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ida4–1, *ida4–2*, and *ida4–3* Are Intron Splicing Mutations Affecting the Locus Encoding p28, a Light Chain of *Chlamydomonas* Axonemal Inner Dynein Arms

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We recently determined the nucleotide sequence of the gene encoding p28, a light chain of inner dynein arms of *Chlamydomonas* axonemes. Here, we show that p28 is the protein encoded by the *IDA4* locus. p28, and the dynein heavy chains normally associated with it, are completely absent from the flagella and cell bodies of three allelic strains of *ida4*, named *ida4–1*, *ida4–2*, and *ida4–3*. We determined the nucleotide sequence of the three alleles of the p28 gene and found in each case a single nucleotide change, affecting the splice sites of the first, second, and fourth introns, respectively. Reverse transcriptase-polymerase chain reaction amplification of RNAs prepared from *ida4* cells confirmed that these mutations prevent the correct splicing of the affected introns, thereby blocking the synthesis of full-length p28. These are the first intron splicing mutations described in *Chlamydomonas* and the first inner dynein arm mutations characterized at the molecular level. The absence in *ida4* axonemes of the dynein heavy chains normally found in association with p28 suggests that p28 is necessary for stable assembly of a subset of inner dynein arms or for the binding of these arms to the microtubule doublets.

INTRODUCTION

Axonemes are microtubule-based organelles that bend as a result of the highly regulated activity of mechanochemical structures, the dynein arms. The role of the different types of arms in axonemal motility may be studied through the analysis of mutants with various deficiencies. *Chlamydomonas* mutants lacking subsets of inner dynein arms have flagella that beat at a normal frequency but with an inefficient waveform. As a result, inner dynein arm mutant cells have swimming velocities much lower than wild-type cells. Because inner dynein arms appear to control the flagellar waveform, the analysis of the composition and arrangement of inner dynein arms within the axoneme will yield important clues concerning the basic axonemal bending mechanism.

An inner dynein arm *Chlamydomonas* mutant, *ida4*, was isolated by Kamiya and his collaborators (Kamiya *et al.*, 1991). Its axonemes lack a polypeptide of molecular weight close to 500,000 (see accompanying paper), and a subset of inner dynein arms whose subunits are p28 (a 28,000 molecular weight protein), actin, and dynein heavy chain (DHC)[1] 2′ and 2A (Kamiya *et al.*, 1991;† Piperno *et al.*, 1992; accompanying paper). It was possible that the *IDA4* locus encoded one of these five proteins. The p28 deficiency is most severe in *ida4* mutant cells, although the amount of p28 found in axonemes is reduced in several inner

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[1] Abbreviations used: bp, base pair; DHC, dynein heavy chain; nt, nucleotide; PCR, polymerase chain reaction; RFLP; restriction fragment length polymorphism; RT-PCR, reverse transcriptase-polymerase chain reaction.

† The DHCs' nomenclature is based on their electrophoretic mobility as described by Piperno *et al.* (1990). We recently obtained evidence suggesting that what was known as DHC 2 may in fact consist of two polypeptides with identical mobilities in the gel system used (see accompanying paper). One, which we proposed to name DHC 2A, is part of complexes containing p28; the other, DHC 2B, is associated with caltractin/centrin.

dynein arm mutants (Piperno *et al.*, 1992). This suggested that p28 may be the gene product of *IDA4*.

In a companion paper, we describe the nucleotide sequence of the single gene encoding p28 and show that p28 has no relevant homology to other proteins of known function. This finding made it important to determine whether p28 was the protein encoded by the *IDA4* locus. If this was the case, the characterization of *ida4* mutations might allow us to identify functional domains within p28. Furthermore, the analysis of the motility of p28 mutants could reveal the role of this protein within the axoneme. Such a study was made more promising by the existence of three distinct *ida4* alleles, of which only one had been characterized so far (see MATERIALS AND METHODS).

We show here that p28 is the gene product of the *IDA4* locus and that p28 is absent from all three *ida4* alleles. The mutations causing the absence of p28 in the *ida4* alleles were shown to affect splice sites of introns in the p28 gene. Because the absence of p28 in the *ida4* mutants results in the absence of p28-containing dynein arms in the axoneme, p28 is likely to be necessary for the stable assembly of these arms or for their binding to the outer doublet microtubules.

MATERIALS AND METHODS

Mutant Strains Used

Three *ida4* alleles, *ida4–1*, *ida4–2*, and *ida4–3* were used in this study. All three were isolated in the laboratory of Dr. Ritsu Kamiya, Tokyo University, Tokyo, Japan. *ida4–1* and *ida4–2* were obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis of an *oda1* strain and isolation of paralyzed double mutants. The *ida4* mutation was then isolated by backcrossing the double mutant to wild type. *ida4–1* (isolation number 166 [Kamiya *et al.*, 1991]) was the only *ida4* mutant analyzed until now, and is referred to as *ida4* in previous publications (Kamiya *et al.*, 1991; Kagami and Kamiya, 1992; Mastronarde *et al.*, 1992; Piperno *et al.*, 1992; Gardner *et al.*, 1994; accompanying paper). *ida4–2* was isolated during the same screen that yielded *ida4–1*. It has the isolation number 168 (Kamiya *et al.*, 1991). *ida4–3* was recently isolated after ultraviolet mutagenesis of the wild-type strain 137c^{+}. The isolation and characterization of slow swimmers was essentially as described (Kamiya and Okamoto, 1985).

General Techniques

Unless otherwise noted, all procedures were performed following the manufacturer's instructions. Conditions for cell culture and labelling, tetrad analysis, dikaryon formation, isolation of flagella and axonemes, and one- and two-dimensional gel electrophoresis have all been described (Luck *et al.*, 1977; Piperno *et al.*, 1981–1992; Piperno, 1988; Harris, 1989; Piperno and Ramanis, 1991). Swimming velocities at 25°C were determined for 50 cells cultured in TAP medium (Harris, 1989) for 3 days. Flagellar beat frequencies were measured with a Fast Fourier Transform analyzer (Kamiya and Hasegawa, 1987).

