

# *regA*, a *Volvox* gene that plays a central role in germ-soma differentiation, encodes a novel regulatory protein

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## SUMMARY

*Volvox* has two cell types: mortal somatic cells and immortal germ cells. Here we describe the transposon-tagging, cloning and characterization of *regA*, which plays a central role as a master regulatory gene in *Volvox* germ-soma differentiation by suppressing reproductive activities in somatic cells. The 12.5 kb *regA* transcription unit generates a 6,725 nucleotide mRNA that appears at the beginning of somatic cell differentiation, and that encodes a 111 kDa RegA protein that localizes to the nucleus, and has an unusual abundance of alanine, glutamine and

proline. This is a compositional feature shared by functional domains of many 'active' repressors. These findings are consistent with the hypothesis that RegA acts in somatic cells to repress transcription of genes required for growth and reproduction, including 13 genes whose products are required for chloroplast biogenesis.

Key words: Active repressor, Chloroplast biogenesis, Green algae, Transcriptional repressor, *regA*, *Volvox carteri*

## INTRODUCTION

*Volvox carteri*, a simple multicellular green alga, is an attractive model for studying the genetic control of germ-soma differentiation, because each spheroid (or 'individual') contains only two cell types: mortal somatic cells and immortal germ cells (Kirk, 1998; Schmitt et al., 1992). A typical asexual spheroid contains 16 large asexual reproductive cells, or 'gonidia', just internal to a surface monolayer of small, biflagellate, *Chlamydomonas*-like somatic cells (Fig. 1A). Gonidia are immotile and specialized for reproduction; each will divide to produce a juvenile containing a new cohort of gonidia and somatic cells (Starr, 1969, 1970). Somatic cells, in contrast, are specialized for motility but have no reproductive potential; once differentiated, they never divide, and they eventually undergo programmed death (Pommerville and Kochert, 1981, 1982).

The *regA* locus plays a central role in establishing this germ-soma division of labor by acting as a negative regulator of reproductive functions in somatic cells. Whereas *regA*<sup>+</sup> somatic cells can not be forced to divide, *regA*<sup>-</sup> somatic cells exhibit all of the reproductive potentials of the species (Huskey and Griffin, 1979; Kirk et al., 1987; Starr, 1970). In the asexual phase of the life cycle, *regA*<sup>-</sup> mutants exhibit the 'somatic regeneration' (or 'Reg') phenotype, in which somatic cells appear to differentiate normally, but then redifferentiate as gonidia and divide to produce juveniles of like phenotype; in the sexual phase *regA*<sup>-</sup> somatic cells produce offspring containing eggs or sperm (Starr, 1970). All of the >100 Reg

mutants that have been analyzed to date (Huskey and Griffin, 1979; Kirk et al., 1987; D. Kirk, M. Kirk and K. A. Stamer, unpublished observations) have lesions that map to *regA*, indicating that this is the only locus that can mutate to yield a Reg phenotype. No effect of *regA* mutations on development of 'true' gonidia can be detected, indicating that the gene is not expressed in gonidia. Molecular studies reinforce the view that *regA* acts as a negative regulator of reproductive functions in somatic cells, because *regA*<sup>-</sup> somatic cells accumulate transcripts that are normally accumulated only by developing gonidia (Tam and Kirk, 1991b). We conclude that *regA* is a master regulatory gene that plays a central role in germ-soma differentiation by acting in somatic cells to prevent expression of functions required for germ cell development.

Prior efforts to clone the *regA* locus by performing a chromosome walk from two different linked polymorphisms (Adams et al., 1990; Harper et al., 1987) did not succeed (D. L. Kirk and J. K. Miller, unpublished). Here we report success in using the cold-inducible *Volvox* transposon, *Jordan*, to tag and recover the *regA* locus, and we show that *regA* encodes a nuclear protein with a combination of features similar to ones found in various transcription factors.

## MATERIALS AND METHODS

### *Volvox* strains and cultivation conditions

Except where otherwise specified, *V. carteri* cultures were maintained in standard *Volvox* medium (SVM) at 32°C, under standardized

conditions (Kirk and Kirk, 1983, 1985). SVM lacking the usual urea or ammonium chloride (N-SVM) was used to select for ability to reduce nitrate.

EVE (Adams et al., 1990), is a subclone of HK10, the standard female lab strain of *V. carteri* (Starr, 1969, 1970). CRH22 (Miller et al., 1993), is a morphologically wild-type, Nit<sup>-</sup> (reduced nitrogen requiring) derivative of EVE. HB11A is a multiply-marked derivative of EVE used in previous mapping studies (Adams et al., 1990); it carries a bleomycin induced *regA* mutation, and a stable *nitA* mutation that causes a splicing defect (Gruber et al., 1996). Progeny analyzed in Fig. 2C came from crosses between HB11A and PM1, a genetically distant, wild-type male (Adams et al., 1990). Strain 153-68, a Reg<sup>-</sup>, Nit<sup>-</sup> member of that progeny cohort, was the recipient in all *regA* transformation attempts; the mutant *regA* locus it inherited from HB11A has a 281 bp deletion in exon 6 (Figs 3C and 6) that deletes 93.67 codons, causes a frame shift that generates a stop codon 12 nt down stream, and should result in a mutant *regA* product only 291 residues long.

Candidate *Jordan*-tagged Reg mutants were isolated by microscopic screening of flask cultures of CRH22 after 7-10 days growth at 24°C. Prospective mutants were cultured at 32°C in individual wells of multi-well plates and examined at intervals to determine the stability of the mutant phenotype. No more than one mutant was saved from any flask. This procedure yielded 36 Reg mutants, seven of which were highly revertible, and 28 of which were subsequently found to exhibit readily detected RFLPs at the *regA* locus.

### Preparation of a *regA* genomic clone

Methods for isolation and Southern blot analysis of *Volvox* DNA were as previously described (Miller et al., 1993). The first fragment representing part of the *regA* locus was cloned as follows. The novel 2.7-kb *Jordan*-containing *SalI* restriction fragment (Fig. 2A) of strain 22Reg5 (Fig. 1B) was gel-purified, cloned in plasmid pJOE533 (a suicide vector, Josef Altenbuchner, personal communication), and used as the source of a genomic fragment flanking the site of *Jordan* insertion that was then subcloned in Bluescript KS<sup>+</sup> (Stratagene), yielding plasmid pVR2-B.

