Molecular Cloning and Sequence Analysis of the *Chlamydomonas* Gene Coding for Radial Spoke Protein 3: Flagellar Mutation *pf-14* Is an Ochre Allele

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Abstract. *Chlamydomonas reinhardtii* flagellar motility mutant *pf-14* fails to assemble radial spokes because of a deficiency for assembly-competent radial spoke protein 3 (Huang, B., G. Piperno, Z. Ramanis, and D. J. L. Luck. 1981. *J. Cell Biol.* 88:80–88). Here, we raise an antiserum to protein 3 and use it to isolate the corresponding structural gene from an expression library. Southern blot analysis indicates that the gene is single copy and has not undergone major rearrangement in mutant *pf-14* cells. Northern blot analysis suggests that wild-type amounts of an apparently normal 2.3-kb transcript accumulate in mutant cells during flagellar regeneration. When this mutant RNA is hybrid selected and translated in vitro, however, it produces a slightly truncated polypeptide 3 with an altered charge. The mutant protein 3 fails to assemble into *pf-14* flagella and is maintained within a cytoplasmic pool of unassembled radial spoke polypeptides, as indicated by immunoblot analysis of proteins from whole cells and isolated axonemes using antisera to several radial spoke polypeptides. Interestingly, amounts of the mutant protein are greatly diminished relative to other spoke components. Complete genomic and cDNA nucleotide sequences were determined, and the *pf-14* mutation was identified. It is a C-to-T transition near the 5′ end of the protein coding region, which changes codon 21 to the ochre termination signal UAA. The size and charge of the mutant protein, and its reduced levels in cells, suggest that it is produced by relatively inefficient translational initiation at codon 42. The unphosphorylated isoform of radial spoke protein 3 is identified, and the sequence similarities between intervening sequences of the radial spoke protein 3 gene and a conserved intervening sequence of the two *Chlamydomonas* β-tubulin genes (Youngblom, J., J. A. Schloss, and C. D. Silflow. 1984. *Mol. Cell. Biol.* 4:2686–2696) are reported.

ULTRASTRUCTURAL, biochemical, and genetic analyses have been performed on *Chlamydomonas reinhardtii* flagellar motility mutants (for review see 14). This approach has yielded information about the function, polypeptide composition, and assembly of complex eucaryotic flagellar substructures, perhaps best exemplified by the analysis of radial spokes. Spokes are attached at precise linear intervals to axonemal outer doublet microtubules. The base of each spoke is a thin, stalk-like projection from the doublet A-subfiber; the distal spoke tip forms an enlargement, or spokehead, which interacts with the central microtubule pair. This interaction apparently converts an intrinsically symmetric flagellar bending pattern into the asymmetric pattern used by *Chlamydomonas* when swimming forward (4, 16). Flagellar mutants lacking radial spokes, or just spokeheads, have been recovered and their flagellar proteins analyzed by two-dimensional gel electrophoresis (29, 30). By correlating the missing structure with the missing proteins, 17 radial spoke polypeptides have been identified (radial spoke proteins 1–17).

We previously have used antibodies to molecularly clone several flagellar radial spoke and dynein arm genes from a *Chlamydomonas* genomic expression library (38). Here we use the same approach to clone the gene coding for radial spoke protein 3. Flagellar mutation *pf-14* occurs in this gene, as indicated by previous pseudo-revertant analysis (15). Mutant *pf-14* cells have paralyzed flagella that completely and specifically lack radial spokes (29, 39). Dikaryon rescue experiments have shown that 16 of the 17 radial spoke proteins are present and assembly competent, but remain unassembled within a cytoplasmic pool in the mutant cells. The only apparent deficiency is for assembly-competent spoke protein 3 (15, 22). It is not known whether protein 3 is simply not synthesized or is present in a form that is not able to coassemble with the other spoke components. Since, to date, only mutations at the *pf-14* genetic locus completely block spoke assembly, it has been suggested that protein 3 may attach the radial spoke to the outer doublet microtubule (22). Protein

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3 is also of interest because it is one of five radial spoke proteins that are phosphorylated in vivo (30).

In this report, we describe production of an antiserum against spoke protein 3, isolation of the corresponding structural gene, and complete sequence analysis of the wild-type and mutant *pf-14* alleles. The antibody and cloned gene are also used to characterize the mutant *pf-14* phenotype. Our results indicate that mutation *pf-14* is a nonsense mutation near the 5′ end of the protein coding region, and that the mutant cells synthesize a slightly truncated protein 3 polypeptide apparently by restarting translation downstream from the mutant termination signal. The truncated protein is not able to assemble into the flagellum and is maintained within the cell body.

Materials and Methods

Chlamydomonas Strains

Wild-type cells were strain 21gr (mt +). Isolates of mutant *pf-14* (mt +) were obtained from D. J. L. Luck and the *Chlamydomonas* Genetics Center (Duke University, Durham, NC).

Isolation and Fractionation of Flagella

These methods were detailed elsewhere (26, 38). Briefly, flagella were released from *Chlamydomonas* using dibucaine and then demembranated with the nonionic detergent NP-40 to produce axonemes. These were either loaded directly onto gels for immunoblot analysis or were further fractionated to enrich for proteins of the basal spoke stalk (30) and then loaded onto preparative gels.

Gel Electrophoresis

Procedures were as previously described (38), except that SDS was supplied by BDH Chemicals Ltd. (Poole, England). Molecular weight markers were β-galactosidase, phosphorylase B, BSA, ovalbumin, and carbonic anhydrase (all supplied by Sigma Chemical Co., St. Louis, MO).

Production of Antiserum to Spoke Polypeptide 3

Protein 3 was excised from preparative two-dimensional gels of fractionated axonemes and then used to immunize rabbit S essentially as described (38).

Affinity Purification of Antibodies

10 mg of fractionated axonemes in coupling buffer (0.5 M NaCl, 0.1 M $NaHCO_2$, pH 8.3) at 0°C was sonicated (three 10-s bursts, 50% duty cycle; Sonifier 200 equipped with a microtip; Branson Sonic Power Co., Danbury, CT). After centrifugation (20 min, 12,000 *g*, 4°C) to remove any intact axonemes, the solubilized axonemal proteins were bound to 1.2 ml of CNBr-activated Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) following procedures recommended by the manufacturer, and the Sepharose beads were then poured into a column fitted with a flow adaptor. The flagellar protein affinity column was prerun with wash and elution buffers (see below) and then equilibrated with PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2). 10 ml of immune serum from rabbit S was diluted threefold in PBS and loaded overnight onto the column. After a 30-ml wash with PBS adjusted to 0.5 M NaCl, the direction of flow was reversed and the antibodies were eluted with 0.1 M glycine, pH 3. Column fractions were immediately neutralized by the addition of 1 M Tris, pH 8, and then tested for antibodies to protein 3 by one-dimensional immunoblot analysis.

Immunoblot Analysis

Procedures were essentially as described (6) except that the secondary and tertiary probes were biotinylated goat anti-rabbit IgG and streptavidin horseradish peroxidase conjugate, respectively (both from Bethesda Research Laboratories, Gaithersburg, MD), and blots were developed with 4-Chloro-1-Napthol (Aldrich Chemical Company, Milwaukee, WI) as the chromophore.

Chlamydomonas whole-cell protein was prepared as described (21).

Isolation of Genomic Clones Encoding Spoke Protein 3

Approximately 10^6 plaques of a *Chlamydomonas* genomic λgt11 expression library were screened with the affinity-purified antiserum S as described previously (38). Two positives showing the strongest reactions were cloned, and their inserts were subsequently subcloned into the Eco RI site of vector SP65, creating plasmids p18S and p18SIII. Both inserts were restriction mapped and found to contain overlapping sequences (Fig. 2 *A*).