To prepare cell bodies, *Chlamydomonas* cells were deflagellated by pH shock. The cell bodies were separated from flagella by differential centrifugation (Piperno and Ramanis, 1991), washed once in R medium (Harris, 1989), and pelleted by centrifugation. Cell body proteins were dissolved in 2% sodium dodecyl sulfate, 1% β-mercaptoethanol after digestion of the cell wall by autolysin (LeDizet and Piperno, 1986) for 1 h at room temperature. ^{35}S and ^{32}P radioactivity was detected and quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Proteins separated by electrophoresis were transferred electrophoretically to Immobilon membranes (Millipore, Bedford, MA) in a buffer consisting of 10 mM 3-cyclohexylamino-1-propane sulfonic acid (CAPS), 10% methanol, pH 11. Immunodetection was performed using a chemiluminescence kit from Amersham (Arlington Heights, IL). The anti-p28 antiserum was diluted 5000-fold while the secondary antibody (anti-rabbit Ig, horseradish peroxydase–linked whole antibody from donkey; Amersham) was diluted 10,000-fold.

Nucleotide Numbering

Nucleotide numbering within the p28 gene follows that shown in the accompanying paper. Briefly, the N-terminal methionine of the mature p28 protein is encoded by the ATG at position 482–484. The termination codon is at positions 1887–1889. The polyadenylation signal TGTAA is at nt 2442–2446. The five introns interrupting the coding sequence span the nt 584–650, 728–796, 912–995, 1149–1253, and 1377–1697.

Molecular Biology Techniques

Genomic DNA was prepared as described (Walther *et al.*, 1994). RNA was isolated by lithium chloride precipitation (Wilkerson *et al.*, 1994). Unless otherwise noted, the cells were deflagellated by pH shock and allowed to regenerate flagella for 45 min before RNA extraction. This procedure increases by several fold the abundance of mRNAs encoding flagellar proteins. For Northern blot analysis, RNA was separated by electrophoresis in a 1.2% agarose, 2.2 M formaldehyde gel in 3-(*N*-morpholino) propane-sulfonic acid buffer at pH 7. RNA and DNA were transferred to GeneScreen Plus membranes by capillarity. The probe D16/Eco corresponds to nt 779-1335 of the p28 gene. Hybridization with the ^{32}P-random–labeled probe was performed at 42°C in a solution containing 50% formamide. The blots were washed to a final stringency of 0.5× SSC, 0.1% sodium dodecyl sulfate at 65°C.

Primers used in this study were the following:
5PR001 (+): GGTACCAACGTCTC (1–14)
N3 (+): ATGATYCCICCICTITCITCICTIGT (482–507)
157 (+): CTAAACTCTATTCTGCC (705–721)
ANT157 (−): GGCAGAATAGAGTTTAG (721–705)
350 (+): ATGAGCTCATCCGCCAGGTGACCAT (1051–1075)
447 (+): GACCCTGTACGAGA (1253–1266)
596 (−): TCGCACTTGAACTTCC (1723–1708)
1218 (−): GTTGCGGTCACGCCCCTTGCGTTGC (2341–2317)

Y stands for C or T, I stands for inosine. A (+) indicates that the sequence is that of the coding strand. A (−) means that the sequence is that of the noncoding strand. The position of the primers in the p28 gene sequence is indicated between parentheses.

To determine the nucleotide sequence of the mutant alleles, we amplified the p28 gene by PCR from wild-type and mutant cells as follows. Nucleotides 1–721 were amplified using the primers 5PR001 and ANT157. Nucleotides 482-1723 were amplified using the primers N3 and 596. Nucleotides 1253–2341 were amplified using the primers 447 and 1218. In all cases, approximately 0.1 μg of genomic DNA was used as template. The cycling parameters were as follows: 4 min at 95°C followed by 35–40 cycles of 45 s at 95°C, 90 s at 48°C, and 4 min at 72°C. All reactions were performed using Perkin-Elmer *Taq* DNA polymerase (Norwalk, CT).

For RT-PCR, 2 μg of polyadenylated RNA or 60 μg of total RNA were reverse-transcribed using 200 U of ALV reverse transcriptase (Life Technologies, Gaithersburg, MD). The primer used consisted of either 60 pmol of oligo(dT)$_{12–15}$ or 10 pmol of primer 596. Ten to fifteen percent of the reaction mixture was used as template in a subsequent PCR amplification using the appropriate PCR primers and the same conditions as above.

Uncloned PCR products were purified from agarose gels using GeneClean II (Bio 101, Vista, CA) and were sequenced following a protocol provided by United States Biochemical (Cleveland, OH): 0.5 pmol of PCR product in water was mixed with 2.5 pmol of the appropriate sequencing primer, incubated at 100°C for 3 min in a thermal cycler, and quickly cooled in an ice bath. This annealed template was then sequenced with Sequenase v2.0 (Amersham) using a standard protocol.

Computer Analysis of DNA Sequences

Nucleotide sequences were retrieved from databases and analyzed using the GCG software package version 8 (Genetics Computer Group, Madison, WI). Intron sequences were extracted manually from GenBank (release 86.0) entries and analyzed using a custom-made Microsoft Excel spreadsheet. Two erroneous splice site positions in the GenBank entries (locus CRODA6DIC, intron 1, 5′ end; locus CRCAH2, intron 8, 3′ end) were detected by the highly unusual splice site sequences they predicted. These were corrected to match the original published data (Fukuzawa *et al.*, 1990; Mitchell and Kang, 1993).