About 10<sup>4</sup> pfu from an unamplified library of randomly sheared EVE DNA fragments in  $\lambda$  Dash II (Stratagene) were plated on XL1-Blue MRA (P2) bacteria, and screened with a *regA* probe, resulting in recovery of 17 distinct genomic clones, one of which ( $\lambda$ 7/1, Fig. 3B) was successfully used for phenotypic rescue of Reg strain 153-68.

### Preparation of pVcRegA1, a plasmid containing the $\lambda$ 7/1 insert

A plasmid, pVcRegA1, containing the 15.3-kb insert of  $\lambda$ 7/1 was prepared as follows. First pSK<sup>-</sup> was digested with *NotI* and *XhoI*, and a three-partner ligation was performed with it, a 2.7-kb *NotI/ClaI* fragment derived from the left end of the  $\lambda$ 7/1 insert, and a 5.8-kb *ClaI/XhoI* fragment derived from the right end of the  $\lambda$ 7/1 insert. The resulting plasmid was reopened by *ClaI* digestion, treated with alkali, and ligated to a 6.8-kb *ClaI* central fragment of the  $\lambda$ 7/1 insert. Cloning sites and the orientation of the central *ClaI* fragment were analyzed by sequencing, and the presence of a complete *regA* gene was verified by transformation rescue of strain 153-68.

### Nuclear transformation

Genomic clones were tested for ability to rescue the Reg phenotype via biolistic co-transformation (Schiedlmeier et al., 1994), with the modified *V. carteri nitA* gene carried on plasmid pVcNR15 (Gruber et al., 1996) used as the selectable marker, and strain 153-68 as the DNA recipient. Following bombardment, samples were cultured in small aliquots in N-SVM in multiwell plates and monitored at intervals for the appearance of green, growing Nit<sup>+</sup> survivors. Survivors were transferred to well plates or tubes containing fresh N-

SVM and scored with respect to their Reg phenotypes. No more than one survivor was saved per well. Putative transformants were subjected to PCR amplification of the region in which strain 153-68 has a deletion, using the primers pR1 (5'GTCACATTCCGGTGCAGTCG3') and pR4 (5'GCCACCAGCTCTTCCAGGTT3') which generate 1038 bp and 757 bp products with the wild-type and mutant templates respectively.

### DNA sequencing

The genomic sequence of *regA* was obtained from plasmid subclones of  $\lambda$ 7/1, using dideoxynucleotide chain-termination sequencing (Sanger et al., 1977) with either the T7 Sequenase Quick Denature plasmid sequencing kit (Amersham Life Science) with  $\alpha$ -<sup>35</sup>S-dATP (Amersham), or the Thermo Sequenase radiolabelled-terminator, cycle-sequencing kit (Amersham) with [ $\alpha$ -<sup>32</sup>P]ddNTPs. All regions of *regA* present on the  $\lambda$ 7/1 insert, including all non-coding regions, were sequenced in both directions to a 99.9% level of accuracy. cDNA clones and RT-PCR products were also sequenced by the dideoxy chain-termination method, either manually (using a kit from United States Biochemical), or with an ABI Prism automated sequencer.

### cDNA library preparation and screening

Because repetitive screenings of existing *V. carteri* cDNA libraries with *regA* genomic probes yielded no bona fide *regA* cDNAs, a new library was constructed in Lambda ZAPII (Stratagene) using poly(A)<sup>+</sup> RNA isolated from young juvenile spheroids of EVE. *regA* cDNAs were present in this library at a frequency of approx. 3 $\times$ 10<sup>-4</sup>, but the longest insert recovered in four rounds of screening was only approx. 2.4 kb long, approx. one third as long as the *regA* transcript detected on northern blots. (Subsequently it was found that the region upstream of the 5' end of the longest cDNA was 63% G+C, and extremely difficult to copy with reverse transcriptase.) Hence RT-PCR amplification was used to complete the analysis of the *regA* transcript.

### RT-PCR analysis of the *regA* transcript

The RNA used as template in RT-PCRs designed to complete the sequencing of the *regA* transcript was isolated as previously described (Kirk and Kirk, 1985) from young juvenile spheroids. First-strand cDNAs were prepared using either oligo(T), random-sequence deoxynucleotide hexamers, or (in a few cases) *regA*-specific oligomers as primers for the reverse transcription. Each PCR used a pair of gene-specific primers (Gibco BRL), and cycling conditions were empirically established for each primer pair. The final step in the RT-PCR analysis of the cDNA involved the use of two nested pairs of gene-specific primers to perform 'circular first-strand cDNA-mediated rapid amplification of cDNA ends' (cRACE) by the method of Maruyama et al. (1995), and thereby define the 5' end of the transcript.

### Semi-quantitative RT-PCR

First strand cDNAs for semi-quantitative RT-PCR were generated (as described above) from total RNA samples isolated from either whole spheroids, purified somatic cells, or purified gonidia/embryos harvested from synchronous cultures at intervals throughout the life cycle. Two primer pairs were used for PCRs: A 237 bp *regA*-specific product was generated using pR62 (5'GAACTCC-CGAAAACCTT-G3'), which corresponds to a region of the *regA* sense strand in exon 5, and pR2 (5'CGACTGCACCCGAATGTGAC3'), which corresponds to a portion of the antisense strand in exon 6, while a 355 bp *S18*-specific control product was generated with primers c38-1 (5'ATGGGCTCTCTGGTCCACGGCG3') and c38-2 (5'CGGATC-TTCTTCAGGCGCTCCA3'). (The C38 RNA, which encodes the S-18 ribosomal protein, is present in approximately constant abundance in both cell types; Tam and Kirk, 1991a.) Each RT product used as a template was diluted to the point that the amount of PCR product produced with each primer pair was proportional to the amount of template used. Aliquots of six *regA*-specific and six *S18*-specific PCR

products generated at the same time from a single RT product were electrophoresed through a 1% agarose gel, stained with ethidium bromide, and visualized by UV transillumination. Digital images of each gel were obtained with a CCD camera (Cosmicar/Pentax), and the intensity of the bands was quantitated with the NIH Image1.61 software. The relative abundance of *regA* message at each time point was then calculated by dividing the mean product yield of the six *regA* PCRs by the mean product yield of the six control (S18) PCRs.

