A *Chlamydomonas* Homologue of the Putative Murine *t* Complex Distorter Tctex-2 Is an Outer Arm Dynein Light Chain

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Abstract. Molecular analysis of a 19,000-M_r protein from the *Chlamydomonas* flagellum reveals that it is homologous to the *t* complex–encoded protein Tctex-2, which is a candidate for one of the distorter products that cause the extreme transmission ratio distortion (meiotic drive) of the murine *t* complex. The 19,000-M_r protein is extracted from the axoneme with 0.6 M NaCl and comigrates with the outer dynein arm in sucrose density gradients. This protein also is specifically missing in axonemes prepared from a mutant that does not assemble the outer arm. These data raise the possibility that Tctex-2 is a sperm flagellar dynein component. Combined with the recent identification of Tctex-1 (another distorter candidate) as a light chain of cytoplasmic dynein, these results lead to a biochemical model for how differential defects in spermiogenesis that result in the phenomenon of meiotic drive might be generated in wild-type vs *t*-bearing sperm.

The murine *t* complex (haplotype) is an alternate form of the proximal 30–40 Mb of chromosome 17. The *t* form of this chromosome contains a series of inversions that serve to suppress recombination with wild type and, therefore, the *t* complex is normally inherited intact. The most striking property of this famous genetic unit is that heterozygous (+/*t*) males pass the *t*-bearing copy of the chromosome to >95% of their progeny. This phenomenon is referred to as transmission ratio distortion or meiotic drive (see Silver [1985, 1993] for detailed discussion).

Genetic studies have revealed that, in mice, ratio distortion derives from the combined effects of four to five gene products: a series of "distorters" (Tcd-1 to Tcd-4) that interact with a "responder" protein (Tcr) which is expressed late in spermiogenesis after the completion of meiosis (Lyon, 1984). All of the genes encoding these proteins are located within the *t* complex. Males containing two complementing *t* haplotypes are sterile. This phenotype is thought to be a consequence of homozygosity for some or all of the genes responsible for meiotic drive (Lyon, 1986). A variety of recessive lethal mutations are also present in different *t* haplotypes, and these result in embryonic death for many *t*/*t* combinations. In spite of these obviously deleterious effects in *t*/*t* animals, the *t* complex is transmitted at such a high ratio that it is found in ~25% of all feral mice. Furthermore, although in heterozygotes the *t* complex reveals no overt phenotype, in the presence of a mutation at the *T* (*Brachyury*) locus it does result in mice that lack tails. This latter phenotype is apparently caused by a defect in neural tube development (for review see Herrmann and Kispert, 1994).

Although much is known about the *t* complex in genetic terms, the molecular mechanisms involved in these disparate effects on spermiogenesis and neural tube development have remained obscure. A number of studies have identified excellent candidates for both the distorters and the responder. These assignments have been based on genetic mapping of the various loci using partial *t* haplotypes derived from rare recombinants, combined with aberrant levels of mRNA expression in the testis, the presence of *t*-specific mutations, and/or phenotypic effects on sperm morphology. Examples include Tctex-1 (*t*-complex testis expressed) (Lader et al., 1989) and Tctex-2 (Huw et al., 1995), which are candidates for the proximal Tcd-1*t* and Tcd-3*t* distorters, and the hybrid sterility loci Hst-4 to Hst-6, which may encode the distal Tcd-2*t* distorter (Pilder et al., 1993). However, none of these proteins have obvious homologies or structural motifs that provide clues as to their function or to the pathways in which they are involved.

Recently, we identified the *t* complex–encoded protein Tctex-1 as a novel light chain (LC)[1] of cytoplasmic dynein that is differentially expressed in various tissues (King et al., 1996*a*). This has raised the possibility that dysfunction of intracellular transport as a result of the *t*-encoded mutations within the Tctex-1 light chain of cytoplasmic dynein might contribute to meiotic drive. This phenomenon is thought to occur through direct effects on spermiogenesis during which the meiotic partners of *t* haplotype–bearing spermatids become disabled. Many sperm produced by +/*t* males are functionally deficient either in their motility or

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1. *Abbreviations used in this paper*: DHC, dynein heavy chain; IC, intermediate chain; LC, light chain; MBP, maltose binding protein.

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acrosome reaction (Olds-Clarke, 1983; Brown et al., 1989). Even though it is not yet clear that these phenotypic effects actually reflect aspects of the meiotic drive phenomenon, they do raise the possibility that defects in sperm dyneins (or other flagellar components) that result in abnormal flagellar beating also might contribute to ratio distortion.

Detailed biochemical analysis of mammalian flagellar dyneins is difficult because of several features inherent in the design of mammalian spermatozoa (discussed in Gatti et al., 1989). Therefore, to gain insight into the molecular structure and function of flagellar dyneins, we have been examining the outer arm from the *Chlamydomonas* flagellum as a model system. This microtubule-based molecular motor consists of three heavy chains (α, β, and γ dynein heavy chains [DHCs]; ~520 kD each), two intermediate chains (IC78 and IC69 of 76 and 63 kD, respectively), and a series of light chains (10–22 kD) and has a total mass of ~2 MD (Pfister et al., 1982; Piperno and Luck, 1979). The DHCs contain the ATPase and motor domains of the complex (for reviews see Mitchell, 1994; Witman et al., 1994), whereas the ICs are involved in cargo attachment (King et al., 1991, 1995) and perhaps also in regulation (Mitchell and Kang, 1993). Recent molecular studies of the flagellar LCs from several systems have revealed a variety of intriguing functional attributes associated with these dynein components including cAMP-dependent phosphorylation (Barkalow et al., 1994), Ca^{2+} binding (King and Patel-King, 1995*a*), and sulphydryl oxidoreductase (Patel-King et al., 1996) activities. Furthermore, an 8,000-M_r axonemal dynein LC (King and Patel-King, 1995*b*) that dimerizes in situ (Benashski, S.E., A. Harrison, R.S. Patel-King, and S.M. King, manuscript submitted for publication) has also been found to occur within both cytoplasmic dynein (King et al., 1996*b*) and the unconventional actin-based motor myosin-V (Espindola, F.S., R.E. Cheney, S.M. King, D.M. Suter, and M.S. Mooseker. 1996. *Mol. Biol. Cell.* 7:372*a*).

In this report we describe the molecular cloning and analysis of a 19,000-M_r LC from the *Chlamydomonas* outer dynein arm. This molecule, which is tightly associated with the β DHC, is a homologue of the mouse *t* complex protein Tctex-2 and is also more distantly related to Tctex-1 (a cytoplasmic dynein LC; King et al., 1996*a*). As Tctex-2 is a strong candidate for the Tcd-3*t* distorter (Huw et al., 1995), this result indicates that defects in outer arm function because of the *t*-specific mutations within Tctex-2 may contribute to meiotic drive and/or to the male sterility phenotype associated with homozygosity of the genes responsible for transmission ratio distortion of the *t* complex. The model for ratio distortion outlined here builds on that proposed by Cebra-Thomas et al. (1991) and suggests that the responder acts as a "gatekeeper" for axoneme assembly. Wild-type sperm become poisoned because the wild-type responder (Tcr^+) allows the incorporation of both wild-type and *t* mutant Tctex-2 into the axoneme. The presence of the mutant dynein LC is predicted to directly affect dynein function in vivo. The *t*-bearing sperm are protected because the *t* mutant responder (Tcr^t) cannot interact with *t* mutant distorters (Cebra-Thomas et al., 1991); i.e., only wild-type Tctex-2 can be incorporated into the sperm tail. The predicted result of this model is that *t*-bearing sperm show normal motility, whereas wild-type sperm are defective as a result of the presence of mutant dynein.