RESULTS

All Three ida4 Alleles Lack p28 in Their Flagella and Cell Bodies

To determine whether the three *ida4* alleles have distinct molecular phenotypes, we performed electrophoretic analyses of axonemal proteins from *ida4–2* and *ida4–3* cells, as previously shown for *ida4–1* (Piperno *et al.*, 1992).

Two-dimensional gel electrophoretic analysis of axonemal proteins revealed that p28 was absent. No new protein species, which could be modified or truncated forms of p28, were detected. Separation of the DHCs by one-dimensional gel electrophoresis on a 3.6–5% polyacrylamide gel showed that *ida4–2* and *ida4–3* lack a polypeptide of molecular weight above 500,000 and DHC2′ and are deficient for the DHC 2A-DHC 2B band (likely because of the absence of DHC 2A). These results are identical to those previously obtained for *ida4–1* (Kamiya *et al.*, 1991; Piperno *et al.*, 1992; accompanying paper).

To determine whether any unassembled p28, or fragments of it, could be detected in *ida4–1*, *ida4–2*, or *ida4–3* cells, we performed a Western blot analysis of flagellar and cell body protein with a polyclonal antibody specific for p28, which was described in the accompanying paper. No p28 was detectable in fractions containing proteins from the three *ida4* alleles under conditions that generate a strong signal from p28 in similar fractions prepared from a wild-type strain (Figure 1).

This evidence indicates that *ida4–1*, *ida4–2*, and *ida4–3* mutant cells have the same molecular phenotype. In agreement with this observation, the three alleles have similar motility. The average velocity of *ida4–1*, *ida4–2*, and *ida4–3* (measured for 50 cells) was 68.3 ± 10.8 μm/s, 81.3 ± 11.4 μm/s, and 62.6 ± 11.3 μm/s, respectively. Their beat frequencies were close to 55–60 Hz.

Dikaryon Rescue Experiments Suggest that p28 Is the Gene Product of the IDA4 Locus

The absence of p28 from *ida4* axonemes may be a direct or an indirect consequence of the *ida4* mutation; p28 may be synthesized in the mutant cells, but be unable to assemble into the axonemes because of the absence of another protein. To determine whether p28 is a putative gene product of *IDA4*, we performed a dikaryon rescue experiment (Luck *et al.*, 1977); in this procedure, radioactively labeled mutant gametes are mated with nonradioactive

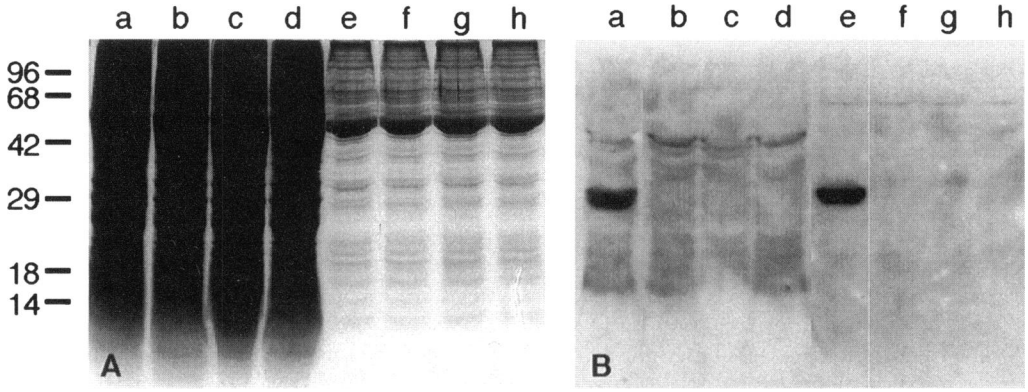

Figure 1. Immunoblot analysis of flagella and cell bodies. Panel A is a Coomassie blue–stained electrophoretogram of approximately 60 μg of cell body proteins (lanes a–d) and 4 μg of flagellar proteins (lanes e–h). The proteins were prepared from wild-type cells (lanes a and e), *ida4–1* cells (lanes b and f), *ida4–2* cells (lanes c and g), and *ida4–3* cells (lanes d and h). Panel B shows an anti-p28 chemiluminescent immunostaining of proteins from a companion electrophoretogram. The samples are presented in the same order as in panel A. The film was overexposed to reveal any weakly reacting product. The proteins were separated on a 7.5–15% polyacrylamide gel. The position of the molecular weight markers (in kilodaltons) is indicated on the left.

wild-type gametes in the presence of protein synthesis inhibitors. Cytoplasmic fusion results in the formation of a temporary quadriflagellated dikaryon. Early after the fusion, the two flagella contributed by the wild-type gamete are motile while the two mutant flagella have an abnormal motility. At later times, axonemal structures missing from the mutant flagella may assemble from the pools of unassembled components present in the cytoplasms of the gametes. All four flagella then exhibit normal motility. If the mutant cytoplasm does not carry an axonemal component, the flagella of the dikaryon will only contain the component contributed by the wild-type, nonradioactive, gamete. This component will be nonradioactive while the other components will be radioactive.

We performed a dikaryon rescue experiment involving *ida4–1* gametes. We found that p28 was not radioactive in the flagella of a wild-type × *ida4–1* dikaryon after rescue of functional activity. In contrast, DHC 2′ was radioactive in the flagella of the dikaryon.

This result ruled out dynein heavy chain 2′ as a putative gene product of the *IDA4* locus, because it was present in an assembly-competent form in the cytoplasm of *ida4–1* gametes. The finding that p28 cannot be rescued from the cytoplasm of *ida4–1* gametes indicates that p28 may be the protein encoded by the *IDA4* locus. Alternatively, unassembled p28 is degraded very rapidly in the *ida4–1* gametes, preventing its incorporation into the flagella of the dikaryon. In retrospect, this result could be expected, because no p28 was detected immunologically in the *ida4* mutants (Figure 1). Our finding that p28 from the cytoplasm of wild-type cells can associate with mutant DHCs can be interpreted in two ways: some wild-type p28 may be found free in the cytoplasm, or it may be part of complexes that remain able to exchange DHCs.