### Computer analysis of the RegA peptide sequence

The deduced RegA peptide sequence was analyzed by the SAPS (Brendel et al., 1992), PSORT (Nakai and Kanehisa, 1992), BLAST (Altschul et al., 1990) and PHDsec (Rost and Sander, 1993a, b) computer programs.

### Protein-accumulation studies with HA-tagged RegA

One copy of the coding sequence for the hemagglutinin (HA) nonapeptide epitope (Atassi and Webster, 1983; Field et al., 1988) was inserted into the plasmid pVcRegA1, using a *KpnI* site at the end of exon 6 (Fig. 3C) and a pair of oligonucleotides designed to have *Volvox* codon bias (Schmitt et al., 1992). The resulting plasmid carrying an HA-tagged *regA* gene (pVcRegA2) was used to co-transform strain 153-68 and generate strain A2-12, a Nit<sup>+</sup> Reg<sup>+</sup> co-transformant that was then used for western blot and immunocytological analyses.

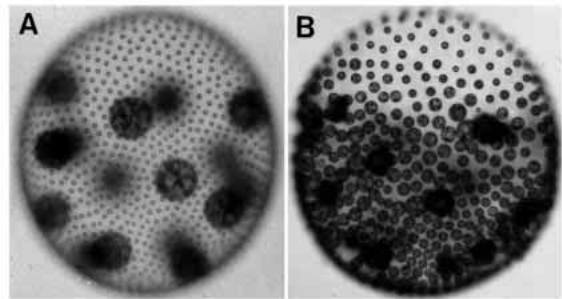
Extracts of A2-12 for western blots were prepared as previously described (Huber et al., 1995), and SDS-PAGE and immunoblotting were carried out according to standard procedures (Sambrook et al., 1989). Immunoblots were reacted for 1 hour at room temperature with a 1:120 dilution of a commercial anti-HA antibody (Boehringer Mannheim), and immunoreactivity was visualized using horseradish peroxidase-linked sheep-anti-mouse antibody (Amersham, 1:1000) and the ECL detection system (Amersham).

Samples of A2-12 spheroids for immunocytologic examination were harvested by filtration, disrupted mechanically, attached to 0.1% polyethylenimine-treated coverslips, fixed for 15 minutes with freshly prepared 3.7% EM grade formaldehyde (Ted Pella Inc.), 0.1% Triton X-100, and 1 mM dithiothreitol (DTT) in phosphate-buffered saline (PBS), extracted for 1 hour with 1% NP40, 1% bovine serum albumin and 1 mM DTT in PBS to remove chlorophyll, exposed for 1 hour to 'blocking solution' (1% BSA and 0.1% Tween 20 in PBS), then overnight to primary antibody (1:10 dilution of anti-HA monoclonal 12CA5, provided by L. Ellis, Washington Univ.) and 4 hours to a mixture of secondary antibodies (2 µg/ml each of Cy<sup>TM</sup>2-labeled goat anti-mouse IgG, and Cy<sup>TM</sup>3-labeled goat anti-rabbit IgG, Amersham). After each of the above steps specimens were washed extensively with 0.1% BSA and 0.1% Tween 20 in PBS. Coverslips were mounted with 1 mg/ml phenylendiamine in 50% glycerol, examined by epifluorescence with a Zeiss Photomikroskop I, and photographed on Hypertech film (Microfluor, Ltd., Stony Brook, NY).

## RESULTS

### Transposon tagging of *regA* and recovery of genomic clones

Transposition of the mobile *Volvox* element *Jordan* can be stimulated by several types of stress (Miller et al., 1993). During cultivation at 22-24°C (approx. the lower limit for growth of *V. carteri*) transposition rates of approx. 10<sup>-4</sup> per gonidium per generation are observed, which is >30-fold higher than the rate at the usual culture temperature of 32°C (S. M. Miller, unpublished). We recovered 36 independent Reg mutants (Fig. 1B) from cultures of CRH22, a morphologically wild-type strain, maintained at 22-24°C and observed that one



**Fig. 1.** Wild-type (CRH22) and Reg (22Reg5) *Volvox carteri* spheroids. (A) Each wild-type adult contains approx. 2,000 small, biflagellate somatic cells at the surface and approx. 16 large asexual reproductive cells, or gonidia, below the surface of a transparent, glycoprotein-rich sphere. In wild-type spheroids somatic cells are <8 µm in diameter at the end of embryogenesis, grow less than two-fold during the next 4-5 days, and then undergo programmed cell death. (B) In Reg ('somatic regenerator') mutants, although somatic cells appear to differentiate normally at first, during the second day of life (pictured here) they become much darker green and begin to grow; then later they divide to produce progeny of similar phenotype. Note that in the spheroid pictured here regeneration has started at the posterior pole of the spheroid (bottom) and is progressing toward the anterior pole.

of them (22Reg5) exhibited a novel, well-resolved *Jordan*-containing genomic DNA fragment (Fig. 2A). DNA flanking the *Jordan* insertion site in this novel fragment was cloned to produce plasmid pVR2-B. When used as a hybridization probe, the pVR2-B insert detected RFLPs in 11 of 23 independent cold-induced Reg mutants that were analyzed on DNA blots (Fig. 2B) and also detected a 281 bp deletion in a bleomycin-induced Reg mutant that co-segregated perfectly with the Reg phenotype among the approx. 50 sexual progeny analyzed (representative data, Fig. 2C).

Two of 17 overlapping clones recovered when pVR2-B was used to probe a lambda library of randomly sheared *V. carteri* DNA fragments are shown diagrammatically in Fig. 3B. When various fragments of these clones were used as probes, they detected RFLPs similar to those illustrated in Fig. 2 in 28 of the 36 Reg mutants that had been isolated following low-temperature growth of CRH22. The distribution of these RFLPs (Fig. 3A) provided the first estimate of the boundaries of the *regA* locus.

