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Cloning of Flagellar Genes in *Chlamydomonas reinhardtii* by DNA Insertional Mutagenesis

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ABSTRACT

Chlamydomonas is a popular genetic model system for studying many cellular processes. In this report, we describe a new approach to isolate Chlamydomonas genes using the cloned nitrate reductase gene (*NIT1*) as an insertional mutagen. A linearized plasmid containing the *NIT1* gene was introduced into *nit1* mutant cells by glass-bead transformation. Of 3000 Nit^+ transformants examined, 74 showed motility defects of a wide range of phenotypes, suggesting that DNA transformation is an effective method for mutagenizing cells. For 13 of 15 such motility mutants backcrossed to nit^- mutant strains, the motility phenotype cosegregated with the Nit^+ phenotype, indicating that the motility defects of these 13 mutants may be caused by integration of the plasmid. Further genetic analysis indicated that three of these mutants contained alleles of previously identified loci: *mbo2* (*m*ove *b*ackward *o*nly), *pf13* (*p*aralyzed *fl*agella) and *vfl1* (*v*ariable *fl*agellar number). Three other abnormal-flagellar-number mutants did not map to any previously described loci at which mutations produce similar phenotypes. Genomic sequences flanking the integrated plasmid in the *mbo2* and *vfl1* mutants were isolated and used as probes to obtain wild-type genomic clones, which complemented the motility defects upon transformation into cells. Our results demonstrate the potential of this new approach for cloning genes identified by mutation in Chlamydomonas.

THE unicellular green alga, *Chlamydomonas reinhardtii*, has been widely used as a model system for studying the genetic and molecular mechanisms of a number of cellular processes. Our focus is on the development and function of the flagellar apparatus. Each Chlamydomonas cell has two flagella that are attached to two basal bodies inside the cell. Common to all eukaryotic flagella and cilia is the flagellar axoneme, which is composed of an array of nine doublet microtubules surrounding a pair of singlet microtubules and a number of accessory structures arranged regularly along the length of the organelle. Flagella are complex structures containing more than 200 different polypeptides (PIPERNO *et al.* 1981). So far, more than 270 mutations covering over 85 genetic loci affecting the function or assembly of this organelle have been identified in Chlamydomonas (DUTCHER and LUX 1989). However, due to the limited approaches that have been available for cloning genes in Chlamydomonas, the protein products of many of these genes remain uncharacterized.

Recently, techniques for efficient transformation of Chlamydomonas using cloned Chlamydomonas genes such as nitrate reductase (FERNANDEZ *et al.* 1989; KINDLE *et al.* 1989; KINDLE 1990), oxygen-evolving enhancer (MAYFIELD and KINDLE 1990), argininosuccinate lyase (DEBUCHY, PURTON and ROCHAIX 1989) and radial-spoke-protein 3 (DIENER *et al.* 1990) have become available. Chlamydomonas can be stably and efficiently transformed by simply agitating cells and DNA with glass beads (KINDLE 1990). The DNA is integrated in low copy number and can be maintained in the absence of selection for an indefinite number of generations (KINDLE *et al.* 1989; KINDLE 1990). Moreover, the integration of the transforming DNA into the nuclear genome occurs almost exclusively at nonhomologous sites (KINDLE *et al.* 1989). If such nonhomologous integration events are random, the transforming DNA may act as an insertional mutagen to disrupt functional genes. Such insertional mutations are potentially very useful because they may facilitate cloning of the genes that are disrupted and tagged by the exogenous DNA. Insertional mutagenesis by DNA transformation has been successfully used for gene isolation in mouse (WOYCHIK *et al.* 1990; SINGH *et al.* 1991), Arabidopsis (FELDMAN *et al.* 1989; YANOFSKY *et al.* 1990), fungi (DIALLINAS and SCAZZOCCHIO 1989; TILBURN, ROUSSEL and SCAZZOCCHIO 1990; KANG and METZENBERG 1993) and Dictyostelium (KUSPA and LOOMIS 1992). Several factors make it highly feasible to test this strategy for isolating genes in Chlamydomonas. First, thousands of transformants can easily be generated. Second, Chlamydomonas has a haploid genome, allowing the immediate phenotypic expression of nonlethal mutations. Third, genetic techniques including tetrad analysis are routine in Chlamydomonas, so that mutations created by DNA transformation can readily be analyzed.

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Here we describe the use of this new approach to disrupt and clone genes required for flagellar assembly and function in *C. reinhardtii*. pMN24, a plasmid containing the wild-type structural gene for nitrate reductase (*NIT1*; FERNANDEZ *et al.* 1989; KINDLE *et al.* 1989), was transformed into a *nit1* mutant strain to generate motility mutants. Genetic analysis of these mutants indicates that DNA transformation is an effective approach for mutagenesis and gene tagging. To demonstrate the procedure, we describe the cloning of two motility genes, *MBO2* (*m*ove *b*ackward *o*nly) and *VFL1* (*v*ariable *fl*agellar number), using the pMN24 inserts as tags.

MATERIALS AND METHODS

Strains and cell cultures: Strain 5D, containing a *nit1-305* mutation in the nitrate reductase structural gene and a *cw15* mutation which confers a cell-wall-less phenotype was used for transformation. Strains L5 (*nit1, apm1-19, mt*$^+$), L8 (*nit1, apm1-19, mt*$^-$) and B22 (*nit2, ac17, mt*$^+$) were used as parents in backcrosses to different motility mutants. (*apm1-19*, which confers resistance to amiprophos-methyl (APM), maps to linkage group XIX; JAMES *et al.* 1988.) For cotransformation experiments to test whether a genomic clone could complement a motility mutation, double mutant strains containing *arg7* (derived from strains described in LUX and DUTCHER 1991) and the mutation of interest were constructed. Strains CC1388 (*vfl1, mt*$^+$), CC1686 (*vfl3, mt*$^+$), CC1687 (*vfl3, mt*$^-$), CC2530 (*vfl2, mt*$^+$), CC2531 (*vfl2, mt*$^-$), CC2376 (*mbo1, mt*$^+$), CC2679 (*mbo1, mt*$^+$), CC2377 (*mbo2, mt*$^-$) and CC2378 (*mbo3, mt*$^+$) were provided by the Chlamydomonas Genetic Center, Duke University, Durham, North Carolina. (All of these strains also carry both *nit1* and *nit2* mutations.) Strain B8 (*pf13, nit1, mt*$^+$) was mated to strain 7-4 (a paralyzed-flagella mutant) for construction of diploids (both strains were provided by R. A. SCHNELL). Unless otherwise stated, cells were grown either in minimal (M) medium I (SAGER and GRANICK 1953) or in TAP (GORMAN and LEVINE 1965) and supplemented with 0.005% arginine when necessary. Nit$^+$ transformants were selected on M medium in which NH_4NO_3 was replaced by 4 mM KNO_3 (MNO_3 medium) and Arg$^+$ transformants were selected on M medium. Nit$^-$ cells were grown in SGII liquid medium (SAGER and GRANICK 1953; KINDLE 1990) and resuspended in SGII-NO_3 medium in which NH_4NO_3 was replaced with 4 mM KNO_3 for use in transformation experiments. For mating, cells were resuspended in M-N medium (KATES and JONES 1964) to induce gametogenesis.