Evidence of Genetic Linkage between the Gene Encoding p28 and the IDA4 Locus

In *Chlamydomonas*, as in other organisms, it is possible to demonstrate a genetic linkage between a DNA sequence and a genetic locus by showing that an RFLP detected by a gene-specific DNA probe cosegregates through meiosis with a given phenotype (Gross *et al.*, 1988; Ranum *et al.*, 1988).

To detect an RFLP linked to the p28 gene, we used the D16/Eco DNA fragment (see MATERIALS AND METHODS) to probe Southern blots of DNA from *Chlamydomonas reinhardtii ida4–1* and from *Chlamydomonas grossi*. Figure 2 shows that the D16/Eco probe hybridized to a 3.6-kb *Sty*I fragment in *C. reinhardtii ida4–1* and to a 2.6 kb *Sty*I fragment in *C. grossi*.

ida4–1 gametes were then crossed with *C. grossi* gametes and tetrads were analyzed. The motility phenotype (wild-type like or slow swimmer) of each of the daughter cells was recorded, and found to segregate 2:2 in 21 tetrads, confirming that the phenotype was due to a single mutation. DNA from each daughter cell of four tetrads was purified, digested with *Sty*I, and analyzed by Southern blot using the same radioactive probe as before. The RFLP found above cosegregated with the *ida4* phenotype in all four tetrads analyzed.

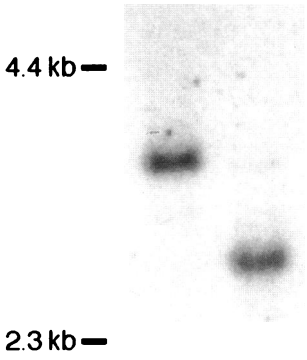

Figure 2. Restriction fragment length polymorphism between *C. reinhardtii* and *C. grossi*. Five micrograms of genomic DNA prepared from *C. reinhardtii* (left lane) or *C. grossi* (right lane) were digested to completion with *Sty*I, separated by electrophoresis, and transferred by capillarity to a GeneScreen Plus membrane. The membrane was then incubated with random-labeled probe D16/Eco specific for p28 and washed at high stringency. The position of DNA molecular weight markers is indicated on the left.

This result proved that there is a genetic linkage between the p28 coding sequence and the *IDA4* locus, because no recombination occurred between the two. An upper limit for the distance separating the *IDA4* locus from the p28 gene can be estimated at 10 centiMorgan. However, a prohibitively large number of tetrads should be analyzed to prove that the two coincide. Instead, we sought to strengthen this point by biochemical methods.

The p28 mRNA Is Larger in ida4–1 Cells

The p28 mRNA was detected in both wild-type and *ida4–1* cells. Figure 3 shows the results of a Northern blot analysis, using the D16/Eco probe specific for the p28 gene.

In wild-type cells, the p28 mRNA is approximately 1.45 kb long (Figure 3, panel A, lane a). In *ida4–1* cells, the p28 mRNA is slightly longer and approximately three times less abundant than in wild-type cells (Figure 3, lane b). When the cells were deflagellated and allowed to regenerate flagella for 45 min, the abundance of the p28 mRNA increased threefold, both in wild-type cells (Figure 3, lane c) and in *ida4–1* cells (Figure 3, lane d). The size of the *ida4–1* p28 mRNA remained the same. During flagellar regeneration, the amount of mRNAs encoding flagellar proteins increases in response to a higher transcription rate and to a lower mRNA turnover (Johnson and Rosenbaum, 1993). Both mechanisms appear to function normally in *ida4–1* cells.

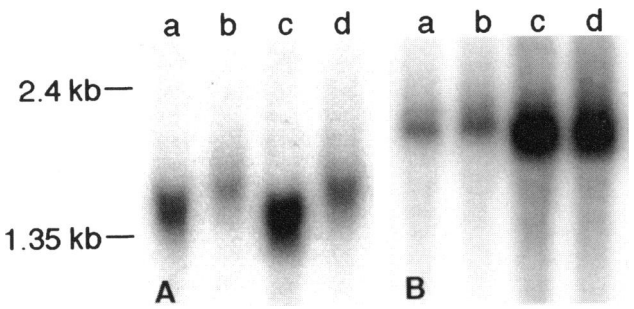

Figure 3. High stringency Northern blot analysis of the p28 mRNA. Approximately 2 μg of oligo(dT)-selected RNA per lane were separated by electrophoresis on a formaldehyde-containing agarose gel. The RNA was prepared from wild-type (lane a) and *ida4–1* cells (lane b) before deflagellation, and from wild-type (lane c) and *ida4–1* cells (lane d) 45 min after deflagellation. The probe used was D16/Eco, specific for the p28 gene (A), or pβ9–12, containing the coding sequence of β-tubulin (B). The position of RNA molecular weight markers (in kb) is indicated on the left.

The difference in size of the p28 mRNA in *ida4* cells was not due to an electrophoretic artifact or to a general defect of RNA processing; when the same blot was re-probed with a fragment of a β-tubulin gene (kindly provided by Dr. Silflow, University of Minnesota, Minneapolis, MN), we found that the β-tubulin mRNA in *ida4–1* cells had a normal size and abundance before and after flagellar regeneration (Figure 3, panel B).

Southern blot analysis of wild-type and *ida4–1* DNA had ruled out the possibility of an insertion within the p28 gene. A difference in size of the p28 mRNA in *ida4–1* cells could therefore result from one of three causes: 1) a different promoter sequence was used during transcription; 2) a different polyadenylation site was used; or, more likely, 3) the p28 mRNA was incorrectly spliced.