### Mutant rescue

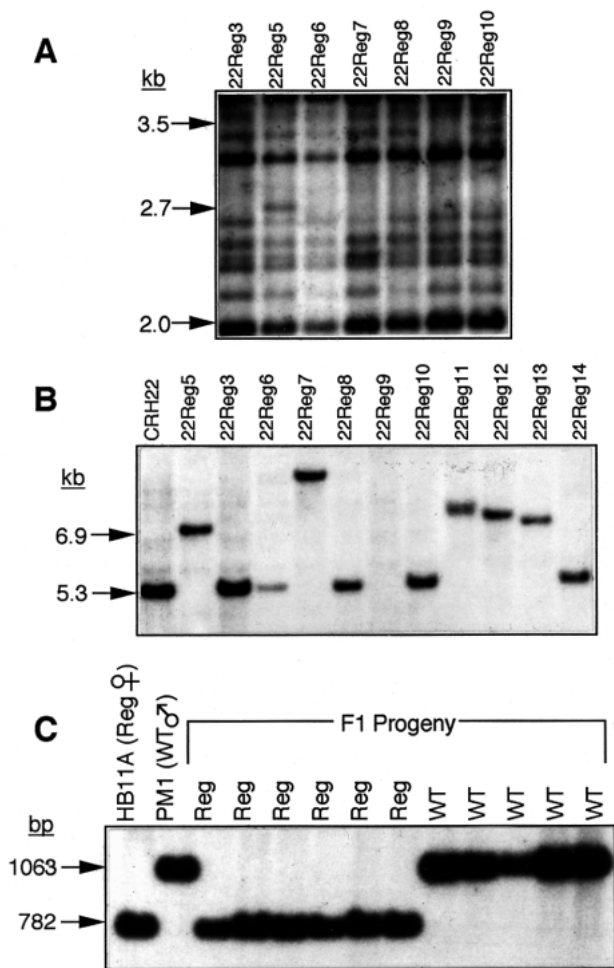
Strain 153-68 (Fig. 4A), which carries a non-revertible mutation of the *nitA* (nitrate-reductase encoding) locus, as well as the 281 bp deletion at the *regA* locus (Fig. 3C), was used as a recipient to test the capacity of putative *regA* genomic clones to achieve mutant rescue via biolistic co-transformation with plasmid pVcNR15 carrying the *Volvox nitA* gene as the selectable marker (Schiedlmeier et al., 1994). When clone λ7/1 (Fig. 3) was used for cotransformation, approx. 30% of all Nit<sup>+</sup> transformants that were recovered had wild-type (*regA*<sup>+</sup>) morphology (Fig. 4B), and Southern blot analysis of four such wild-type strains revealed that in each case the putative *regA* transformant had stably incorporated the transgene (Fig. 4C). In contrast, no amelioration of the Reg phenotype was exhibited by any of the 130 Nit<sup>+</sup> transformants recovered following co-bombardment of

the same recipient strain with clone  $\lambda 4/2$ , the insert of which is only 300 bp shorter at the 5' end than the  $\lambda 7/1$  insert is (Fig. 3). This suggested that the 5' end of the *regA* functional unit might be located near the left end of the  $\lambda 7/1$  insert.

A pSK-based plasmid, pVcRegA1, carrying the  $\lambda 7/1$  insert was prepared and found to be capable of rescuing Reg mutants even more efficiently than  $\lambda 7/1$  (data not shown). Studies using variants of this plasmid carrying *regA* genes lacking one or more introns are in progress.

### Transcription of *regA*

Previous studies (Huskey and Griffin, 1979; Tam and Kirk, 1991a,b; Tam et al., 1991) led to the conclusion that *regA* first



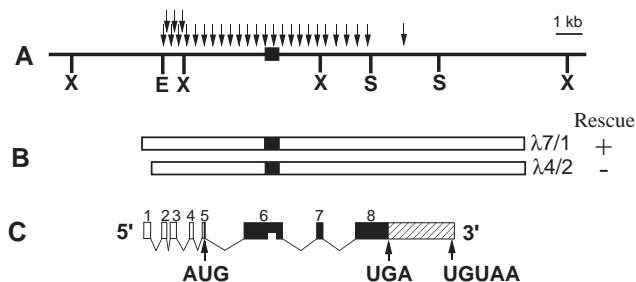
**Fig. 2.** Restriction fragment length polymorphisms (RFLPs) of Reg strains. (A) Southern blot of *SalI*-digested genomic DNAs of three cold-induced Reg strains probed with *Jordan*. A fragment adjacent to the *Jordan* insertion in the 2.7 kb fragment of 22Reg5 was subcloned to produce pVR2-B. (B) Southern blot of *ScaI*-digested DNAs of the wild-type parental strain (CRH22) and eleven independent cold-induced Reg mutants derived from it, probed with pVR2-B. Five strains possess inserts within the size range of *Jordan* elements known to be present in the *V. carteri* genome (Miller et al. 1993); a sixth RFLP was detected in 22Reg9 in a region of the gel not shown. (C) Southern blot of *SalI*-digested genomic DNAs of parental and progeny strains probed with pVR2-B, showing a deletion in HB11A that cosegregated with the Reg phenotype. Fragment sizes shown at the left were determined by sequencing.

acts while presumptive somatic cells are in the early stages of cytodifferentiation, many hours before the Reg phenotype can be detected microscopically. When developmental northern blots were probed with cloned *regA* fragments, an approx. 7 kb transcript was detected in very young somatic cells (Fig. 5A), but never in gonidia (data not shown). However, the amount of *regA* transcript often was so near the limit of detection on RNA blots that expression patterns analyzed this way were not very reproducible. Therefore, semi-quantitative RT-PCR was used to examine temporal and spatial patterns of accumulation of the *regA* transcript. These studies indicated that the transcript appears in very young somatic cells immediately after the end of embryogenesis, and then is present in fluctuating (but generally increasing) abundance in somatic cells throughout most of the life cycle (Fig. 5B). In contrast, the transcript was not detected by RT-PCR in gonidia.

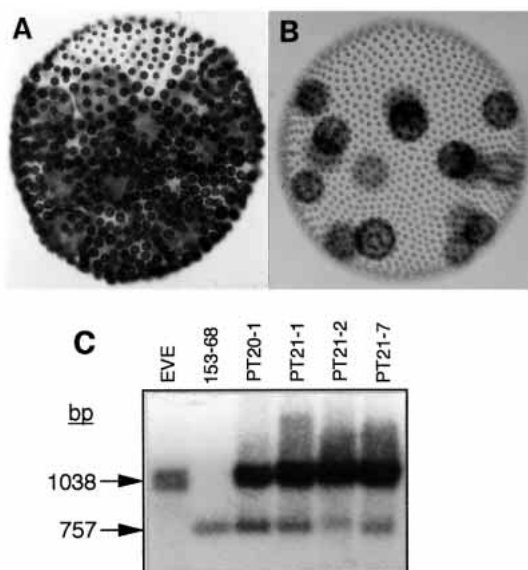
### Structure of the *regA* transcription unit

Repetitive screenings of a somatic-cell cDNA library produced several categories of *regA* cDNA clones that all terminated downstream of a canonical algal polyadenylation signal (TGTA; Schmitt et al., 1992), but differed in their 5' ends. But because the longest cDNA thus recovered was only approx. one third the length of the transcript detected on northern blots, RT-PCR (using primers based on the genomic sequence) was used to amplify and sequence the rest of the transcript. The 5' end of the transcription unit was then identified by the 'cRACE' method of Maruyama et al. (1995).

