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TECHNICAL ADVANCE

A promoter trap for *Chlamydomonas reinhardtii*: development of a gene cloning method using 5' RACE-based probes

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Summary

A promoterless radial spoke protein *RSP3* gene has been used to identify promoter regions in the genome of *Chlamydomonas reinhardtii*. The acceptor strain *pf-14 arg7* was transformed with a linearized vector containing the ARG 7.8 gene as a selection marker and a promoterless *RSP3* gene. The frequency at which the motility was restored in transformants varied from 2–3%. Several of these were motile only in ammonium-free medium, indicating that the procedure could be used to select inducible promoters. Transformation of nitrogen-starved cells produced about twice as many transformants which were only motile in ammonium-free medium. Since one of the tagging vectors contained an *RSP3* gene with a hybridization flag in its 3' untranslated region, it was possible to estimate the size of the new *RSP3* transcripts in transformants. The results suggested that in most cases a hybrid RNA was generated consisting of the tagged gene transcript and reporter gene RNA. By 5' RACE, these parts of the new transcripts were amplified and it was shown that the generated DNA fragments could be used to clone a tagged gene. One such example, gene *2BC9*, is predicted to code for a mitochondrial matrix protein. The tagging procedure will be optimized for cloning genes induced by nitrogen starvation, the cue for gametogenesis.

Introduction

Chlamydomonas reinhardtii has been studied extensively with regard to gametogenesis and sexual recognition processes (Goodenough, 1995; Beck and Haring, 1996). When vegetative cells are transferred to medium without nitrogen, they differentiate into gametes. Gametogenesis requires light and the expression of several new proteins (Treier and Beck, 1991), and is completed within 6–8 h.

Differential screening has resulted in the cloning of several gamete-specific genes (Von Gromoff and Beck, 1993). However, it has been difficult to assign a function to these genes. Consequently, a program to obtain sexual differentiation mutants was initiated. It has produced mutants in four independent loci involved in the light-dependent step of gametogenesis (Buerkle *et al.*, 1993; Gloeckner and Beck, 1995). While it should be possible to identify the genes by map-based cloning, the insertional mutagenesis method of Tam and Lefebvre (1993) may be a faster way to generate mutants and clone the gene (Campbell *et al.*, 1995). However, this procedure only permits the identification of gamete-specific genes whose disruption causes a clear phenotype. To be able to clone other genes that are induced by nitrogen deprivation, the cue for gametogenesis, we developed a promoter tagging strategy for *C. reinhardtii*. In nematodes (Hope, 1991), *Drosophila* (Bier *et al.*, 1989) and plants (Koncz *et al.*, 1989; Teeri *et al.*, 1986), promoter-tagging has identified genes that are developmentally regulated. In these cases, reporter genes such as *LacZ*, *NPTII* or *uidA* (GUS) were used, but these heterologous reporter genes are not functional in *C. reinhardtii*. We therefore used an algal gene, *RSP3* (radial spoke protein, a flagellar protein) as a reporter of promoter activity. The *RSP3* gene product complements the *pf-14* mutation of *C. reinhardtii* and restores motility, which can easily be scored by microscopic observation. We combined this method with a strategy for rapidly cloning the tagged gene by generating an 5' RACE-derived probe.

Results

Construction of a *C. reinhardtii* transformation vector suitable for promoter tagging

A promoter-trapping vector should contain a selection marker and a promoterless reporter gene. For *C. reinhardtii*, the pARG7.8 plasmid containing the argininosuccinate lyase gene is a very efficient transformation vector (Debuchy *et al.*, 1989). As a reporter gene, the promoterless *RSP3* gene coding for the radial spoke protein 3 was chosen. This gene complements the paralyzed flagella *pf-14* mutation and thereby restores motility to this strain, which can be scored visually. In order to assess the complementation frequency, we used as control the

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Figure 1. Genomic organization of the *RSP3* gene and the construction of tagging vectors.