Complete genomic clones for the protein 3 gene were isolated from two separate EMBL 4 libraries; screening was accomplished as described (1) using radiolabeled RNA synthesized from p18SIII using SP6 polymerase (Promega Biotec, Madison, WI). The first library, a generous gift of J. Youngblom and C. Silflow (University of Minnesota, St. Paul, MN), was constructed from *Chlamydomonas* wild-type DNA (41). The second library was constructed essentially as described (23) from DNA that was isolated from cells carrying the mutant *pf-14* allele. Clone 8D(wt) from the wild-type library and clone 4C (*pf-14*) from the mutant *pf-14* library were found to contain the complete gene (Fig. 2 *A*). A 3.8-kb Kpn I–Eco RI fragment from wild-type clone 8D(wt) (Fig. 2 *B*) and a 4-kb Hind III–Bam HI fragment (not shown) from *pf-14* clone 4C (*pf-14*) each contain the entire coding region, and each fragment was subcloned for sequence analysis into Bluescript vector KS- (Stratagene, La Jolla, CA) to create plasmids pKE-RS3(wt) and pHB-RS3 (*pf-14*), respectively.

Isolation of cDNAs

To screen for cDNAs that are complete or close to full length from an oligo-dT-primed library, we first identified restriction fragments corresponding to the 5′ end of the protein 3 transcript to use as hybridization probes. This was accomplished by deducing the direction of transcription and then mapping the 5′ end of the transcription unit. Orientation of the transcription unit was deduced by comparing a restriction map of the genomic insert in pKE-RS3(wt) with a restriction map of a partial cDNA clone that was isolated from the oligo-dT-primed library described by Schloss et al. (34) (data not shown). It was assumed that the partial cDNA corresponded to the 3′ end of the mature transcript. The deduced orientation was later confirmed by complete sequence analysis of a full-length oligo-dT-primed cDNA (see Results). Next, the 5′ end of the transcript was roughly mapped by hybridizing gel-purified and nick-translated restriction fragments from the genomic insert in subclone pKE-RS3(wt) to blots of size-fractionated whole-cell RNA isolated 25 min after flagellar amputation (not shown). Fragments that overlapped the transcription unit hybridized to a single 2.3-kb RNA. By comparison of several different fragments, it was determined that transcription initiates somewhere within the 290-bp interval defined by a Hind III site and a Pst I site (Fig. 2 *B, right half of probe C*).

A *Chlamydomonas* cDNA library, generously provided by W. S. Adair (Tufts University School of Medicine, Boston, MA), was screened as described (1) with a nick-translated Bgl I fragment (Fig. 2 *B, probe B*) that spans most of the 5′ half of the gene. Several positive clones were identified and subsequently tested for hybridization to a Kpn I–Pst I restriction fragment (Fig. 2 *B, probe C*) that overlaps the first ~200 bp of the transcription unit. Clone cRS3-2 hybridized to the Kpn I–Pst I fragment. Its 2.3-kb insert was subcloned into the Eco RI site of plasmid vector Bluescript KS-, creating plasmid pcRS3-2(wt). A restriction map for cDNA cRS3-2(wt) is shown in Fig. 2 *C*.

In Vitro Transcription

Bam HI–linearized pcRS3-2(wt) was transcribed with T3 polymerase (Stratagene) following procedures recommended by the supplier. The transcription reaction was phenol extracted and ethanol precipitated before translation in vitro.

Hybridization Selection

Plasmid p18SIII was denatured, bound to nitrocellulose, and hybridized to *Chlamydomonas* whole-cell RNA as described previously (38). The whole-cell RNA for these experiments was isolated from cells 25 min after deflagellation.

In Vitro Translation

RNAs synthesized from cDNA subclone pcRS3-2(wt) or hybrid selected using genomic insert 18SIII were translated in the rabbit reticulocyte lysate system with [^{35}S]methionine (28). The translation products were processed for two-dimensional electrophoresis as described (20) and then coelectrophoresed with 240 μg of unlabeled fractionated axonemal proteins. Gels were stained with Coomassie blue to reveal the unlabeled axonemal proteins. The ^{35}S-labeled translation products were subsequently visualized by direct autoradiographic exposure of dried gels to XAR-5 film (Eastman Kodak Co., Rochester, NY) at −70°C. Gels containing translation products of hybrid-selected RNA were treated with Autofluor (National Diagnostics, Inc., Somerville, NJ) before they were dried and exposed as above.

Isolation of Nucleic Acids from Chlamydomonas

DNA was prepared according to the method of Chiang and Sueoka (7), except that after RNAse and protease digestions the cell lysate was extracted three times with an equal volume of phenol/choroform/isoamyl alcohol (50:48:2), and then DNA was ethanol precipitated and collected by spooling onto a glass rod.

Whole-cell RNA was isolated from cells before and at various times after mechanical deflagellation (32) by phenol extraction and purification on CsCl gradients as described (34).

Southern Blot Analysis

3 μg of *Chlamydomonas* DNA was digested with Ava I, Pst I, Sal I, or Bam HI. DNA fragments size fractionated on a 1% agarose gel were denatured and transferred to nylon membrane (Nytran; Schleicher & Schuell, Inc., Keene, NH) essentially as described by Southern (37), and then hybridized to nick-translated Bgl I–Pvu II restriction fragment A (Fig. 2 *B*) in a solution containing 50% formamide, 5× Denhardt solution (8), 100 μg/ml sonicated calf thymus DNA, and 0.1% SDS. The hybridization conditions were 42°C for 36–48 h. Filters were first washed at room temperature for 20 min in two changes of a solution containing 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS, and then at 68°C for 2 h in three changes of 0.2× SSC plus 0.1% SDS.

Northern Blot Analysis

Whole-cell RNA was size fractionated on a horizontal 1.75% agarose gel containing 6% formaldehyde and transferred to nitrocellulose essentially as described (35). Blots were initially hybridized to a nick-translated Pvu II–Bgl I restriction fragment (Fig. 2 *B, probe C*) that was gel purified from genomic subclone KE-RS3(wt). Subsequently, blots were stripped of the original probe by soaking for 2 min in H_2O at 100°C, and then rehybridized to nick-translated plasmid pcf2-40 (34), which contains a cDNA corresponding to a constitutively expressed RNA. For size markers, a lane was loaded with Hind III fragments of λ DNA, transferred to nitrocellulose, and hybridized separately with nick-translated λ DNA. Hybridization was in a solution containing 50% formamide, 5× SSPE (180 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4), 0.5% nonfat dry milk, 100 μg/ml poly(A), 100 μg/ml sonicated calf thymus DNA, 50 μg/ml heparin, and 0.1% SDS. Hybridization conditions were 42°C for 36–48 h. Filters were first washed at 45°C for 40 min in two changes of a solution containing 50% formamide, 5× SSPE, and 0.5% SDS. Second and third washes were also at 45°C for two changes each; the second wash solution contained 0.1% SSPE plus 0.2% SDS, and the third contained 10 mM $NaHPO_4$ plus 0.1% SDS.

DNA Sequencing

A set of overlapping exonuclease III deletions were made for wild-type and *pf-14* genomic subclones pKE(wt) and pHB(*pf-14*), respectively, and for wild-type cDNA subclone pcRS3-2(wt), as described by Henikoff (13). These constructions are in M13 phagmid vector Bluescribe KS- (Stratagene), which allowed recovery of single-strand DNA that was then sequenced by the dideoxynucleotide chain termination method (2, 33) using 7-deaza-dGTP (Pharmacia Fine Chemicals) to reduce compression. Both strands of the cDNA insert in pcRS3-2(wt) were sequenced at least once. One strand of genomic inserts in pKE(wt) and pHB(*pf-14*) was completely sequenced, and parts of the second strand for the pKE(wt) insert were sequenced so that there was complete data from both strands of the entire transcription unit.

Searches for protein sequence similarity were conducted with the National Biomedical Research Foundation (Washington, DC) Protein Identification Resource, Data Base Release 16, using the WORDI(WORDSEARCH) program (9) and the FAST A program (27) on BIONET. The whole amino acid sequence and 100-residue segments were used as queries. The resulting matches were very short and of low similarity and thus were judged insignificant.