Comparison of the cDNA sequence with the genomic sequence revealed that the 12,477 nt-long *regA* transcription unit commences 23 nt downstream of a putative TATA box, and only 70 nt downstream of the left end of the  $\lambda 7/1$  insert (the



**Fig. 3.** Diagram of the *regA* locus, with subfigures aligned vertically. (A) Partial restriction map of the *regA* region of the genome; E, *EcoRI*; S, *SacI*; X, *XhoI*. These particular restriction sites are shown because they define the fragments that were screened for RFLPs in 36 independent cold-induced Reg mutants. The arrows above the line indicate the numbers of strains that exhibited RFLPs in each of these regions, but not the precise locations of all RFLPs. The black rectangle indicates the region represented by the insert in pVR2-B. (B) Two clones from the lambda DNA library that were tested extensively for their ability to rescue a Reg mutant by transformation; black rectangle as in (A). Whereas  $\lambda 7/1$  rescued with high efficiency,  $\lambda 4/2$  provided no rescue. (C) The *regA* transcription unit. Inverted carets: introns; open boxes: exons in the 5' UTR; filled boxes: coding exons; hatched box: the 3' UTR; AUG, UGA, UGUAA: initiation codon, termination codon and polyadenylation signal, respectively. The notch in the box representing exon 6 indicates the location of the 281-bp deletion present in the *regA* gene of HB11A and its Reg progeny (see Fig. 2C).



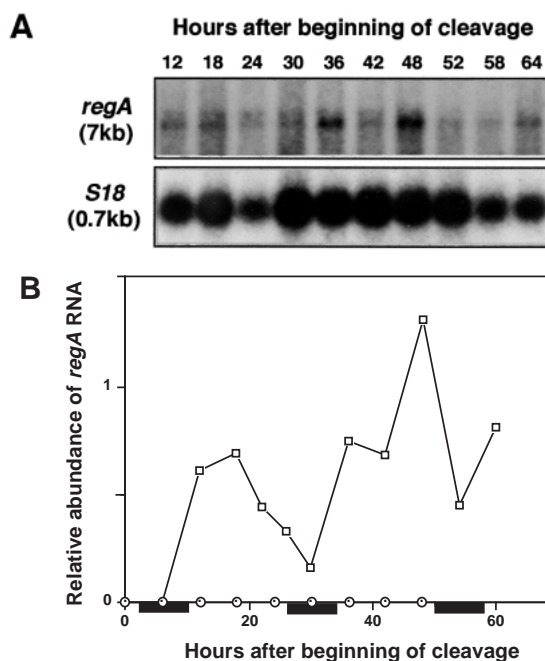
**Fig. 4.** Phenotypic rescue of a mutant by transformation. (A) 153-68, the recipient Reg strain. (B) PT21-1, a wild-type strain recovered following transformation of 153-68 with  $\lambda$ 7/1. (C) Southern blot of PCR products generated from DNA of a wild-type strain (EVE), the recipient strain (153-68) and four independent putative transformants, probed with pVR2-B. Note that each transformant possesses multiple copies of *regA* DNA of wild-type length, as well as the allele with the 281-bp deletion that is present in strain 153-68.

genomic clone that rescues the Reg phenotype of mutants with high efficiency; Fig. 3B). The transcription unit comprises eight exons and seven introns, with four of the introns being located in the 5' UTR (Fig. 3C). The mature *regA* transcript as depicted is 6,725 nt long and consists of a 940-nt 5' UTR, a 3,147-nt coding region, and a 2,638-nt 3' UTR that contains a canonical green-algal polyadenylation signal (UGUAA) 13 nt upstream of the polyadenylation site.

Although all objective evidence presently indicates that the transcription unit has the structure depicted in Fig. 3B, we cannot rule out the possibility that one or more alternative exon 7-intron 7 (E7-I7) splice sites may be used. Although in each of several experiments with independent RNA samples the only RT-PCR product recovered so far has had the E7-I7 structure illustrated, reverse transcription of this region has consistently been exceptionally difficult. Whereas the intron depicted exhibits the same deviation from the canonical border sequence (5'GC...AG3') as three previously described volvoclean introns (Fabry et al., 1993; Harper and Mages, 1988; Kropat et al., 1995), multiple in-frame, canonical potential splice sites are present downstream, in an open reading frame potentially capable of encoding a peptide region very similar in amino acid composition to the rest of the predicted protein. Studies to resolve this issue are in progress.

### Sequence of the deduced RegA protein

The *regA* transcript shown in Fig. 3B is predicted to encode a 1,049-residue, approx. 111 kDa polypeptide that is unusually hydrophilic, and that has a net charge of  $-47$  and a predicted pI of approx. 4.8 (Fig. 6). Computer-aided evaluation of this sequence revealed the following features. RegA is in the 99%



**Fig. 5.** *regA* transcript-abundance patterns in cultures of EVE synchronized with a 16:8 hour light:dark cycle. Timing of the life cycle begins with hour zero, when gonidia begin to cleave, which (in EVE) is approx. 2 hours before the end of a light period; embryogenesis is then completed at approx. hour 8, but differentiation of the juvenile somatic cells and gonidia only begins after the lights come back on at approx. hour 10. (A) A northern blot of RNAs extracted from EVE somatic cells at ten different stages of development, probed simultaneously with a cDNA covering exons 1-6 of *regA* (upper box), and, as a loading control, with a cDNA encoding the S18 ribosomal protein (lower box). No *regA* signal was detected on parallel blots of gonidial RNA samples (not shown). (B) Semi-quantitative RT-PCR analysis of *regA* RNA abundance in EVE somatic cells (squares) and gonidia (circles). Each plotted value is the average amount of PCR product obtained in six independent PCR reactions with *regA*-specific primers divided by the average amount of PCR product obtained at the same time, from the same RT product, in six reactions using the control (*S18*) primers. Black rectangles below the abscissa indicate the periods of darkness during each 24 hour light:dark cycle.