(a) The complete *RSP3* fragment was cloned as an *EcoRI* fragment into the pARG7.8 vector, resulting in the complementation control vector pMH214. The promoterless *NheI* fragment was cloned into the *NheI* site of pARG7.8 resulting in pMH213. Upon linearization with *EcoRI*, the 5' end of the *RSP3* gene is facing inward, preventing fusion to *C. reinhardtii* promoters. Digestion with *BamHI* results in the tagging vector. Insertion of a ϕ X174 hybridization flag into the *Scal* site of the *RSP3* gene (shaded triangle) results in vector pMH223. Introns (I1, I2 and I3) are indicated by shaded boxes. Arrows indicate transcription directions of *C. reinhardtii* genes.

(b) DNA sequence of the leader of the *BamHI*-linearized tagging vector pMH213. The pBR322 part of the leader is depicted in italics. The *RSP3* coding region, starting with an ATG, is in bold face. Introns and exons are indicated, as well as the position of primers used in PCR experiments (sense primers above, anti-sense primers below the sequence).

complete *RSP3* gene, cloned as a 4 kb *EcoRI* fragment into the ARG7.8 vector pCB412, yielding vector pMH214 (Figure 1a). A promoterless *RSP3* fragment was obtained by *NheI* digestion of the genomic clone. This 2.7 kb fragment contained 23 bp of 5' leader sequence, the coding sequence and the complete 3' end of the *RSP3* gene. It was cloned into the *NheI* site of vector pCB412 in such an orientation that the 5' end of the gene would be exposed when this plasmid (pMH213) was digested with *BamHI*. This created a promoterless *RSP3* gene with a synthetic leader of 120 bp pBR322 sequence (Figure 1b), which was used to distinguish newly inserted *RSP3* copies from the endogenous gene which lacks this piece of DNA. When pMH213 is linearized with *EcoRI*, the 5' end of the *RSP3* gene faces 'inward', making fusion with *C. reinhardtii* promoter sequences impossible and thus served as a no-promoter control for our system. To detect new *RSP3* transcripts, a hybridization flag, the 394 bp ϕ X174 *HpaI* fragment (Gumpel *et al.*, 1994), was inserted in the *Scal* site of the 3' untranslated region of the *RSP3* gene. This plasmid, pMH223, was otherwise identical to the promoter-tagging vector pMH213.

Transformation of *C. reinhardtii*: frequency of *RSP3* gene-activation

C. reinhardtii strain *pf-14 arg7* was transformed with equal amounts of linearized vectors (Table 1). Arginine-

Table 1. Promoter tagging using the *RSP3* reporter gene

Transformation vector	Transformants tested	Swimming transformants (Frequency)
pARG7.8 (pCB412) $\times$ <i>BamHI</i>	384	0 (-)
pMH214 $\times$ <i>BamHI</i> (intact <i>RSP3</i>)	96	66 (70 %)
pMH213 $\times$ <i>EcoRI</i> (NO promoter control)	576	0 (-)
pMH213 $\times$ <i>BamHI</i> (tagging vector)	576	15 (2.5 %)
pMH223 $\times$ <i>BamHI</i> (ϕ X-tagging vector)	456	2 (0.4 %)
pMH223 $\times$ <i>BamHI</i> ^a (ϕ X-tagging vector)	1212	26 (2.1 %)

^aDouble amount of DNA used for transformation.

independent transformants were individually analyzed for motility. No motile transformants were obtained by transformation with the ARG7.8 vector (pCB412), confirming that the reversion frequency of the *pf-14* mutation is low. The complete *RSP3* gene (vector pMH214) restored motility in 70% of the transformants. When the promoter region

Table 2. Pre-treatment enhances detection of Nitrogen-starvation induced motility

Transformation vector	Transformants tested	Swimming transformants (frequency)	Swimming in TAP-N only (frequency)
Cells grown in complete TAP medium			
pMH213 × <i>Bam</i> HI (tagging vector)	2400 ^a	48 (2%)	8 (0.33%)
Cells incubated in TAP medium without nitrogen for 18 h			
pMH213 × <i>Bam</i> HI (tagging vector)	1150 ^b	18 (1.6%)	7 (0.61%)

