Two enhancers and one silencer located in the introns of *regA* control somatic cell differentiation in *Volvox carteri*

Klaus Stark,¹ David L. Kirk,² and Rüdiger Schmitt^{1,3}

¹Lehrstuhl für Genetik, University of Regensburg, D-93040 Regensburg, Germany; ²Department of Biology, Washington University, St. Louis, Missouri 63130, USA

The *regA* gene plays a central role in germ-soma differentiation of *Volvox carteri* by suppressing all reproductive functions in somatic cells. Here we show that the minimal promoter of *regA* consists of only 42 bp immediately upstream of the transcription start site, and that it contains no discernible regulatory elements. However, introns 3 and 5 are both required for *regA* expression in somatic cells, and intron 7 is essential for silencing *regA* in gonidia (asexual reproductive cells). A *regA* gene lacking intron 7 rescues the normal phenotype of mutant somatic cells, but also results in gonidia that reproduce only weakly and soon die out. The same phenotype is observed when a *regA* gene containing intron 7 is placed under control of a constitutive promoter, suggesting that the silencing activity of intron 7 is promoter specific. Intron 7 is unusual in that it contains a potential ORF that is in frame with exons 7 and 8, and some transcripts are produced in which intron 7 is retained. However, a regulatory role for the intron 7 translation product can be ruled out, because a construct in which intron 7 must be translated, and one in which it cannot be translated, both result in wild-type development of both cell types. Furthermore, intron 7 is unable to act in *trans* to silence *regA*, but is able to exert its normal effect when placed in a different location within the gene. Therefore, it appears that intron 7 functions in gonidia as a classical cell-type-specific and promoter-specific enhancer, of the inhibitory type that is often referred to as a silencer.

[Key Words: *Volvox carteri*; *regA*; cell-type-specific gene regulation; enhancer; silencer; *fruitless*]

Received December 15, 2000; revised version accepted March 29, 2001.

Volvox carteri is a multicellular green alga with a complete division of labor between somatic cells and asexual reproductive cells (gonidia) (Fig. 1A). Gonidia are immobile and specialized for reproduction (Starr 1969, 1970). In contrast, somatic cells are specialized for motility and phototaxis, but have no reproductive potential. Once differentiated, they never divide, and they eventually undergo senescence and programmed cell death (Pommerle and Kochert 1981, 1982).

Genetic analyses identified the *regA* gene as a master gene that plays a key role in establishing the germ-soma dichotomy by suppressing all aspects of reproductive activity in somatic cells. In the first published discussion of *V. carteri* developmental genetics, it was reported that whereas wild-type somatic cells lack any reproductive potential whatsoever, the fertile somatic cells of what is now called a *regA*, or Reg, mutant (Fig. 1B) are able to participate in both asexual and sexual reproduction (Starr 1970). Such studies led to the working hypothesis

that *regA* encodes a negative regulator that is active in somatic cells — but not in gonidia — and that represses genes required for reproductive development (Kirk 1988). Evidence consistent with that hypothesis came when *regA* was cloned, characterized, and found to encode a protein with features of a transcriptional repressor that appears early in somatic cell development, is present in somatic cell nuclei through most of the life cycle, but can never be detected (at either the RNA or the protein level) in gonidia (Kirk et al. 1999). Further support for such a hypothesis came when 16 genes that are expressed abundantly in both gonidia and Reg somatic cells, but not in wild-type somatic cells (and therefore are putative targets of *regA* regulation), were shown to be nuclear genes encoding chloroplast proteins. This study led to the newer, more specific hypothesis that *regA* blocks reproductive activity in somatic cells by preventing their growth (Meissner et al. 1999).

All such prior studies of *regA* have led to the rather obvious question that we address here: What is the molecular basis for the cell-type-specific expression of *regA*?

Previously, we established that a cloned genomic fragment that provides efficient phenotypic rescue of a non-revertible Reg mutant has a rather unusual structure in

³Corresponding author.

E-MAIL rudolf.schmitt@biologie.uni-regensburg.de; FAX 49-941-9433163. Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.195101>.

Stark et al.

Figure 1. Mutant phenotype, map, and promoter analysis of the *regA* gene. (A) An adult wild-type *Volvox carteri* spheroid containing a surface monolayer of tiny, terminally differentiated somatic cells (arrow) and 16 juvenile spheroids that are about ready to hatch out and become free-swimming individuals; $\sim 20\times$ magnification. Each juvenile contains a surface monolayer of small somatic cells and ~ 16 large asexual reproductive cells called gonidia. (B) A representative spheroid of strain 153-68, the non-revertible Reg/Nit (*regA*⁻/*nitA*⁻) mutant used as the DNA recipient in all cotransformation studies reported here; $\sim 20\times$ magnification. This spheroid is about the same age as the wild-type spheroid shown to its left, but its somatic cells (arrow) have begun to grow and redifferentiate rather than remaining small and terminally differentiated; they will all become fully functional gonidia. (C) The recombinant plasmid, pVcRegA1, containing *regA* in pBlueScript SK(-). The vector portion is shown in grey. Also shown are cloning sites *NotI* and *XhoI* at the 5' and 3' ends, respectively, and the *EcoRI* site used for cloning of promoter deletions. Filled segments symbolize exons, open segments the promoter, introns, 3' UTR and the 3' region downstream of *regA*. (D) Map of the *regA* locus. Open boxes symbolize noncoding exons, solid boxes represent coding exons, and lines between exons represent introns. The transcription start point (+1), start codon (ATG), stop codon (TGA), and poly(A) signal (TGTA) are positioned according to scale. The 70-nt sequence upstream of +1 and the extent of six deletions (Δ Pr1 to Δ Pr6), each with the number of deleted nucleotides in parentheses, are shown below the map. Symbols + or - at the bottom, right indicate whether a construct with that deletion did or did not provide cotransformation rescue of strain 153-68, restoring it to the wild-type morphology depicted in A. The denominators of the quotients to the right of these symbols indicate the total number of Nit⁺ transformants recovered in each case, and the numerators indicate the number of these Nit⁺ strains that were rescued with respect to the Reg phenotype. The start of the minimal promoter defined by these deletion constructs is indicated by a broken line at T42. Putative TATA and initiator (Inr) elements within this promoter are in bold letters.

that it contains only ~ 70 bp of DNA upstream of the transcription start site, has four introns within the 5' UTR (as well as three in the coding region), and a rather long 3' UTR (Kirk et al. 1999). We now have performed systematic deletions of portions of the 3' and 5' regions, and each of the introns, to determine which of these regions may contain *cis*-regulatory elements that are required for normal cell-type-specific expression of *regA*. Here we show that introns 3 and 5 contain enhancers

that are required for expression of *regA* in somatic cells, whereas intron 7 contains a silencer that is required to prevent *regA* expression in gonidia.