Plasmid and phage DNA: pMN24, a plasmid containing the cloned wild-type nitrate reductase gene (FERNANDEZ *et al.* 1989; KINDLE *et al.* 1989), was digested to completion with *Eco*RI, cutting the plasmid twice to produce a 14.5-kb fragment that contained the entire *NIT1* coding sequence plus the pUC119 vector, and a 3-kb fragment (see Figure 2A). pARG7.8, which carries a 7.8-kb insert of the Chlamydomonas argininosuccinate lyase gene (DEBUCHY, PURTON and ROCHAIX 1989), was linearized with *Bam*HI for use in cotransformation experiments. After digestion with restriction endonucleases, plasmid DNA was extracted twice with phenol/chloroform (1 : 1), precipitated in ethanol and resuspended in 10 mM Tris, 1 mM EDTA. Genomic clones were obtained from a library constructed with DNA from the wild-type strain 21gr *mt*$^+$ (SCHNELL and LEFEBVRE 1993) in the λ phage vector λFixII (Stratagene). These clones were tested for complementation of the motility defects by cotransformation with pARG7.8 into motility mutants. Genomic inserts containing the putative genes were then subcloned into the plasmid vector Bluescript KS$^+$ (Stratagene) using restriction enzymes indicated in Figure 5. CsCl-purified plasmids were prepared by the alkaline-lysis method (SAMBROOK, FRITSCH and MANIATIS 1989). Bacteriophage DNA was prepared from 25-ml cultures by PEG precipitation as described (TAM and KIRK 1991) and resuspended in 100 μl of 10 mM Tris, 1 mM EDTA.

Transformation protocol: Cells were transformed using the glass-bead procedure of KINDLE (1990). Specifically, 1 μg of pMN24 DNA digested with *Eco*RI was agitated with 4×10^7 *nit1-305* cells in the presence of 5% PEG-8000 (Sigma) and 0.3 g of acid-washed glass beads (0.7–1.2 mm in diameter; Sigma), for 45 sec on a Fisher Vortex Genie II mixer. Cells were spread on 1% MNO_3 agar plates to select for Nit$^+$ transformants. For cotransformation experiments, 1 μl of λ DNA or 1 μg of plasmid DNA, along with 1 μg of pARG7.8 DNA digested with *Bam*HI, was transformed into *arg7* cells which had been treated with autolysin for 1 hr. Cells were plated on 1% M agar to select for Arg$^+$ transformants. Autolysin was prepared using strains L5 and L8 as follows. Cells were grown on 1% M agar plates, resuspended in M-N medium to a cell density of 1×10^8 cells/ml and incubated under bright lights for 3–4 hr to induce gametogenesis. Equal numbers of L5 and L8 gametes were then mixed together and placed under bright lights for 30 min. Cells were removed by centrifugation and the supernate was aliquoted and stored at −80°.

Motility assay: Transformant colonies were allowed to grow on plates for 7–10 days, then they were transferred by toothpick into liquid medium (0.2 ml) in 96-well microtiter plates. After 2 days in liquid culture, the motility of each transformant line was examined using a stereo microscope (Zeiss DR-C) at 80 × magnification. Samples containing putative motility mutants were fixed in 10% glutaraldehyde and observed for flagellar-length defects using phase-contrast microscopy. Motility mutants were then single-colony isolated again for examination to ensure that the motility phenotype was stably maintained.

Genetic analysis: Techniques for mating and tetrad analysis were as described (JAMES *et al.* 1988). Meiotic progeny were scored for nitrate or arginine auxotrophy, motility phenotype and APM resistance. Dominance/recessiveness tests and complementation tests of mutations were performed in stable diploids which were constructed by mating complementing auxotrophic strains (*arg7* × *nit1 nit2* or *nit1* × *nit4*) and selecting diploids on MNO_3 medium. Diploid colonies were identified as prototrophs; diploid cells were larger than haploid and were always mating-type minus. For each cross producing diploids, 16–24 diploid lines were recovered and analyzed.

DNA-blot analysis and library screening: Genomic DNA was isolated by an adaptation of the method of WEEKS, BEERMAN and GRIFFITH (1986) as described by SCHNELL and LEFEBVRE (1993). Genomic-DNA blotting, plaque lifts and hybridization were performed using Nytran membrane, following protocols provided by the manufacturer (Schleicher & Schuell). After hybridization, filters were washed according to TAM and KIRK (1991). ^{32}P-Labeled DNA probes were prepared by the random priming method (FEINBERG and VOGELSTEIN 1983). Approximately 2×10^5 plaques from an amplified wild-type *C. reinhardtii* genomic library were screened with the cloned DNA fragment from each mutant.

Isolation of genomic sequence flanking the integrated plasmid: A 1.2-kb genomic DNA fragment flanking the

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integrated plasmid in the *mbo2* mutant (strain 2G12) was cloned by plasmid rescue. Five micrograms of mutant DNA were digested with *Sal*I for 5 hr at 37° and the reaction was stopped by heating at 65° for 15 min. Ligation of DNA was performed with 2 units of T4 DNA ligase (BRL) in a total volume of 450 μl to favor circularization of DNA fragments. After phenol/chloroform extraction and precipitation of DNA in 70% ethanol, 1 μg of the DNA was transformed into frozen competent DH5α cells by electroporation using the BTX transfector 100 (Biotechnologies and Experimental Research, Inc.) following protocols in the Bio-Rad Gene Pulser Application Guide. The transformed cells were then plated on LB agar plates containing 60–80 μg/ml ampicillin to select for resistant colonies.

A 4.4-kb junction fragment containing the 5′ portion of the integrated *NIT1* gene and its flanking host genomic sequence in the *vfl1* mutant (strain 5E8) was cloned by screening a partial library constructed with DNA from mutant 5E8. Twenty micrograms of genomic DNA from 5E8 were digested with *Pst*I and fractionated on a 1% SeaPlaque agarose gel (FMC Corporation). After electrophoresis, 1-mm slices were cut out from the gel around the 4.4-kb region and DNA was extracted from the gel slices by melting at 65°, followed by three phenol extractions and three ether extractions. The fraction containing the junction fragment was identified using DNA-blot hybridization: 0.1 volume of each recovered DNA fraction was electrophoresed on an agarose gel, transferred to Nytran filters and hybridized with a labeled probe prepared with a 0.9-kb *Xho*I fragment isolated from pMN24 (Figure 2A). The DNA fraction containing the junction fragment was ligated (using T4 DNA ligase) to 0.1 μg of *Pst*I-digested Bluescript DNA which had been dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim) under conditions described by MANIATIS, FRITSCH and SAMBROOK (1982). The ligation mixture was transformed into DH5α cells as described above. Ampicillin-resistant colonies were transferred to nitrocellulose membranes (SAMBROOK, FRITSCH and MANIATIS 1989) and hybridized with the ^{32}P-labeled 0.9-kb *Xho*I fragment from pMN24 under conditions similar to those used for plaque lifts.