^aResult of three independent transformations.^bResult of two independent transformations.

was removed and the 5' end of the *RSP3* gene was not exposed (pMH213×*Eco*RI), no motile transformants were found. However, when the 5' end of the *RSP3* gene is available for fusion with a *C. reinhardtii* promoter (pMH213×*Bam*HI), about 2% of the the transformants were motile. This frequency was reproducibly achieved when 200 ng of vector was used for transformation. Transformation with higher concentrations of pMH213 DNA (up to 1 µg) increased the frequency of motile transformants (maximum 3.2%). The vector with the hybridization flag (pMH223) reproducibly yielded a lower number of motile transformants although the transformation frequency was similar to that of vector pMH213. Again the number of motile transformants was enhanced when the amount of vector pMH223 DNA was increased (Table 1).

To extend the application of the promoter-tagging system to the detection of inducible promoters, we adapted the assay for the detection of those promoters that are induced by nitrogen-deprivation. Transformants were first analyzed after overnight incubation in medium without nitrogen, then complete TAP medium was added and motile strains were analyzed again after overnight incubation. Approximately one in six motile transformants stopped swimming in complete TAP medium (Table 2), suggesting that the *RSP3* gene had been fused to a promoter induced by nitrogen deprivation. Support for this suggestion came from the transformation of cells that had been incubated in nitrogen-free medium before transformation. This pre-treatment resulted in a significant increase in the number of transformants that were motile only in medium without nitrogen (Table 2).

Molecular analysis of motile transformants obtained with the promoter-tagging vector

As shown previously, homologous recombination can occur with a low frequency (0.01–0.7%) when *C. reinhardtii*

is transformed (Gumpel *et al.*, 1994; Nelson and Lefebvre, 1995; Sodeinde and Kindle, 1993). We therefore assumed that some of the motile strains obtained with the tagging vector contained an *RSP3* gene that had been restored by homologous recombination. Southern blot analysis was used to investigate the nature of the integration events in motile transformants. DNA cut with *Sst*I yielded a 4.4 kb endogenous fragment when hybridized with a 5' *RSP3* probe (Figure 2). The presence of extra bands in most transformants suggests that extra copies of the *RSP3* gene have integrated elsewhere in the *C. reinhardtii* genome. Only transformant number 10 contained a single *RSP3* band, suggesting a homologous recombination event. The copy number of extra *RSP3* inserts ranged from one to two.

Transformants with an extra *RSP3* insert were analyzed by cDNA PCR, to determine whether the RNA of the newly introduced *RSP3* genes contained the predicted synthetic leader (Figure 3). The primer combination for this assay consisted of an antisense *RSP3* primer (2954, see Figure 1b), used for making the first-strand cDNA, and a PCR primer based on the pBR322 leader sequence (primer 3009). Primer 2954 is an exon 2 primer allowing the distinction between DNA products (with intron I, 294 bp) and RNA products (190 bp). This is illustrated by the PCR reaction products obtained with DNA or RNA (cDNA) from the same transformant (Figure 3, lanes DNA 01 and 01). From all motile transformants, the expected cDNA PCR product was amplified. Some of the RNA samples were evidently contaminated with DNA since the 294 bp band also appeared in these samples (Figure 3, lanes 06, 04 and 13). Non-motile strains, acceptor strain *pf*-14 *arg7* and transformant 10 did not yield cDNA PCR products (data not shown). Apparently, the newly introduced *RSP3* gene of motile transformants was transcribed with its synthetic leader, arguing against homologous recombination.

Figure 2. Southern blot analysis of motile *C. reinhardtii* transformants. A DNA gel blot of total DNA digested with *Sst*I was used for hybridization. Numbers above the lanes correspond to individual transformants; *pf*-14 is the acceptor strain. The blot was hybridized with a 1.4 kb *Sst*I *RSP3* cDNA fragment. The arrow points to the expected 4.4 kb endogenous *RSP3* fragment. Additional copies are visible as extra bands.