Materials and Methods

Axoneme Isolation and Dynein Purification

Flagella were prepared from *Chlamydomonas reinhardtii* using standard methods and demembranated with NP-40 (King, 1995; Witman, 1986). Outer arm dynein was subsequently extracted with 0.6 M NaCl, purified by centrifugation on a 5–20% sucrose density gradient (King et al., 1986), and concentrated in a Centricon 30 unit (Amicon Corp., Danvers, MA) that had been incubated overnight with 5% Tween-20 to reduce nonspecific protein binding.

For most experiments and for peptide sequencing, the wild-type strain 1132D− was used. To investigate the presence of the 19,000-M_r protein in other axonemal structures, axonemes also were prepared from mutants lacking the outer (*oda9*) or different subsets of inner dynein arms (*ida1*, *ida2*, *ida4*), the radial spokes (*pf14*), and central pair microtubule complex (*pf18*).

Peptide Sequencing

Concentrated dynein components were separated by electrophoresis in a 5–15% acrylamide gradient gel and blotted to polyvinylidene difluoride membrane (Immobilon P^{sq}; Millipore Corp., Woburn, MA) in 10 mM $NaHCO_3$, 3 mM Na_2CO_3, 0.01% SDS, and 20% methanol. The 19,000-M_r LC band was identified by staining with amido black, excised, and incubated with trypsin. Peptides eluting from the membrane were purified by reverse phase chromatography on a C_8 column. Two peptides were sequenced using an Applied Biosystems 492A sequencer (Foster City, CA) in the Protein Chemistry Facility at the Worcester Foundation for Biomedical Research (Shrewsbury, MA).

Molecular Cloning

To amplify a region of the 19,000-M_r LC for use as a probe, we used a 3′ rapid amplification of cDNA ends procedure (Frohman et al., 1988). The initial reaction used a series of blunt gene-specific primers designed from peptide sequences derived from several LCs. The reverse primer was the standard oligo(dT) adaptor primer (5′-GCGCGTCGACTCGAGT$_{20}$V-3′) that contains SalI and XhoI sites at the 5′ end. The 100 μl PCR was performed in 10 mM Tris-Cl, pH 8.85, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, and 0.2 mM dNTP and contained 1 μg of each primer. A λZapII cDNA library made from mRNA derived from cells actively regenerating flagella (Wilkerson et al., 1995) was used as the template. 2 U of Pwo DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added and, after denaturation at 96°C for 5 min, the sample was subjected to the following thermal regime: 96°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 30 cycles. This was followed by a final 10-min incubation at 72°C. Products were reamplified with a fourfold degenerate gene-specific primer (5′-GCGCGAATTCAAGGGYCTSTACTACGAG-3′; based on the peptide sequence [K]GLYYE and incorporating an EcoRI site and GC clamp at the 5′ end) and the oligo(dT) adaptor primer using Pfu DNA polymerase and were subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA).

Amplification products were sequenced, and one judged to encode part of the 19,000-M_r LC was used to screen the λZapII cDNA library made from mRNA derived from cells actively regenerating flagella (Wilkerson et al., 1995). Phagemids were rescued using helper phage and the longest clone sequenced on both strands using both single- and double-stranded DNA templates. Northern and Southern blots were prepared by standard methods (Sambrook et al., 1987) and probed with the 19,000-M_r cDNA using the conditions described in King and Patel-King (1995*a*).

Computational Methods

Sequence assembly was performed using the GCG suite of software (Devereux et al., 1984). Searches of the Genbank™ and Expressed Sequence Tag databases were made using BLAST (Altshul et al., 1990). Pairwise sequence comparisons were generated using GAP (Devereux et al., 1984); multiple alignments were constructed with CLUSTALW (Thompson et al., 1994); and secondary structure was predicted using PHD (Rost and Sander, 1993). Helical segments were analyzed using HELICALWHEEL (Devereux et al., 1984) and COILS (Lupas et al., 1991). The phylogenetic tree was calculated with DISTANCES and plotted with GROWTREE.

Fusion Protein Expression and Antibody Preparation

The coding region for the 19,000-M_r LC was obtained by the PCR using primers that resulted in a blunt 5′ end and an XbaI site at the 3′ end after the stop codon. This product was subcloned across the XmnI/XbaI sites in pMal-c2 (New England Biolabs, Beverly, MA) and resulted in the COOH-terminal fusion of the LC to maltose binding protein (MBP) via a short hydrophilic linker that contains a Factor Xa proteolytic cleavage site immediately NH_2-terminal to the first Met residue of the LC. Protein expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside. The soluble fusion protein fraction was purified by affinity chromatography on amylose resin and eluted with 10 mM maltose in 20 mM Tris-Cl, pH 7.4, 1 mM EDTA, and 200 mM NaCl. The intact purified fusion protein was used as the immunogen for polyclonal antisera production, and a 19,000-M_r LC-specific antibody fraction was obtained by blot purification using the minor modifications to the method of Olmsted (1986) described in King et al. (1996*b*).

Electrophoresis and Immunoblotting

All samples were separated in 5–15% acrylamide gradient gels and either stained with Coomassie blue or blotted to nitrocellulose using the buffer system described above and probed as described in King et al. (1996*b*). Briefly, blots were blocked with 5% dry milk and 0.1% Tween-20 in Tris-buffered saline and probed with purified antibody diluted ~1:50 and a peroxidase-conjugated secondary antibody. After a wash with 0.5% Triton X-100 in Tris-buffered saline, antibody reactivity was visualized using an enhanced chemiluminescent system (ECL; Amersham Corp., Arlington Heights, IL) and RX film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

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   1 CTATACTGGTTCATACATACCCCGATAGGCCGAAGTTGAGGCGACATTCCGCTCGAAGTT   60
  61 TCAGTCATTTGGTAGTGCCCGCCACCGCTAGCCAGGATTCGCTGTCGCAAGGTAACCAAT  120

   1                         M  D  D  M  P  F  Q  S  G  G  I  K    12
 121 AGAAATTAGCATTCATTAGGCACCATGGACGACATGCCATTCCAGTCTGGCGGCATTAAG  180

  13  E  V  V  Y  E  N  T  F  I  T  D  P  E  G  Y  G  P  N  T  K   32
 181 GAGGTTGTCTATGAGAACACCTTCATCACAGACCCCGAGGGATACGGCCAAACACAAAA  240

  33  F  E  R  H  K  V  Q  A  V  L  K  Q  V  L  K  E  R  I  E  K   52
 241 TTTGAGCGGCACAAGGTTCAGGCGGTGCTGAAGCAAGTTCTGAAGGAGCGCATTGAGAAG  300

  53  Q  K  Y  D  P  V  K  G  A  Q  I  S  K  Q  L  A  D  D  L  R   72
 301 CAAAAATATGACCCCGTGAAGGGCGCGCAAATATCCAAGCAGCTAGCAGACGACCTGCGG  360

  73  E  K  V  K  A  L  G  Y  D  R  Y  K  L  V  I  Q  V  T  V  G   92
 361 GAAAAAGTCAAGGCGCTGGGCTATGACCGGTACAAGCTGGTGATCCAGGTCACCGTAGGG  420

  93  Q  K  Q  G  Q  A  M  R  I  I  S  R  C  L  W  D  Q  T  N  D  112
 421 CAAAAGCAGGGTCAGGCCATGCGGATTATCTCCCGCTGTCTGTGGGACCAGACGAACGAC  480

 113  N  F  A  S  E  Y  Y  E  N  E  S  M  Y  C  V  C  Q  V  Y  G  132
 481 AACTTTGCGTCGGAGTACTACGAGAATGAGAGCATGTACTGTGTATGCCAGGTGTATGGG  540

