic DNA. There was a two-part PCR cycle. The first PCR cycle, for higher annealing, was repeated 5 times, and the cycling conditions were as follows: denaturation at 94 °C for 45 s, primer annealing at 45 °C for 1 min, and primer extension at 72 °C for 1 min. The second PCR cycle, for lower annealing, was repeated 40 times. Cycling conditions were as follows: denaturation at 94 °C for 30 s, primer annealing at 35 °C for 1 min, and primer extension at 72 °C for 1 minute.

A2: 5'-GGGCGAATTC[TG][AG]TT[AG]AA[TC]TC[AG]TC[AG]AA[AG]CA-3'
S2: 5'-CCCCGGATCCTGCTGG[TCAG]AC[TCAG]GG[TCAG]AA[GA]AC-3'

Figure 2: Degenerate oligonucleotide primers, A2 and S2. Degenerate base pairs are illustrated in the square brackets. (Asai and Criswell, 1995).

Cloning of PCR Product and Transformation into *Escherichia coli*

The PCR product, the amplified dynein gene fragment, was cloned into a vector to isolate individual products. The cloning was done using a TOPO TA Cloning kit, following manufacturer's instructions (Invitrogen, Inc.). This kit utilizes the enzyme topoisomerase to form a covalent linkage between the PCR product insert and the pCR4-TOPO vector. The constructs were then transformed into One Shot TOP10 *E. coli* cells following manufacturer's instructions, in order to isolate and amplify individual ligation products (Invitrogen, Inc.). The cells were grown on plates containing ampicillin in order to select for transformed cells. The alkaline lysis "miniprep" protocol was used to prepare the plasmid DNA from each colony, as outlined by Ausubel et al. (Ausubel et al., 1995). The DNA was digested with EcoRI and run on an agarose gel to determine whether each vector contained an insert.

Sequencing and Sequencing Analysis

Plasmids were prepared for sequencing using the Qiagen Purification System as recommended by manufacturer (QIAGEN, inc.). DNA sequencing was performed using the DNA sequencing kit with BigTye™ terminator as recommended by the manufacture (PE Biosystems). The vector and PCR insert sequences were first examined using a computer program, DNA Strider, which translated the DNA sequences. Then, the following web site, National Center for Biotechnological Information (National Institutes of Health) was used to compare the sequences to other known sequences: <http://www.ncbi.nlm.nih.gov/BLAST/>. The final sequences were also compared to partial sequences of dynein heavy chains, cited in the literature, that were also amplified with the S2 and A2 primers (figure 3) (Asai and Criswell, 1995).

Consensus Sequence:

GPAGTGKTETTKDLGKALGXCVVFNCSXXDYKXMGKXFKGLAQSGAWGCFDEFNR

Figure 3: The consensus sequence of the region of the dynein heavy chain that is encoded by the PCR amplified region of DNA studies in this work. The letter X is inserted wherever there were multiple possibilities for the amino acid at that position. (Asai and Criswell, 1995).

Results

Isolation of Giardia PCR Product

PCR was used to amplify regions of different *Giardia* dynein heavy chain genes that encode for the conserved catalytic P-loop, using degenerate primers A2 and S2 (figures 2 and 3). PCR was performed first on *S. cerevisiae* and *Tetrahymena* genomic DNA to perfect the PCR program for use on *Giardia* genomic DNA. Agarose gel electrophoresis was used to test whether the appropriate size fragments (180bp) were produced (figure 4).

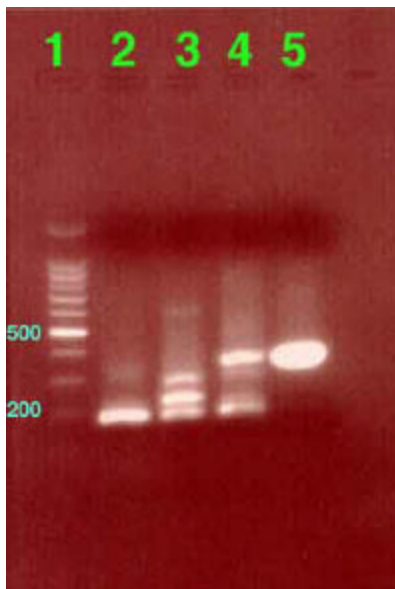


Figure 4: Agarose gel used to confirm the isolation of the correct PCR product. Lane 1 is an hundred base pair marker. Lanes 2 and 3 are the *Giardia* PCR products. Lanes 4 and 5 were used as controls: lane 4 contains yeast genomic DNA amplified with the A2 and S2 primers, and lane 5 contains yeast DNA amplified with different primers.