Isoelectric points were calculated from deduced amino acid sequences using the CHARGEPRO program of PC/GENE (IntelliGenetics, Inc., Mountain View, CA).

Results

Identification of a Novel Isoform of Spoke Protein 3

Protein 3 is phosphorylated in vivo (30) and displays microheterogeneity in two-dimensional protein gels; it appears as two closely apposed spots in a gel loaded with wild-type axonemes (Fig. 1 *A*, *3* and *small arrows*). A minor previously unidentified protein, which will be referred to as polypeptide 3a, is also positioned in this region of the gel (Fig. 1 *A*, *3a*). It has a slightly faster mobility in the SDS dimension and a slightly more basic isoelectric point than the two prominent protein 3 isoforms. To test the possibility that protein 3a is a radial spoke component, we ran an identical gel loaded with "spokeless" axonemes isolated from mutant *pf-14* cells (Fig. 1 *B*). Comparison clearly shows that polypeptide 3a is missing from the mutant axonemes along with previously identified radial spoke proteins (*open arrows*). Since polypeptide 3a fits the genetic definition of a radial spoke component and is slightly more basic than the major protein 3 isoforms, it is reasonable to propose that it is simply the unphosphorylated polypeptide. This possibility was confirmed by in vitro translation of hybrid-selected protein 3 RNA (see below).

Production and Characterization of an Antiserum against Spoke Protein 3

To molecularly clone the protein 3 structural gene from a *Chlamydomonas* expression library it was first necessary to raise antibodies against the polypeptide. Gel fragments containing the two major flagellar isoforms of protein 3 were used as immunogens, and the resulting antiserum was purified on an axonemal protein affinity column before final characterization by two-dimensional immunoblot. Fig. 1 *C* shows an immunoblot of wild-type axonemal polypeptides stained with the affinity-purified antiserum; direct comparison with a corresponding Coomassie blue–stained gel (Fig. 1 *A*) shows that the antibodies react with the major protein 3 isoforms, and cross react with unmodified isoform 3a as expected. To verify that the antiserum does not react with any axonemal polypeptides fortuitously comigrating with any of the protein 3 isoforms, it was also used to stain an immunoblot of axonemal polypeptides from mutant *pf-14* cells. As expected, no staining is visible (Fig. 1 *D*).

Genomic and cDNA Clones for the Protein 3 Structural Gene

The affinity-purified anti–protein 3 serum was used to isolate two overlapping partial genomic clones for the wild-type

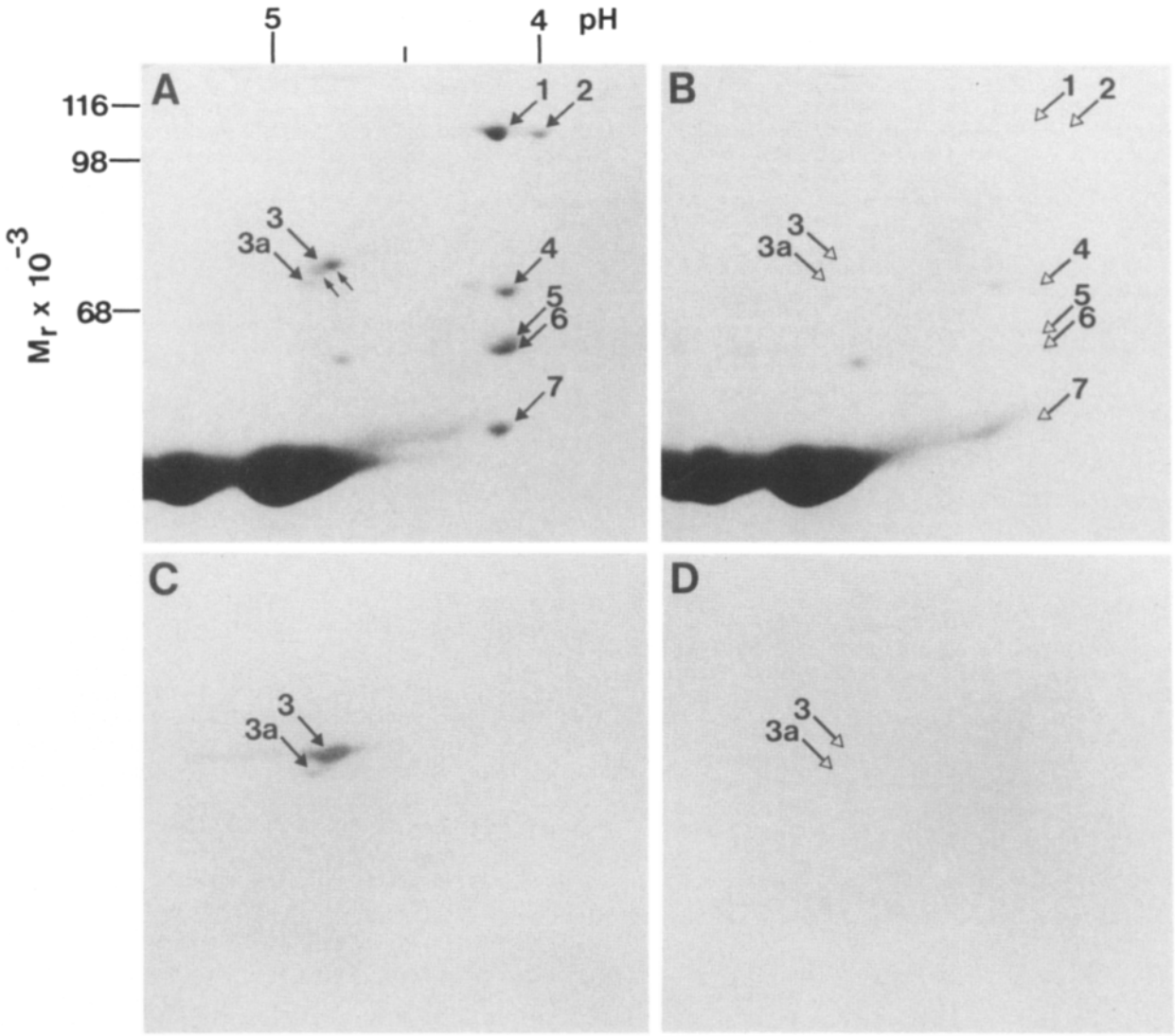

Figure 1. Wild-type and *pf-14* axonemal polypeptides resolved by two-dimensional gel electrophoresis and stained with Coomassie blue (*A* and *B*) or, after transfer to nitrocellulose, stained instead with an affinity-purified antiserum raised against gel-purified spoke protein 3 (*C* and *D*). Only a portion of each gel and blot is shown. (*A*) Wild-type axonemes with Coomassie blue stain. Numbered solid arrows point to radial spoke proteins 1–7, which are visible in this region of the gel. The major protein 3 isoforms appear as two closely apposed spots (*small arrows*); a third, minor isoform 3a, is better resolved and is labeled separately here and in subsequent figures. (*B*) *pf-14* axonemes with Coomassie blue stain. Open arrows indicate that spoke proteins 1–7 are missing, including isoform 3a. (*C*) Wild-type axonemes in an immunoblot stained with anti-protein 3. Comparison of *C* with *A* shows that the antiserum recognizes the major protein 3 isoforms and minor isoform 3a (*C*, *arrows*). (*D*) *pf-14* axonemes in an immunoblot stained with anti-protein 3. Major protein 3 isoforms and minor isoform 3a are not detected (*open arrows*).

protein 3 gene from a *Chlamydomonas* λgt11 expression library (Fig. 2 *A*, *18S* and *18SIII*), and one of these clones (18SIII) was subsequently used as a hybridization probe to isolate complete wild-type genomic clone 8D(wt) from an EMBL 4 genomic library (Fig. 2 *A*). A Kpn I–Eco RI fragment containing the entire transcription unit was then subcloned, creating plasmid pKE-RS3(wt) (Fig. 2 *B*). The orientation of transcription was deduced, and the 5′ and 3′ ends of the transcription unit were roughly mapped (see Materials and Methods). Restriction fragments spanning the 5′ half of the transcription unit (Fig. 2 *B*, *probes B and C*) were used to isolate a 2.3-kb cDNA from a *Chlamydomonas* cDNA library in λgt11. A map of cDNA insert cRS3-2(wt) is shown in Fig. 2 *C*.