 133  L  Y  Y  E  *                                                136
 541 CTGTACTACGAGTAATGGTGCGGCCAAGCTGCCACGGCGCGGAGGAGGGGAGATAGTTAG  600
 601 GCTTGCGCTGCCAGCTGACAGCGTGCATAGCACTATCGGGGACGTCGTGGTATCACCAGC  660
 661 ATGGGACACAACAAAGGGATGGGGACAGTCCTATGCGCAGTCAACGTAGGCGGGCGCATC  720
 721 AAACAGGTCTATCGGTCGCGGGATGGGCAAGCGCAGACGAGAGGCTGTGCAGCGGTGAAG  780
 781 AGGCAGGTCCTCGATTTGTACCATGGCAAGCTAGCTGAACGAACCAGATGTGGTCGGGAC  840
 841 CATGAGATGTTACTTTTGTTTGTGGTAGGACTCACACTACAACGAAGGCGGCGAAAATAC  900
 901 ATCGGTGTGATAGGGAGTTTGCTTGAGCTCACCATGCCAGTCATATGTGGGCGGCATGGC  960
 961 GGCTTGCCGTTGCAAGGCTGTAACGGTGTATGCGCGGAAAAAAAAAAAAAAAAAAAAAAA 1020
1021 AAAAAAAAAAAAAAAAAAAAAAAAAAA 1047
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Figure 1. Sequence of the 19,000-M_r LC cDNA clone. The nucleotide and deduced amino acid sequences for the LC are shown. Residues indicated in bold type were identified by peptide sequencing. A perfect copy of the putative *Chlamydomonas* polyadenylation signal is underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number U89649.

Results

The *Chlamydomonas* outer dynein arm contains eight LCs (Pfister et al., 1982). Chemical dissection of outer arm dynein has indicated that the 19,000-M_r LC is tightly associated with the β DHC (Mitchell and Rosenbaum, 1986). Furthermore, after salt extraction from the axoneme, this LC cosediments with the β DHC (and other dynein components) at ~18S in sucrose density gradients; it also copurifies with the β DHC after both hydroxylapatite and ion exchange chromatography. The electrophoretically purified LC was blotted to polyvinylidene difluoride membrane, and the excised band was incubated with trypsin. Peptides eluting from the membrane were purified by reverse phase chromatography. Two peptides were sequenced and yielded a total of 23/25 unambiguous residue assignments: namely, XLXDQTNDNFASEYYENESM and GLYYE. Note that the thiohydantoin derivatives of Cys and Trp residues were not readily identifiable under the conditions used. The shorter peptide (GLYYE) was present in relatively high yield, and the sequence ended abruptly after the Glu residue, suggesting that it represented the COOH terminus of the molecule.

A gene-specific primer based on the sequence (K)GLYYE and an oligo(dT) adaptor primer were used in the PCR and yielded a product of ~460 bp. Upon sequencing, this product was found to encode the stop codon TAA immediately after the primer. As the peptide upon which this primer was based had been predicted to be at the COOH terminus, this product potentially encoded the 19,000-M_r LC and therefore was used to screen a λZapII cDNA library made from RNA derived from *Chlamydomonas* actively regenerating flagella (Wilkerson et al., 1995). Multiple clones were obtained and the longest one was sequenced.

The longest clone is 1,047 bp in length (Fig. 1) and contains a single open reading frame of 408 bp that encodes a protein of 136 residues with a predicted molecular weight of 15,825 daltons and an isoelectric point (pI) of 7.11. The reading frame is preceded by a 144-bp 5′-untranslated region that contains three in-frame stop codons before the first Met residue. The reading frame terminates with a single stop codon followed by 445 bp of 3′-untranslated region before the poly A tail. There is a perfect copy of the putative *Chlamydomonas* polyadenylation signal located near the end of the clone. Both peptide sequences obtained from the authentic molecule are found within the predicted protein (23/25 residues correct with two unknown). The two uncalled residues in the longer peptide are a Cys and a Trp, neither of which would have been identified under our sequencing conditions. This peptide also is preceded by a basic (Arg) residue as predicted for the product of a tryptic digest. However, the GLYYE peptide is preceded by a Tyr. Thus, this peptide presumably derives from tryptic cleavage at a noncanonical site, as was also found for a peptide from the 16,000-M_r *Chlamydomonas* LC (Patel-King et al., 1996). As only the GLYYE sequence was used to obtain the original PCR product, the above data fully confirm that this clone indeed encodes the 19,000-M_r LC.

Southern blot analysis of *Chlamydomonas* genomic DNA revealed single bands in both BamHI and SmaI digests (Fig. 2 *a*), suggesting that a single gene for this protein exists within *Chlamydomonas.* On Northern blots of total RNA derived from nondeflagellated cells and from cells actively undergoing flagellar regeneration, one message of ~1.6 kb was found (Fig. 2 *b*). The amount of this RNA was greatly upregulated in regenerating cells, as expected for an integral axonemal component.

The secondary structure of the 19,000-M_r LC was predicted using PHD (Fig. 3 *a*; Rost and Sander, 1993). The

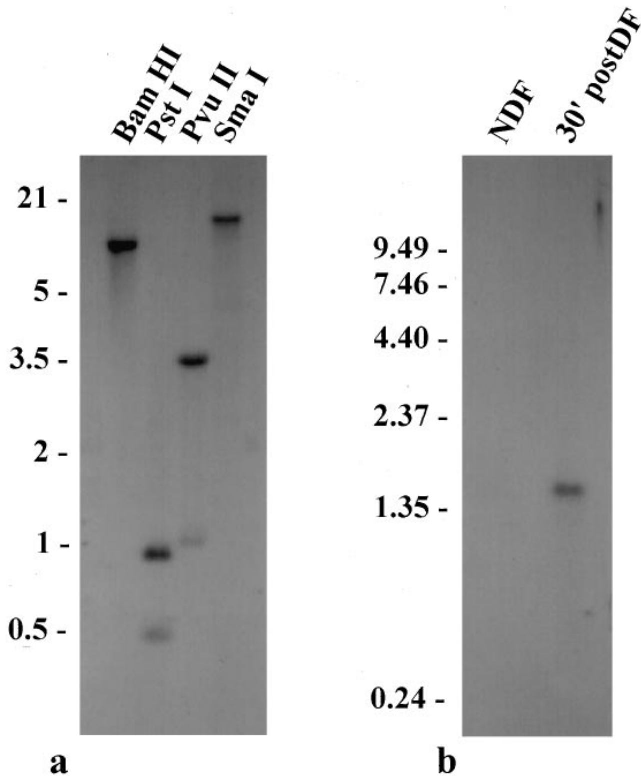


Figure 2. Southern and Northern blot analysis. (*a*) Southern blot of 10 μg genomic DNA from *Chlamydomonas* strain S1D2 digested with BamHI, PstI, PvuII, and SmaI. Single bands are observed in the BamHI and SmaI lanes, suggesting that there is only a single gene for this LC within *Chlamydomonas.* Standards are indicated at left (kb). (*b*) Northern blot of 20 μg total RNA obtained from nondeflagellated cells (*NDF*) and from cells that had been deflagellated and allowed to regenerate flagella for 30 min (*30′ postDF*). Standards are shown at left (kb). A single message of ~1.6 kb that was greatly upregulated after deflagellation is evident.