Detection of hybrid RNAs by 'ϕx-flag' hybridization and 5' RACE

Integration of the promoterless *RSP3* gene into a *C. reinhardtii* gene could lead to the production of fusion RNAs. We used two approaches to test whether this was true. First, we analyzed transformants that were obtained by transformation with the ϕx-flag tagging vector pMH223. RNA was isolated from actively swimming transformants and the RNA gel blot was hybridized with the *RSP3* probe (Figure 4a) and the ϕx-flag probe (Figure 4b). As well as the major transcript of the endogenous gene (2.4 kb), additional weaker signals were detected with the *RSP3* probe. The abundance of the new transcripts was estimated to be 2- to 10-fold lower than that of the endogenous *RSP3* transcript. Using transformants that have the full-length *RSP3* gene (with its own promoter) containing one or two copies of the ϕx-flag in their 3' untranslated region as references (Figure 4, lanes ϕ1 and ϕ2), the size of the new transcripts could be estimated. Some of the transcripts (Figure 4b, lane 3) had a leader sequence that was just as long that of the endogenous *RSP3* transcript. Others were approximately 200 bp (Figure 4b, lanes 2, 4 and 6) or even 400 bp (Figure 4b, lane 1) longer. These results suggested

Figure 3. RNA PCR analysis of motile *C. reinhardtii* transformants. Agarose gel electrophoresis of PCR products generated on cDNA made of RNA isolated from motile transformants. Numbers above the lanes correspond to individual transformants; *pf*-14 is the acceptor strain. PCR products originating from DNA, contaminating RNA preparations, can be distinguished from cDNA-based products because they contain intron I. Arrows represent the PCR primers 3009 and 2954, respectively.

Figure 4. RNA gel blot analysis of motile *C. reinhardtii* transformants obtained with the ϕx174 hybridization flag containing vector pMH223. (a) Hybridization with a 2.3 kb *RSP3* cDNA probe. (b) Hybridization with the 387 bp *Hpa*I fragment of ϕx174. ϕ1 and ϕ2 are lanes with RNA from transformants obtained with the complete *RSP3* gene, tagged with one (ϕ1) or two (ϕ2) copies of the hybridization flag, which serve as size standards (2.8 and 3.1 kb).

that the 5' part of the tagged gene transcript was fused to the *RSP3* reporter gene.

If one could amplify the 5' ends of these fusion products by PCR, one could rapidly generate probes for cloning the tagged genes. Five pMH213 transformants that yielded cDNA PCR products (Figure 3, lanes 01, 03, 06, 08 and 11) were used to develop a 5' RACE protocol. Using the antisense primer 2954 (Figure 1b), first-strand cDNA was

Figure 5. Schematic presentation of the 5' RACE products obtained from motile *C. reinhardtii* transformants.

The *Bam*HI site of the linearized tagging vector pMH213 is indicated. The dark areas represent pBR322 sequence, the pale area the 23 bp *RSP3* leader. The length of the tagged transcript sequence is indicated above the thin white bar.

made on total RNA of these transformants. These cDNAs were equipped with poly(A) tails and used for PCR amplification with primer 2954 and an oligo dT primer. Part of each PCR mixture, which also contained 5' RACE products of the endogenous *RSP3* gene, was then re-amplified with the pBR322 leader-specific antisense primer 3205 (Figure 1b) and the oligo dT primer. The products of this reaction were separated by gel electrophoresis and the hybrid fragments were identified by hybridization with the *Bam*HI-*Nhe*I pBR322 leader probe. Hybridizing fragments were reamplified, cloned into a vector and sequenced (Figure 5). From each transformant, we obtained a single hybrid fragment indicating that only one copy was expressed. The length of the extra leader varied from 76 to 192 bp. The fragment from transformant 2AE7 did not contain any extra leader sequence. The DNA sequence of the four new leader fragments did not have any homology with sequences in current databases, suggesting that previously unknown *C. reinhardtii* genes were tagged by the *RSP3* vector.