RESULTS

Isolation of motility mutants: To generate motility mutants, a plasmid (pMN24) containing the nitrate reductase structural gene (*NIT1*) was digested with *Eco*RI and transformed into *nit1* mutant cells. Nit$^+$ transformants were selected by growth on MNO_3 medium in which nitrate is the sole source of nitrogen. To minimize multiple insertion events, the ratio of plasmid DNA to cell number in transformation experiments was maintained at a relatively low level (see MATERIALS AND METHODS); several hundreds of transformants were typically obtained per microgram of plasmid DNA. Each transformed cell line was grown in liquid culture and scored for motility defects first by observation with a stereo microscope, then by phase-contrast microscopy. Of 3000 transformants screened, 74 independent cell lines showing abnormal flagellar motility were recovered. These motility mutants belonged to a wide range of phenotypic classes (Table 1). In addition to the many mutants with phenotypes similar to those of previously described strains (HUANG 1986), there was an interesting class of mutants which displayed an unusual new phenotype: unequal-length flagella. While most of the cells in a population were flagella-less or had stumpy flagella, a small fraction of the cells had two flagella of different lengths (Figure 1). These cells with unequal-length flagella usually spun very slowly at the bottom of liquid cultures.

TABLE 1

Classes of motility mutants

Motility phenotype	No. of strains	Strains analyzed
1. Paralyzed flagella	12	12C10, 4F1
2. Aberrant motility	29	2E4, 3C10, 4G1
3. Move backward only	1	2G12
4. Flagella-less	11	
5. Abnormal flagellar number	7	5E8, 8E4, 17D4, 12A10
6. Short/stumpy flagella	8	1C6
7. Long flagella	1	28D11
8. Unequal-length flagella	3	1H3, 12D9
9. Monster dividers	2	

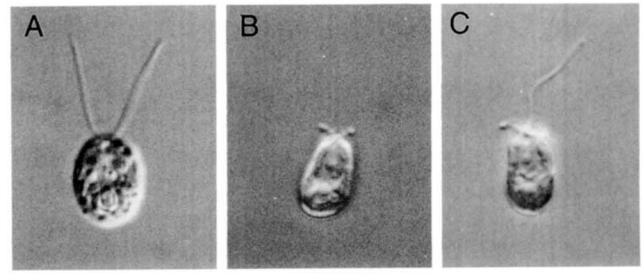

FIGURE 1.—Flagellar defects of unequal-length flagella mutants visualized by differential interference contrast microscopy. (A) A wild-type cell showing normal flagella, (B) cells of strain 1H3 showing stumpy-flagella or (C) a pair of unequal-length flagella (3000× magnification).

To determine whether these motility mutants contained DNA sequences derived from the pMN24 plasmid integrated into the genome, DNA from 18 of these mutants was analyzed on blots using pMN24 DNA as the labeled hybridization probe. Under these conditions, three major *Pvu*II fragments (Figure 2B, fragments A, B and C) from the untransformed strain 5D hybridized with the probe. The transformants contained these three fragments, which represented the endogenous *nit1* gene, plus additional fragments which were derived from the integrated *NIT1* plasmid (Figure 2B, 10 representative examples are shown). Some of these new fragments may represent the junctions between the integrated plasmid and host genomic sequence. One of the extra fragments (D in Figure 2, A and B) was derived exclusively from the vector DNA and was present in most cases. The pattern of fragments was simple in most mutants analyzed, suggesting that these mutants contained only one or a few copies of the integrated plasmid.

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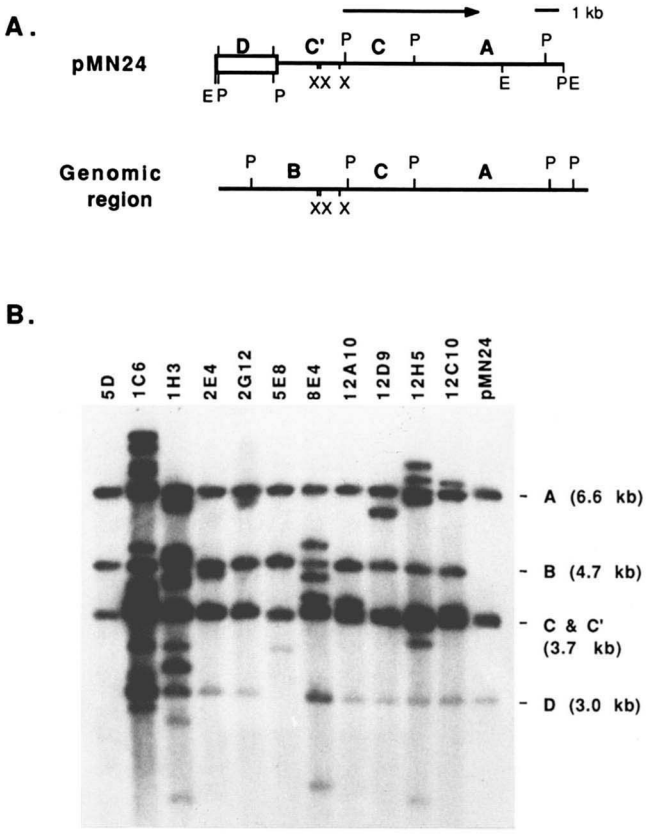

FIGURE 2.—Genomic-DNA analysis of 10 different transformants with motility defects. (A) A restriction map of pMN24 showing *Pvu*II (P), *Xho*I (X) and *Eco*RI (E) sites. pMN24 is shown linearized at an *Eco*RI site within the pUC119 vector (open rectangle). The *NIT1* coding region is indicated by the arrow above the map (D. ZHANG and P. A. LEFEBVRE, unpublished data). Restriction-endonuclease sites of the corresponding genomic region are illustrated on the lower map. Fragment D was derived exclusively from the vector and represented most of the vector sequence. (B) DNA (3 μg) from the untransformed strain (5D) and from 10 different motility mutants, plus 0.6 ng (1 copy equivalent) of pMN24 DNA were digested with *Pvu*II and probed with ^{32}P-labeled pMN24 DNA. In strain 5D, three major bands were identified with the probe. In the transformants, extra fragments in addition to these three bands were detected.

Linkage of the motility mutations to the integrated *NIT1* plasmid: To determine whether the motility phenotypes of the transformants were due to single mutations caused by the insertion of the *NIT1* plasmid, 14 of the motility mutants were backcrossed to strains carrying the *nit1* mutation. Also included in this analysis was a paralyzed-flagella mutant (strain 7-4) generated by insertion of the cloned *NIT2* gene (R. A. SCHNELL and P. A. LEFEBVRE, unpublished results); the mutant 7-4 was backcrossed to a *nit2* mutant (strain B22). Progeny from these crosses were scored for their motility phenotype and for the ability to grow on MNO_3 medium.