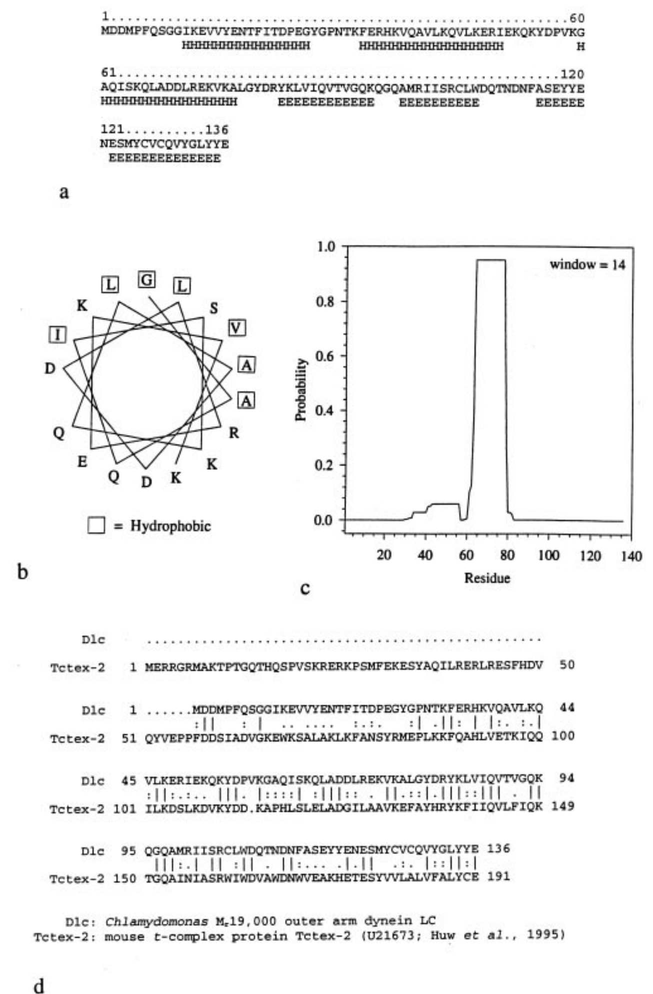


Figure 3. Sequence analysis of the 19,000-M_r LC. (*a*) The secondary structure of the 19,000-M_r LC was predicted using PHD (Rost and Sander, 1993). *E*, extended sheet; *H*, helix. (*b*) The helical segment formed by residues 60–76 is amphiphilic and displayed using HELICALWHEEL. Hydrophobic and hydrophilic residues cluster to opposite sides of the helix. (*c*) COILS output for the *Chlamydomonas* LC using a window size of 14. The plot displays the probability of coiled-coil formation vs residue number. The region of high probability coincides with the amphiphilic helix predicted for residues 60–76. (*d*) Sequence comparison between the *Chlamydomonas* 19,000-M_r LC (U89649) and the mouse *t* complex protein Tctex-2 (U21673; Huw et al., 1995). The alignment was generated by GAP using the default parameters. These proteins share 35% identity (56% similarity) with the smallest Poisson probability $P_{(n)} = 5.2 \times 10^{-22}$ (calculated by BLAST).

NH_2-terminal portion of the molecule likely contains three helical segments, one of which (residues 60–76) is predicted to be amphiphilic (Fig. 3 *b*). This same region has a very high probability of forming a coiled-coil when analyzed using the program COILS with window size = 14 (Fig. 3 *c*). The COOH-terminal region of this LC is predicted to consist of extended sheet structures (Fig. 3 *a*).

Examination of the Genbank™ and Expressed Sequence Tag databases using BLAST (Altshul et al., 1990) revealed that the 19,000-M_r LC is a homologue of the mouse *t* complex protein Tctex-2 (Huw et al., 1995). A comparison between the 19,000-M_r LC and mouse Tctex-2 generated by GAP using the default parameters is shown in Fig. 3 *d.* These proteins share 35% identity and 56% similarity; the smallest Poisson probability $P_{(n)} = 5.2 \times 10^{-22}$ (calculated by BLAST), indicating that the match is highly significant.

Further examination of the databases using the 19,000-M_r LC and Tctex-2 sequences revealed several additional matches of high statistical significance. These include another *t* complex–encoded protein Tctex-1 that is a candidate for the Tcd-1*t* distorter and that we have recently identified as an LC of cytoplasmic dynein that is differentially expressed in various tissues (King et al., 1996*b*), as well as several other mammalian and nematode proteins, at least one of which (rp3; Roux et al., 1994) also is a cytoplasmic dynein LC (King, S.M., E. Barbarese, S.E. Benashski, J.F. Dillman III, and K.K. Pfister, manuscript in preparation). The smallest Poisson probabilities ($P_{(n)}$) for the comparisons between the 19,000-M_r LC vs human Tctex-1 and the nematode open reading frame D1009-5 are 2.2×10^{-7} and 1.8×10^{-10}, respectively. An alignment of these proteins generated by CLUSTALW is shown in Fig. 4 *a.* This analysis revealed that the COOH-terminal

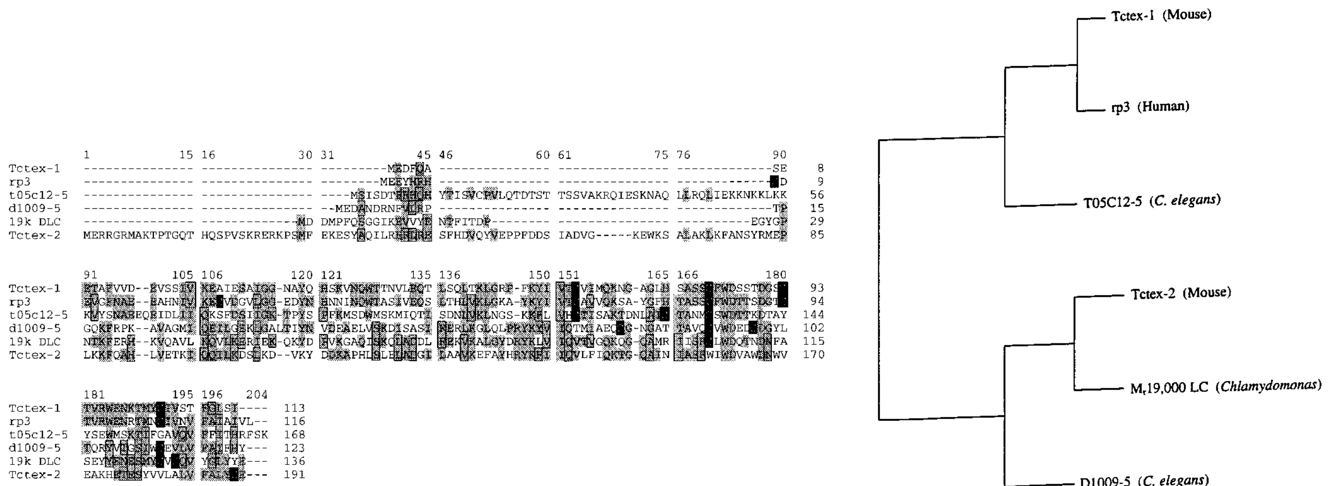


Figure 4. The 19,000-M_r LC and Tctex-2 are members of the Tctex-1 protein family. (*a*) Alignment of the Tctex-1 protein family generated by CLUSTALW. Residues conserved between two or more polypeptides are shaded (and boxed where necessary). The Cys residues are marked by black boxes. The invariant Trp-Asp motif is at residues 172/173 of the alignment. Sequences used in the alignment are: murine Tctex-1 (A32995; Lader et al., 1989), human rp3 (U02556; Roux et al., 1994), *C. elegans* ORF T05C12-5 (Z66500), *C. elegans* ORF D1009-5 (U40938), *Chlamydomonas* 19,000-M_r LC (U89649), and murine Tctex-2 (U21673; Huw et al., 1995). (*b*) Phylogenetic analysis of the members of the Tctex-1 protein family. The relationship was calculated with DISTANCES using the Kimura protein option and plotted with GROWTREE (UPGMA option). Murine Tctex-2 is most closely related to the *Chlamydomonas* outer arm LC.

portions of these molecules are most highly conserved and that the NH_2-terminal regions are much more divergent. The former region includes a Trp-Asp dipeptide motif that is invariant in all known members of this protein family and a number of Cys residues that are conserved between several of these polypeptides.