Cloning the tagged gene from transformant 2BC9

The cloned 5' RACE fragments could potentially be used for cloning the tagged gene. Transformant 2BC9 was taken as an example for this approach. Insertion of the *RSP3* gene probably would cause a change in the genomic structure of the 2BC9 gene. This could be visualized by comparing hybridization patterns of 2BC9 DNA with those of other transformants. Two probes were used on a blot of *Sst*I-digested DNA from transformants 2AE2 and 2BC9

Figure 6. The 5' RACE fragment of transformant 2BC9 is derived from the *RSP3*-tagged gene.

(a) Southern blot analysis of an *Sst*I digest of transformant 2AE2 and 2BC9 using the *RSP3* probe and the 5' RACE fragment of 2BC9 as a probe. (b) RNA gel blot hybridization of the acceptor strain *pf*-14 and transformants 2AE2 and 2BC9. The 2BC9 cDNA fragment was used as a probe first, then the membrane was reprobed with a random cDNA (no. 41), which recognizes a transcript that is similar in size (2.4 kb).

(Figure 6a). The *RSP3* probe gave the same pattern as described in Figure 2 (lanes 01 and 06, respectively). The additional *RSP3* band in 2AE2 is approximately 23 kb, while the 2BC9 has an additional band of 4.4 kb, which overlaps with the endogenous *RSP3* band. This 4.4 kb band also hybridizes with the 5' RACE 2BC9 probe, which confirms the hybrid nature of this fragment. However, transformant 2AE2 does not have this 4.4 kb band but a band of approximately 29 kb, which is larger than the band detected by *RSP3* hybridization. Other transformants and the acceptor strain *pf*-14 also have this large band, suggesting that this is the wild-type *Sst*I fragment that harbors the 2BC9 gene. Insertion of the *RSP3* gene resulted in the band-shift to 4.4 kb in transformant 2BC9.

The 5' RACE 2BC9 probe was then used to screen a *C. reinhardtii* cDNA library. The cloned cDNA was used to probe an RNA gel blot with RNA isolated from *pf*-14, 2BC9 and 2AE2 cultures (Figure 6b). The 2BC9 cDNA probe

MGMAAGMDRSSARRTTTRQRRRGCSRRSRNR**TS**Q**Q**APALAKQ
AACTCQSRSRWPGANTHGGGRKAGS**DREGRFHAPRRQSCEGDRG**
AQRVAKKLAPNGQMDLAGVEAVDIHAGALDEYRFNMFMRD**LM**AE
KRTDILCKGVLNMQGYGDTKTFVFKGAHEAICYGPAEQPWKPDET
RFSHVVF**IGRGLDKKALKEGLSSCLWKPPPPAGRRSATL**TRSCPS
MSIRRRARRRGCVLRRQHKCTASMR**AAFVRHAGAALRLGGCWGHP**
APWEAHNGAFNFMKALSLDCLALREQWNTDYEWGKCGCAR**SRTQ**
GQGAAFEAGLMVRAITHQVTVPNAGMRCICAPSSTCALQSDAGLHV
YHALCLTIWTRSLVAARRRLSLLVCRDARCTNETGWGASDTWLLW
LPGLRAIHSTCVASWHSYNSFHCIRPWAC* (435 AA)

Figure 7. Amino acid sequence deduced from the 2BC9 cDNA sequence. The protein is predicted to be a mitochondrial matrix space protein. The potential transit peptide (1–52, italics), mitochondrial targeting sequence (GRKAGS, outlined) and potential transmembrane region (underlined) are indicated.

recognized a 2.5 kb transcript in both the acceptor strain *pf*-14 and the transformant 2AE2. No transcript was visible in the RNA sample of transformant 2BC9 although the RNA amounts loaded were approximately equal (Figure 6b, probe 41). The cloned cDNA must therefore originate from the gene that was knocked-out by the integration of the tagging vector.