To determine gene copy number and identify rearrangements that may have been introduced by the *pf-14* mutation, a Bgl I–Pvu II restriction fragment from the 3′ half of the *pf-14* transcription unit (Fig. 2 *B*, *probe A*) was nick translated and hybridized to blots of restricted DNA from a wild-type strain and a strain carrying the *pf-14* mutant allele. As shown in Fig. 3, the probe hybridized to a single band of characteristic size for each restriction digest, and no differences in the size or number of bands was seen between wild-type and mutant samples. These results demonstrate that the protein 3 gene is single copy and suggest that it has not undergone major rearrangement in mutant *pf-14* cells.

Data supporting the identification of the cloned gene are provided by restriction fragment-length polymorphism anal-

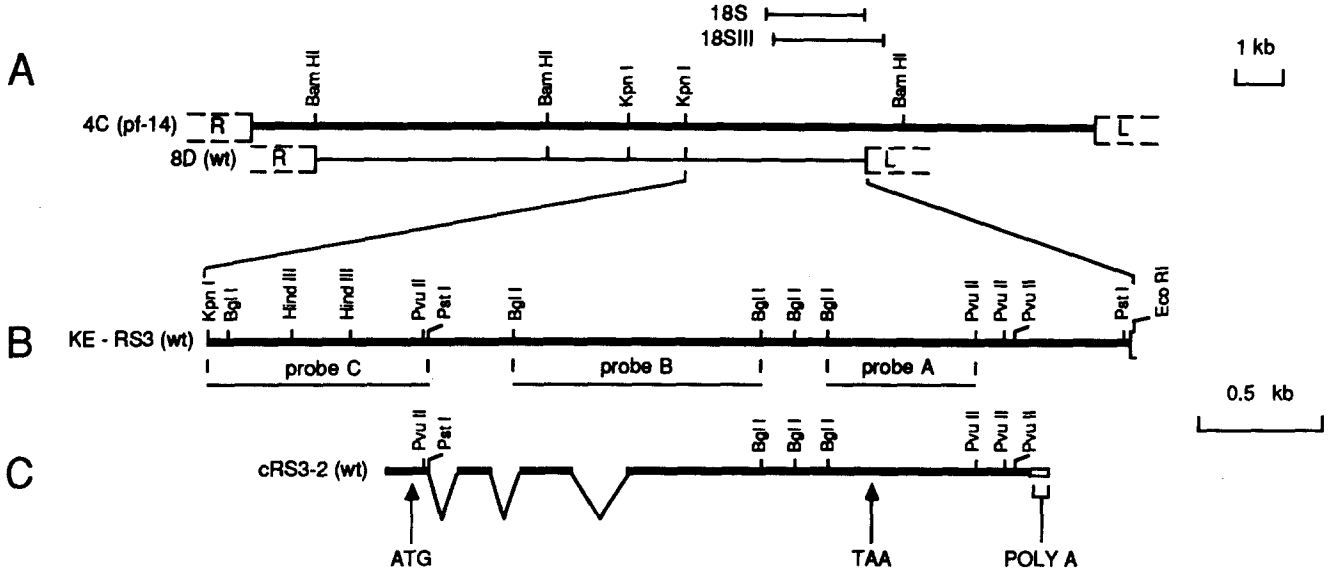

Figure 2. Restriction maps of genomic and cDNA clones coding for radial spoke protein 3. (*A*) 18S and 18SIII, genomic inserts recovered from λgt11 expression library using an antiserum against gel-purified spoke protein 3. 8D(wt) and 4C(*pf-14*), genomic clones recovered from EMBL 4 libraries of wild-type and mutant *pf-14* DNA, respectively, by hybridization to insert 18SIII. *R* and *L*, the right and left arms of the λ vector DNA, respectively. (*B*) KE-RS3(WT), Kpn I–Eco RI restriction fragment of clone 8D(wt) containing the entire transcription unit. Restriction fragment designated probe A was gel-purified, nick-translated and used to probe Southern and Northern blots shown in Figs. 3 and 6. Fragments designated probe B and probe C were treated similarly and used to screen a *Chlamydomonas* cDNA library in λgt11. (*C*) cRS3-2(wt), full-length cDNA insert corresponding to the wild-type transcript. The cDNA map is aligned with the genomic map in *B*; gaps indicate placement of IVSs as determined by DNA sequencing (see text). Positions corresponding to translation initiation (*ATG*), termination (*TAA*), and polyadenylation (*POLY A*) are also indicated.

ysis. The cloned sequence maps to the right arm of linkage group VI, a position that corresponds to the *pf-14* genetic locus (31).

Polypeptide 3a Is the Unmodified Product of the Protein 3 Gene

To verify the identity of the cloned gene and confirm that cDNA insert cRS3-2(wt) contains the complete protein coding region, the insert was subcloned into transcription vector Bluescript KS-, transcribed with T3 polymerase in vitro, and the resulting RNA translated in vitro in the rabbit reticulocyte lysate. The [^{35}S]methionine-labeled translation products were coelectrophoresed with unlabeled wild-type axonemal proteins on two-dimensional gels. Comparision of the Coomassie blue–stained gel (Fig. 4 *A*) with the autoradiograph (Fig. 4 *B*) shows that the major translation product comigrates with polypeptide 3a. Interestingly, a small amount of translation product that comigrates with the two unresolved major flagellar isoforms of protein 3 (Fig. 4 *B*, *3*) is also evident and is apparently the fraction of unmodified product that has been phosphorylated by an endogenous kinase in the rabbit reticulocyte lysate. Phosphorylation of a heterologous protein synthesized in the reticulocyte lysate has been reported previously (10).

Essentially identical results were obtained when RNA coding for protein 3 was hybrid selected from whole-cell RNA, using genomic subclone pl8SIII, and then translated in vitro (Fig. 4 *C*). Together these results suggest that we have cloned the structural gene for spoke protein 3, and that polypeptide 3a is the unmodified gene product.

Transcript from the Mutant pf-14 Allele Codes for an Altered Polypeptide 3

Dikaryon rescue experiments with mutant *pf-14* cells (15, 22) indicate that they lack assembly-competent protein 3, but whether the polypeptide is not synthesized or, alternatively, an assembly-incompetent protein is produced has not been determined. To see if the mutant *pf-14* allele codes for a protein with altered primary structure, we hybrid selected the mutant transcript with subclone pl8SIII and then translated it in vitro. Fig. 4 *D* shows an autoradiograph of a two-dimensional gel that was loaded with a mixture of ^{35}S-labeled translation products from the wild-type and mutant hybrid-selected RNAs. Comparison to the translation products of the wild-type RNA alone (Fig. 4 *C*) shows that a novel polypeptide, labeled 3m, is produced by the mutant transcript. This polypeptide has an isoelectric point of 4.85, 0.1 pH units more acidic than wild-type protein 3a, and an apparent molecular mass of 69 kD, ~5.5 kD lower than the value measured for wild-type polypeptide.

A small amount of second polypeptide is also produced in the translation products of the mutant protein 3 RNA (Fig. 4 *D, small arrow*). As already described for the synthesis of wild-type protein 3 in vitro, this may be a small fraction of mutant protein 3m that has been phosphorylated by a heterologous kinase of the rabbit reticulocyte lysate.