quantile of proteins in the database with respect to exceptionally high content of glutamine, alanine and proline, and in the 5% quantile with respect to exceptionally low content of major hydrophobic amino acids. There are 113 'multiplets' (homo-oligopeptides, such as P<sub>2</sub>, A<sub>7</sub>, Q<sub>12</sub>, etc.; all overlined in Fig. 6), seven of which (at residues 246-50, 604-11, 661-7, 789-95, 798-802, 847-58, and 936-45) are unusually long. No N-terminal signal sequence, potential transmembrane segments, or mitochondrial- or chloroplast-localization signals were detected; however, six nuclear localization signals of the SV40 large T antigen type (one each at residues 461-4, 911-14, and 1016-19 and three overlapping ones at residues 798-803), plus one nuclear localization signal of the *Xenopus* nucleoplasmin type (at residues 618-34) and two potentially significant helix-loop-helix domains (at residues 639-708 and 781-826) were detected.

A BLAST search (Altschul et al., 1990) did not identify any

protein in the database with a highly significant level of overall sequence similarity to RegA. However, three glutamine-rich regions, two alanine-rich regions and two proline-rich regions of RegA (boldfaced in Fig. 6) were identified as having significant similarity (sum probabilities of  $10^{-10}$  to  $10^{-22}$ ) to various sequence elements found in a diverse array of proteins – primarily, but not exclusively, transcription factors – that have been implicated in developmental regulation in other model organisms (see Discussion).

### Accumulation of RegA protein

Strain A2-12 carrying a transgene encoding the RegA protein tagged with the influenza-virus hemagglutinin (HA) epitope was generated by co-transformation of strain 153-68 with a suitably modified plasmid. Anti-HA-stained western blots of stage-specific extracts of A2-12 revealed that HA-tagged RegA protein can first be detected by this method approx. 4 hours after the lights come back on after the completion of embryogenesis (Fig. 7A). In contrast, the *regA* transcript can be detected several hours earlier, at the very beginning of the light period (Fig. 5 and other data not shown). Whether this represents a difference in the threshold sensitivity of the RNA and protein detection methods, or reflects a delay in the translation of the *regA* message is currently unknown. After its initial appearance, HA-tagged RegA protein accumulates progressively for several hours, and then it remains moderately abundant throughout the life of the somatic cells, but it is never detected in gonidia (data not shown).

Indirect immunofluorescence examination of A2-12 with anti-HA antibody clearly reveals, as predicted by PSORT analysis of the sequence, that RegA is a nuclear protein (Fig. 7B-D). No nuclear staining with the anti-HA antibody could be detected in gonidia of strain A2-12, or in somatic cell nuclei of a control strain (EVE) not carrying the HA-tagged transgene.

## DISCUSSION

Ever since Richard Starr described the first mutant with a 'fertile somatic cell,' or 'somatic regeneration' phenotype more than a quarter century ago (Starr, 1970), the *regA* locus, to which all such mutations map, has fascinated *Volvox* developmental biologists. It has long been appreciated that this locus must act as a master regulatory gene that suppresses all aspects of reproductive behavior in the somatic cells of *V. carteri*, because whereas *regA*<sup>+</sup> somatic cells have no reproductive potentials, *regA*<sup>-</sup> somatic cells redifferentiate as germ cells that exhibit all of the asexual and sexual reproductive potentials of the species. The central role played by *regA* in programming the germ-soma dichotomy of *Volvox carteri* motivated our efforts to clone the locus in order to begin an analysis of its regulation and function.

### Jordan as a gene-tagging agent

The present study confirms the prediction made at the time that we initially described the transposon *Jordan*

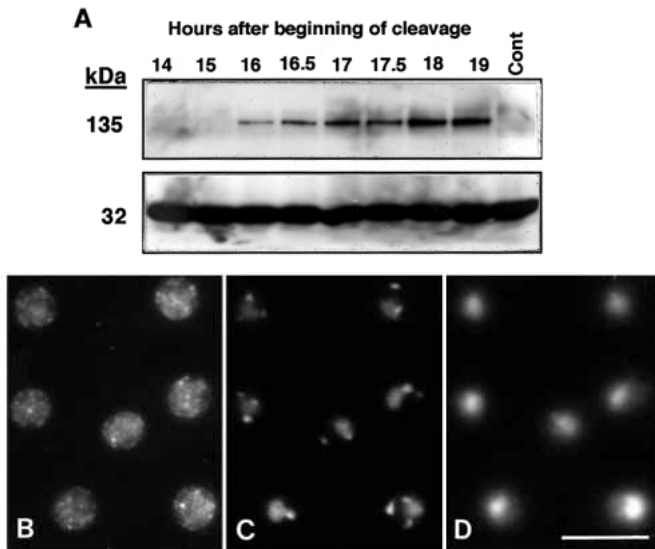
(Miller et al., 1993), namely that it has properties that should make it highly suitable for tagging and recovering *Volvox* genes of developmental interest. Of particular importance is the fact that *Jordan* can be induced to transpose at a substantially elevated rate by any one of a variety of mild environmental stresses (Miller et al., 1993; S. M. Miller, unpublished observations). The stress used here (cultivation at reduced temperature) is clearly the simplest and most convenient for routine use. Of the 36 Reg mutants that were isolated following cultivation of a wild-type strain at 24°C, 28 (80%) have readily detected RFLPs at the *regA* locus (Fig. 3), and of these at least 24 have lesions consistent with the hypothesis that they have been inactivated by a *Jordan* insertion (data not shown).