Cloning and Sequencing of Dynein PCR Product was Successful

To facilitate sequencing, the PCR products were inserted into the TOPO vector. Then, plasmids were isolated from select transformants, followed by a digestion with EcoRI, to determine whether the plasmid of each transformant contained the insert, the PCR product (figure 5). If the insert is present, both a 180bp band and a 400bp band are expected. If the insert is not present, only the 400bp band is expected. Out of initial eight plasmids analyzed, seven gave bright defined 180bp bands upon examination of the agarose gel. One gave a slightly lighter band, most likely due to the fact that it had less DNA than the other samples. The second set of analyzed plasmid DNA, showed seven out of eight plasmids containing inserts.

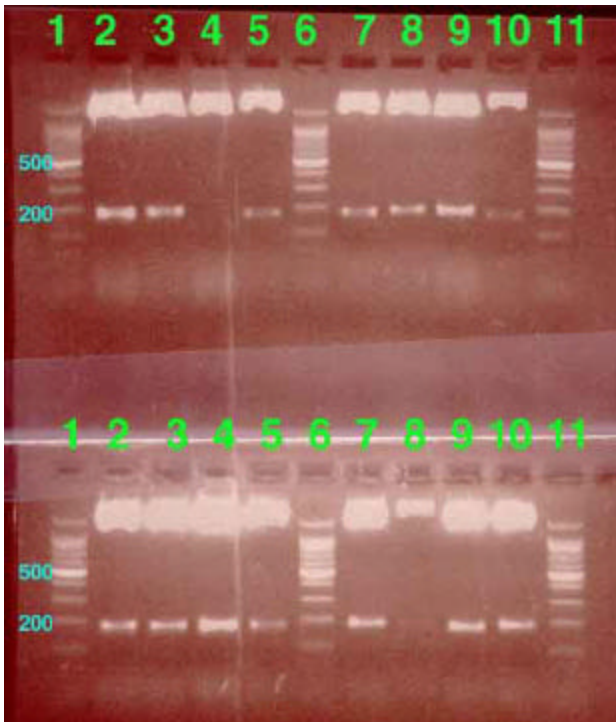


Figure 5: Agarose gel of alkaline miniprep analysis and digestion with EcoRI. This gel used to determine whether each vector contained the insert, the PCR product. Top gel: Lanes 1, 6, and 11, contain a 1kb marker. Lanes 2, 3, 4, 5, 7, 8, 9, and 10, contain vector and insert segments, and therefore, all of the plasmids contain inserts. The insert in lane 8 is not clearly visible here, since it contained a lighter band than the others. Bottom gel: shows the results of the second set of plasmid digestions with EcoRI. Lanes 1, 6, and 11 contain a 1kb marker. Lanes 2, 3, 5, 7, 8, 9, and 10 contain vector and insert segments, and therefore, all of the plasmids contain inserts.

Once it was confirmed that the vectors did indeed contain the PCR products, the samples were prepared for sequencing using the Qiagen Purification System. Overall, 26 plasmids were sequenced. The sequence analysis resulted in four novel dynein sequences (figure 6).

(a)

Sequence 1: SM1, SM3, JR5, JR9, JR11, and JR13:
ETTKDLAKAISIQCLIFNCSEGLDYKSLGRMFSGLCQTGAWS

Sequence 2: SM4, JR16, and JR19:
ETTKDLAKALAMPICIVFNCSFSLDYRIMGRFFAGYLSQVGAWI

Sequence 3: SM5 and JR17:
EISKAFARSLGRFCIVLNCTEALDYSTVVRIFMGMAATGSIL

Sequence 4: SM8, JR6, JR10, and JR12:
ESTKDLAKAMSIQCLVFNCSEGLNVAAMGKFFIGLVMCGAWS

(b)