In all 15 cases the motility phenotype segregated as a single Mendelian trait, as indicated by the equal ratio of normal to abnormal swimmers among the progeny (Table 2). If the motility mutations were caused by insertion of the *NIT1* transgene, then in backcrosses all of the progeny that displayed abnormal motility should also be able to grow on MNO_3 medium. Cosegregation of the motility and the Nit$^+$ phenotypes was indeed observed for 13 of 15 mutants analyzed (Table 2). To determine whether the integrated plasmid DNA also cosegregated with the motility and Nit$^+$ phenotypes, *Pvu*II-digested genomic DNA of six to eight progeny from backcrosses of six different mutants (2G12, 5E8, 8E4, 17D4, 7-4, 4F1) was analyzed on blots hybridized with ^{32}P-labeled pUC119 or pMN24 DNA. For all six mutants, the integrated plasmid DNA was detected only in progeny that were Nit$^+$ and showed abnormal motility (two examples are shown in Figure 3). Therefore, the results from both genetic and DNA analyses are consistent with the conclusion that insertion of the plasmid DNA into the nuclear genome disrupted a gene that was involved in flagellar motility in 13 of 15 mutants analyzed.

Among the 13 mutants which showed cosegregation of the Nit$^+$ and the defective motility phenotypes, the Nit$^+$ phenotype also behaved as a single Mendelian trait in all but one case (Table 1). In mutant 1C6, which appeared to have more than 10 copies of the plasmid (Figure 2B), the number of Nit$^+$ to Nit$^-$ progeny in the F_1 generation was 63 to 30, far from the 1:1 ratio indicative of a single Mendelian trait.

TABLE 2

Cosegregation tests for the motility and Nit phenotypes

Mutant strain	Parental progeny		Recombinant progeny	
	Mot$^+$ Nit$^-$	Mot$^-$ Nit$^+$	Mot$^-$ Nit$^-$	Mot$^+$ Nit$^+$
1H3	52	60	0	0
12D9	56	64	0	0
5E8	207	192	0	0
8E4	61	51	0	0
17D4	125	131	0	0
12A10	145	102	0	0
2G12	80	80	0	0
12C10	46	37	0	0
7-4	65	66	0	0
4F1	68	68	0	0
4G1	42	36	0	0
3C10	37	46	0	0
1C6	70	88	0	15
2E4	19	19	25	25
28D11	42	13	18	7

Abbreviations: Mot$^+$ = normal motility; Mot$^-$ = abnormal motility; Nit$^+$ = growth on nitrate; Nit$^-$ = no growth on nitrate. The motility phenotypes of these mutants are listed on Table 1. Viability of the meiotic progeny for most of the crosses was lower than 20%. Similarly poor meiotic viability was also observed in crosses using the strain 5D (from which these mutants were derived) to other wild-type strains. Meiotic viability generally improved in subsequent backcrosses, except in cases involving mutants with abnormal flagellar number. The results presented were derived from all surviving progeny from both incomplete and complete tetrads from one or more backcrosses and do not represent random-progeny data.

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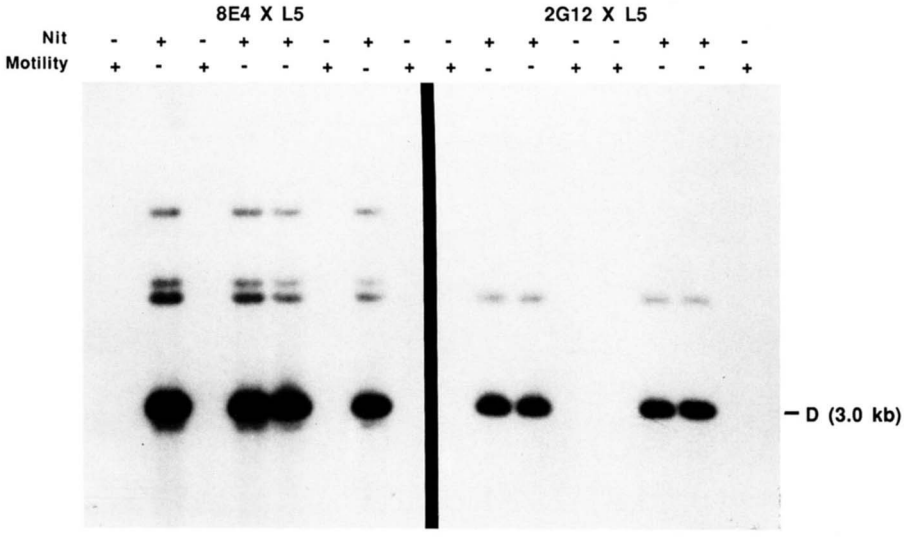

FIGURE 3.—Genomic-DNA blots of progeny from 2G12 and 8E4 crossed to L5 showing cosegregation of the integrated plasmid with the phenotypic markers. Each lane contains 2 μg of *Pvu*II-digested genomic DNA hybridized with ^{32}P-labeled probes derived from pUC119. pUC119 has two *Pvu*II sites flanking the polylinker region (Figure 2A). Fragment D is the major fragment derived from the integrated plasmid vector whereas the faint bands are restriction fragments containing the remaining pUC119 sequence in junction with *NIT1* or other genomic sequences. For abbreviations, see Table 2.

Moreover, some of the Nit$^+$ progeny were normal swimmers. It was possible, however, to obtain F_1 Nit$^+$ progeny with the motility defect, which in subsequent backcrosses segregated as expected for a single insertion event (two Nit$^+$ to two Nit$^-$ progeny in 20 tetrads). Therefore, in this mutant at least two separate integration events of the pMN24 plasmid must have occurred, only one of which was linked to the mutation giving rise to the motility phenotype.

Three mutants contain alleles of previously identified loci: Among the 13 mutants that appeared to be tagged with the *NIT1* plasmid, one of them (2G12) was unable to swim forward, but it could move backward slowly. Mutants with a very similar "*m*ove-*b*ackward-*o*nly" phenotype have been previously described, and they define three distinct *MBO* loci (NAKAMURA 1979; SEGAL *et al.* 1984). In these mutants, the ciliary (forward) beat pattern is defective while the flagellar (backward) beat pattern is at least partly functional.

Two classes of mutations that have defective control of flagellar number have been identified previously. Mutations in three different *VFL* loci (KUCHKA and JARVIK 1982; WRIGHT, CHOJNACKI and JARVIK 1983; ADAMS, WRIGHT and JARVIK 1985) produce a "*v*ariable number of *fl*agella" phenotype. Uniflagellar mutations, all of which mapped to a single locus closely linked to *apm1* on linkage group XIX, produce cells with a single flagellum (HUANG *et al.* 1982). Both *vfl* and uniflagellar mutants are found in our collection (Table 1) and four of them have been analyzed genetically. Mutants 5E8 and 8E4 possessed 0–5 flagella with more than 10% of cells showing three or more flagella. Mutants 12A10 and 17D4 were similar to uniflagellar mutants; most cells in each population had 0, 1 or 2 flagella with fewer than 2% of cells having three or more flagella.