### Unusual features of the *regA* locus

Comparison of the complete cDNA sequence with the genomic sequence revealed two unusual features of the *regA* transcription unit that may be related: (1) the presumed TATA

MEIGFRKEDS	DIAPVAETQQ	SPQPEQQEQK	DQDMPQPQQL	QPQALQLAAQ	50
<u>QHLQSSAQQP</u>	<u>RPLEQMALDQ</u>	NTELASAATP	AGGSNGGYDA	VKPLSHIRVQ	100
SPGRGSTPLA	LRPILYSMTV	RNADGTPQTA	RQLPSISAPC	SALSAYGPVT	150
SGELPYDESI	EPPLTSVAAY	PRSGRVGGSS	GGGAAGPNGE	DDGLSQGSPS	200
EPELRPATTA	TLQSAIGSLM	VTEGPATSTA	GGELLGADLP	SNEGVQQQQQ	250
<u>IKQQEEQEQEE</u>	<u>QQEEQEQEEQ</u>	<u>EKQEQEQEE</u>	<u>QEQQDQQLA</u>	<u>GPHSASPPEA</u>	300
VAVSAVSDSE	EEVGRGSVSS	GTARPQPSSP	TAAEVTGEAI	EVAAVPPVQP	350
<u>QNIQEYQSSP</u>	<u>PPPALPQQSP</u>	<u>PPPGQPKRAS</u>	AGQSPEASDD	AVQPENAGQ	400
GAGSSADGG	DVAGANTTDT	CAEAPGQPNQ	DLAQNLLELV	AALPPGPIQV	450
TVAVRVGAGG	RRPRGGGSRV	PEEDTRSGYM	RGHACGVFDV	PRYLAGRDCI	500
HNGSKWMSRS	QFEKVGGSKM	AKWYRSIRVL	PDLEPLGEWL	ERHGMPVTKG	550
PARRSRKRAA	ESGDGEEQQG	QGAEQEGVAA	SPDDVQGGV	<u>ADPTAALPAV</u>	600
<u>PPGPPPPPPP</u>	<u>PSQVPQARRA</u>	LSGMALPNGA	RVRRVEPSES	<u>AALDPASAGF</u>	650
<u>GAVASAATRS</u>	<u>AAAAAATAG</u>	GPPPRQERRL	PPPLPSAPLQ	QASALVLDAA	700
TLARGLAALQ	SLDVAGGGLS	GHSRDHLTRT	PASVLNQYLG	QSYTGQRRYR	750
PHAPEPEHIS	ASGDLGYGRS	DTYHGEGSRR	<u>AAASNAATAA</u>	<u>AAAAASLRRR</u>	800
RRPWEDEEEE	DEAEEDDVW	RQRESVCEAP	SRLFRLPAE	LKPNLLQQQQ	850
<u>QQQQQQQLP</u>	HKRQRLGEGE	CQPPFPQLNG	HDPIDVDPYT	VRQHMARLQD	900
PPPSMLLQQQ	HRRRAHAVFA	AAADELGEDD	EHQEGEEEE	EEEEEGGGVA	950
EYWADDTRRH	AYRSGFNAVQ	DAKGEHPQVP	PLAMSLPAVA	ARRPAYYTPQ	1000
RPVMAANREG	AASSWRRPRG	GPVLQGESQG	RRTDWPQQR	RSGDDSLSD*	1050

Fig. 6. Deduced sequence of the RegA polypeptide. Arrowheads indicate locations at which introns interrupt the coding sequence; overlining identifies 113 multiplets present in the sequence; underlining indicates the location of the 281-bp deletion (which ends after the first two nucleotides of codon 381) that is present in strains HB11A and 153-68; bold type indicates the regions that exhibit the most significant similarities to peptide sequences in the database. Both the genomic and the cDNA sequences have been submitted to GenBank (accession numbers: AF106962 and AF106963, respectively).



**Fig. 7.** Accumulation of HA-tagged RegA protein. (A) Western blot analysis. Extracts prepared from A2-12 cultures harvested at the indicated times were applied to each of the first eight lanes. In strain A2-12, cleavage begins approx. 4 hours before the end of a light period (defining hour 0); thus the dark period occurs between hours 4 and 12, and the 16-hour time point occurs approx. 4 hours after the lights come back on. The control sample in the last lane was prepared from an asynchronous culture of strain 153-68, from which strain A2-12 had been derived by transformation. (Asynchronous cultures of A2-12 always exhibit a strong signal at 135 kDa under such conditions.) The signal at approx. 32 kDa constitutes a cross-reacting antigen of unknown nature that the Boehringer Mannheim anti-HA antibody detects (at relatively constant abundance) in all *Volvox* and *Chlamydomonas* samples tested; it serves as a loading control. (This cross reaction is not observed with the 12CA5 antibody used for immunocytochemistry.) (B-D) Fluorescence images of seven somatic cells of strain A2-12 treated with Cy<sup>TM</sup>3-labeled goat anti-rabbit IgG (which stains the cytoplasm lightly and non-specifically) to reveal cellular outlines (B), with DAPI to visualize DNA (C), and with anti-HA monoclonal antibody followed by Cy<sup>TM</sup>2-labeled goat anti-mouse IgG to reveal the location of the HA-tagged RegA (D). Note that whereas DAPI stains the chloroplast nucleoids (small bright spots) in addition to the nuclei, the anti-HA antibody does not; it stains only the nuclei. Scale bar indicates 10  $\mu$ m.

box is located only 42 bp downstream – and the transcription start site is located only 70 bp downstream – of the end of the  $\lambda$ 7/1 insert, and (2) the 940-nt-long 5' UTR is spread out over >2.3 kb of genomic sequence and is interrupted by four introns.

The high frequency with which a Reg mutant can be restored to a fully wild-type phenotype by  $\lambda$ 7/1 DNA (approx. 30%), or by the pVcRegA1 plasmid carrying the same insert (>40%), clearly indicates that this genomic clone must contain all of the elements required for normal cell-type-specific expression of *regA*, despite its short (70 bp) upstream region. We speculate, therefore, that at least some of the *cis*-regulatory elements necessary for proper regulation of *regA* expression may be located within one or more of the four introns that interrupt the 5' UTR. This speculation is consistent with the fact that the region where disabling mutations have been recovered at the highest density is in the 5' UTR (Fig. 3A). We are in the process of testing this hypothesis by evaluating the ability of plasmids lacking one or more of the introns in the 5' UTR

region to provide transformation rescue of mutants. Parallel studies of plasmids carrying inserts truncated at the 3' end will be used to test for possible regulatory elements in the 3' UTR.