Results

The minimal *regA* promoter is only 42 bp long

Bioblastic cobombardment with a selectable marker was used to test the capacity of various *regA* deletion con-

structs to achieve phenotypic rescue of a Reg strain (153–68; Fig. 1B), which carries non-revertible mutations of both the *regA* and *nitA* (nitrate reductase-encoding) loci. The selectable marker was the *nitA* gene carried on the plasmid pVcNR15, which is able to restore Nit⁺ recipients to Nit⁺ (able to grow on nitrate as a sole source of nitrogen). Transforming plasmids integrate into the *Volvox* genome at random locations, frequently in the form of tandem repeats (Babinger et al. 2001). The frequency with which Nit⁺ transformants recovered in such an experiment exhibit cotransformation of an unselected marker such as *regA* (and hence transformation rescue of the mutant phenotype) typically ranges between 30% and 80% (Schiedlmeier et al. 1994; Kirk et al. 1999).

The starting point for making a set of six nested 5'-deletion mutants was plasmid pVcRegA1, which contains the native *regA* locus (Fig. 1C,D), and which was used as the positive control for all cotransformation experiments reported in this work. Four of the 5'-deletion clones, ΔPr1 through ΔPr4, all of which retained at least 42 bp upstream of the transcription start point (+1), yielded an average of 35% cotransformants with wild-type morphology (Fig. 1D). In contrast, no rescue of the Reg phenotype was achieved with clones bearing deletions that extended any further downstream (ΔPr5 and ΔPr6; Fig. 1D). This defines the 42-bp sequence upstream of +1 as the minimal promoter of the *regA* gene. As indicated in Figure 1D, this sequence includes a plausible TATA box (TAATTGA) starting at position -28, and an initiator element (CACTCAT) overlapping the transcription start site at an A, but it contains no obvious regulatory elements. Therefore, other noncoding regions of the *regA* region were systematically screened for regulatory sequences.

There are no regulatory elements downstream of the 3' UTR

A set of five clones with nested deletions covering the 2-kb region immediately downstream of the poly(A) site had no effect on the capacity of the cloned *regA* gene to achieve mutant rescue (data not shown). Therefore, we concluded that the *cis*-regulatory elements controlling *regA* expression must be located within the transcription unit.

Enhancer elements are present in introns 3 and 5

It has been shown that a variety of volvocalean genes, such as *nitA* of *V. carteri*, and *CAH1*, *ODA6*, and *RBCS2* of the closely related unicellular alga *Chlamydomonas reinhardtii*, carry regulatory elements in their introns (Gruber et al. 1996; Kang and Mitchell 1998; Lumbraeras et al. 1998; Kucho et al. 1999). Therefore, we screened the seven introns of *regA* for such elements. Individual introns were deleted (in frame) from plasmid pVcRegA1 by use of overlap extension PCR (Horton et al. 1989). All seven deletion constructs (Fig. 2A) were tested for their ability to rescue the Reg mutant phenotype. Constructs

lacking introns 1, 2, 4, or 6 (ΔI₁, ΔI₂, ΔI₄, and ΔI₆), all rescued the Reg phenotype of strain 153–68 with an efficiency in the 60% range (Fig. 2A), and PCR analysis revealed that in each tested case the transgene had been stably incorporated into the transformant genome (data not shown). In contrast, although PCR analysis revealed that more than half of the Nit⁺ transformants recovered after cobombardment with the *regA* constructs lacking introns 3 and 5 (ΔI₃ or ΔI₅) had stably integrated the *regA* transgene, no amelioration of the Reg phenotype was observed with either of these derivatives (Fig. 2A). This result suggested that deletion of either intron 3 or intron 5 prevents *regA* expression. All attempts to detect transcripts from stably integrated copies of the ΔI₃ or the ΔI₅ transgenes by RT-PCR failed. It was therefore concluded that introns 3 and 5 must both contain positive regulatory elements (enhancers) that are required for *regA* gene transcription.

This concept was further supported by functional analysis of three constructs bearing three intron 5 variants that are depicted in Figure 2B. If a central 937-bp *EcoRV* fragment was deleted from the 1816-bp intron 5, no *regA* mutant rescue was observed among 21 Nit⁺ transformants. However, if this same *EcoRV* fragment was reintegrated in reverse orientation, mutant rescue was observed at the same level as with the wild-type construct (Fig. 2B). Furthermore, a construct from which intron 5 had been deleted regained the capacity to rescue the Reg phenotype with high efficiency when the intron 5 *EcoRV* fragment was inserted into intron 3 (Fig. 2B). This sort of ability to function in both orientations and in different locations is a classical feature of enhancer elements (Banerji et al. 1981). However, database searches using BLAST (Altschul et al. 1990), PLACE (Higo et al. 1999), and TRRD (Kolchanov et al. 2000) failed to identify any previously defined enhancer motif in the 937-bp fragment that had been inverted, or in the rest of intron 5, or in intron 3.

A construct lacking intron 7 causes a lethal fruitless phenotype

Nine of 19 Nit⁺ transformants recovered following cobombardment of strain 153–68 with the *regA* derivative lacking intron 7 (ΔI₇; Fig. 2A), resulted in a completely novel phenotype that we have termed *fruitless*. The somatic cells of the *fruitless* strains exhibit all of the features of phenotypic rescue that are seen following transformation with the control *regA* plasmid. That is, at a developmental age when the somatic cells of the recipient Reg strain have resorbed their eyespots and flagella and have begun to grow rapidly and redifferentiate as reproductive cells (Fig. 1B), the somatic cells of *fruitless* strains exhibit no tendency to grow or redifferentiate. They remain tiny, with typical wild-type somatic features such as eyespots and flagella (Fig. 3A,B). The novel aspect of the *fruitless* strains was that their gonidia grew and reproduced extremely slowly at first, but then invariably died out after no more than two or three asexual generations. At a developmental stage by which the

Figure 2. Intron deletions and modifications that were tested for their ability to achieve cotransformation rescue. (A) Maps of constructs with deletions of intron 1 to 7 (ΔI_1 – ΔI_7), which led to the fusion of neighboring exons as shown. Symbols and quotients to the right of these maps are as defined in Fig. 1D. The term *fruitless* denotes spheroids containing wild-type somatic cells and nonviable gonidia (see text). (B) The role of a 937-bp *EcoRV* fragment of intron 5 in marker rescue was tested by deletion (ΔRV), inversion (RVinv), and transposition into the singular *XhoI* site of intron 3 in a ΔI_5 background ($I_3RV\Delta I_5$). Symbols and quotients to the right of these maps are as defined in Fig. 1D.

gonidia should have completed their growth, developed typical gonidial morphology, and divided, most gonidia of a *fruitless* strain were small, dense, misshapen, and clearly moribund (Fig. 3A). We were unable to recover enough cells from these mutants before they died to perform any molecular analyses.