To test whether the mutations produced in this study represent new alleles of previously identified loci, we performed pairwise crosses of the putative *mbo* mutant 2G12 to the three previously described *mbo* mutants; we also crossed the four mutants with abnormal flagellar number to the three genetically characterized *vfl* mutants. In addition, we crossed the four abnormal-flagellar-number mutants in our collection *inter se* to determine if they are allelic. Nonallelism of two mutations is indicated by the appearance of wild-type recombinant progeny in a cross, whereas the absence of recombinant progeny indicates that the two mutations are either allelic or tightly linked. The results of these allelism tests are shown in Table 3. No recombinants were observed in a cross of 2G12 to *mbo2*, suggesting that these mutations are either allelic

TABLE 3

Pairwise crosses for tests of allelism

Parents		No. of progeny	
		Mot$^-$	Mot$^+$
1. 2G12	× *mbo1*	69	23
	mbo2	78	0
	mbo2	70	24
2. 5E8	× *vfl1*	83	0
	vfl2	53	22
	vfl3	43	15
3. 8E4	× *vfl1*	25	6
	vfl2	116	32
	vfl3	70	15
	17D4	87	38
	12A10	54	30
4. 17D4	× *vfl1*	46	12
	vfl2	82	10
	vfl3	39	12
	12A10	55	24
5. 12A10	× *vfl1*	103	41
	vfl2	42	17
	vfl3	85	35

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or closely linked. Mutant 5E8 produced no wild-type recombinants in a cross to *vfl1*. On the other hand, our results indicate that the mutations in strains 8E4, 17D4, 12A10 were nonallelic to any of the three *vfl* mutations and to each other. In addition, they were unlinked to *apm1* (data not shown), which is 6.5 cM from *uni1* (JAMES *et al.* 1988). Therefore these three mutants contained lesions at three separate loci other than the *VFL* and *UNI* loci that have been identified before. Strain 7-4, which had paralyzed flagella, was found to be closely linked to *nit1*. Since *pf13* is less than 0.5 cM from *nit1* (HARRIS 1989), we suspected that mutant 7-4 might be a *pf13* allele.

To determine whether 2G12, 5E8 and 7-4 were alleles of *mbo2*, *vfl1* and *pf13*, respectively, we performed complementation tests in stable vegetative diploids. Mutants 2G12, 5E8 and 7-4 were first crossed to wild-type cells to generate heterozygous diploids to determine whether the mutations are recessive. Then 16–24 diploid lines from each cross were analyzed and they all showed wild-type motility and flagellar number, indicating that each mutation is recessive. Next we constructed stable diploids between 2G12 and an *mbo2* mutant, 5E8 and a *vfl1* mutant, and 7-4 and a *pf13* mutant. In all cases, the diploids displayed the abnormal motility characteristic of the haploid parents, suggesting that each pair of mutations tested is allelic. We have designated these new alleles as *mbo2-3* (2G12), *vfl1-2* (5E8) and *pf13-3* (7-4).

Cloning of the *MBO2* gene: To test whether the insertion of pMN24 plasmid DNA will allow us to clone the affected genes in these mutants, we first cloned the *MBO2* gene, starting with the mutant 2G12. Analysis of genomic DNA digested with a variety of restriction enzymes indicated that only a single copy of the plasmid was present in the genome of 2G12 and that the pUC119 vector sequence was intact and juxtaposed to an integration junction (as illustrated in Figure 4A). This configuration of the integrated plasmid allowed us to clone the sequence flanking one side of the integration site by plasmid rescue. DNA of 2G12 was digested with *Sal*I, which cut in the polylinker site of pUC119, to release the junction fragment containing pUC119 and a 1.2-kb genomic sequence adjacent to the site of insertion (Figure 4A). After circularization and transformation into *Escherichia coli*, ampicillin-resistant clones containing the rescued plasmid sequence were selected. The recovered plasmid, p2G12S1, was used as a hybridization probe for analysis of genomic DNA. It had no homology to *NIT1* DNA, as expected, but it contained a repetitive sequence (data not shown). A 0.6-kb *Alu*I-*Pvu*II fragment (probe b in Figure 4A) was isolated from p2G12S1 and shown to hybridize to a single genomic fragment. The probe recognized restriction

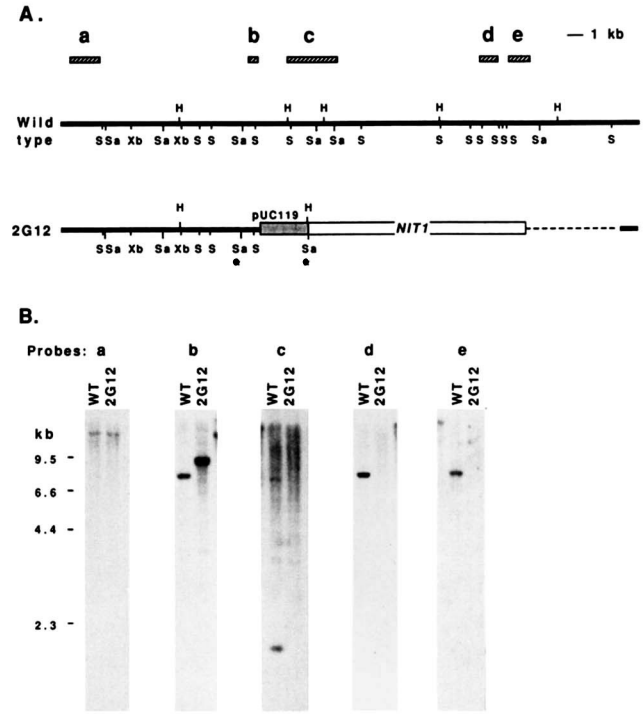

FIGURE 4.—(A) Top: Restriction map of the wild-type genomic region which was disrupted in the mutant 2G12. Abbreviations: H *Hin*dIII; S, *Sst*I; Sa, *Sal*I; Xb, *Xba*I. Five different fragments (shaded boxes) that were used as probes in (B) are indicated above the map. Bottom: A proposed map of the corresponding genomic region in 2G12. The junction fragment that was cloned by plasmid rescue is the fragment bordered by the two *Sal*I sites marked with asterisks. The extent of deletion determined by genomic-DNA analysis as shown in (B) is represented by the combined span of the integrated plasmid and the dotted line. (B) Genomic-DNA analysis of the region around the integration junction in the mutant 2G12. *Hin*dIII-digested DNA from a wild-type strain and from mutant 2G12 was hybridized with labeled probes corresponding to the five different fragments indicated in (A). Sequences corresponding to probes c, d and e were not detected in the mutant DNA. Digestion with other restriction endonucleases gave similar results (not shown).

fragments of different sizes in wild-type DNA and DNA from mutant 2G12, confirming that p2G12S1 was derived from the site of insertion (Figure 4B). A genomic library containing DNA from wild-type cells was screened with labeled probe b; nine different overlapping clones spanning a 30-kb region were obtained.