Mutant Protein 3m Is Maintained in a Cytoplasmic Pool of Unassembled Radial Spoke Precursors

Mutant protein 3m is not detected in immunoblots of axonemal polypeptides from mutant *pf-14* cells (Fig. 2 *D*) or in

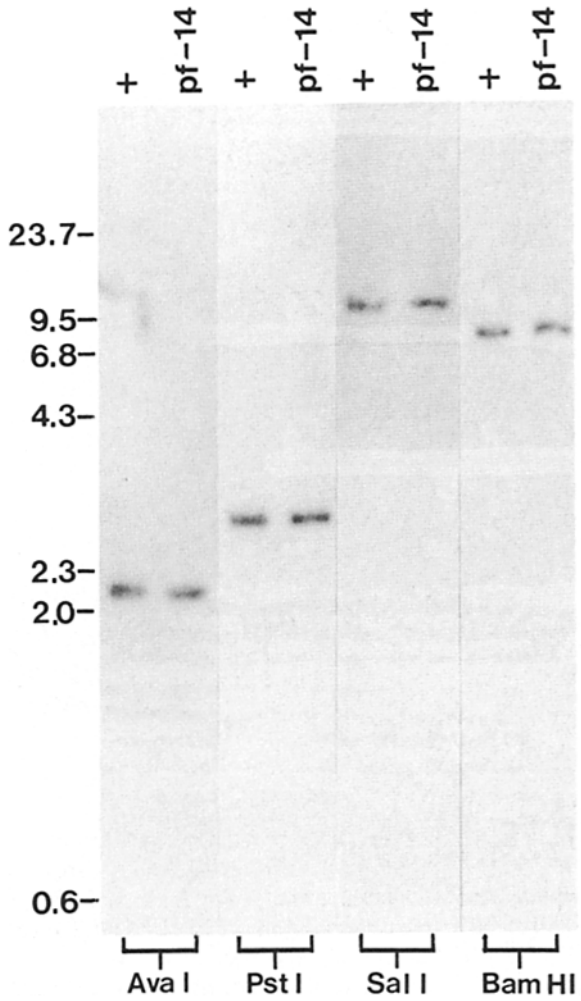

Figure 3. Southern blots of total DNA from wild-type and *pf-14* cells hybridized to a radiolabeled restriction fragment of the gene coding for radial spoke protein 3. Parallel digests of wild-type and mutant DNA were performed with single restriction enzymes. The restricted DNA was size fractionated in adjacent lanes of a 1% agarose gel, blotted to nitrocellulose, and then hybridized to a gel-purified and nick-translated Bgl I–Pvu II fragment (Fig. 2, *probe A*). Source of total DNA is shown at the top of each lane: (+) wild type; (*pf-14*) mutant *pf-14*. The restriction enzyme is shown at the bottom of the lane. Note that a single hybridizing band is detected for each digest, and there is no discrepancy in band size between adjacent lanes loaded with wild-type and mutant *pf-14* DNA. Size markers indicated are Hind III restriction fragments of λ DNA.

similar immunoblots prepared from mutant whole flagella (data not shown). These results suggest that if protein 3m is actually synthesized by the mutant cells, it does not assemble into the flagellum. Since dikaryon rescue experiments have shown that the 16 radial spoke polypeptides not directly affected by the *pf-14* mutation are maintained unassembled in the cell body, we decided to try and detect mutant protein 3m in this cytoplasmic pool. To do this we used the antiserum against spoke protein 3 to probe immunoblots of total cell protein. Fig. 5, *A* and *B*, shows identically prepared immunoblots from wild-type and mutant *pf-14* cells, respectively, that were probed simultaneously with affinity-purified anti–protein 3 serum and with an affinity-purified antiserum A (38), which recognizes spokehead proteins 4 and 6. Since proteins 4 and 6 should be unassembled and in the mutant *pf-14* cell body, their detection in the mutant blot serves as a positive internal control for gel loading and for protein transfer to nitrocellulose. Although detected in the blot of wild-type proteins (Fig. 5 *A*), the major flagellar isoforms of protein 3 and unmodified isoform 3a are specifically absent from the mutant blot (Fig. 5 *B*). They are apparently replaced by a small amount of mutant protein 3m. This result demonstrates that protein 3m is actually synthesized by *pf-14* cells, and since it is not detected in mutant axonemes (Fig. 2, *B* and *D*) or whole flagella (data not shown), it apparently cannot assemble.

The relatively small amount of protein 3m that is present in mutant whole-cell protein is intriguing and may indicate that the unassembled protein is either unstable or is synthesized inefficiently (see below).

Accumulation of the Mutant Transcript Is Normal during Flagellar Regeneration

When *Chlamydomonas* is experimentally deflagellated it immediately begins to regrow two new flagella and, during the ~90-min flagellar regeneration response, cells transiently accumulate elevated levels of RNA coding for flagellar proteins (5, 20, 25, 34, 35, 38). To address the possibility that the relatively low amount of protein 3m detected by immunoblot (Fig. 5 *B*) is due to a defect in accumulation of the mutant transcript, a Bgl I–Pvu II restriction fragment (Fig. 1 *B*, *probe A*) from wild-type genomic subclone pKE-RS3(wt) was nick translated and hybridized to blots of size-fractionated whole-cell RNA isolated from wild-type and mutant *pf-14* cells before and 25 min after flagellar amputation. Fig. 6 *A* shows that after flagellar amputation, wild-type and *pf-14* cells accumulate similar amounts of a single 2.3-kb transcript from the protein 3 gene. The blots were reprobed with nick-translated constitutive cDNA clone pcf2-40 (34) to demonstrate that approximately equal amounts of RNA were loaded in each lane (Fig. 6 *B*). Analysis of time points spanning the entire 2-h flagellar regeneration response (data not shown) indicates no significant differences between the wild-type and *pf-14* cells and shows that the RNA coding for spoke protein 3 accumulates and then declines with kinetics very similar to those previously measured for transcripts of other radial spoke, dynein arm, and tubulin genes (38).

Since synthesis and accumulation of transcripts coding for protein 3 do not appear to be significantly altered by mutation *pf-14*, an alternative explanation for the small amounts of mutant protein 3m in *pf-14* cells must be sought.

Sequence of Wild-type and pf-14 Alleles of the Protein 3 Gene Reveals a Nonsense Mutation

Sequences were determined for the Hind III–Eco RI fragment of clone 8D(wt), containing the wild-type allele of the protein 3 gene, and the cDNA insert cRS3-2(wt), corresponding to the wild-type transcript. The sequence data is presented in Fig. 7, including the deduced primary structure of wild-type protein 3.

An open reading frame in the cDNA sequence extends from the first ATG triplet (codons will be written with T residues instead of U residues for the remainder of the paper in as much as the sequence data presented here were deter-