The ida4 Mutations Affect Splice Sites of the p28 Gene

The results presented above constituted compelling genetic and biochemical evidence that p28 was the gene product of the *IDA4* locus. To confirm this, we set out to identify nucleotide differences within the p28 gene between wild-type and *ida4* cells. The p28 gene was amplified by PCR, using DNA prepared from wild-type, *ida4–1*, *ida4–2*, or *ida4–3* cells as template. For each strain, amplification products from several individual reactions were pooled before their nucleotide sequence was determined. This procedure eliminates errors due to PCR mistakes and cloning artifacts. The DNA sequences of the mutant alleles were then compared with that of the wild-type allele.

The sequence of the *ida4–1* p28 gene was determined from nt 31 (451 nt upstream of the initiation codon) to nt 2335 (436 nt downstream from the termination codon). The only difference found was a T to A transversion affecting nt 585, the second nucleotide of the first intron (see Figure 4).

The sequence of the *ida4–2* p28 gene was compared with that of wild type from nt 31 to 2300. Only one difference was found, a G to A transition affecting nt 728, the first nucleotide of the second intron (see Figure 4).

Finally, the sequence of the *ida4–3* p28 gene was determined from nt 31 to 2300. The only nucleotide change found was a G to A transition affecting nt 1253, the last nucleotide of the fourth intron (Figure 5).

In all organisms studied, the two nucleotides found at the 5′ end of introns are almost always GT and the 3′ terminal nucleotides are usually AG, thus giving rise to the "GT-AG rule." These four nucleotides are present in over 99% of all introns (Senapathy *et al.*, 1990). Mutations affecting them have been described in a variety of organisms and always prevent correct splicing. However, because the *ida4* mutations were

		Exon			Intron					
Position		-3	-2	-1	+1	+2	+3	+4	+5	+6
	A	43	91	8	0	0	28	92	3	16
	C	78	33	8	0	3	9	44	2	47
	G	26	17	135	157	0	120	20	148	22
	T	10	16	6	0	154	0	1	4	72
***Chlamydomonas* Consensus**		**A/C**	**A/C**	**G**	**G**	**T**	**G**	**A/C**	**G**	**C/T**
Eukaryotic Consensus			**A**	**G**	**G**	**T**	**A/G**	**A**	**G**	
First intron's splice site (584)		A	A	G	G	T	T	A	G	C
First intron (*ida4-1*)		A	A	G	G	a	T	A	G	C
First intron's cryptic splice site (567)		A	A	G	G	C	A	C	G	C
Second intron's splice site (728)		A	A	G	G	T	A	A	G	C
Second intron (*ida4-2*)		A	A	G	a	T	A	A	G	C

Figure 4. 5′ splice sites in *Chlamydomonas reinhardtii* nuclear genes coding for proteins. One hundred fifty-seven introns were found in release 86.0 of GenBank. The numbers represent the number of sequences where a given nucleotide is found at a given position relative to the splice site. A *Chlamydomonas* consensus sequence was derived by selecting, when possible, one nucleotide present in over 60% of the sequences or two nucleotides present in over 75% of the sequences. We also show a consensus derived from eukaryotic intron sequences (Senapathy *et al.*, 1990) using the same selection rules. The p28 splice site sequences of the first and second introns (in wild-type, *ida4–1*, and *ida4–2* cells) are aligned below the consensus sequence. The number between parentheses indicates the position in the p28 gene sequence of the first nucleotide of the intron. Nucleotides printed in lowercase letters are those affected by the *ida4–1* and *ida4–2* mutations.

Position		Intron -7	-6	-5	-4	-3	-2	-1	Exon +1
	A	14	20	6	48	4	157	0	20
	C	85	69	89	15	148	0	0	24
	G	13	29	11	79	2	0	157	99
	T	45	39	51	15	3	0	0	14
***Chlamydomonas* Consensus**		C/T		C/T	A/G	C	A	G	G
Eukaryotic Consensus		C/T	C/T	C/T		C	A	G	A/G
Fourth intron's splice site (1253)		C	G	T	G	C	A	G	A
Fourth intron (*ida4-3*)		C	G	T	G	C	A	a	A
Fourth intron's cryptic site (1272)		A	G	C	G	C	A	G	T

Figure 5. 3′ splice sites in *Chlamydomonas reinhardtii* protein-coding nuclear genes. One hundred fifty-seven introns were found in release 86.0 of GenBank. The numbers represent the number of sequences where a given nucleotide is found at a given position relative to the splice site. A *Chlamydomonas* consensus sequence was derived by selecting, when possible, one nucleotide present in over 60% of the sequences or two nucleotides present in over 75% of the sequences. We also show a consensus derived from eukaryotic intron sequences (Senapathy *et al.*, 1990) using the same selection rules. The p28 sequences are those surrounding the wild-type 3′ splice site for the fourth intron and the analogous sequence in the *ida4–3* allele. The number between parentheses indicates the position in the p28 gene sequence of the last nucleotide of the intron. The nucleotide printed in lowercase is the one affected by the *ida4–3* mutation.

the first affecting splicing sites found in *Chlamydomonas*, we verified that the splice site consensus sequences described in other eukaryotes were valid in *Chlamydomonas*. We found that this was the case, both for 5′ and for 3′ splice sites. Figures 4 and 5 show that the consensus sequences derived from *Chlamydomonas* splice site sequences is only slightly different from that derived from other eukaryotes (Senapathy *et al.*, 1990).