Constitutive expression of *regA* also causes a *fruitless* phenotype

The foregoing result led to the working hypothesis that the *fruitless* phenotype was a consequence of the fact that in the absence of intron 7 *regA* is expressed at some level in gonidia, in which its product, RegA, functions as it normally does in somatic cells; that is to say, it represses chloroplast biogenesis, which leads eventually to a failure of growth and reproduction (Meissner et al.

1999). This hypothesis led to the prediction that any alternative means of eliciting *regA* expression in gonidia should result in the *fruitless* phenotype—even in the presence of intron 7. To test this prediction, we developed a construct, $P_{Tub}RegA$ (Fig. 3C), in which intron 5 of the *regA* gene was deleted (to abolish its usual expression pattern; see above) and then the rest of the *regA* coding region was fused to a constitutive promoter and the 3' UTR of the *V. carteri* $\beta 2$ -tubulin gene, which is known to be expressed in both gonidia and somatic cells (Mages et al. 1995).

When $P_{Tub}RegA$ was tested in mutant-rescue experiments, 8 of the 23 Nit^+ transformants recovered exhibited the *fruitless* phenotype described above with wild-type somatic cells and small, dense gonidia that soon ceased dividing. Gonidia and somatic cells harvested from the eight transformants with *fruitless* morphology yielded sufficient RNA for RT-PCR tests. As shown in

Figure 3. The *fruitless* phenotype and evidence that it involves expression of *regA* in gonidia. (A) A *fruitless* transformant; $\sim 20\times$ magnification. Three generations are included. The outermost sphere contains the somatic cells of the first generation. Most of the spheroids within it are second generation individuals, but their differences in size attest to the fact that they developed asynchronously (compare the synchronous development depicted in Fig. 1A). Within these second generation spheroids, only one gonidium has grown enough to divide and produce a third generation offspring (arrow, bottom, left). The rest of the gonidia are small, pycnotic, and moribund. (B) Three somatic cells of the first generation individual seen in A viewed at $\sim 1000\times$ magnification. Eyespots, a typical marker of differentiated somatic cells, are indicated by arrowheads. At the same age, somatic cells of the DNA-recipient strain, 153–68, would have resorbed their eyespots, grown manyfold, and would have divided to produce progeny spheroids. (C) Construct *P_{Tub}RegA* that was used to obtain constitutive *regA* expression. It contains the coding region of *regA*, minus intron 5, fused to the promoter (*P_{Tub}*) and 3' UTR of $\beta 2$ -tubulin. Parts of the pBlueScript SK(–) vector are symbolized by broken lines and tubulin gene elements by grey rectangles. Transcription start site (+1), start codon (ATG), stop codon (TGA), and poly(A) signal (TGTA) are shown. Exons (solid boxes) and introns (lines) are numbered as in Fig. 1. A notch indicates the 281-bp region that is deleted in the strain 153–68 *regA* mutant allele (Kirk et al. 1999). Arrows indicate the regions complementary to the oligonucleotides used as primers for RT-PCR as discussed under D. The expected sizes of RT-PCR products are indicated. (D) RT-PCR analysis of *P_{Tub}RegA* transformants that had a phenotype such as shown in A and B. Cell-cycle-arrested gonidia and somatic cells were isolated from eight independent transformants and used for RNA isolation and RT-PCR. Oligonucleotide *pregA* served as primer for first-strand synthesis. In subsequent PCR, the primer pair *pregB/pregC* were used to amplify a transformant-specific 1622-nt transcript extending from the tubulin 5' UTR to *regA* exon 7 (left two lanes). The absence of intron 6 rules out a potential artifact arising from inadvertent amplification of a genomic DNA contaminant. The *pregLE6/pregRE6* primer pair amplifies products (right two lanes) templated by transcripts of both the endogenous mutant allele (469 bp) and the transgene (750 bp) in the somatic cell sample, but only the product templated by the transgene transcript in the gonidial sample.

Figure 3D, both the gonidial and the somatic samples yielded a 1622-bp RT-PCR product with the *pregB/pregC* primer pair, and a 750-bp product with the *pregLE6/pregRE6* primer pair. These are the sizes of the RT-PCR products that should have been produced in the presence of a transcript of the *P_{Tub}RegA* transgene (see Fig. 3C). In contrast, only the somatic cell sample generated a product of the size (469 bp) that should have been produced in the presence of a transcript of the endogenous *regA* gene of the host strain, 153–68, which has a 281-bp deletion in

the region that is amplified by use of the *pregLE6/pregRE6* primer pair (Fig. 3C,D). Thus, these data provide clear evidence that the *regA* transgene driven by the tubulin promoter is transcribed in gonidia, whereas the native *regA* gene under the control of its own promoter is not. Furthermore, these results also are fully consistent with the twin hypotheses that (1) expression of *regA* in gonidia extinguishes the reproductive potentials of these cells, and (2) that intron 7 normally prevents *regA* from being expressed in gonidia.

A spliced transcript retaining intron 7 is present at a low level

The above observations provided strong motivation for exploring possible mechanisms by which intron 7 might act in the gonidia to silence *regA*. In this context, we were intrigued by our earlier observation that the 1194-bp intron 7 is extremely unusual, in that it constitutes an ORF that is fully in frame with the ORFs of the two flanking exons (Kirk et al. 1999), raising the question of whether intron 7 is ever retained in a mature transcript. This possibility was reinforced by the knowledge that intron 7 of *regA* (like three other volvoclean introns; Harper and Mages 1988; Fabry et al., 1993; Kropat et al. 1995) has a GC, instead of the canonical GT, at the donor splice site, which could lead to inefficient splicing. However, our earlier Northern-blot analysis had revealed the presence of only one kind of processed transcript, a 7-kb component lacking intron 7 (Kirk et al. 1999). Therefore, we decided to use a more sensitive RT-PCR method to determine whether we could detect any processed transcript that retained intron 7. To that end, we designed two primers, *pregE* and *pregF*, that are complementary to the ends of exons 6 and 8 (Fig. 4A). RT-PCR with these primers was predicted to yield a 310-bp product with an RNA template from which both exon 6 and exon 7 had been spliced out, a 2845-bp product with an unprocessed nuclear RNA (or a DNA) template, and a 1504-bp product with an RNA molecule from which intron 6 had been completely removed, but intron 7 had been retained (Fig. 4A).