Phylogenetic analysis (Fig. 4 *b*) indicates that the six known members of the Tctex-1 family fall into two main groups. (There are at least two additional members of this protein family exemplified by partial sequences in the Expressed Sequence Tag database.) Both known cytoplasmic dynein LCs and one of the *Caenorhabditis elegans* proteins (T05C12-5) comprise one group, whereas the second *C. elegans* protein (D1009-5), Tctex-2, and the *Chlamydomonas* outer arm light chain form the second. This indicates that mouse Tctex-2 is much more closely related to the *Chlamydomonas* flagellar protein than it is to mouse Tctex-1.

Even though it lacks a signal sequence, murine Tctex-2 has previously been described as a peripheral sperm membrane protein (Huw et al., 1995). This assignment was based on immunological staining of air-dried spermatozoa and on extraction of Tctex-2 after treatment of intact sperm with 100 mM Na_2CO_3 (pH ~10.5). To determine whether all of the homologous 19,000-M_r protein within *Chlamydomonas* flagella is actually associated with the outer dynein arm, the coding region for this molecule was fused to maltose binding protein, and the entire fusion protein was used for polyclonal antibody production (antibody R5391). An antibody fraction specific for the 19,000-M_r protein was then obtained by blot purification vs the recombinant molecule after separation of the LC from MBP by digestion with Factor Xa (Fig. 5 *a*). The blot-purified antibody is highly specific for the 19,000-M_r LC and does not recognize several other members of the Tctex-1 protein family (Fig. 5 *b*).

The blot-purified R5391 antibody was then used to follow immunoreactive proteins during flagellar fractionation (Fig. 5 *c*). Importantly, two proteins are recognized within intact flagella: the original 19,000-M_r polypeptide and a second molecule of M_r ~15,000. This is completely consistent with the previous sequence analysis of murine Tctex-2 that revealed two alternatively spliced variants within the testis that encode proteins of similar M_r to those observed in *Chlamydomonas* (see Huw et al., 1995). After treatment of *Chlamydomonas* flagella with detergent to remove the membrane, all of both immunoreactive bands were obtained in the insoluble axoneme fraction. This indicates that, in *Chlamydomonas*, the Tctex-2 homologue is not membrane-associated. After axoneme extraction with 0.6 M NaCl, ~90% of the 19,000-M_r band was solubilized as expected for an integral component of the outer dynein arm. Interestingly, the immunoreactive species of lower M_r was not extracted under these conditions and remained tightly bound to the axonemal remnants.

To determine whether all of the extracted 19,000-M_r protein was associated with the outer arm, a high salt axonemal extract was sedimented through a sucrose density gradient, and the fractions were probed for the presence of both the 19,000-M_r protein (antibody R5391) and IC78 using mAb 1878A (King et al., 1986). All of the 19,000-M_r protein precisely comigrated with IC78 as expected for an integral component of the outer dynein arm (Fig. 6 *a*).

To gain further insight into the axonemal location of the smaller immunoreactive species, axonemes were prepared from mutants lacking the outer (*oda9*) or inner (*ida1*, *ida2*, *ida4*) arms, the radial spokes (*pf14*), and the central pair microtubule complex (*pf18*) and probed with the R5391

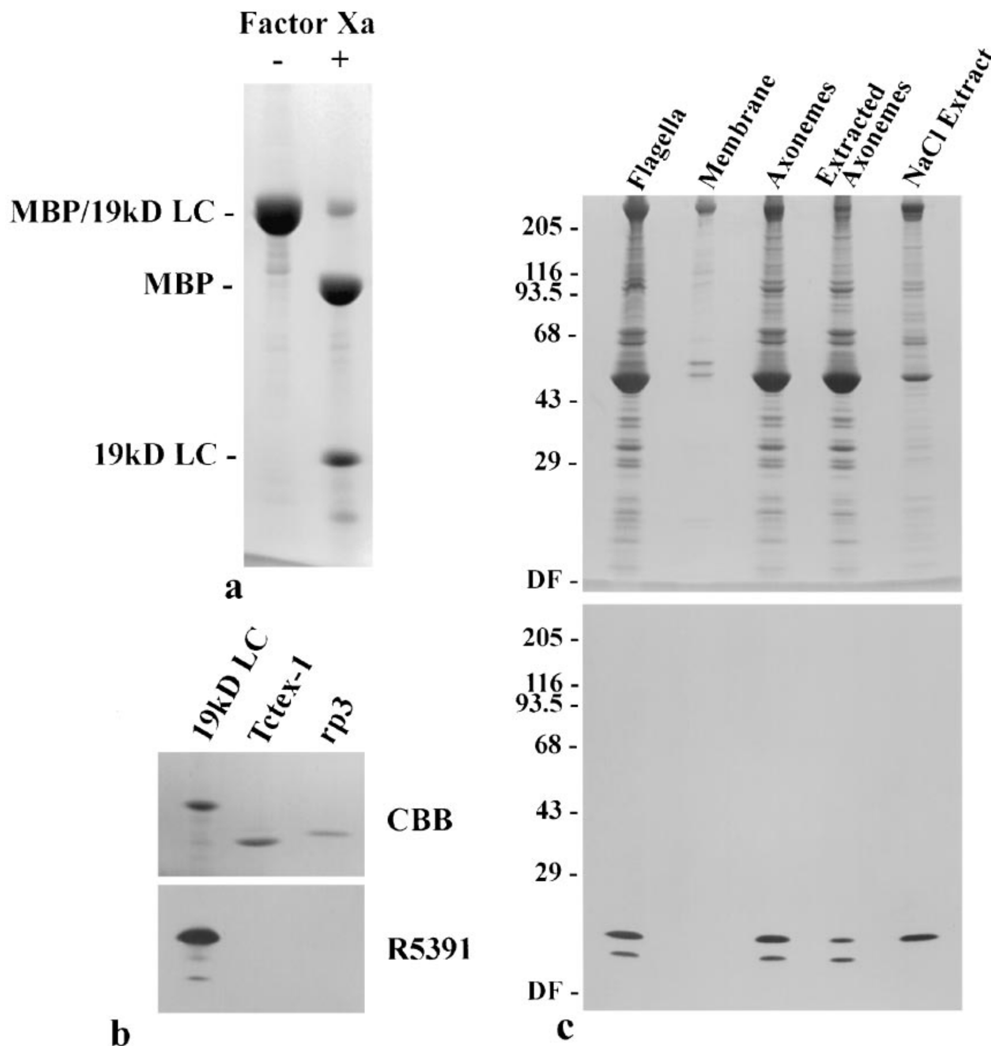


Figure 5. Distribution of the 19,000-M_r LC in *Chlamydomonas* flagella. (*a*) 30 μg of the MBP/19,000-M_r LC fusion protein and of a similar sample digested with 60 ng Factor Xa were electrophoresed in a 5–15% acrylamide gradient gel and stained with Coomassie blue (*CBB*). The intact fusion protein was used for immunizations, and the resulting antiserum (R5391) was blot purified using the electrophoretically purified 19,000-M_r LC band. (*b*) (*Upper panel*) Recombinant *Chlamydomonas* 19,000-M_r LC and two members of the Tctex-1 family, namely, murine Tctex-1 and human rp3, after staining with Coomassie blue. (*Lower panel*) Nitrocellulose blot of similar samples probed with purified R5391 antibody. The antibody preparation is highly specific and only recognizes the *Chlamydomonas* LC. (*c*) Flagella (185 μg) were extracted with 1% NP-40 to remove the membrane, and the resulting axonemes were treated with 0.6 M NaCl to solubilize the outer arm. Equivalent amounts of each sample were separated in a 5–15% acrylamide gradient gel and either stained with Coomassie blue (*upper panel*) or blotted to nitrocellulose and probed with the blot-purified R5391 antibody (*lower panel*). The locations of the M_r markers and the dye front (*DF*) are indicated at left. Two immunoreactive bands are evident. Approximately 90% of the upper (M_r19,000) band was extracted with 0.6 M NaCl, whereas the lower band remained tightly associated with the axonemal remnant. Neither immunoreactive species was solubilized upon detergent extraction, indicating that these homologues of Tctex-2 are not peripheral membrane proteins.

antibody. The 19,000-M_r band was absent from axonemes prepared from *oda9* as expected for an outer arm component (Fig. 6 *b*). However, the lower band was present in all mutants examined, suggesting that this protein is not a component of either row of dynein arms nor is it associated with the radial spokes or central pair microtubule complex (not shown).