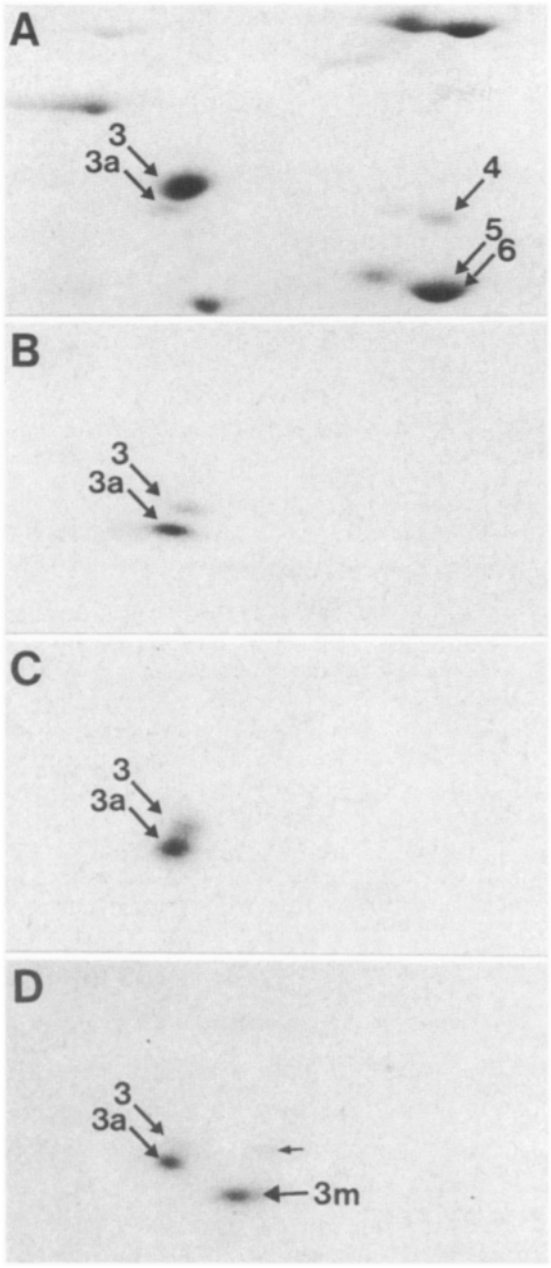

Figure 4. In vitro synthesis of radial spoke protein 3 from a full-length wild-type cDNA and from RNA isolated by hybridization selection from wild-type and mutant *pf-14* cells. cDNA cRS3-3(wt) was subcloned into transcription vector Bluescript KS- and transcribed in vitro with T3 polymerase. The resulting RNA was translated in vitro in the rabbit reticulocyte lysate, and the ^{35}S-labeled translation products were subsequently coelectrophoresed with unlabeled axonemal proteins that served as a standard. For hybridization-selection experiments, RNA was selected by hybridization to a portion of the cloned protein 3 gene, translated, and the products were electrophoresed as described above. (*A*) Coomassie blue stain showing positions of unlabeled radial spoke proteins. The two major isoforms of spoke protein 3 are not clearly resolved from each other and appear as a single large spot; minor isoform 3a is resolved and appears as a single small spot. Positions of spoke proteins 4, 5, and 6 are also indicated. (*B*) Autoradiograph showing radiolabeled translation products of RNA transcribed in vitro from plasmid pcRS3-3(wt). Comparison of *B* with protein-stained gel in *A* shows that the predominant translation product comigrates with isoform 3a. A small amount of product that comigrates with the two

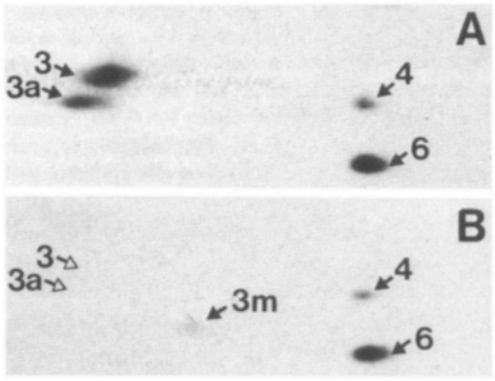

Figure 5. Total cell protein from wild-type and mutant *pf-14* cells resolved by two-dimensional gel electrophoresis, blotted to nitrocellulose, and stained with a mixture of antibodies to spoke proteins 3, 4, and 6. Only a portion of each blot is shown. (*A*) Wild-type whole-cell protein. All three isoforms of spoke protein 3 are detected: the two major flagellar isoforms (these are not well resolved from each other and appear as a single spot) and isoform 3a. Spoke proteins 4 and 6 are also detected. (*B*) Mutant *pf-14* whole-cell protein. The major isoforms of protein 3 and minor isoform 3a are not detected, and apparently replacing them is a relatively small amount of mutant protein 3m. Note that signals for radial spoke proteins 4 and 6 do not vary significantly between the blots of wild-type and mutant proteins.

mined from DNA), which occurs in a context favorable for translational initiation (17, 18), and codes for a 516-residue polypeptide with a calculated molecular mass of 56,748 D and a calculated isoelectric point of 4.62. Although the calculated isoelectric point is relatively close to value of 4.95 measured for isoform 3a on two-dimensional gels, the calculated molecular mass is significantly lower than the measured value of 74.5 kD. This discrepancy suggests that protein 3a migrates anomalously on SDS-acrylamide gels.

Computer searches of the National Biomedical Research Foundation's Protein Identification Resource failed to find any significant similarities between the protein 3 amino acid sequence and sequences previously published (see Materials and Methods).

To identify the molecular lesion in the *pf-14* mutant allele, we constructed an EMBL 4 genomic library for a *Chlamydomonas* strain carrying the *pf-14* mutation, and used genomic clone 18SIII as a hybridization probe to recover phage 4C(*pf-14*) (Fig. 1 *A*). A 4-kb Hind III–Bam HI fragment that contains the entire protein 3 transcription unit was subcloned and sequenced. Comparison with the wild-type sequence re-

unresolved major flagellar isoforms of 3 is also evident. (*C*) Fluorograph showing radiolabeled translation products of hybrid-selected RNA from wild-type cells. As in *B*, the predominant product comigrates with isoform 3a, and a small amount of protein comigrating with the major isoforms of 3 is also present. (*D*) Fluorograph showing coelectrophoresis of translation products of RNA hybrid selected from wild-type and mutant *pf-14* cells. Comparison of *D* with *C* shows that the predominant product from the mutant RNA is a novel polypeptide, 3m, that is more acidic than isoform 3a and has a lower apparent molecular mass. The mutant RNA also produces a small amount of a second novel polypeptide (*small arrow*).

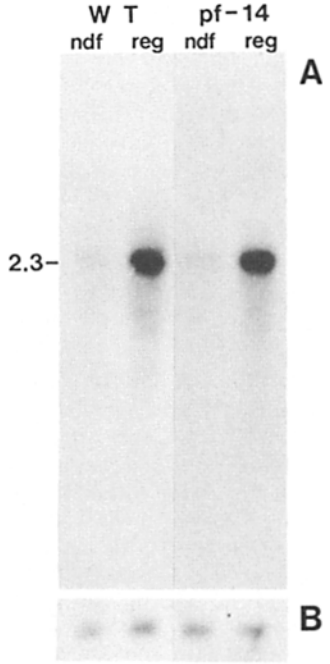

Figure 6. Relative abundance of protein 3 transcripts before and after deflagellation in wild-type and mutant *pf-14* cells. (*A*) Total RNA was isolated from nondeflagellated cells (*ndf*), and deflagellated cells that were allowed 25 min to begin regenerating new flagella (*reg*). 10 μg of each RNA sample was size fractionated on an agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized to a gel-purified and nick-translated Bgl I–Pvu II fragment from the protein 3 gene (Fig. 2, *probe A*). The pair of lanes on the left (*WT*) were loaded with the wild-type RNA samples; the pair of lanes on the right (*pf-14*) were loaded with RNA from *pf-14* cells. A single 2.3-kb RNA that greatly increases in abundance during flagellar regeneration is detected in both wild-type and mutant *pf-14* cells. Migration of the 2.3-kb Hind III fragment of λ DNA is indicated on the left. (*B*) To verify that lanes were loaded with approximately equivalent amounts of RNA, the blot shown in (*A*) was stripped and reprobed with radiolabeled plasmid pcf2-40, which hybridizes to a constitutively expressed RNA (34). Only the region of the autoradiograph showing hybridization to the constitutive RNA is shown.