The results of this experiment were unambiguous (Fig. 4B): The major product templated by RNA from somatic cells of both wild-type and *Reg* spheroids is the 310-bp product. However, lesser amounts of the 1504-bp product are also present in each of these samples, indicating the presence of a minor RNA component that lacks intron 6, but retains intron 7. However, it is important to note that no product of ei-

ther size was templated by RNA from either gonidia or embryos.

Translation of intron 7 has no discernible effect on germ-soma differentiation

The demonstration that a processed *regA* transcript that retains intron 7 is present as a minor RNA component in wild-type somatic cells immediately raised the question of whether translation of the intron 7 ORF at some level is required to obtain normal cell-type-specific expression of *regA* (either expression of *regA* in somatic cells or—more likely—silencing of *regA* in gonidia).

This question was addressed by testing the ability of five different types of *regA* variants produced by in vitro mutagenesis (Fig. 5A) to rescue the *Reg* phenotype as follows: (1) In *CCI*₇, the unconventional GC donor splice site was replaced with a CC, making it impossible to remove intron 7 by splicing. (2) In *GCI*₇ 5'Stop, intron 7 was left in a spliceable form, but a stop codon was introduced near its 5' end, making it possible to generate two kinds of mRNAs, but precluding translation of most of intron 7. (3) In *CCI*₇ 5'Stop, the two types of mutations described above were combined, precluding the translation of both intron 7 and exon 8. (4) In *GCI*₇ 3'Stop, intron 7 was left in a spliceable form, but a stop codon was introduced near its 3' end, making it possible to generate two kinds of processed transcripts, and to translate most of intron 7 when it was present. (5) In *CCI*₇ 3'Stop, the loss of the intron 7 donor splice site was combined with the stop codon near the end of intron 7, assuring that most of intron 7 would be—but exon 8 would not be—translated.

To our great surprise, the results (Fig. 5A) clearly indicated that the translation of intron 7 is neither necessary for, nor detrimental to, the differentiation of either cell type. Spheroids with normal, wild-type somatic cells and gonidia were recovered with similarly high frequen-

Figure 4. RT-PCR analysis of intron 7 splicing. (A) Maps of the exon 6 to exon 8 region of *regA*, showing the sizes of the RT-PCR products to be expected by use of *pregD* as a primer for first strand synthesis and the primer pair *pregE*/*pregF* for subsequent PCR if the template RNA lacks intron 7 (*top*), or retains it (*bottom*). (B) RT-PCR analysis of *regA* transcripts from cells of wild-type and mutant (153–68) spheroids. (St) Size standard; (lane 1) wild-type embryos in early cleavage; (lane 2) 1-day-old wild-type gonidia; (lane 3) 1-day-old wild-type somatic cells; and (lane 4) 2-day-old wild-type somatic cells; and (lane 5) 2-day-old *Reg* mutant (153–68) spheroids. The 310-bp band is the major processed transcript that lacks intron 7, whereas the 1504-bp band contains the complete intron 7 sequence, as was subsequently confirmed by sequence analysis.

Figure 5. Variants of intron 7 tested in transformation–rescue experiments. (A) Nucleotide and peptide sequences near the 5' and 3' ends of wild-type (wt) and modified versions of intron 7. Exon sequences are shown in lower case and intron sequences in upper case letters. Nucleotide numbering is according to Fig. 1D. (CCI₇) A single-nucleotide was changed (GC to CC) at the 5' splice site of intron 7 to prevent its splicing. (GCI₇ 5'Stop) A single-nucleotide was changed to introduce a stop codon near the 5' end of intron 7. (CCI₇ 5'Stop) An inactivated 5' splice site as in CCI₇ was combined with a stop codon as in GCI₇ 5'Stop. (GCI₇ 3'Stop) A stop-codon oligonucleotide was inserted into an *EcoRV* site near the 3' end of intron 7. (CCI₇ 3'Stop) An inactivated 5' splice site as in CCI₇ was combined with a stop codon as in GCI₇ 3'Stop. Donor and recipient splice motifs are underlined (wt), substitutions and additions are shown in bold lettering. Symbols and quotients to the right are as defined in Fig. 1D. (B) Map of the exon 6 to 8 region of *regA* with the G to C substitution that inactivates the donor site of intron 7 (CCI₇). To exclude PCR amplification of the transcript from endogenous *regA*, primer pregG corresponding to a position within the region that has been deleted in the mutant allele (notch) was used for RT–PCR. The expected size of the amplification product (including intron 7) is shown. (C) RT–PCR analysis of RNA extracted from cells of four independent CCI₇ transformants in either the first or second day of the 2-day asexual life cycle. (St) Size standard; (lane 1) 1-day-old gonidia of strain CCI₇ #5; (lane 2) 1-day-old somatic cells of strain CCI₇ #5; (lane 3) 2-day-old somatic cells of strain CCI₇ #5; (lane 4) 2-day-old somatic cells of strain CCI₇ #6; (lane 5) 2-day-old somatic cells of strain CCI₇ #7; (lane 6) 2-day-old somatic cells of strain CCI₇ #8; (lane 7) control, 2-day-old spheroids of strain 153–68 (the DNA recipient). (D) Construct P_{Tub}I₇ used to obtain expression of *regA* intron 7. The recombinant plasmid contains the β2-tubulin promoter (P_{Tub}) and 3' UTR as described for P_{Tub}RegA (Fig. 3C) fused to the sequence of intron 7, in frame, to allow potential translation. The CCI₇ splice site variant (A) was used to inhibit potential splicing and to allow accumulation of intron 7 RNA. Nucleotide and peptide sequences near the 5' and 3' ends of intron 7 are shown below. Start and stop codons from β2-tubulin are shown in small letters, intron sequences are shown as in A.

cies when the Reg recipient was transformed with constructs carrying either the wild-type *regA* gene (pVcRegA1), the version of the gene in which intron 7 cannot be removed by splicing, and must be translated (CCI₇), or two versions of the gene in which intron 7 could be removed by splicing, but could not be fully translated if it were not removed (GCI₇ 5'Stop and GCI₇ 3'Stop). In striking contrast, no rescue of the Reg phenotype was obtained with either of the two constructs that contained a stop codon that precluded translation of exon 8 (CCI₇ 5'Stop and CCI₇ 3'Stop).