The cDNA sequence was determined, which suggested that the full-length transcript (2452 bp) was cloned. The 2BC9 gene codes for a polypeptide of 435 amino acids (Figure 7). No homology with other proteins in the current databases was found. The 2BC9 protein is predicted to be localized in the mitochondrial matrix space, based on the presence of the mitochondrial targeting sequence GRKAGS and the long potential signal peptide (52 amino acids). However, the phenotype of the transformant did not suggest that mitochondrial function was disturbed, since it was capable of heterotrophic growth in the dark.

Discussion

Following the example of promoter trapping in other organisms, we have tried to establish such a system for the green alga *C. reinhardtii*. For this purpose, a reporter gene that originates from *C. reinhardtii* itself was used, because heterologous genes are poorly expressed in this alga (Stevens *et al.*, 1996). The radial spoke protein gene *RSP3* complements the *pf*-14 mutation and restores motility. The advantage of the *RSP3* system is that one does not need to incubate the transformants with a substrate; rather, the phenotype may be scored directly. We could easily test 3000 transformants in one week, illustrating the effectiveness of the system. Our results show that a promoterless *RSP3* gene can function as a reporter for promoter activity. When the 5' end of the promoterless *RSP3* gene is not available for fusion to

C. reinhardtii genomic DNA, no motile transformants were found. This confirms that the gene construct does not have any residual promoter activity and indicates that homologous recombination is not a major factor in restoring gene activity. Still, because the frequency of motile transformants (2–3%) was close to the frequency of homologous recombination reported for *C. reinhardtii* (0.01–0.7%; Gumpel *et al.*, 1994; Sodeinde and Kindle, 1993), this possibility had to be considered. Most motile transformants had one or two additional *RSP3* fragments integrated in their genome and an indication for exchange with the endogenous *RSP3* gene was only found in one of the nine cases analyzed. Furthermore, all motile strains (again with the exception mentioned above) expressed a fusion RNA that contained part of the artificial pBR322 leader. If homologous recombination were to be a dominant factor in the creation of motile strains, at least some of the transformants should have lost these sequences in the process. Finally, the 5' RACE products that were characterized confirm that the expressed reporter gene integrated at different positions in these transformants. Therefore, homologous recombination is apparently not interfering with the promoter tagging approach.

If we compare the frequency of restored marker gene activity in *C. reinhardtii* (2–3%) with that of higher plant systems (between 5 and 50%; Koncz *et al.*, 1989; Teeri *et al.*, 1986), there is a striking difference. This may be due to the marker gene used. When the *uidA* gene was used, high frequencies of promoter tagging were reported (Goldsborough and Bevan, 1991; Kertbundit *et al.*, 1991), perhaps because the enzyme is very stable and can be detected even if expressed in only a few cells within the tissue. Use of a selection marker such as the *NPTII* gene reduced the frequency to 5% (Fobert *et al.*, 1991), reflecting more closely the results with the *RSP3* gene. When the expression of such genes is too low or restricted to a certain period of the cell cycle, the amount of gene product may be insufficient to complement the *pf*-14 mutation. It will be interesting to see whether a reporter gene such as arylsulfatase, which can be scored by enzymatic conversion of a chromogenic substrate, could improve the detection of tagging events.

An alternative explanation for the lower tagging frequency could be the difference in DNA delivery used. The T-DNA transfer is relatively predictable as everything between the borders will be transferred in most cases. The tagging procedure for *C. reinhardtii* relies on the integration of linearized plasmid DNA. Degradation of the *RSP3* gene would thus be a realistic possibility and could account for the reduction in tagging frequency.