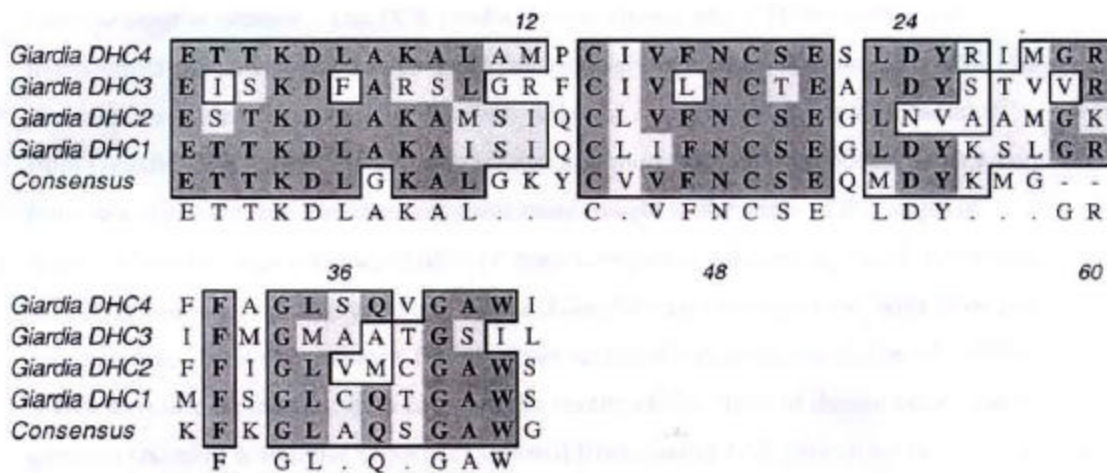


Figure 6: (a) The different dynein heavy chain sequences isolated from *Giardia* genomic DNA. This sequence is represented by the single letter amino acid codes; the sequence encoded by the primers is excluded. (b) The alignment of the four newly discovered dynein sequences aligned with the consensus sequence. The sequence encoded by the primer is excluded.

The number of times that each sequence was found among the different PCR products varied considerably for each gene; the final numbers are noted here in parenthesis: Sequence 1 (6), Sequence 2 (3), Sequence 3 (2), and Sequence 4 (4). All of the sequences shown above were analyzed using the NIH BLAST database and were found to have homology with only previously described dynein heavy chain sequences. While these sequences were similar to other dynein sequences, none of them were exactly the same as any of the sequences found in the database. This is significant because it suggests that products isolated in this experiment were indeed a *Giardia* dynein gene. Therefore, this experiment has succeeded in isolating four novel *Giardia* dynein sequences.

Discussion

This study used degenerate PCR to amplify the P-loop encoding fragments of dynein heavy chains in an effort to determine the number of dynein proteins encoded in the *Giardia lamblia* genome. The PCR products were cloned into a TOPO vector. The plasmid inserts were then sequenced and four novel dynein heavy chain genes were isolated from *Giardia*. The reason *Giardia* was chosen as a model organism is because it is a very primitive organism, and therefore may add more insight to the study of the origin of dynein sequences than previous studies of more complex organisms such as *S. cerevisiae* or *Chlamydomonas*. According to one source, *Giardia*'s common ancestor with yeast and man may have been very ancient, for it had not acquired mitochondria (Nasmyth, 1996). It will therefore be interesting to compare the results of this study of dynein heavy chain genes in *Giardia*, with those sequences isolated from studies of *S. cerevisiae* or *Chlamydomonas*.

The number of times that each sequence was found among the different PCR products varied considerably. This difference in the number of isolated sequences was probably due to the small number of plasmids sequenced, and therefore with continuation of this study, the numbers may equalize. The fact that only four sequences have been isolated at this point lends itself to interesting conclusions; there are two possibilities at this point. First, the fact that only four sequences have been isolated may be indicative of the small number of plasmids sequenced, and possibly sequencing more plasmids in the future will reveal additional *Giardia* dynein heavy chain genes. The second possibility is that there actually exists only four dynein sequences in *Giardia*. Either result will lead to valuable information as to the evolution of the dynein gene.

If the second possibility is correct, and it is concluded that there are indeed only four dynein heavy chain sequences in *Giardia*, while there are approximately twelve in *Tetrahymena*, we can conclude two things. First, most likely only four of the twelve *Tetrahymena* dynein heavy chain genes are actually needed for the basic functioning of the dynein genes. Second, the eight other dynein genes are most likely encode proteins with more evolved functions, such as fine-tuning mechanisms for the basic dynein functions.

Further research will have to be done to make any final conclusion as to the significance of these four novel sequences. First, to verify these sequences, genomic clones for each of the sequences would have to be isolated from a *Giardia* genomic library. Also, a Southern blot of genomic *Giardia* DNA, probed with either one of the previously isolated sequences at a time, or a mixture of the four, would hopefully provide some information about the size of the dynein gene family in *Giardia*. Upon the retrieval of these final results, more definite conclusion will be made.

References

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