

Translational control of *regA*, a key gene controlling cell differentiation in *Volvox carteri*

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The complete division of labour between the reproductive and somatic cells of the green alga *Volvox carteri* is controlled by three types of genes. One of these is the *regA* gene, which controls terminal differentiation of the somatic cells. Here, we examined translational control elements located in the 5' UTR of *regA*, particularly the eight upstream start codons (AUGs) that have to be bypassed by the translation machinery before *regA* can be translated. The results of our systematic mutational, structural and functional analysis of the 5' UTR led us to conclude that a ribosome-shunting mechanism – rather than leaky scanning, ribosomal reinitiation, or internal ribosome entry site (IRES)-mediated initiation – controls the translation of *regA* mRNA. This mechanism, which involves dissociation of the 40S initiation complex from the message, followed by reattachment downstream, in order to bypass a secondary structure block in the mRNA, was validated by deleting the predicted 'landing site' (which prevented *regA* expression) and inserting a stable 64 nucleotide hairpin just upstream of this site (which did not prevent *regA* expression). We believe that this is the first report suggesting that translation of an mRNA in a green eukaryote is controlled by ribosome shunting.

KEY WORDS: Germ-soma differentiation, *regA*, Translation control, Upstream AUGs, Ribosome shunting, *Volvox carteri*

INTRODUCTION

Volvox carteri (Fig. 1B) is a multicellular green alga with a complete division of labour between two cell types: several thousand small somatic cells on the surface of the spheroid and 16 large reproductive cells (gonidia) inside. Gonidia are immotile and specialized for reproduction (Starr, 1969; Starr, 1970). By contrast, the somatic cells are specialized to provide flagellar motility and have no reproductive potential: once differentiated they never divide, and after ~96 hours they die (Kirk, 1997; Kirk, 1998; Kirk, 2001; Pommerville and Kochert, 1982; Pommerville and Kochert, 1981; Schmitt, 2003).

Three types of genes – *gls*, *lag* and *regA* – are responsible for cell differentiation in *V. carteri*. The *regA* gene is a master control gene that is expressed only in somatic cells, where it suppresses all reproductive activity. If the *regA* gene is defective, the Reg phenotype (Fig. 1C) appears, in which the somatic cells appear to differentiate normally at first, but then redifferentiate as fully functional reproductive cells. RegA, the *regA* gene product, is a protein with features of a transcriptional repressor that is present in somatic nuclei through most of the life cycle, but can never be detected in gonidia (Kirk et al., 1999; Stark et al., 2001). Sixteen putative targets of RegA repression were found to be nuclear genes encoding important chloroplast proteins; this led to the hypothesis that *regA* blocks reproductive activity in somatic cells by preventing chloroplast biogenesis, and thereby preventing their growth (Meissner et al., 1999).

All of these aspects led to the belief that *regA* must be regulated in a very exact manner. Previous studies had shown that the transcription of *regA* is regulated primarily by two enhancers and one silencer located in the introns (Stark et al., 2001). But there were also indications that *regA* might be regulated at the translational

level. First, it was observed that the synthesis of RegA protein lags behind the appearance of the *regA* mRNA by 4 hours (Kirk et al., 1999). Second, the level of RegA protein is much lower than might be expected from the mRNA level. Third, there are eight potential start codons (AUGs) (Fig. 2) in the 940 nucleotide 5' UTR of the *regA* mRNA that would have to be bypassed by the translation initiation complex in some manner, in order to reach the bona fide initiation codon.

In eukaryotes, three different mechanisms in addition to normal ribosomal scanning are known to regulate translation initiation: (1) leaky scanning (Kozak, 2002); (2) reinitiation (Kozak, 2002); and (3) internal ribosome entry site (IRES)-mediated translation initiation (Hellen and Sarnow, 2001). In leaky scanning the translation initiation complex does not recognize a potential start codon on the mRNA, because the surrounding sequence deviates strongly from the ideal, so-called Kozak sequence (Kozak, 2002). Thus the complex moves on, and translation starts at a subsequent start codon. Reinitiation takes place on mRNAs with short upstream open reading frames (ORFs); after the translation of such ORFs, the 60S ribosome subunit dissociates from the mRNA, but the 40S subunit remains bound and continues scanning until it is again loaded with all initiation factors and initiator tRNA, whereupon translation initiation can start as soon as the complex reaches the next start codon (Kozak, 2002). Such reinitiation can occur up to five times under optimal conditions (Wang and Rothnagel, 2004). Both leaky scanning and reinitiation reduce the translation efficiency of the downstream ORF, because the upstream start codons serve as ribosome traps. IRESs (Hellen and Sarnow, 2001) appear in long non-coding regions of virus mRNAs, but also in certain cellular mRNAs, such as the *Gtx* mRNA of mouse (Hu et al., 1999). The translation initiation complex is assembled along these non-coding regions, but not at the 5' cap. So far, no consensus sequence has been established for the known cellular IRESs (Hellen and Sarnow, 2001).