The presence of the complete *MBO2* gene in one of these genomic clones was demonstrated by transformation as follows. Previous studies have shown that when a plasmid containing a selectable marker such as *NIT1* and a second plasmid with an unselected gene were transformed together into cells, 10–70% of the Nit^+ transformants also expressed the gene carried by the second plasmid (DIENER *et al.* 1990; KINDLE 1990). We constructed an *arg7*, *mbo2-3* double mutant and performed cotransformation experiments in which genomic clones to be tested were transformed into cells along with the plasmid pARG7.8 (containing the

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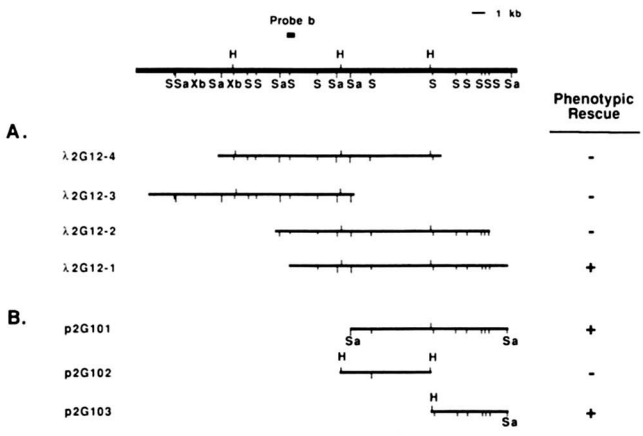

FIGURE 5.—Complementation of the *mbo2-3* mutation by transformation. Upper line represents the restriction map of the putative *MB02* gene region (see Figure 4A for abbreviations). Probe b, which was used to screen a wild-type genomic library, is indicated above the map. In (A), four different genomic clones covering this region were cotransformed (with pARG7.8 as the selected marker) into *arg7*, *mbo2-3* recipient cells. Only λ2G12-1 was able to restore wild-type motility to *mbo* mutant cells, as seen in 5% of the Arg^+ transformants. In (B), three subclones of λ2G12-1 were tested for the ability to complement the *mbo* mutation.

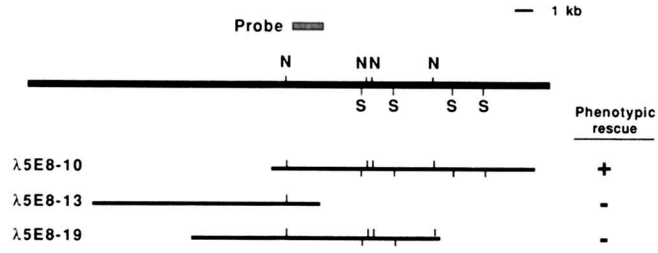

FIGURE 6.—Complementation of the *vfl1-2* mutation by transformation. *Sst*I (S) and *Not*I (N) sites of the wild-type genomic region corresponding to the region of plasmid integration in the mutant are shown. The 2-kb *Pst*I-*Xho*I fragment used to isolate the genomic clones is illustrated above the map. Three different lambda clones were tested by cotransformation into *vfl1-2*, *arg7* cells. Only λ5E8-10 could restore wild-type motility to *vfl1-2* cells, as seen in about 5–15% of the Arg^+ transformants.

wild-type argininosuccinate lyase gene) as selectable marker. Arg^+ transformant colonies were selected, grown in liquid medium, and their motility was scored to test for the expression of the wild-type *MBO2* gene in the transformants. Among the four overlapping λ clones tested, only cotransformation with λ2G12-1 gave rise to swimming cells (Figure 5A). Eleven of 196 Arg^+ transformants tested showed restoration of flagellar function. Analysis of DNA from five of these swimming transformants using DNA fragments from λ2G12-1 as probes showed that they all contained DNA derived from the λ clone (data not shown). Backcrosses of four motile transformants to an *mbo2-3* strain indicated that the normal motility phenotype segregated as a single Mendelian trait (2:2 segregation in 20–24 tetrads for each cross). By crossing one of these Mbo^+ transformants to the original *mbo2-1* mutant isolated by SEGAL *et al.* (1984), we found that the wild-type and the Mbo phenotypes segregated 2:2 among the tetrad progeny (six tetrads), indicating that the putative *MBO2* transgene was able to restore normal motility to the *mbo2-1* mutant. Therefore, we conclude that a functional *MBO2* gene must be contained within clone λ2G12-1.

To further define the extent of the functional *MBO2* gene, a 13-kb fragment (p2G101), a 7.5-kb (p2G102) fragment and a 6.4-kb fragment (p2G103) from λ2G12-1 were subcloned into the vector Bluescript and tested for phenotypic rescue of *mbo2-3* in cotransformation experiments (Figure 5B). We found that both p2G101 and p2G103 were able to correct the Mbo defect in 15–35% of Arg^+ transformants but p2G102 could not. Thus the functional *MBO2* gene has been localized to a 6.4-kb genomic region.

Cloning of the *VFL1* gene: We have also cloned the wild-type copy of the *VFL1* gene starting with mutant 5E8. Analysis of the mutant DNA with a number of restriction endonucleases and hybridization probes indicated that one copy of the pMN24 plasmid had integrated into the genome, but the pUC119 vector sequence was completely lost in this mutant. The genomic sequence flanking the site of insertion was therefore cloned as follows. A 4.4-kb *Pst*I fragment was predicted by restriction mapping to contain the 5′ portion of the integrated *NIT1* gene and several kilobases of DNA adjacent to the insertion. A partial library of genomic fragments was prepared (MATERIALS AND METHODS) and screened with a 0.9-kb *Xho*I fragment derived from the 5′ portion of the *NIT1* gene (Figure 2A). A 4.4-kb clone containing the junction fragment was isolated and shown to contain a 2-kb *Pst*I-*Xho*I fragment (Figure 6) comprising only the genomic sequence adjacent to the site of insertion. This fragment was used as a hybridization probe to screen the genomic library to identify three overlapping clones. When tested for the ability to complement the *vfl1-2* mutation upon transformation, only genomic clone λ5E8-10 was able to complement the variable-flagellar-number phenotype (Figure 6).

Host DNA deletion accompanied DNA integration: Host sequence rearrangements have been commonly observed in association with nonhomologous integration of exogenous DNA into the genome of mouse or fungi (ROTH and WILSON 1988; ASCH *et al.* 1992). To investigate whether rearrangement of host genomic sequences occurred upon integration of pMN24 in strain 2G12, we used five different fragments (Figure 4A) from the wild-type genomic clones as hybridization probes for analysis of DNA from a wild-type strain and from 2G12 (Figure 4B). Probes a and b, which are on the left of the integration junction, hybridized to genomic fragments in both strains, although the band hybridizing to probe b in 2G12 was larger than that in wild type. In contrast, probes c, d and e, which are on the right side of the integra-

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tion junction (Figure 4A), failed to hybridize to any sequence in the mutant strain (Figure 4B), suggesting that a large region to the right of the integration junction had been deleted. We have mapped the endpoint of this deletion and determined the total size of the deletion to be 23 kb. The deletion covered the entire span of the *MBO2* gene as established by the phenotypic rescue experiments described above.