Discussion

Here we identify a *Chlamydomonas* flagellar outer arm dynein LC as a homologue of the murine *t* complex–encoded protein Tctex-2. Tctex-2 is of interest as it is a candidate for one of the three to four distorter products that interact with a cell-specific responder and lead to the extreme meiotic drive exhibited by the *t* complex. The data presented here strongly suggest that Tctex-2, which is known to be a sperm tail-specific protein (Huw et al., 1995), is a flagellar dynein LC. This is particularly intriguing as the *t*-encoded forms of Tctex-2 may thus represent the first defined mammalian flagellar dynein mutations. Furthermore, these data raise the possibility that flagellar dynein dysfunction contributes to meiotic drive and/or to the male infertility phenotype associated with homozygosity for the *t* complex.

Transmission Ratio Distortion of the t Complex: A Biochemical Hypothesis

The most puzzling aspect of the meiotic drive of the *t* complex is that only those sperm bearing the wild-type chromosome 17 are poisoned. Analysis of chimeric mice containing both +/*t* and +/+ spermatocytes indicates that *t* sperm only show distortion over wild-type sperm that derived from the +/*t*, and not those from the +/+, spermatocyte (Seitz and Bennett, 1985). Cebra-Thomas et al. (1991) proposed that this could be achieved if the *t*-encoded responder protected *t*-bearing sperm from the *t*-encoded distorters by failing to interact with those components while

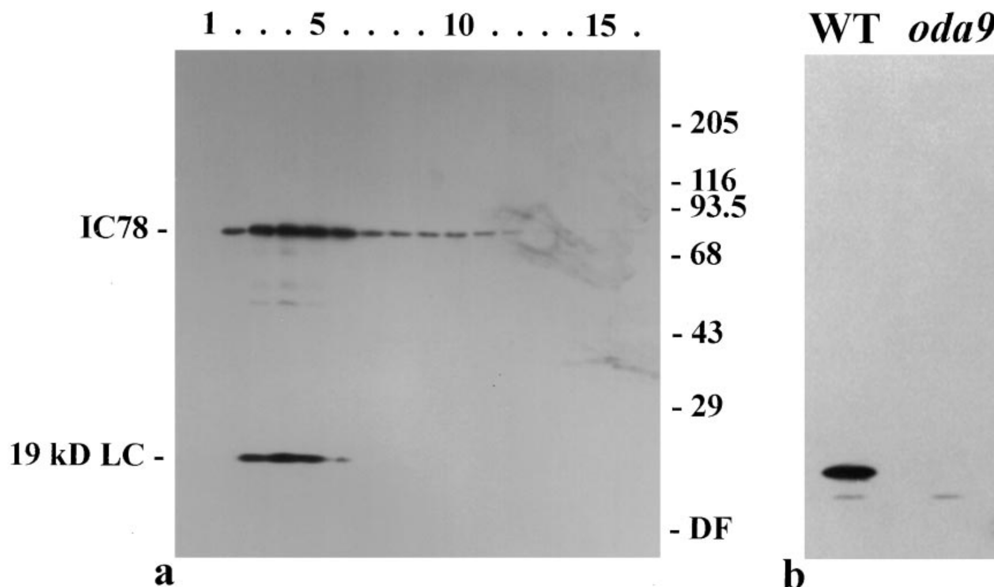


Figure 6. All of the 19,000-M_r protein is associated with the outer dynein arm. (*a*) Proteins extracted from the axoneme by high salt were separated by sucrose density gradient centrifugation. Equal volumes of each fraction were electrophoresed in a 5–15% acrylamide gradient gel and blotted to nitrocellulose. The bottom of the gradient is at left. The blot was first probed with the R5391 antibody and subsequently with 1878A to reveal the 19,000-M_r protein and IC78, respectively. Finally, the blot was stained with Ponceau S to reveal the location of individual lanes and the M_r markers. All of the 19,000-M_r protein comigrates with the outer dynein arm at ~18S. (*b*) Axonemes (~150 μg) prepared from wild-type and *oda9* strains were electrophoresed in a 5–15% acrylamide gradient gel, blotted to nitrocellulose, and probed with the R5391 antibody. The 19,000-M_r protein is completely absent from the outer arm–less axonemes. However, the lower band is present in both samples, indicating that it is not associated with outer arm dynein.

still able to act on its downstream targets. The distorters are thought to be diffusible between meiotic products and to act in a concerted fashion such that the degree of ratio distortion is directly affected by the distorter alleles present in a given haplotype. However, to achieve distortion, the responder must act only within the cell in which it is synthesized. If Tctex-2 is indeed the Tcd-3*t* distorter, it may well represent the final step in the distortion pathway, as the *t*-encoded mutations within Tctex-2 could lead to assembly, regulatory or targeting defects in the outer dynein arm, and, consequently, to the observed aberrant sperm motility phenotype. This hypothesis readily explains how different Tcd-3*t* distorter alleles might result in different levels of ratio distortion by simply producing differential effects on sperm dynein and thus having greater or lesser consequences for functional motility.

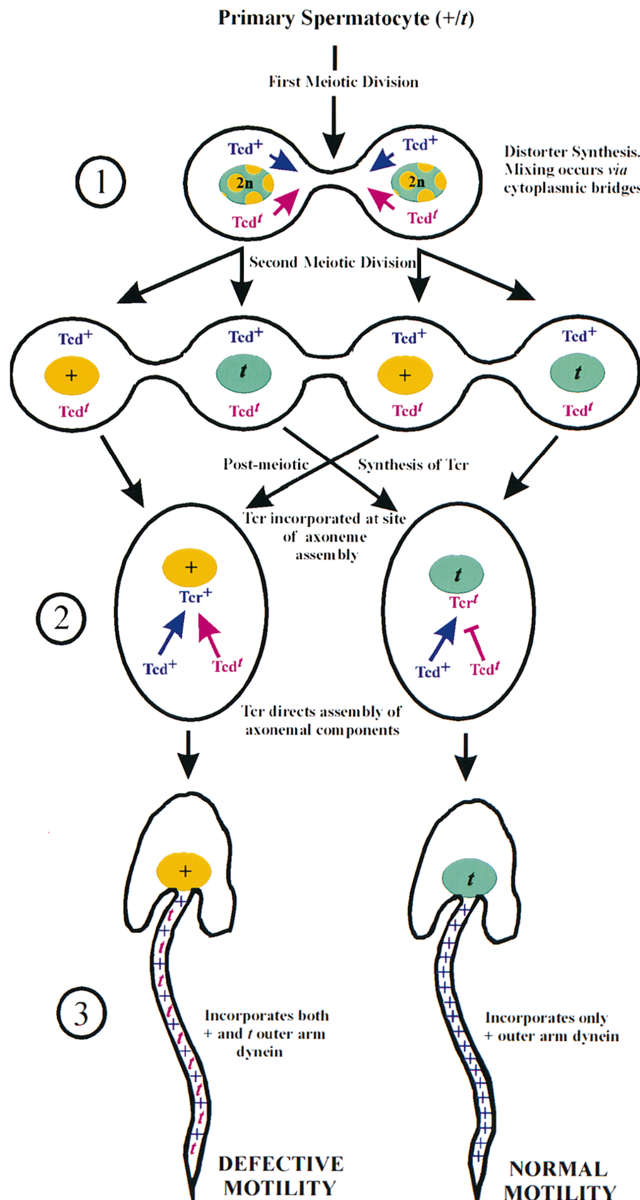
If Tctex-2 dysfunction itself results in nonmotile sperm and thus represents one end of the pathway, the question then becomes how can the ratio of wild-type to *t*-encoded Tctex-2 protein be distorted during spermiogenesis? Here our previous observation that Tctex-1 (a candidate for the Tcd-1*t* distorter) is an LC of cytoplasmic dynein (King et al., 1996*a*) potentially becomes of significance, as it suggests that alterations in intracellular transport activities during spermiogenesis also may underlie distortion.