Although we have evidence for tagging promoters that are induced by nitrogen starvation, this needs confirmation. Nonetheless, it is striking that cells transformed after nitro-

gen starvation produced twice as many transformants that were motile in nitrogen-free medium only, as those transformed after growth in complete medium (see Table 2). This suggests that the frequency of tagging promoters that are induced by nitrogen starvation is enhanced by growing the recipient cells under inducing conditions. This result provides evidence for the hypothesis that illegitimate recombination takes place preferentially in expressed regions of the genome (Gheysen *et al.*, 1991).

An important feature of our method of promoter tagging is that transformants analyzed express the inserted *RSP3* gene as a fusion transcript with the tagged gene. Indeed, in most cases, both RNA gel blot analyses and the results of the 5' RACE protocol (Figures 4 and 5) revealed the presence of new RNA fused to the *RSP3* transcript. This allowed us to develop a new and fast gene-cloning procedure based on the generation of probes through 5' RACE. Four of the five transformants tested did have a new piece of RNA fused to the *RSP3* transcript. Analysis of transformant 2BC9 showed that the controls at the DNA level (a band-shift, Figure 6a) and the RNA level (knock-out of 2BC9 transcription, Figure 6b) are sufficient to identify the cloned fragment as belonging to the tagged gene. This, together with the speed of the 5' RACE method and the fact that only the expressed copy of multiple *RSP3* gene inserts may be cloned, make this approach superior to the generally applied plasmid rescue and inverse-PCR (Does *et al.*, 1991) methods. Because we have generated a knock-out strain for the 2BC9 gene, we could potentially reintroduce this gene with a peptide epitope and establish whether the protein is localized to the mitochondria.

The promoter tagging procedure for *C. reinhardtii* described here will allow the characterization of genes that are induced by changes in the physiological state of the cell, while mutants are generated simultaneously by insertion of the tagging vector.

Experimental procedures

Construction of transformation vectors containing the *RSP3* gene

For the construction of a promoter-tagging vector, we used a modified version of plasmid pARG7.8, pCB412 (Kropat *et al.*, 1995). This vector has unique *Bam*HI, *Eco*RI and *Nhe*I sites. The complete radial spoke protein *RSP3* gene was cloned as an 3984 bp *Eco*RI fragment from plasmid pEK*RSP3* (Williams *et al.*, 1989) into the *Eco*RI site of pCB412, resulting in plasmid pMH214. The 2674 bp *Nhe*I fragment of the *RSP3* gene, which contains the coding region plus 23 bp of 5' leader and the complete 3' untranslated region, was cloned into the *Nhe*I site of pCB412. This plasmid was named pMH213. Plasmid pMH223 contains the *Nhe*I *RSP3* fragment with a 'flag', the 394 bp *Hpa*I fragment of ϕ X174, cloned into the *Sal*I site of the 3' untranslated region. Plasmids pMH217 and pMH215 contain the complete *RSP3* gene with a single or a double 'flag', respectively, inserted into the *Sal*I site.

C. reinhardtii cultures, transformation, DNA and RNA isolation

C. reinhardtii strains were cultured as described before (Kropat *et al.*, 1995). The strain *pf-14 arg7* (obtained from John Jarvik, Pittsburgh, USA) was used for the glass-bead transformation method as described by Kindle (1990) and Kropat *et al.* (1995). Autolysin-treated cells were transformed with 200 ng of linearized vector DNA and then selected for arginine-independent growth. Isolation of RNA and DNA from *C. reinhardtii* has been described before (Von Gromoff and Beck, 1993).

Phenotypic assay: restoration of swimming activity

Transformants were picked from TAP (tris, acetate, phosphate pH 6.0) plates with sterile toothpicks and resuspended in 100 μ l TAP medium without nitrogen, in the wells of a microtitre plate. After overnight (16 h) incubation in the light, cultures were observed with a stereo microscope (Leica, 400 $\times$ magnification). To score whether transformants swam only in nitrogen-free medium, 100 μ l complete TAP medium was added and scoring performed again after overnight incubation in the light. Although many cells were going through their first division cycle, we scored for motility, when most cells of strains that were always motile were swimming again. Strains that were not swimming in TAP medium were monitored for swimming activity during the rest of the day. Transformants that swam were transferred to TAP plates for further analyses.