RT–PCR analysis was used to confirm that construct CCI₇ did, in fact, generate a mature RNA that contained all of intron 7 (Fig. 5B,C). The observation that this construct rescued the Reg phenotype about as well as the wild-type gene leads to the astonishing conclusion that two versions of the RegA polypeptide that differ by the

presence of nearly 400 residues—near the middle of the polypeptide—have indistinguishable biological activities!

Intron 7 RNA has no detectable trans-regulatory effect on regA gene expression

Having been unable to detect any regulatory role for the peptide product of intron 7, we wished to determine whether the RNA product of this intron might be involved in *regA* regulation. To that end, we used our standard cotransformation system to test the effects of a construct, P_{Tub}I₇, in which intron 7 had been placed under the control of the β2-tubulin promoter (Fig. 5D). Eleven of the twenty-nine Nit⁺ transformants that were recovered when Reg strain 153–68 was cobombarded with the *nitA*, ΔI₇ and P_{Tub}I₇ constructs exhibited the *fruitless*

phenotype that had first been observed in spheroids transformed with ΔI_7 in the absence of $P_{Tub}I_7$. No transformants were recovered in which gonidial development was modified in any discernible way that could be attributed to the presence of the $P_{Tub}I_7$ construct. Thus, we found no evidence that the intron 7 transcript could act in *trans* to silence *regA* in gonidia. Nor did we find any evidence that it could affect somatic cell development in any way.

Intron 7 acts in a position-independent manner

To determine whether intron 7, like intron 5, can exert its effect from more than one position (thereby meeting one of the criteria of a classical enhancer; Banerji et al. 1981), we used cotransformation to test the behavior of two constructs in which intron 7 had been placed where intron 6 is normally located (Fig. 6A). In one of these ($GCI_7\Delta I_6$; Fig. 6A), the GC splice site of intron 7 was left intact, so that the intron could be spliced out in transformants, whereas in the other construct ($CCI_7\Delta I_6$) this splice site had been changed to CC, and thereby inactivated.

The $GCI_7\Delta I_6$ construct provided phenotypic rescue of the Reg recipient that was nearly as efficient as that obtained with the wild-type gene; 14 of 24 Nit^+ transformants were converted to wild type, and no *fruitless* cotransformants were recovered. This clearly indicated that intron 7 was able to silence *regA* in gonidia just as well when it was in the intron 6 location as when it was in its usual location.

However, it is noteworthy that the $CCI_7\Delta I_6$ construct, in which splicing of intron 7 had been precluded, provided no phenotypic rescue (Fig. 6A), even though its ORF was in frame with the exons on either side, just as it is in its usual location. Although the RegA protein apparently is fully functional when the peptide encoded by intron 7 is incorporated downstream of the peptide encoded by exon 7, it is nonfunctional when the order of these two peptide regions is reversed (Fig. 6A).

Introns 3 and 5 are required for expression of *regA* in gonidia, just as they are in somatic cells

To assess possible epistatic relationships among the three *cis*-regulatory elements that are present in *regA* introns, we next used cotransformation to evaluate double and triple deletion constructs lacking intron 7 plus one or both of the introns that are required for somatic cell expression of *regA*. As anticipated, none of these constructs lacking introns 3 and/or 5 was able to rescue the phenotype of the Reg somatic cells (Fig. 6B). However, of greater importance was the fact that no gonidia with the *fruitless* phenotype could be detected in any of the 78 Nit^+ transformants that were recovered in these experiments, despite careful microscopic examination. The significance of this will be discussed below.

Discussion

Differential gene expression is an essential prerequisite for cellular differentiation in all multicellular organisms.

Figure 6. Intron rearrangements tested in transformation-rescue experiments. [A] Maps of the wild-type *regA* gene (wt), a variant in which intron 7 with an intact splice site replaces intron 6 ($GCI_7\Delta I_6$), and a similar variant in which the intron 7 splice site has been inactivated ($CCI_7\Delta I_6$). Note that the ORF of intron 7 is in frame with exon 6 in both of these constructs. Symbols and quotients to the right are as defined in Fig. 1D. [B] Double and triple intron deletions tested in transformation rescue experiments. Symbols and quotients to the right are as defined in Fig. 1D. No *fruitless* transformants were detected, despite the absence of intron 7 in each construct.

The *regA* gene has been shown to play a critical role in *V. carteri* germ-soma differentiation, by acting as a master regulatory gene that normally is expressed exclusively in somatic cells, and that represses functions required for cellular growth and reproduction (Kirk et al. 1999; Meissner et al. 1999). This immediately raised a question similar to that raised in the case of any other important regulatory locus, how is the master regulator itself regulated in a cell-type-specific manner? Here we show that at least a partial answer to this question is provided by three cell-type-specific *cis*-regulatory elements that reside within three of the seven *regA* introns.

Two considerations had led us to suspect a priori that important *cis*-regulatory elements might reside within the *regA* introns. The first was our observation that the amount of upstream DNA that had to be included in a *regA* genomic clone to achieve high-efficiency phenotypic rescue of Reg mutants was surprisingly small. We had shown initially that a genomic clone that included only 70 bp of DNA upstream of the transcription start point was capable of rescuing a Reg mutant via cotransformation (Kirk et al. 1999). Here we have shown that no decrease in transformation efficiency is observed when this upstream region is trimmed down to a 42-bp sequence that contains no obvious regulatory elements (Fig. 1D). The second consideration was prior evidence that introns in both *V. carteri* and the closely related unicell, *C. reinhardtii*, contain regulatory information. Introns within the *C. reinhardtii* genes encoding dynein (Kang and Mitchell 1998) and a rubisco small subunit (Lumbreras et al. 1998) contain classical type enhancer elements that exert strong positive effects on transcription. Introns within the *Volvox nitA* gene, on the other hand, appear to exert their positive influences on *nitA* expression at the post-transcriptional level (Gruber et al. 1996), as do many introns of higher-plant genes (Koziel et al. 1996).

We found that introns 3 and 5 of *regA* must both be present in order to accumulate a detectable amount of *regA* transcript, or to achieve phenotypic rescue of a Reg mutant. We also found that a central 937-bp fragment of intron 5 that is essential for phenotypic rescue functions equally well in either orientation or in either of two locations (Fig. 2B). Such orientation- and position-independent functioning is, of course, a hallmark of classical enhancer elements (Banerji et al. 1981), and there is presently no reason to suspect that introns 3 and 5 are anything other than classical enhancers.