To obtain ratio distortion, the responder must be retained in close proximity to the nucleus that encoded it. As spermatids form a syncitium after meiosis, it has been difficult to envisage how this might be achieved. However, identification of axonemal and cytoplasmic dynein LCs as candidate distorters provides a potential clue. Axoneme assembly occurs at a specific perinuclear site that is defined by the basal body. This structure and its many associated proteins are <u>not</u> freely diffusible, being retained in close association with each sperm nucleus. Therefore, we suggest that the responder is a protein (possibly basal body associated) that is involved in directing the assembly of the sperm axoneme; i.e., it acts as a gatekeeper to determine what can and cannot enter the growing flagellum. A general scheme for how distortion might then arise is outlined in Fig. 7 *a*.

Ratio distortion would be achieved through a combination of (*i*) the ability of Tcr^t to interact only with wild-type distorters and thus to incorporate only wild-type flagellar dynein into the axonemes of *t* spermatozoa, which are thus protected from the deleterious effects of the *t*-encoded distorters; and (*ii*) poisoning of wild-type sperm through the axonemal incorporation of *t* mutant flagellar dynein allowed by Tcr^+. Assuming wild-type and *t* mutant distorters are present in equimolar amounts within the syncitium, wild-type axonemes would be predicted to incorporate ~50% of the *t* mutant flagellar dynein, which could be more than sufficient to cause motility defects. Distorter allele-specific effects could be readily understood in the following terms: (*i*) alterations in the relative affinities of distorters for each other and/or for Tcr^+ and Tcr^t could lead to subtle alterations in the amount and potency of *t* mutant flagellar dynein incorporated into a given sperm flagellum; and (*ii*) direct consequences for dynein function. The presence of Tctex-2 in sperm from *t*/*t* mice (Huw et al., 1995) may then simply derive from a low affinity interaction between *t* mutant Tctex-2 and Tcr^t in the absence of the competing high affinity interaction with wild type.

Possible Roles for Cytoplasmic Dynein

Newly synthesized flagellar components need to be directed to the site of flagellar assembly at the perinuclear basal body. Assuming that cytoplasmic microtubules within spermatids adopt the standard orientation with plus ends toward the cell periphery, this activity would presumably require a minus end–directed motor such as cytoplasmic



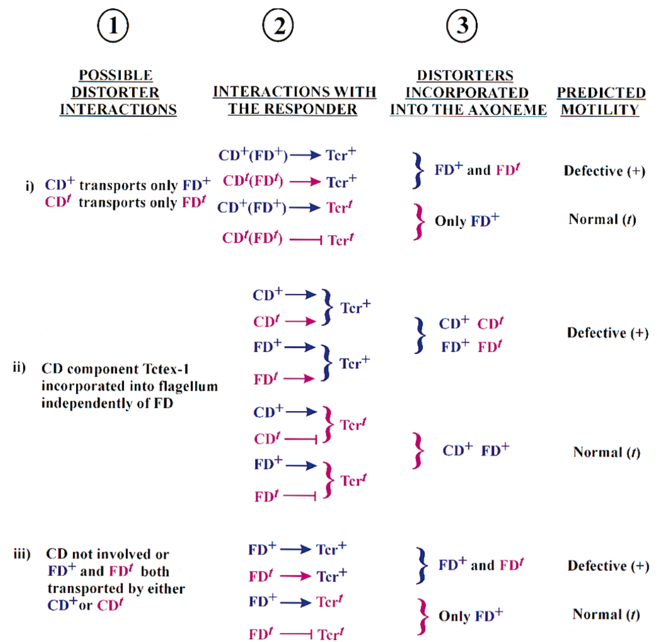


Figure 7. A model for dynein-mediated meiotic drive. Recent studies have revealed that candidates for the Tcd-1*t* and Tcd-3*t* distorters are LCs of cytoplasmic dynein (King et al., 1996*a*) and outer arm dynein (this work), respectively. This suggests a model in which ratio distortion arises as a result of the differential incorporation of wild-type and mutant dynein into sperm axonemes. The responder (Tcr) is hypothesized to be associated with the sperm nucleus (possibly at the basal body) and to be involved as a gatekeeper in determining which components are assembled into the flagellum. This would satisfy the requirement for Tcr to be retained within the cell in which it is synthesized and also suggests several mechanisms through which cytoplasmic and flagellar dynein LCs might interact to achieve ratio distortion. (*a*) The biological consequences are illustrated (*1–3* in *a* indicate the points at which the different interactions shown in *b* occur). Distortion arises as a result of two features: (*i*) the inability of the *t*-encoded responder (*Tcr^{t}*) to incorporate *t* mutant flagellar dynein into the axoneme (it thus incorporates only the wild-type enzyme); and (*ii*) assembly of both wild-type and *t* dynein into axonemes by Tcr^{+}, which leads directly to motility defects. Thus, in this model, axonemes of *t* haplotype–bearing sperm contain only wild-type flagellar dynein and would exhibit normal motility, whereas sperm containing the wild-type chromosome would be defective as a result of axonemal incorporation of *t* mutant flagellar dynein. (*b*) The results from three possible interactions between the two putative distorters (Tctex-1 [a cytoplasmic dynein LC] and Tctex-2 [a flagellar dynein LC]) and the responder are shown. In *i*, cytoplasmic dynein (*CD*) transports flagellar dynein (*FD*) to the assembly site and interacts with Tcr to determine which FD (+ or *t*) is incorporated. In this scenario, wild-type CD^{+} (the motor) must transport only wild-type FD^{+} (the cargo) and, likewise, *t* mutant CD^{t} transports only FD^{t}. (*ii*) Immunological analyses of both mouse sperm (O'Neill and Artzt, 1995) and *Chlamydomonas* (Harrison, A., and S.M. King, unpublished results) indicate that Tctex-1 (or a close homologue) also may be present within the flagellum. This suggests a second possibility in which CD (i.e., Tctex-1) and FD interact independently with Tcr. In *iii*, CD is not involved in distortion, which arises simply because of interactions between FD and Tcr.

dynein of which the putative distorter Tctex-1 is an integral component. There are several possible scenarios for the role that cytoplasmic dynein containing *t*-specific mutations in the Tctex-1 LC might play in ratio distortion. These are illustrated in Fig. 7 *b*.