*Table I. Codon Usage in the Gene for Radial Spoke Protein 3**

Ala	GCT	5	His	CAT	0	Ser	TCT	1
	GCC	31		CAC	6		TCC	2
	GCA	5					TCA	0
	GCG	51	Ile	ATT	4		TCG	4
				ATC	12		AGT	0
Arg	CGT	4		ATA	0		AGC	5
	CGC	20						
	CGA	1						
	CGG	13	Leu	TTA	0	Thr	ACT	0
	AGA	0		TTG	2		ACC	9
	AGG	1		CTT	0		ACA	6
				CTC	2		ACG	21
Asn	AAT	1		CTA	0			
	AAC	7		CTG	32			
						Trp	TGG	3
Asp	GAT	5						
	GAC	24	Lys	AAA	0			
				AAG	27	Try	TAT	2
Cys	TGT	0					TAC	8
	TGC	0						
			Met	ATG	11			
Gln	CAA	5				Val	GTT	2
	CAG	21					GTC	5
			Phe	TTT	6		GTA	2
Glu	GAA	0		TTC	6		GTG	33
	GAG	65						
						End	TAA	1
Gly	GGT	2	Pro	CCT	1		TGA	0
	GGC	15		CCC	13		TAG	0
	GGA	1		CCA	0			
	GGG	0		CCG	14			

* Number of times each codon occurs.

veals only one discrepancy: the C residue at position +61 in the wild-type allele is replaced with a T residue in the mutant *pf-14* allele (Fig. 7, *pf-14*). This transition alters codon 21 from CAA to TAA, so that instead of specifying glutamine, it terminates translation.

Mutant protein 3m has an apparent molecular mass of 69 kD on two-dimensional protein gels (see above). Ribosomes could synthesize a protein of this size from the mutant transcript by initiating translation at an ATG codon downstream from the nonsense mutation. The first downstream ATG, at position +197 (Fig. 7, *bold underline*), is in a favorable context for translational initiation (17, 18), but can be eliminated from consideration because it is not in the correct reading frame. Ribosomes initiating at this point can only elongate 17 codons before encountering a termination signal. The next downstream ATG, at codon 42 (Fig. 7, *box*), also occurs in a favorable context for translational initiation and is in the correct protein 3 reading frame. Ribosomes initiating here should produce a truncated polypeptide missing the first 41 amino-terminal residues. Since these residues have a net charge of +2, the truncated protein should be more acidic. The calculated isoelectric point and molecular mass for the truncated protein are 4.51 and 52.1 kD, respectively, which are 0.1 pH units more acidic and 4.8 kD smaller than the calculated values for the complete wild-type polypeptide. These differences correspond quite closely to those measured between wild-type protein 3a and mutant protein 3m on two-dimensional protein gels (Δ pI = 0.1, M_r = 5.5 kD, see above), suggesting that codon 42 is the probable initiation site of protein 3m. In fact, codon 42 is the only likely candidate because all remaining downstream ATG codons start open reading frames that are too short to code for the mutant polypeptide.

It should be noted that translational initiation at codon 42 is likely to be relatively inefficient (see Discussion). This may explain the reduced levels of mutant protein 3m observed in *pf-14* cells (Fig. 5) even though the mutant RNA appears to be present at normal levels (Fig. 6).

Codon Usage and Gene Structure

Codon usage for the protein 3 gene is biased (Tables I and II); most notably, a G or a C residue occurs much more frequently in the third position than an A or a T residue. This correlates with the high overall G and C content (64%) of *Chlamydomonas* nuclear DNA (7). A similar bias has been

Table II. Percentage of Each Base at Each Codon Position

	Codon position		
Base	1	2	3
	%	%	%
A	20.1	33.2	4.0
T	6.7	22.6	6.3
G	47.5	12.5	57.6
C	25.5	31.5	31.9

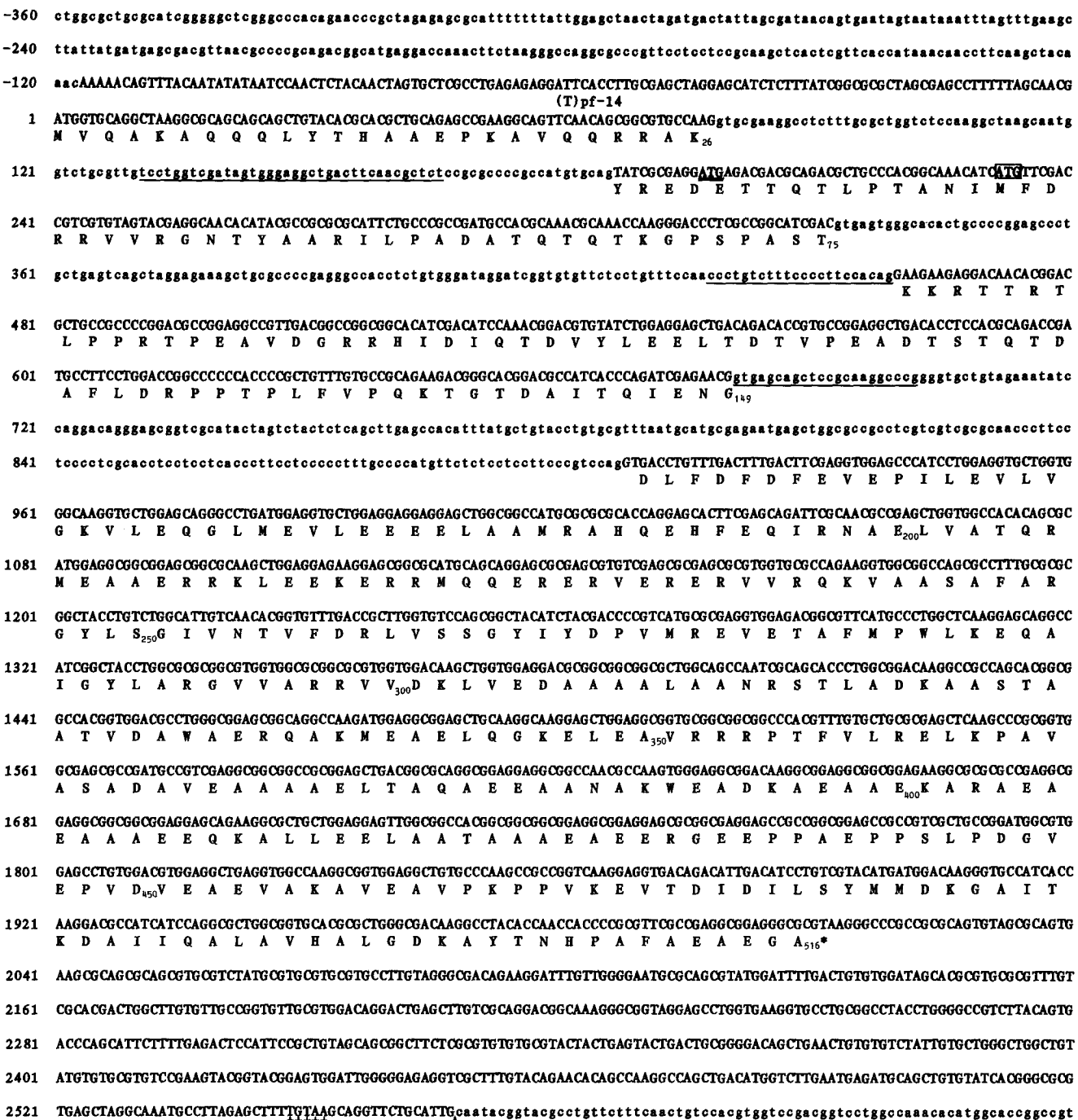

Figure 7. Nucleotide sequence of the wild-type and mutant *pf-14* alleles of the radial spoke protein 3 gene and deduced amino acid sequence. Data are derived from analysis of genomic and cDNA clones; upper case indicates sequences contained in cDNA cRS3-2(wt). The nucleotide designated +1 corresponds to the first residue of the putative translation initiation site. Comparison of complete genomic sequences for the wild-type with mutant *pf-14* alleles reveals only a 1-bp difference: the C residue at position +61 in the wild-type gene is replaced by a T residue in the mutant gene (*[T] pf-14*). This change converts codon 21 from CAA to the ochre termination signal TAA. The first ATG triplet downstream from the mutation is underscored (*thick line*). The second ATG triplet, codon 42 (*box*), corresponds to the postulated site for translational initiation of mutant protein 3m. IVS sequences similar to conserved IVS 3 of *Chlamydomonas* β-tubulin genes (40) (*thin lines*), a possible polyadenylation signal (+), and the site of polyadenylation (*arrow*) are also highlighted. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide data bases under the accession number X14549.

found in other *Chlamydomonas* nuclear genes (11, 24, 36, 40, 41).