By classical enhancers, we mean *cis*-regulatory elements lying outside of the basal promoter region of a locus that bind specific *trans*-acting factors (transcriptional regulators), and thereby act to initiate, increase, decrease, or prevent transcription of the locus. The use of the term enhancers for such elements is unfortunate, of course, because although the term was deliberately introduced to indicate that such elements act only to stimulate transcription of loci that would otherwise be transcribed at some lower level (Banerji et al. 1981), it is now well established that a single such element can exert both qualitative and quantitative, and both positive

and negative effects on transcription of the locus of which it is a part (for review, see Novina and Roy 1996; Arnone and Davidson 1997). As one example of the mechanistic ambiguity of the term enhancer, it has now been established that the stripe 3 and 7 enhancer of the *Drosophila even-skipped* (*eve*) locus functions exclusively in a negative manner (i.e., by repression) to establish the 3rd and 7th stripes of *eve* transcription in the fly embryo. When fused to a reporter gene, this enhancer silences (represses) the gene in the regions anterior to stripe 3, posterior to stripe 7, and between the two stripes, but permits expression within both stripes (Small et al. 1996). A different illustrative example is a well-studied enhancer of sea urchins that acts to silence the *endo 16* gene in mesomeres under one set of conditions, but to activate transcription of *endo 16* in mesomeres under other conditions (Yuh et al. 1998).

The novel *fruitless* phenotype that was observed when Reg mutants were transformed with a *regA* gene lacking intron 7 (Fig. 3A,B) indicated that this intron is required to silence *regA* in gonidia. This interpretation was reinforced when the identical phenotype was observed in Reg mutants that had been transformed with a *regA* gene under the control of a constitutive promoter that caused it to be transcribed in gonidia (Fig. 3C,D). Certain unusual structural features of intron 7 suggested initially that it might exert its effects by some rather novel mechanism. Like a few other *Chlamydomonas* and *Volvox* introns that have been described, *regA* intron 7 has a non-canonical splice junction, which we suspected might cause a splicing defect. This suspicion was confirmed when we detected a minor fraction of the processed *regA* transcript that retained intron 7 (Fig. 4B). We had shown earlier that intron 7 consisted entirely of an ORF that was perfectly in frame with the exons on either side, and that encoded a polypeptide very similar in amino acid composition to the rest of the RegA protein (Kirk et al. 1999). Thus, we speculated that the translation product of intron 7 might play some important role in regulating *regA* expression. However, this possibility can now be ruled out by the observation reported here that both cell types differentiate in a completely normal, wild-type manner both in transformants carrying a *regA* gene in which intron 7 cannot be translated (GCI₇ 5' Stop; Fig. 5A), and in transformants in which intron 7 must be translated (CCI₇; Fig. 5A).

These results deserve additional consideration. RT-PCR analysis of four independent transformants that had been rescued with the CCI₇ construct confirmed that (as anticipated) only one form of the *regA* transcript can be detected in the somatic cells of these strains, the transcript containing all of intron 7 (Fig. 5C). But all of these strains were indistinguishable in phenotype from wild-type *V. carteri* as are the transformants carrying the GCI₇ 5' Stop construct, in which a stop codon precludes translation of most of intron 7. These astonishing results indicate that the RegA protein must be able to function in the same manner in either of two versions that differ in length by the 398 amino acid residues that are encoded by intron 7 and that are located nearly in the

middle of the RegA polypeptide. We are unaware of any precedent for such an observation.

These studies appeared to rule out the possibility that intron 7 exerts its silencing effect on *regA* in gonidia via its polypeptide product. Therefore, we next reconsidered the possibility that it might do so via its RNA product. Two of our earlier observations made it seem to us that this possibility was rather unlikely. The first was that we had never been able to detect any *regA* transcripts of any kind in wild-type gonidia (see Figs. 4B and 5C, for example). The second was that an experiment reported here established that intron 7 is unable to silence *regA* in gonidia when it is present in a transgene that is being transcribed under the control of a constitutive promoter (Fig. 3C,D). That is to say, it appears to function in a promoter-specific manner, which is usually taken as a hallmark of transcriptional, rather than post-transcriptional, regulation. Nevertheless, we performed one more experiment to test the hypothesis that intron 7 exerts its silencing effect in gonidia at a post-transcriptional level, via its RNA product. We cotransformed Reg spheroids with a *regA* construct lacking intron 7, together with a construct in which intron 7 was expressed (by itself) under the control of the constitutive β 2-tubulin promoter (Fig. 5D). We found no evidence that when intron 7 was transcribed in this way — in *trans* to an intron 7-less gene — it had any effect on development of either cell type.

The fact that intron 7 could not exert its effect in *trans* established that intron 7 is (by definition) a *cis*-regulatory element and strongly suggested that it exerts its effect at the transcriptional level. Transcriptional control that is mediated by a *cis*-regulatory element in a promoter-specific manner is a defining feature of classical enhancers, so we next performed an experiment to determine whether intron 7 met another defining feature

of an enhancer: position-independent function. We found that it did. Intron 7 is as able to silence *regA* in gonidia when it is located in the position where intron 6 is normally located as it is when it is in its native location (Fig. 6A). Therefore, we are led to conclude that the unusual structural features of intron 7 are purely coincidental, and that its cell-type-specific regulatory activity is due to the fact that it contains a classical enhancer element, but of the inhibitory type sometimes called a silencer.

However, the transformations we performed with double and triple intron deletion constructs led to an important additional insight about regulation of *regA* in gonidia. Although a *regA* construct lacking only intron 7 is expressed in gonidia and causes all transformants to exhibit the *fruitless* gonidial phenotype, no such effect is seen if either intron 3 or intron 5 has also been deleted (Fig. 6B). This clearly indicates that both intron 3 and intron 5 must be required for transcription of *regA* in gonidia, just as they are in somatic cells. This, in turn, indicates that all of the *trans*-acting factors required for the functioning of the intron 3 and intron 5 enhancers must be present in gonidia, just as they are in somatic cells. Under normal conditions, however, intron 7 obviously overrides the enhancer activity of introns 3 and 5 in gonidia. A model consistent with all of these observations is presented in Figure 7.

Identification of *cis*-regulatory elements regulating cell-type-specific transcription of a gene of interest is, of course, only the first step in defining the complete regulatory network at play in the developmental system of interest. The obvious next step is to identify the cell-type-specific transcription factors for which the newly identified *cis*-regulatory elements serve as targets. Efforts to identify these *trans*-acting factors are presently being initiated, using now-standard techniques.