Figure 4 shows the results of a compilation of the 5′ splice sites of 157 introns found in *C. reinhardtii* protein-coding nuclear sequences present in the GenBank database (as of 1/9/95). The exact position of the splice site cannot always be determined unambiguously by comparing a cDNA with a genomic DNA sequence. When this was the case, the splice site position was set to best fit the eukaryotic consensus. The 9-residue *Chlamydomonas* consensus was derived from the data by selecting one nucleotide present in over 60% of the sequences or two nucleotides present in over 75% of the sequences. One hundred thirty-seven introns of 157 match the proposed consensus at seven or more positions. The first nucleotide of *Chlamydomonas* introns analyzed here is always a G and the second is almost always a T. In three cases, a C in present instead of a T at position +2. Such a sequence has been described in other instances (Jackson, 1991), and shown to allow splicing in vitro, although more slowly than the canonical GT sequence (Aebi *et al.*, 1987). As Figure 4 shows, the 5′ splice sites of the first and second introns of the p28 gene fit the *Chlamydomonas* consensus at eight of nine positions. The nucleotides affected by the *ida4–1* and *ida4–2* mutations are printed in lowercase letters.

Figure 5 shows the result of an analogous compilation of 157 *Chlamydomonas* 3′ splice sites. All *Chlamydomonas* introns analyzed here end with the dinucleotide AG. One hundred thirty-three of 157 sequences match the proposed 7-base consensus at six or more positions. The 3′ splice site of the fourth intron matches six of seven residues of the consensus. The nucleotide affected by the *ida4–3* mutation is printed in lowercase.

The ida4 Mutations Prevent the Correct Splicing of the Affected Introns

To test whether the *ida4* mutations resulted in aberrant splicing, we amplified p28 cDNAs prepared from wild-type and mutant cells. RNA was prepared from *Chlamydomonas* cells 45 min after deflagellation to increase the abundance of the p28 mRNA. cDNAs were synthesized, using as primer either oligo(dT)$_{15}$ or the p28-specific primer 596 as described in MATERIALS AND METHODS. Figure 6 shows the location of the

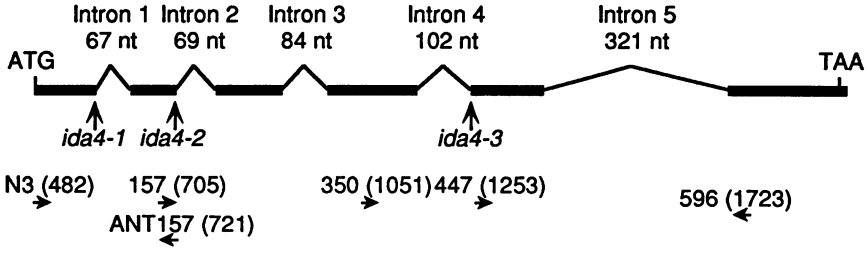

Figure 6. Experimental design of the RT-PCR amplification of p28 cDNAs. The structure of part of the p28 gene is indicated in the top part of the figure. The thick lines represent exons and the thin broken lines represent introns. ATG and TAA indicate the positions of the initiation and termination codons, respectively. The horizontal arrows (not drawn to scale) represent the primers used in the PCR reactions. Above each arrow is the name of the primer and, in parentheses, the position in the p28 gene of the primer's 5′ end. The sequences of the primers are located entirely within exons. The vertical arrows indicate the position of the *ida4–1*, *ida4–2*, and *ida4–3* mutations.

primer pairs used in the subsequent PCR reaction. In all reactions, there was clearly one (or two in the case of *ida4–2*) major amplification product. Small amounts of primer 596 leftover from the reverse transcription sometimes caused the appearance of traces of contaminating products after PCR amplification.

Panel A of Figure 7 shows the amplification products obtained with the primers N3 and ANT157, located on either side of the first intron (see Figure 6). When DNA from *ida4–1* or wild-type cells was used as template, one amplification product was obtained (Figure 7, lane c), approximately 240-bp long (its expected size is 243 bp). Amplification of wild-type cDNAs yielded one product (Figure 7, lane a) with the size and nucleotide sequence predicted from the cDNA sequence (expected size 186 bp). Amplification of *ida4–1*–derived cDNAs yielded two products, with approximate sizes of 240 and 175 bp. The nucleotide sequence of both products was determined: the larger, more abundant, product derived from mRNA molecules where intron 1 was not spliced (the sequence of the cDNA is identical to that of the DNA); and the smaller, rarer, product derived from mRNA molecules where the intron 1 was spliced incorrectly, using a 5′ splice site 13 bp upstream from the wild-type site. The 240-bp amplification product was not derived from genomic DNA contaminating the *ida4–1* RNAs; amplification of *ida4–1* RNA with the primers N3 and 596 yielded a product whose size was clearly different from that observed when genomic DNA was amplified. The cryptic site used in *ida4–1* cells matches the *Chlamydomonas* consensus sequence for 5′ splice sites at seven of nine positions (Figure 4). DNA sequence analysis shows that if the first intron is not spliced, translation of the p28 mRNA will terminate after 86 residues. If the cryptic site is used, translation is predicted to terminate after 57 residues. Amplification reactions using other pairs of primers showed that all other introns were spliced correctly in *ida4–1* cells (not shown).

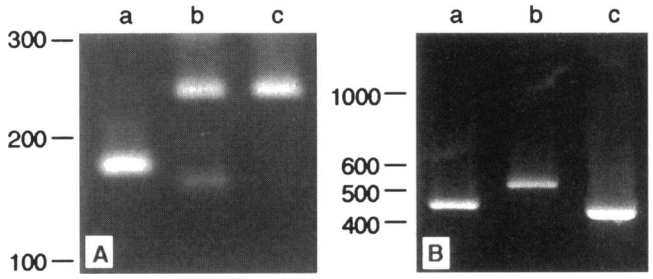

Figure 7. RT-PCR amplification of p28 mRNAs using the primers N3 and ANT157 (A) or 157 and 596 (B). The templates used were the following. (A) Lane a, wild-type oligo(dT)-selected RNA; lane b, *ida4–1* oligo(dT)-selected RNAs; lane c, *ida4–1* genomic DNA. (B) Lane a, wild-type oligo(dT)-selected RNA; lane b, *ida4–2* total RNA; lane c, *ida4–3* total RNA. The cDNAs were synthesized using 596 as primer. The amplification products were separated on a 2% (A) or 1.5% (B) agarose gel and visualized with ethidium bromide. The position of molecular weight markers (in bp) is indicated on the left of each panel.