(*i*) Cytoplasmic dynein is involved in the interaction with Tcr to determine whether wild-type or mutant flagellar dyneins are incorporated. In this case, to achieve distortion, cytoplasmic dynein containing *t* mutant Tctex-1 would have to transport only flagellar dynein containing *t* mutant Tctex-2 to the site of axoneme assembly. Likewise, wild-type cytoplasmic dynein would transport wild-type flagellar dynein. Which cargo(s) are allowed to dock and enter the growing flagellum would be determined by the interaction of the responder with the cytoplasmic dynein motor; i.e., cytoplasmic dynein containing *t* mutant Tctex-1 would cause distortion because it would transport flagellar dynein containing only *t* mutant Tctex-2 that would be incorporated by the wild-type responder but not by the *t* mutant responder. Note that the interaction between cyto-

plasmic and flagellar dyneins need not be direct here, and indeed this coupling could provide a site of action for one or more additional distorter products.

(*ii*) Although all Tctex-1 within mammalian brain is cytoplasmic dynein associated (King et al., 1996), immunological analysis has suggested that sperm tails also contain Tctex-1 (or a related homologue) (O'Neill and Artzt, 1995). We have recently observed that a 14-kD *Chlamydomonas* protein recognized by an antibody directed against human Tctex-1 is an integral axonemal component (Harrison, A., and S.M. King, unpublished results). Thus it is possible that Tctex-1 exerts a direct effect on flagellar function because either flagella contain a "cytoplasmic-like" dynein or Tctex-1 is also a component of some other flagellar complex. In this case, the incorporation of wild-type and *t* mutant Tctex-1 and Tctex-2 proteins could be distorted independently simply through differential interactions with Tcr^{+} and Tcr^{t}.

(*iii*) This cytoplasmic motor complex might play no direct part in ratio distortion; i.e., both wild-type and *t* mutant cytoplasmic dynein either are not involved at all or they both transport flagellar components (+ and *t* mutant) to the site of axoneme assembly, but have no role in the interaction with Tcr.

Predictions of the Model

The model presented above makes a number of predictions, at least some of which are readily testable. For example, (*i*) flagellar axonemes from motile (*t*-bearing) sperm should contain only wild-type Tctex-2 (and perhaps also Tctex-1), whereas those from immotile (+-bearing) sperm should have both the wild-type and mutant proteins. (*ii*) For the model to be correct, defective Tctex-2 must lead to aberrant motility. Therefore, *Chlamydomonas* strains lacking the 19,000-M_r Tctex-2 homologue should exhibit a detectable swimming defect. (A *Chlamydomonas* mutant lacking the 19,000-M_r protein exhibits an outer arm assembly defect; consequently, such cells swim more slowly than wild-type; Pazour, G., A. Koutoulis, H. Sheng, R.S. Patel-King, S.M. King, and G.B. Witman, unpublished results.) The same should also be true for Tctex-1 if that protein's distorter phenotype derives from its function within the flagellum rather than the cytoplasm. (*iii*) The responder (Tcr) (for which there is a good candidate; Cebra-Thomas et al., 1991) should localize to the base of the growing flagellum within spermatids.

Intraflagellar Associations of Tctex-2

The characterization of Tctex-2 as a putative *t* complex distorter is based on gene location (mapping of rare partial haplotypes places it near Tcd-3), testis-specific expression, and the presence of several *t*-specific mutations that are predicted to cause significant alteration in protein structure (see Huw et al., 1995; Lader et al., 1989; O'Neill and Artzt, 1995). Previously, Tctex-2 was considered to be a peripheral sperm membrane protein (Huw et al., 1995). This assignment was based on several observations. First, histochemical staining of air-dried spermatozoa with an anti–Tctex-2 antiserum yielded a positive signal. However, the mammalian sperm membrane is a very fragile structure, and this treatment is known to disrupt it, thereby allowing antibodies access to intracellular components (see Phillips et al. [1991] for discussion of the effects of various treatments on sperm membrane integrity). Moreover, it is not possible to distinguish between external and internal sperm antigens at the light microscopic level. Second, Tctex-2 was released after treatment of intact sperm with 100 mM Na_2CO_3 (pH ~10.5). Again this treatment could have disrupted membrane integrity as could the multiple centrifugations to which sperm are necessarily subject during extraction procedures (Phillips et al., 1991). Finally, high pH has been found to be very effective in solubilizing flagellar axonemes and thus releasing both outer and inner arm dynein components (Gatti, J.-L., S.M. King, and G.B. Witman, unpublished observations).

As noted by Huw et al. (1995), Tctex-2 does not have a canonical signal sequence that would predict transmembrane passage. Indeed the NH_2-terminal domain is very hydrophilic with 10 charged and 8 polar amino acids within the first 26 residues and is thus a very unlikely candidate for passing a hydrophobic barrier. Although there are mechanisms for transporting specific proteins lacking signal sequences across lipid bilayers such as interleukin-1β and various growth factors (Rubertelli et al., 1990), the details of how this is achieved remain unclear and are likely highly specialized for specific molecules. There is no evidence to suggest that Tctex-2 is transported in such a fashion. Our identification of a close homologue of this protein within the *Chlamydomonas* outer arm raises the strong possibility that murine Tctex-2 also is a flagellar dynein LC and that dysfunction of this protein as a result of the *t*-specific mutations contributes to ratio distortion through a direct effect on flagellar motility.

An Extended Family of Dynein LCs

Detailed sequence analysis revealed that this *Chlamydomonas* LC and Tctex-2 are both related to recently identified LCs (Tctex-1 and rp3) within the mammalian cytoplasmic dynein complex (see King et al., 1996*a*). Together with several nematode homologues, these proteins form a diverse gene family of dynein LCs (there are at least two additional mammalian members of this protein family exemplified by partial sequences in the Expressed Sequence Tag database). Intriguingly, some of these proteins are differentially expressed in various tissues, suggesting the existence of dynein isoforms based on LC complement and raising the possibility that these LCs play a role in tissue-specific targeting or regulatory events.

Within the *Chlamydomonas* flagellum, the 19,000-M_r LC is tightly associated with the β DHC (Mitchell and Rosenbaum, 1986). Based on analysis of dynein from a mutant (*oda*4-s7) expressing a truncated form of the β DHC, the site of interaction between these molecules must be located within the NH_2-terminal ~160 kD of the DHC, i.e., in the stem domain of the structure (Sakakibara et al., 1993). The functional role played by this LC within outer arm dynein remains unclear at present. However, as multiple members of this protein family have been identified within both cytoplasmic and flagellar dyneins, they may be involved in assembly, targeting dyneins to specific cargoes, or regulating their activity in response to specific signals.

Immunoblot analysis of *Chlamydomonas* flagella revealed two protein bands recognized by the R5391 antibody. All of the upper band was associated with the outer arm. However, the smaller protein was tightly associated with the flagellar axoneme remnant after high salt extraction and was not missing in mutants lacking the outer or inner arms, radial spokes, or central pair complex. Thus, the axonemal location of this protein remains unknown but, given the highly specific nature of the antibody used, it likely represents an additional member of this protein family. In mice, an alternatively spliced cDNA that encoded a smaller variant of Tctex-2 also was identified (Huw et al., 1995); intriguingly, this smaller variant was not detected in whole testis protein samples. In *Chlamydomonas*, only single bands were observed on both Southern and Northern blots with the 19,000-M_r LC probe. Thus, the smaller immunoreactive band is either the product of a separate gene or the result of posttranslational processing of the 19,000-M_r LC.

In conclusion, we demonstrate here that a *Chlamydomonas* homologue of the putative murine *t* complex distorter Tctex-2 is an LC of outer arm dynein. Our results suggest that the *t*-encoded mutations in Tctex-2 lead to dysfunction of the flagellar dynein molecular motor. This in turn may contribute to non-Mendelian chromosome segregation by disrupting normal flagellar motility of +-bearing sperm. Further detailed functional analysis of this LC in both *Chlamydomonas* and mice will provide additional clues as to the important role it plays in dynein activity and to the mechanisms responsible for meiotic drive.

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