Figure 7. A minimal model for cell-type-specific regulation of *regA* transcription by the *cis*-regulatory elements located in introns 3, 5, and 7. In somatic cells, introns 3 and 5 bind complementary *trans*-acting factors, which then cooperate to activate the *regA* promoter. Introns 3 and 5 bind these *trans*-acting factors in gonidia also, but in gonidia, intron 7 binds one or more gonidium-specific *trans*-acting factor(s), which enables it to block the effects of introns 3 and 5 and prevent activation of the *regA* promoter.

Materials and methods

Construction of regA vectors

The complementing genomic *regA* clone pVcRegA1 (15.3 kb; GenBank accession no. AF106962) has been described previously (Kirk et al. 1999). Deletion and mutagenesis constructs were generated, as described below, with suitable subclones of pVcRegA1. All cloning sites and the orientation of inserts were analyzed by sequencing with an ABI Prism automated sequencer. PCR products used for cloning were verified by full-length sequencing, to exclude PCR errors. All oligonucleotides were obtained from Metabion (Martinsried, Germany).

Constructs ΔPr1 to ΔPr6 were generated with PCR by use of six oligonucleotides complementary to the upstream transcription initiation site for the *regA* sequence. Each contained an artificial *NotI* restriction site on the 5' end. Oligomer pE2RI (5'-ACTTCAGGAATTCTTCTTG-3'), overlapping the *EcoRI* site in exon 2, was used as the second primer in PCR. Intron deletions were constructed by overlap extension PCR (Horton et al. 1989). That method was also used to generate a nonfunctional intron 7 splice site (CCI₇) and for insertion of a stop codon near the 5' splice site (GCI₇ 5'Stop and CCI₇ 5'Stop). Downstream stop codons were inserted into intron 7 by use of complementary oligomers pI7Stop1 (5'-TGATAGTAACTGACCTGATAG-3') and pI7Stop2 (5'-CTATCAGGTCAGTTACTATCA-3') that were ligated to the *EcoRV* restriction site at position 8220 to generate plasmids GCI₇ 3'Stop and CCI₇ 3'Stop.

The recombinant plasmid P_{Tub}RegA contains, within the pBlueScript SK(-) vector, the constitutive promoter and 3' UTR of the *V. carteri* β2-tubulin gene (Mages et al. 1995; GenBank accession no. L24547) fused to a *regA* coding region from which intron 5 had been deleted. This construct was prepared as follows: First, a fragment that extends from 338 bp upstream to 119 bp downstream of the β2-tubulin transcription start site (and that therefore terminates with the tubulin initiation codon) was amplified by PCR, by use of appropriate primers containing artificial *NotI* and *NcoI* restriction sites. Next, this fragment was fused in frame to the PCR-generated *regA* coding region using the *NcoI* site. Then a 355-bp PCR product representing the β2-tubulin 3' UTR, including its polyadenylation signal, was attached downstream of the *regA* coding region, exploiting a unique *XhoI* site that is located just downstream of the *regA* stop codon. The same tubulin gene elements were used to generate recombinant plasmid P_{Tub}I₇ with the splicing-deficient version of *regA* intron 7.

Other constructs were made by use of appropriate restriction sites of pVcRegA1 and suitable subclones.

Volvox strains, cultivation conditions, and nuclear transformation

V. carteri cultures were maintained in standard *Volvox* medium (SVM) at 30°C under a 16:8-h light:dark cycle (Kirk and Kirk 1983, 1985). SVM lacking reduced nitrogen (SVMN) was used to select for clones able to reduce nitrate after genetic transformation. Nuclear transformation of *Volvox* was performed according to Schiedlmeier et al. (1994) with modifications. Cells of strain 153-68 (which carries nonrevertible mutations in both the *nitA* and *regA* loci; Kirk et al. 1999) were cobombarded with plasmid pVcNR15 (which carries the selectable marker, *nitA*; Gruber et al. 1996) and various *regA* constructs that are described elsewhere in the text. Equimolar quantities of each construct were used. The cotransformation efficiency observed previously with this system has varied between 30% and 80% (Schiedlmeier et al. 1994; Kirk et al. 1999).

Analysis of *Volvox* genomic DNA and RT-PCR analysis of the regA transcript

Volvox genomic DNA was prepared as described (Miller and Kirk 1999). Integration of *regA* constructs after transformation was tested for by PCR, by use of oligonucleotides pregLE6 (5'-TTGGGAGCCGACCTGCCAT-3') and pregRE6 (5'-AGCGTGACCTCGCATGTATC-3') as primers. This PCR system generates a 750-bp product from the wild-type *regA* allele and a 469-bp product from the *regA* allele present in strain 153-68, which carries a 281-bp deletion. Production, electrophoretic separation, and visualization of PCR products were as described (Kirk et al. 1999). The Stratagene kilobase DNA ladder was used as a size marker.

Published protocols (Kirk and Kirk 1985; Hallmann and Sumper 1994) were used to prepare RNA. First-strand cDNA was generated by reverse transcription by use of *regA*-specific oligomers pregA (5'-GCACTGCCGGAAGAGCCG-3') and pregD (5'-CGACGCTCCTGTCTGAGGC-3'), respectively, as primers. The subsequent PCR used gene-specific primers (pregB, 5'-CGCCTCGTGATCCCGGAC-3'; pregC, 5'-GATGACCCGGTACCACTTG-3'; pregE, 5'-CGCAGTTCGAAAAG-3'; pregF, 5'-TGCGACGGTGGCGGCG-3'; pregG, 5'-GCAGC CGAAGCGAGCATC-3'), as shown in Figures 3, 4, and 5. Cycling conditions were empirically established for each primer pair. Expression of P_{Tub}RegA in transformants was analyzed by RT-PCR of purified RNA from somatic cells and gonidia.