### Is RegA a transcriptional repressor?

Conventional genetic reasoning leads to the conclusion that the function of the *regA* gene product, RegA, is to act as a negative regulator of reproductive functions. Therefore, we have long anticipated that *regA* might encode a transcriptional repressor of genes whose products are required for reproductive development. A BLAST search (Altschul et al., 1990) did not identify any protein in the database that exhibits a significant level of overall sequence similarity to the deduced peptide sequence of RegA. Nevertheless, many proteins were identified that possess sequence elements with strong similarity (sum probability scores  $10^{-10}$  to  $10^{-22}$ ) to certain RegA sequence elements, particularly three glutamine (Q)-rich regions, two alanine (A)-rich regions, and two proline (P)-rich regions. Among the proteins so identified were a few that are involved in important aspects of developmental regulation, but that are not transcription factors. However, the majority of the proteins that have sequence elements with significant similarity to elements in RegA are known or suspected transcription factors, including 19 different types from yeast, slime molds, plants, insects and humans. This set of relationships is hardly surprising, since it has been reported previously that 82% of the proteins in the SwissProt data bank with the most significant polyQ motifs, and 78% of those with the most significant polyP stretches, are known or suspected transcription factors (Gerber et al., 1994). The possibility that RegA might function as a transcription factor, is reinforced by the fact that the PSORT program assigned it a nuclear location with a 'certainty' value of 0.96, and that immunocytochemistry confirmed this prediction (Fig. 7B-D).

Further consideration of the amino acid composition of RegA supports the concept that it might function as a repressor: RegA falls in the 99th quantile with respect to its A+Q+P content (34.5% of all residues) and possesses several regions of substantial length (12-50 residues) that are more than 50% A and/or Q and/or P. Abundance of A, Q and/or P in key functional domains is a unifying feature of a group of transcription factors that often have little discernible structural similarity otherwise, but that all function as 'active' repressors – that is, factors that can inhibit transcription initiation directly, as opposed to down-regulating the activity of transcriptional activators (Cowell, 1994; Hanna-Rose and Hansen, 1996). Among the most well-known and most studied active repressors are several of the segmentation genes that act to pattern the early *Drosophila* embryo: namely, *Krüppel* (*Kr*), *knirps* (*kni*), *even-skipped* (*eve*), *engrailed* (*en*) and *paired* (*prd*). Each such active repressor possesses a discrete 'repression domain' that can be attached to the DNA-binding domain of a transcriptional activator (such as yeast GAL4) to convert the latter into an active repressor (Han and Manley, 1993; Hanna-Rose and Hansen, 1996). No two minimal repression domains yet analyzed have significant sequence similarities, but most of them can be categorized in terms of amino acid composition. For example, the repression domains of *Kr*, *kni* and *en* are all A-rich, whereas the *prd* repression domain is P-rich, the *eve* repression domain is A+P-rich, the repression domain of DR1 (a general repressor of transcription)

is A+Q-rich, and although the Wilms tumor repressor, WT1, is said to fall into none of these categories, and to have a 'unique' type of minimal repression domain (Hanna-Rose and Hansen, 1996), it is 35% A+Q+P (Wang et al., 1995), and mutations in P+Q-rich areas flanking its minimal repression domain reduce WT1 repressor activity (Madden et al., 1993).

Based on the above considerations, combined with the conclusion drawn from earlier developmental studies that *regA* encodes a negative regulator of reproduction, we propose that RegA functions as an active transcriptional repressor.

### What genes might be the targets of RegA regulation?

The 18 families of 'maturation-abundant' gonidial cDNAs of *V. carteri* (Tam and Kirk, 1991a) define a set of genes that are candidate targets of RegA regulation. In wild-type spheroids transcripts of these genes begin to accumulate at a low rate in very young somatic cells and gonidia, but then, at about the time that *regA* is believed to act, their transcripts disappear from somatic cells while they accumulate at accelerating rates in developing gonidia (Tam and Kirk, 1991a). In Reg mutants, by contrast, these genes are never down-regulated in the somatic cells; rather, their transcripts accumulate progressively, much as they do in wild-type gonidia (Tam and Kirk, 1991b).

When three of these genes were subsequently characterized by cDNA sequencing, it was learned that they were nuclear genes encoding three key chloroplast proteins (Choi et al., 1996). Recently we have extended this unexpected finding by sequencing the remaining 15 maturation-abundant gonidial cDNAs and determining that all 13 of the genes in this category that encode polypeptides of known function are nuclear genes encoding important chloroplast proteins (M. Meissner, K. Stark, D. L. Kirk and R. Schmitt, unpublished data). These observations lead to the working hypothesis that the mechanism by which RegA prevents somatic cells from entering the reproductive pathway is by preventing transcription of genes that are required for chloroplast biogenesis and (indirectly) cell growth. *Volvox* is an obligate photoautotroph, in which growth is photosynthesis-limited. During embryogenesis, the chloroplast of a gonidium is subdivided among its >2,000 progeny cells, so that each somatic cell begins life with a very modest chloroplast endowment. But if unable to add to this endowment, a somatic cell would have very limited growth potential. And if unable to grow, it would also be unable to reproduce.

One of the more attractive features of the above hypothesis is that it facilitates visualizing how this single gene product, RegA, is able to act as a negative regulator of two complete developmental pathways: the pathways leading to asexual and sexual reproduction. In both green algae and higher plants it has been clearly established that the nuclear and chloroplast genes whose products are required for chloroplast biogenesis are subject to coordinate regulation in response to a wide variety of different intrinsic and extrinsic signals (Kuhlemeir et al., 1987; Sheen, 1994). Of particular relevance in the present context is the fact that in various higher plants, regulatory elements capable of effecting cell-type-specific silencing of chloroplast-specific nuclear genes are both ubiquitous and varied in nature (Kuhlemeir et al., 1987; Sheen and Bogorad, 1986; Simpson et al., 1986). Clearly, the

'photoregulon' of plants and algae is highly receptive to the imposition of new levels of negative transcriptional control that have adaptive value. Our current hypothesis is that the *regA* gene plays its central role in *Volvox* germ-soma differentiation by exploiting this propensity of algal photogenes. The most attractive feature of this hypothesis is that it is testable.

This paper is dedicated to the memory of Richard Starr, in whose laboratory *Volvox* was first brought into continuous culture, who isolated the *V. carteri* strains that served as the progenitors of all of the strains used here, who described the first Reg mutant, and who was a cherished friend and colleague of ours until his sudden death on February 3, 1998. We thank Lisa Ellis for providing the anti-HA monoclonal antibody, Ichiro Nishii for advice regarding preparation of *V. carteri* embryos for immunocytology and Leonard Duncan for extremely useful discussions and advice during late stages of the research and preparation of the manuscript. This work was supported by grants from the NSF (no. MCB-9304447) and USDA (no. 97-3504-4626) to D. K., a DAAD travel grant to K. S., and a grant from the Deutsche Forschungsgemeinschaft (SFB521/B1) to R. S.

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