Comparison of the cDNA with genomic sequences reveals three intervening sequences (IVSs)[1] positioned in the 5′ half of the transcription unit and having lengths of 108 (IVS 1), 127 (IVS 2), and 227 (IVS 3) bp. IVS 1 is positioned between codons 26 and 27, while IVSs 2 and 3 interrupt codons 75 and 149, respectively. The splice junctions for each IVS are homologous to the eucaryotic (3) and *Chlamydomonas* (41)

1. *Abbreviation used in this paper*: IVS, intervening sequence.

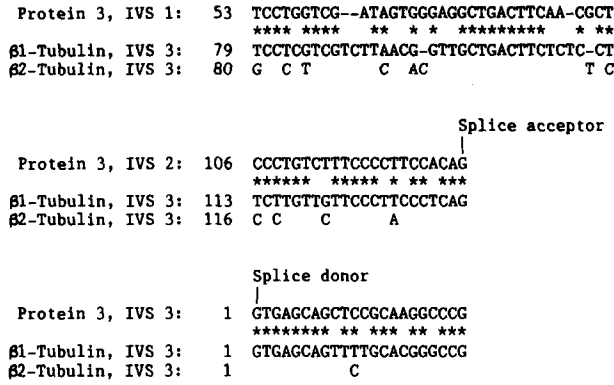

Figure 8. Sequence similarities between IVSs of the radial spoke protein 3 gene and the highly conserved IVS 3 of the Chlamydomonas β1- and β2-tubulin genes. Only the relevant part of each IVS is shown. Sequences are oriented 5′ to 3′ with respect to the sense strand, and the number to the left indicates the position within the IVS of the first nucleotide shown. Both 5′ and 3′ splice junctions are indicated where appropriate. The tubulin gene sequences are taken from Youngblom et al. (40). The β1-tubulin sequence is listed; only deviations from this sequence are indicated for the β2-tubulin gene. Asterisks indicate a match between the protein 3 gene sequence and either the β1- or β2-tubulin sequences. Dashes indicate gaps inserted to maximize sequence alignment.

consensus sequences. Other than these conserved regions, no sequence similarities were seen between the three IVSs.

Surprisingly, each IVS of the protein 3 gene contains a different short region of sequence similarity to the highly conserved IVS 3 of the two *Chlamydomonas* β-tubulin genes (40) (Fig. 7, *thin underlines*). The region of similarity in IVS I of the protein 3 gene (35 bp) starts 56 bp upstream from the splice acceptor site and closely resembles the sequence starting the same distance upstream from the splice acceptor site within the conserved β-tubulin IVS. This region is also similar to a short conserved sequence occurring at an analogous position within IVS 2 of the two *Chlamydomonas* α-tubulin genes (36).

The regions of similarity in IVS 2 (22 bp) and IVS 3 (22 bp) of the protein 3 gene are directly adjacent to the splice acceptor and donor sites, respectively, and resemble the sequences occurring at analogous positions within the conserved β-tubulin IVS.

Fig. 8 shows a direct comparison of the regions of similarity between the protein 3 gene IVSs with the conserved β-tubulin IVS 3. Existence of these similarities between unrelated genes suggest they may have some specific function in transcript processing or in transcriptional regulation.

Comparison of the genomic and cDNA sequences of the protein 3 gene reveals that the site of 3′ polyadenylation occurs 554 bp downstream from the termination codon, and 15 bp downstream from the putative polyadenylation signal TGTAA (Fig. 7), which is conserved in every *Chlamydomonas* nuclear gene that has been sequenced to date (11, 24, 36, 40, 41).

Discussion

Since mutation *pf-14* introduces a translation termination signal at codon 21 of the protein 3 gene, translational "reinitiation" as described by Kozak (19) offers the simplest explanation for the slightly truncated mutant protein we have detected. Her analysis suggests that when eucaryotic ribosomes terminate translation they remain associated with the mRNA, "scan" downstream, and can then reinitiate protein synthesis at an ATG triplet in the appropriate sequence context. Kozak's analysis also indicates that reinitiation occurs at the first appropriate ATG triplet unless it is too close to the termination signal (efficiency of reinitiation apparently begins to decline when the distance between the termination signal and the ATG triplet is <79 bp). In the latter case a significant fraction of the ribosomes apparently fail to reinitiate at this point and can continue scanning downstream where they reinitiate at the next appropriate ATG triplet. This second scenario provides a model for the synthesis of the mutant protein 3m. After translation is terminated at the mutant nonsense codon (creating a 21-residue polypeptide) ribosomes may scan downstream, and then some may pass over the intervening ATG triplet, just 28 bp away from the mutation, and subsequently reinitiate synthesis at codon 42, the next ATG triplet. This triplet is the best candidate for the reinitiation site because it occurs in the appropriate sequence context for translational initiation (17, 18), and the size and charge alterations of the mutant protein closely match those that would be introduced by deleting the first 41 amino acids of the wild-type polypeptide (see Results).

Since the efficiency of reinitiation at codon 42 is likely to be low due to the intervening ATG and to codon 42's proximity to the mutant termination signal (19) (only 60 bp separates the two in the mature mRNA), the translational reinitiation model also provides an explanation for the dramatically reduced levels of the mutant protein in spite of apparently normal amounts of the corresponding transcript that accumulates when mutant *pf-14* cells regenerate flagella (Fig. 6).

Alternatively, the small amount of mutant protein 3m may be due to its instability within the *Chlamydomonas* cell body. This possibility cannot be excluded by the data presented here, but independent lines of experimental evidence suggest that unassembled radial spoke proteins are normally quite stable within a cytoplasmic pool of flagellar precursors. First, wild-type cells maintain a pool of unassembled flagellar proteins sufficient to assemble two functional half-length flagella in the absence of protein synthesis (32). This result indicates that the pool contains all of the radial spoke proteins, including protein 3. Second, results from dikaryon rescue experiments suggest that mutant *pf-14* cells maintain the unassembled radial spoke proteins in the cell body (15, 22). This was demonstrated directly for two of the radial spoke proteins in immunoblot experiments reported here. No significant difference was detected between wild-type and *pf-14* cells in the amount of spoke proteins 4 and 6 in whole-cell protein blots (Fig. 5), and since components 4 and 6 are not assembled into the mutant flagella (15, 29) this result indicates that they simply accumulate within the cell body when radial spoke assembly is blocked. Although the data just summarized suggest that an unassembled mutant protein 3 should be maintained by the cell, they do not rule out the possibility that some novel characteristic of the mutant protein targets is specifically for degradation.

As mentioned in the introduction, it has been suggested that spoke protein 3 may attach the radial spoke to the outer doublet microtubule (22). Since our data show that a mutant protein 3 is actually synthesized and maintained within the cell body, albeit at reduced levels, it is tempting to speculate

that the complete failure of *pf-14* cells to assemble radial spokes is due to the inability of the mutant protein 3 to bind to the outer doublet microtubule A-subfiber and to the consequential failure of the more distal radial spoke proteins to assemble. This conclusion is premature, however, since there is no direct evidence to date that protein 3 actually attaches the spoke to the microtubule. Alternatively, it is possible that protein 3 does not form the attachment but is simply required to form a stable spoke structure (22). The anti-protein 3 serum generated in this study may be useful in resolving this issue since quick-freeze, deep-etch electron microscopy can provide detailed views of radial spoke structure (12) and may allow visualization of antibodies bound to radial spokes. This approach could be used to directly determine the position of protein 3 within the spoke structure.

It should be noted that the *pf-14* mutation is the first characterized example of an ochre nonsense mutation in a *Chlamydomonas* nuclear gene. Screens for revertants may allow recovery of ochre tRNA suppressor mutations that would be generally useful for genetic analysis in this organism.

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