Acknowledgments

We thank Antje Machetanz, André Rusch, and Uschi Zeiser for technical assistance. This work was supported by a travel grant from Deutscher Akademischer Austauschdienst (DAAD) to K.S., a grant from the National Science Foundation (IBN-9904739) to D.L.K., and a grant from the Deutsche Forschungsgemeinschaft (SFB521/B1) to R.S.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**: 403-410.
- Arnone, M.I. and Davidson, E.H. 1997. The hardwiring of development: Organization and function of genomic regulatory systems. *Development* **124**: 1851-1864.
- Babinger, P., Kobl, I., Mages, W., and Schmitt, R. 2001. A link between DNA methylation and epigenetic silencing in transgenic *Volvox carteri*. *Nucleic Acids Res.* **29**: 1261-1271.
- Banerji, J., Rusconi, S., and Schaffner, W. 1981. Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. *Cell* **27**: 299-308.
- Fabry, S., Jacobsen, A., Huber, H., Palme, K., and Schmitt, R. 1993. Structure, expression, and phylogenetic relationships of a family of *ypt* genes encoding small G-proteins in the green alga *Volvox carteri*. *Curr. Genet.* **24**: 229-240.
- Gruber, H., Kirzinger, S.H., and Schmitt, R. 1996. Expression of the *Volvox* gene encoding nitrate reductase: Mutation-dependent activation of cryptic splice sites and intron-enhanced gene expression from a cDNA. *Plant Mol. Biol.* **31**: 1-12.

- Hallmann, A. and Sumper, M. 1994. Reporter genes and highly regulated promoters as tools for transformation experiments in *Volvox carteri*. *Proc. Natl. Acad. Sci.* **91**: 11562–11566.
- Harper, J.F. and Mages, W. 1988. Organization and structure of *Volvox* beta-tubulin genes. *Mol. Gen. Genet.* **213**: 315–324.
- Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T. 1999. Plant cis-acting regulatory DNA elements (PLACE) database. 1999. *Nucleic Acids Res.* **27**: 297–300.
- Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. 1989. Engineering hybrid genes without the use of restriction enzymes: Gene splicing by overlap extension. *Gene* **77**: 61–68.
- Kang, Y. and Mitchell, D.R. 1998. An intronic enhancer is required for deflagellation-induced transcriptional regulation of a *Chlamydomonas reinhardtii* dynein gene. *Mol. Biol. Cell* **9**: 3085–3094.
- Kirk, D.L. 1988. The ontogeny and phylogeny of cellular differentiation in *Volvox*. *Trends Genet.* **4**: 32–36.
- Kirk, D.L. and Kirk, M.M. 1983. Protein synthetic patterns during the asexual life cycle of *Volvox carteri*. *Dev. Biol.* **96**: 493–506.
- Kirk, M.M. and Kirk, D.L. 1985. Translational regulation of protein synthesis, in response to light, at a critical stage of *Volvox* development. *Cell* **41**: 419–428.
- Kirk, M.M., Stark, K., Miller, S.M., Mueller, W., Taillon, B.E., Gruber, H., Schmitt, R., and Kirk, D.L. 1999. *regA*, a *Volvox* gene that plays a central role in germ-soma differentiation, encodes a novel regulatory protein. *Development* **126**: 639–647.
- Kolchanov, N.A., Podkolodnaya, O.A., Ananko, E.A., Ignatieva, E.V., Stepanenko, I.L., Kel-Margoulis, O.V., Kel, A.E., Merkulova, T.I., Goryachkovskaya, T.N., Busygina, T.V., et al. 2000. Transcription regulatory regions database (TRRD): Its status in 2000. *Nucleic Acids Res.* **28**: 298–301.
- Kozziel, M.G., Carozzi, N.B., and Desai, N. 1996. Optimizing expression of transgenes with an emphasis on post-transcriptional events. *Plant Mol. Biol.* **32**: 393–405.
- Kropat, J., von Gromoff, E.D., Muller, F.W., and Beck, C.F. 1995. Heat shock and light activation of a *Chlamydomonas* HSP70 gene are mediated by independent regulatory pathways. *Mol. Gen. Genet.* **248**: 727–734.
- Kucho, K., Ohyama, K., and Fukuzawa, H. 1999. CO₂-responsive transcriptional regulation of *CAH1* encoding carbonic anhydrase is mediated by enhancer and silencer regions in *Chlamydomonas reinhardtii*. *Plant Physiol.* **121**: 1329–1338.
- Lumbreras, V., Stevens, D.R., and Purton, S. 1998. Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *Plant J.* **14**: 441–447.
- Mages, W., Cresnar, B., Harper, J.F., Brüderlein, M., and Schmitt, R. 1995. *Volvox carteri* alpha 2- and beta 2-tubulin-encoding genes: Regulatory signals and transcription. *Gene* **160**: 47–54.
- Meissner, M., Stark, K., Cresnar, B., Kirk, D.L., and Schmitt, R. 1999. *Volvox* germline-specific genes that are putative targets of RegA repression encode chloroplast proteins. *Curr. Genet.* **36**: 363–370.
- Miller, S.M. and Kirk, D.L. 1999. *glsA*, a *Volvox* gene required for asymmetric division and germ cell specification, encodes a chaperone-like protein. *Development* **126**: 649–658.
- Novina, C.D. and Roy, A.L. 1996. Core promoters and transcriptional control. *Trends Genet.* **12**: 351–355.
- Pommerville, J.C. and Kochert, G.D. 1981. Changes in somatic cell structure during senescence of *Volvox carteri*. *Eur. J. Cell Biol.* **24**: 236–243.
- Pommerville, J. and Kochert, G. 1982. Effects of senescence on somatic cell physiology in the green alga *Volvox carteri*. *Exp. Cell Res.* **140**: 39–45.
- Schiedlmeier, B., Schmitt, R., Müller, W., Kirk, M.M., Gruber, H., Mages, W., and Kirk, D.L. 1994. Nuclear transformation of *Volvox carteri*. *Proc. Natl. Acad. Sci.* **91**: 5080–5084.
- Small, S., Blair, A., and Levine, M. 1996. Regulation of two pair-rule stripes by a single enhancer in the *Drosophila* embryo. *Dev. Biol.* **175**: 314–324.
- Starr, R.C. 1969. Structure, reproduction and differentiation in *Volvox carteri* f. *nagariensis* IYENGAR, strains HK 9 & 10. *Arch. Protistenk.* **111**: 204–222.
- Starr, R.C. 1970. Control of differentiation in *Volvox*. *Symp. Soc. Dev. Biol.* **29**: 59–100.
- Yuh, C.H., Bolouri, H., and Davidson, E.H. 1998. Genomic cis-regulatory logic: Experimental and computational analysis of a sea urchin gene. *Science* **279**: 1896–1902.

Two enhancers and one silencer located in the introns of *regA* control somatic cell differentiation in *Volvox carteri*

Klaus Stark, David L. Kirk and Rüdiger Schmitt

Genes Dev. 2001, **15**:

Access the most recent version at doi:[10.1101/gad.195101](https://doi.org/10.1101/gad.195101)

References

This article cites 31 articles, 8 of which can be accessed free at:
<http://genesdev.cshlp.org/content/15/11/1449.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).