Similar studies of the region around the integration junctions in mutant 5E8 indicated that a 3–4-kb region adjacent to the integrated junction was either rearranged or deleted in the mutant (data not shown). Preliminary studies of the genomic DNA around the site of plasmid insertion in a third mutant, 8E4, indicated that at least 12 kb of genomic DNA adjacent to an integration junction was deleted in the mutant (data not shown). Therefore, host DNA rearrangement may be a common phenomenon associated with nonhomologous DNA integration in Chlamydomonas.

DISCUSSION

In the present study, by introducing a plasmid containing the nitrate reductase gene into the nuclear genome of Chlamydomonas, 74 motility mutants were recovered. Motility mutants were also observed in transformants which resulted from integration of the cloned *NIT2* gene (R. A. SCHNELL, personal communication), and one of these mutants is an allele of *pf13*. With a similar approach, a mutant with defective splicing of chloroplast RNA transcripts was obtained by insertion of the pARG7.8 plasmid (M. GOLDSCHMIDT-CLERMONT, personal communication).

Several of our observations suggest that DNA transformation is a feasible and effective approach for tagging and cloning genes in Chlamydomonas. First, a very high percentage (2.5%) of the Nit^+ transformants showed abnormal motility. Backcrosses of 15 of these mutants suggest that each was due to a single nuclear mutation. Second, the 74 motility mutants displayed a wide spectrum of phenotypes, representing virtually all classes of motility mutants that have been described before (HUANG 1986), as well as a new class of mutants that produce unequal-length flagella. Recombination and diploid complementation tests of six mutants showed that three of them contained lesions at previously identified loci: *MBO2*, *VFL1* and *PF13*, and the others, which displayed abnormal flagellar number, did not map to any of the previously described *VFL* or *UNI* loci. We conclude that the transforming DNA can integrate into many different sites in the genome, and that disruption of the nuclear genome by integration of exogenous DNA may be an efficient way to produce random mutations. Third, the tight linkage of the motility phenotype to the Nit^+ phenotype in 13 of the 15 mutants analyzed is consistent with the conclusion that most of the motility mutants obtained in this study were generated by the integration of the plasmid DNA. As a demonstration of the usefulness of the plasmid DNA as both a mutagen and a tag for cloning, we cloned two motility genes by different approaches. Since the majority of transformants contain only a few copies of the integrated plasmids, subsequent recovery of the sequences flanking the sites of integration should be straight forward. Moreover, as shown by the cloning of *MBO2*, those mutants which retain a complete copy of the vector DNA will allow cloning of the flanking sequence by simple plasmid rescue.

Studies of the genomic map around the sites of integration in three motility mutants revealed extensive deletion of the host sequence in two of the mutants and some sequence rearrangement or deletion in the third mutant. This observation raises the possibility that integration of exogenous DNA into the Chlamydomonas nuclear genome may often be accompanied by host DNA rearrangement. These sequence rearrangements may provide an explanation for the unexpectedly high frequency of motility mutants observed among the Nit^+ transformants. That is, deletions associated with integration events would be expected to inactivate a larger region of the genome than simple integration. In mouse, the sequence rearrangements and deletions which accompany plasmid insertion has complicated the isolation of genes in putative insertional mutants (MEISLER 1992). However, with the ease of genetic analysis and the ability to easily complement mutant phenotypes by transformation using cloned DNA in Chlamydomonas, deletions should not be a serious problem. Genes of interest should be readily isolated using clones with large DNA inserts for phenotypic rescue experiments. Our experience using genomic clones from a lambda library (average size 18 kb; R. A. SCHNELL, personal communication) suggests that a few micrograms of a genomic DNA clone in λ phage vector can be used directly for transformation without subcloning into plasmids. Alternatively, it should be possible to use chromosome walking to isolate the affected genes from mutants with large deletions. The ease and efficiency of cotransformation experiments makes it possible to quickly test each set of overlapping clones for the ability to complement mutant phenotypes.

Not all genes of interest will be clonable using this procedure. Genes in which null mutants are lethal could not be obtained by this method because the insertion of plasmid DNA and the accompanying sequence rearrangements will likely produce the null phenotype. There may also be genes which map close enough to essential genes that they will be difficult to clone because of the effect of sequence deletion on the neighboring genes.

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One critical observation for others planning to use this technique is that the majority of mutants arising during transformation do appear to be caused by insertion of the transforming DNA. Transformation itself does not appear to be very mutagenic in the absence of insertion of the functional *NIT* gene. The results of many more mutant screens will be needed to establish with confidence that most Nit$^+$ transformants with a new phenotype of interest are caused by integration of the transforming plasmid. If so, however, tedious or difficult mutant screens would be justified, as would screens for rare mutants occurring in a single gene.

An advantage to isolating mutants by insertional mutagenesis is the ease of genetic mapping using restriction fragment length polymorphism (RFLP) procedures. An RFLP map of *C. reinhardtii* has been constructed as a joint project of our laboratory and Carolyn Silflow's laboratory (RANUM *et al.* 1988) and it now contains markers covering most of the genome. Cloned fragments from mutants of interest can readily be placed on this map, making unnecessary the many strain constructions and crosses needed to map mutations to the 17 linkage groups of this organism.

Our results have demonstrated the effectiveness of using insertional mutagenesis to disrupt and clone genes involved in flagellar motility. The mutants we isolated in this study will provide a basis for understanding many different aspects of flagellar function and assembly, and we believe that this method should also be applicable for studying other problems in Chlamydomonas. This approach should greatly accelerate molecular genetic analysis of various processes in Chlamydomonas, and the nature of a large number of gene products of interest will soon be revealed as a consequence.

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LITERATURE CITED

ADAMS, C. M. W., R. L. WRIGHT and J. W. JARVIK, 1985 Defective temporal and spatial control of flagellar assembly in a mutant of *Chlamydomonas reinhardtii* with variable flagellar number. J. Cell Biol. **100:** 955–964.

ASCH, D. K., G. FREDERICK, J. A. KINSEY and D. D. PERKINS, 1992 Analysis of junction sequences resulting from integration at nonhomologous loci in *Neurospora crassa*. Genetics **130:** 737–748.

DEBUCHY, R., S. PURTON and J.-D. ROCHAIX, 1989 The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the ARG7 locus. EMBO J. **8:** 2803–2809.

DIALLINAS, G., and C. SCAZZOCCHIO, 1989 A gene coding for the uric acid-xanthine permease of *Aspergillus nidulans*: inactivational cloning, characterization, and sequence of a *cis*-acting mutation. Genetics **122:** 341–350.