RT-PCR amplification of *ida4–2* and *ida4–3* p28 mRNAs with the primers N3 and ANT157 yielded a product of the same size as wild type (≈175 bp), indicating that the first intron was spliced correctly (Figure 6). Similarly, amplification with primers 447 and 596 showed that the fifth intron was correctly spliced (Figure 6).

Amplification of the *ida4–2* mRNA using the primers 157 and 596 yielded a product larger than that obtained from wild-type mRNA (≈530 bp vs. 460 bp; Figure 7, panel B, lanes a and b). In contrast, a product of the same size as wild type was obtained when using the primers 350 and 596 (≈250 bp). This clearly suggested that the second intron was not spliced (Figure 6). The nucleotide sequence of the amplification products derived from *ida4–2* mRNAs confirmed that the second intron was not spliced and that all other introns were spliced correctly. Analysis of the p28 gene sequence predicts that if the second intron is not removed, p28 translation terminates after the polymerization of 60 amino acids.

RT-PCR amplification of *ida4–3* mRNAs yielded a product smaller than wild type when using the primers 157 and 596 (≈430 bp vs. 460 bp; Figure 7, panel B, lanes a and c) or 350 and 596 (≈230 bp vs. 250 bp). However, a product of wild-type size was obtained when using the primers 447 and 596 (≈175 bp). This suggested that the splicing of the fourth intron was abnormal (Figure 6) and that the splicing resulted in the removal of an intron larger than in wild-type cells. Nucleotide sequencing of the *ida4–3* amplification products showed that the fourth intron was spliced using a cryptic 3′ splice site 19 nt downstream from the normal site, resulting in the ligation of nt 1148 to nt 1273. The cryptic site used in *ida4–3* cells matches the *Chlamydomonas* consensus at five of seven positions (Figure 5). DNA sequence analysis indicates that if the fourth intron is spliced using this cryptic site, translation of the resulting mRNA yields a protein of 194 residues. However, use of the cryptic splice site results in a shift of the reading frame and only the first 149 residues are identical to the wild-type p28 protein sequence.

In all the reactions described above, the sizes of the products obtained after PCR amplification ruled out the possibility that contaminating DNA was being amplified.

In summary, all three *ida4* mutations affect splice sites within the gene encoding p28, preventing the correct splicing of the p28 mRNA. In all cases, this prevents the synthesis of the p28 protein. The absence of p28 is therefore the primary cause behind

the lack of a subset of inner dynein arms observed in *ida4* cells.

DISCUSSION

p28 Is the Gene Product of the IDA4 Locus

The following arguments show that the *IDA4* locus encodes p28, a light chain of the inner dynein arms: 1) p28 is absent in the cells of three *ida4* mutants; 2) p28 cannot be rescued from the cytoplasm of *ida4–1* gametes during the formation of dikaryons; 3) the p28 gene is genetically linked to the *IDA4* locus; 4) the p28 mRNA is larger in *ida4–1* cells; and finally 5) the p28 gene contains mutations in all three *ida4* alleles.

We compared the wild-type genomic sequence with that of the three *ida4* alleles over a region encompassing the entire p28 coding region and part of the flanking sequences. The mutations described here were the only nucleotide differences found. We can rule out the possibility that the nucleotide changes observed here are the consequence of mistakes made by the *Taq* DNA polymerase; the nucleotide sequences were determined from uncloned PCR products, and therefore represent an average of all PCR products generated. A mistake made in the very early PCR cycles could be amplified and be predominant in the final products. However, such a possibility does not affect our results because the products of several independent PCR amplifications were pooled before their nucleotide sequences were determined. Furthermore, there is no reason to believe that the mutant phenotype may be due to other mutations; p28 is encoded by a single-copy gene in *Chlamydomonas* (see accompanying paper) and the nucleotide changes we found are by themselves sufficient to explain the splicing defects of the p28 mRNA and the absence of p28 in the mutant cells.

Consequences of the ida4 Mutations

Nucleotide sequencing of three p28 alleles revealed that all three *ida4* mutations affect intron splicing sites. To our knowledge, these are the first intron splicing mutations found in a *Chlamydomonas* nuclear gene. We confirmed by RT-PCR that the *ida4–1*, *ida4–2*, and *ida4–3* mutations prevent the correct splicing of the first, second, and fourth introns, respectively. The destruction of normal splice sites may have several different outcomes. Interestingly, the most common consequence in mammals, namely exon skipping (Nakai and Sakamoto, 1994), is not observed in *ida4* cells. On the other hand, intron retention, which we observe in *ida4–1* and *ida4–2* cells, is the most infrequent occurrence in mammals (Nakai and Sakamoto, 1994). This may reflect the possibility that the rules governing splice site selection are different in *Chlamydomonas* and in mammals.

The most obvious consequence of the *ida4* mutations is that the unspliced or incorrectly spliced p28 mRNAs cannot be translated into full-length p28, as the reading frame is interrupted by premature termination codons. Immunostaining with a polyclonal antiserum specific for p28 failed to detect any truncated protein in the axonemes or the cell bodies of the *ida4* mutants. In the case of *ida4–1* and *ida4–2* cells, translation of the p28 mRNA could result in the synthesis of very small proteins (57–86 residues), which may be too small to be detected by the procedure we used. However, the *ida4–3* p28 mRNA could direct the synthesis of a 192-amino acid protein, whose first 149 residues are identical to the p28 sequence. Our failure to detect such a product may indicate that our anti-p28 antiserum only recognizes C-terminal epitopes. More likely, this protein would be rapidly degraded after failing to fold properly.