DNA and RNA gel blot analyses

Genomic *C. reinhardtii* DNA (2–3 μ g) was digested with *Sst*I, separated on a 0.7% agarose gel and blotted onto Hybond N membrane (Amersham). Total RNA (20 μ g) was separated on a denaturing formaldehyde–1.2% agarose gel and blotted onto Hybond N membrane. Hybridization conditions were as recommended by the manufacturer, using random primer [α^{32} P]dATP-labeled DNA probes. Autoradiographs were made using Kodak XAR-1 films and intensifying screens at -70°C .

RNA PCR and 5' RACE protocols

Total RNA (2–10 μ g) was used to synthesize first-strand cDNA in 20 μ l reaction buffer with 50 ng anti-sense primer 2954 (TGCCTCGTTGTACACGACGG, see Figure 1b) and Superscript II reverse transcriptase (Gibco BRL) at 45°C . A 5 μ l aliquot of this mixture was used in a PCR reaction with primer 2954 and the sense primers 2953 (AGTAGCGAAGCGAGCAGG) or 3009 (GCATCAACGCATATAGCGC). PCR conditions were: 94°C for 5 min, 80°C for 5 min, addition of Taq polymerase (Gibco BRL) and 25 cycles of 94°C for 50 sec, 55°C for 60 sec, 72°C for 30 sec. After RNase treatment, products were analyzed by electrophoresis on 2% agarose TBE gels.

For the 5' RACE protocol, 20 μ g of total RNA was used to synthesize first-strand cDNA with 5 ng of primer 2954. The cDNA/RNA mixture was separated from the primer by precipitation with 0.5 vol NH_4ac and 1.5 vol ethanol at room temperature. The pellet was resuspended in 50 μ l H_2O , denatured at 94°C for 5 min, then put on ice and a tailing reaction carried out with 15 units of terminal transferase (Boehringer Mannheim), 200 μ M dATP in a 70 μ l end-volume for 10 min at 37°C . The reaction was terminated by incubation at 70°C for 10 min. After ethanol precipitation, the tailed-cDNA/RNA mixture was resuspended in 20 μ l TE (tris 10

mM/EDTA 1 mM, pH 8.0). A 10 µl aliquot was used for a PCR reaction with primer 2954 and an oligodT primer (GCT-CTAGAGATCC(T)₁₅) in 50 µl. PCR conditions were: 94°C for 5 min, 80°C for 5 min, addition of Taq polymerase and 30 cycles of 94°C for 60 sec, 55°C for 60 sec, 72°C for 60 sec. A 5 µl aliquot of this reaction was used to amplify specific products using the nested primer 3205 (GGCTCGCTAGCGCTATGCGTTGATGC), the oligodT primer and the same PCR conditions. The 5' RACE products were separated on a 3% Metaphor (FMC) TBE (tris, borate, EDTA) buffer gel, purified, reamplified by PCR, digested with *Bam*HI and *Nhe*I and ligated into pBR322 cut with *Bam*HI and *Nhe*I. Using primer 389 (CGGATATCGTCCATTCCGAC); cloned fragments were sequenced with the T7 polymerase sequence kit (Pharmacia).

cDNA cloning and sequence analysis of the 2BC9 insert

A λnm1149 cDNA library of *C. reinhardtii* (a kind gift of Dr J.-D. Rochaix, Geneva, Switzerland) was screened using the 2BC9 5' RACE fragment as a probe. Two positive plaques were processed and their inserts subcloned into pBluescript SK (Stratagene). The double-stranded DNA sequence was determined by sequence analysis of unidirectional deletion clones. The predicted amino acid sequence was compared with the available databases using the BLAST program at NCBI (Bethesda, USA). The PSORT program at GenomeNet (Japan) was used to predict the targeting of the 2BC9-encoded protein.

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