DIENER, D. R., A. M. CURRY, K. A. JOHNSON, B. D. WILLIAMS, P. A. LEFEBVRE, K. L. KINDLE and J. L. ROSENBAUM, 1990 Rescue of paralyzed-flagella mutant of *Chlamydomonas* by transformation. Proc. Natl. Acad. Sci. USA **87:** 5739–5743.

DUTCHER, S. K., and F. G. LUX III, 1989 Genetic interactions of mutations affecting flagella and basal bodies in *Chlamydomonas*. Cell Motil. Cytoskel. **14:** 104–117.

FEINBERG, A. P., and B. VOGELSTEIN, 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6–13.

FELDMANN, K. A., M. D. MARKS, M. L. CHRISTIANSON and R. S. QUATRANO, 1989 A dwarf mutant of *Arabidopsis* generated by T-DNA insertion mutagenesis. Science **243:** 1351–1354.

FERNANDEZ, E., R. SCHNELL, L. P. W. RANUM, S. C. HUSSEY, C. D. SILFLOW and P. A. LEFEBVRE, 1989 Isolation and characterization of the nitrate reductase structural gene of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA **86:** 6449–6453.

GORMAN, D. S., and R. P. LEVINE, 1965 Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA **54:** 1665–1669.

HARRIS, E. H., 1989 *The Chlamydomonas Sourcebook*, p. 454. Academic Press, New York.

HUANG, B. P.-H., 1986 *Chlamydomonas reinhardtii*: a model system for the genetic analysis of flagellar structure and motility. Int. Rev. Cytol. **99:** 181–215.

HUANG, B., Z. RAMANIS, S. K. DUTCHER and D. J. L. LUCK, 1982 Uniflagellar mutants of *Chlamydomonas*: evidence for the role of basal bodies in transmission of positional information. Cell **29:** 745–753.

JAMES, S. W., L. P. W. RANUM, C. D. SILFLOW and P. A. LEFEBVRE, 1988 Mutants resistant to antimicrotubule herbicides map to a locus on the *uni* linkage group in *Chlamydomonas reinhardtii*. Genetics **118:** 141–147.

KANG, S., and R. L. METZENBERG, 1993 Insertional mutagenesis in *Neurospora crassa*: cloning and molecular analysis of the *preg*$^+$ gene controlling the activity of the transcriptional activator NUC-1. Genetics **133:** 193–202.

KATES, J. R., and R. F. JONES, 1964 The control of gametic differentiation in liquid cultures of *Chlamydomonas*. J. Cell Comp. Physiol. **63:** 157–164.

KINDLE, K. L., 1990 High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA **87:** 1228–1232.

KINDLE, K. L., R. A. SCHNELL, E. FERNANDEZ and P. A. LEFEBVRE, 1989 Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. J. Cell Biol. **109:** 2589–2601.

KUCHKA, M. R, and J. W. JARVIK, 1982 Analysis of flagellar size control using a mutant of *Chlamydomonas reinhardtii* with a variable number of flagella. J. Cell Biol. **92:** 170–175.

KUSPA, A., and W. F. LOOMIS, 1992 Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. Proc. Natl. Acad. Sci. USA **89:** 8803–8807.

LUX, F. G., III, and S. K. DUTCHER, 1991 Genetic interactions at the FLA10 locus: suppressors and synthetic phenotypes that affect the cell cycle and flagellar function in *Chlamydomonas reinhardtii*. Genetics **128:** 549–561.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning. A Laboratory Manual*, pp. 133–134. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MAYFIELD, S. P., and K. L. KINDLE, 1990 Stable nuclear transformation of *Chlamydomonas reinhardtii* by using a *C. reinhardtii* gene as the selectable marker. Proc. Natl. Acad. Sci. USA **87:** 2087–2091.

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MEISLER, M. H., 1992 Insertional mutation of 'classical' and novel genes in transgenic mice. Trends Genet. **8:** 341–344.

NAKAMURA, S., 1979 A backward swimming mutant of *Chlamydomonas reinhardtii*. Exp. Cell Res. **123:** 441–444.

PIPERNO, G., B. HUANG, Z. RAMANIS and D. J. L. LUCK, 1981 Radial spokes of *Chlamydomonas* flagella: polypeptide composition and phosphorylation of stalk components. J. Cell Biol. **88:** 73–79.

RANUM, L. P. W., M. D. THOMPSON, J. S. SCHLOSS, P. A. LEFEBVRE and C. D. SILFLOW, 1988 Mapping flagellar genes in *Chlamydomonas* using restriction fragment length polymorphism. Genetics **120:** 109–122.

ROTH, D., and J. WILSON, 1988 Illegitimate recombination in mammalian cells, pp. 621–654 in *Genetic Recombination*, edited by R. KUCHERLAPATI and G. R. SMITH. American Society for Microbiology, Washington, D.C.

SAGER, R., and S. GRANICK, 1953 Nutritional studies in *Chlamydomonas reinhardtii*. Ann. N.Y. Acad. Sci. **56:** 831–838.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

SCHNELL, R. A., and P. A. LEFEBVRE, 1993 Isolation of the Chlamydomonas regulatory gene *NIT2* by transposon tagging. Genetics **134:** 737–747.

SEGAL, R. A., B. HUANG, Z. RAMANIS and D. J. L. LUCK, 1984 Mutant strains of *Chlamydomonas reinhardtii* that move backward only. J. Cell Biol. **98:** 2026–2034.

SINGH, G., D. M. SUPP, C. SCHREINER, J. MCNEISH, H.-J. MERKER, N. G. COPELAND, N. A. JENKINS, S. S. POTTER and W. SCOTT, 1991 *legless* insertional mutation: morphological, molecular, and genetic characterization. Genes Dev. **5:** 2245–2255.

TAM, L.-W., and D. L. KIRK, 1991 Identification of cell-type-specific genes in *Volvox carteri* and characterization of their expression during the asexual life cycle. Dev. Biol. **145:** 51–66.

TILBURN, J., F. ROUSSEL and C. SCAZZOCCHIO, 1990 Insertional inactivation and cloning of the *wA* gene of *Aspergillus nidulans*. Genetics **126:** 81–90.

WEEKS, D. P., N. BEERMAN and O. M. GRIFFITH, 1986 A small-scale five-hour procedure for isolating multiple samples of CsCl-purified DNA: application to isolations from mammalian, insect, higher plant, algal, yeast, and bacterial sources. Anal. Biochem. **152:** 376–385.

WOYCHIK, R. L., MAAS, R. ZELLER, T. F. VOGT and P. LEDER, 1990 "Formins": proteins deduced from the alternative transcripts of the *limb deformity* gene. Nature **346:** 850–853.

WRIGHT, R. L., B. CHOJNACKI and J. W. JARVIK, 1983 Abnormal basal-body number, location and orientation in a striated fiber-defective mutant *Chlamydomonas reinhardtii*. J. Cell Biol. **96:** 1697–1707.

YANOFSKY, M. F., H. MA, J. L. BOWMAN, G. N. DREWS, K. A. FELDMANN and E. M. MEYEROWITZ, 1990 The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. Science **346:** 35–38.

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