The lower abundance of the p28 mRNA is likely to be explained by degradation of nontranslatable mRNAs. It has been shown before that mRNAs carrying a premature termination codon have a much lower steady-state abundance than their wild-type counterparts (Losson and Lacroute, 1979; Daar and Maquat, 1988; Urlaub *et al.*, 1989; Hirschhorn *et al.*, 1994). This remains true whether the termination codon results from a single nucleotide change or from aberrant splicing. Although the mechanism by which faulty mRNAs are recognized is unknown, it appears that defective mRNAs are rapidly degraded. *Trans*-acting genes necessary for this degradation have been isolated in yeast and in *Caenorhabditis elegans* (Leeds *et al.*, 1991; Pulak and Anderson, 1993). Such a mechanism is probably at work in *Chlamydomonas ida4–1* cells, thus explaining the lower abundance of the p28 mRNA (Figure 3). A very similar phenomenon appears to occur in the *Chlamydomonas* mutant *ac-208* (Quinn *et al.*, 1993). In this mutant, a frameshift mutation in the gene encoding preapoplastocyanin results in a steady-state level of mRNA approximately 2–3% of that observed in wild-type cells.

An additional phenomenon preventing the synthesis of fragments of p28 in *ida4* mutants is that unspliced mRNAs typically remain in the nucleus, where they are either spliced or rapidly degraded. Our RNA preparation method does not distinguish nuclear from cytoplasmic RNA. It is possible that most of the unspliced p28 mRNA is sequestered in the nucleus and therefore not in contact with ribosomes. It has been shown that when the accumulation of unspliced mRNA is the result of intron splice site mutations, a fraction of the pre-mRNAs may be exported into the

cytoplasm (Legrain and Rosbash, 1989). However, this is not always the case and may require *trans*-acting factors similar to the *rev* protein of HIV (Chang and Sharp, 1989; Malim and Cullen, 1993). In any case, part of the p28 pre-mRNAs appears to be polyadenylated: p28 sequences could be amplified from oligo(dT)-primed cDNAs (see RESULTS section), and p28 sequences were detectable by Northern blot hybridization in a population of oligo(dT)-selected RNAs (Figure 3).

In summary, although the presence of premature termination codons is sufficient to prevent the synthesis of full-length p28, there are two mechanisms that may act together to prevent the synthesis of fragments of p28: 1) the mRNA may be degraded and therefore fail to accumulate to wild-type levels, and 2) it may be sequestered within the nucleus, thereby never being in contact with ribosomes. An intron-splicing mutation may therefore be more effective than a nonsense mutation in preventing the synthesis of fragments of a protein, which may be advantageous if such fragments are toxic to the cells.

The finding that all three *ida4* mutations affect intron splice sites may appear surprising. As mentioned above, intron splicing mutations may be more effective in preventing the synthesis of protein fragments. In the case of p28, moreover, splice site mutations are rendered more likely by the small size of the coding region (253 codons), and the comparatively large number of introns (five). More surprising is the fact that none of the *ida4* mutations are missense mutations, where one amino acid is replaced by another. One possible explanation is that the *ida4* mutants were isolated using screening procedures discriminating between slow-swimming and paralyzed cells (for *ida4–1* and *ida4–2*) and between wild-type and slow-swimming cells (for *ida4–3*). It is conceivable that missense mutations within the p28 gene cause swimming defects too subtle to be recognized.

The Role of p28 in Motility

Flagellar motility results from the interplay of several hundreds of proteins. The understanding of this phenomenon will require the identification of molecular defects in various motility mutants and the analysis of the consequences of these changes. Different mutations within the same protein may differently affect the various functions of this protein and result in different phenotypes. Flagellar proteins affected by mutations characterized at the molecular level until now are as follows: β2-tubulin (Lee and Huang, 1990), centrin (Taillon *et al.*, 1992), radial spoke protein 3 (Williams *et al.*, 1989), as well as the β DHC (Porter *et al.*, 1994), and one intermediate chain (Mitchell and Kang, 1993) of the outer dynein arms. *ida4* is the first mutation characterized at the nucleotide level that specifically affects the inner dynein arms.

All three *ida4* mutations result in the same molecular phenotype, namely the complete absence of p28. We therefore did not identify important functional domains within p28. We can only conclude that the N-terminal fragments, if they are synthesized at all, (see DISCUSSION above) are not sufficient to restore a normal axonemal structure. However, it is important to note that in *ida4–1*, *ida4–2*, and *ida4–3* cells, the DHCs normally associated with p28 (DHC 2′ and 2A) are missing from the axonemes. This shows that p28 is necessary to promote the stable assembly of the DHCs into arms, or to bind the arms to the microtubule backbone. This situation is similar to that observed in the *oda6* mutant cells of *Chlamydomonas* (Mitchell and Kang, 1993). In *oda6* cells, a frameshift mutation prevents the synthesis of a 70,000 molecular weight intermediate chain of the outer dynein arms. As a result, the outer dynein arms do not assemble. The ODA6 polypeptide was also found to regulate the activity of the DHCs independently of its role in assembly (Mitchell and Kang, 1993). Although more studies are clearly needed, this suggests a critical role of the intermediate and light chains associated with the DHCs. Although the DHCs are the actual ATPases (and are sufficient to move microtubules in in vitro assays [Mazumdar *et al.*, 1994]), the light chains may be required to bind them to the outer doublet microtubules and to regulate their activity. Thus, flagellar motility may be equally dependent on the various DHCs found in the dynein arms and in the intermediate and light chains regulating their assembly and activity.

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