A fourth mechanism of translational regulation that operates on various viral messengers and at least one eukaryotic mRNA is ribosome shunting (Ryabova et al., 2002; Yueh and Schneider, 1996; Yueh and Schneider, 2000). In it, scanning of the 5' UTR starts normally and proceeds until the initiation complex encounters a

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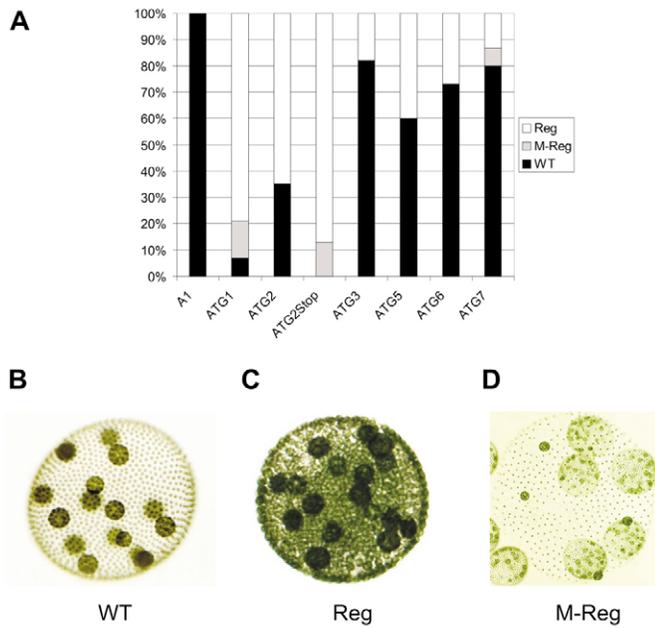


Fig. 1. Histogram showing the ratio and distribution of phenotypes among *V. carteri* 153-68 transformed with WT (A1), mutant ATG (ATG1 – ATG7) and mutant ORF2 (ATG2Stop) *regA* DNA, respectively, and the observed phenotypes. (A) A histogram indicating the abundance – relative to the WT control, A1 (100%) – of co-transformants exhibiting three different phenotypes: wild-type (WT), somatic regenerator (Reg) and modified somatic regenerator (M-Reg). **(B)** An adult WT *Volvox carteri* spheroid containing terminally differentiated somatic cells on the surface and 16 reproductive cells (gonidia) inside the spheroid [picture from Schmitt (Schmitt, 2003)]. **(C)** The Reg mutant phenotype is characterized by somatic cells that dedifferentiate, grow and redifferentiate to become fully functional gonidia (Kirk et al., 1999). **(D)** An adult M-Reg spheroid with juveniles ready for hatching. Just a few somatic cells have begun to grow and to redifferentiate, suggesting suboptimal expression of the *regA* gene.

stable secondary structure in the mRNA, whereupon the complex dissociates from the mRNA but then binds to it again at a downstream ‘landing site’, from which it continues scanning (Xi et al., 2004). In certain cases, such as the 35S mRNA of cauliflower mosaic virus, translation of a small ORF is required before shunting can occur

(Pooggin et al., 2000). Here we report the results of transformation experiments with wild-type (WT) and modified *regA* constructs that lead us to propose that ribosome shunting controls the translation of *regA* mRNA during normal development of *V. carteri*.

MATERIALS AND METHODS

Construction of *regA* plasmids

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 pVcRegA1 and all cloning sites and the orientation of inserts were analysed by sequencing. PCR products used for cloning were also controlled by full-length sequencing to exclude PCR errors. Oligonucleotides were obtained from Qiagen (Hilden, Germany) and Operon Biotechnologies (Köln, Germany).

Constructs ATG1 to ATG7 were generated by overlap extension PCR (Higuchi, 1989) with primers containing ATG to TGG mutations. The ATG2Stop construct was generated with primers containing two in-frame stop codons (ATGGCGTATCCTTGC → ATGGCGTAACTTGA). The Δ S18 construct was obtained by mutational insertion of two *Clal* cloning sites (base 1237–base 1242: AAAGCC → ATCGAT; base 1289–base 1294: ATCTTG → ATCGAT) and subsequent deletion of base 1240 to base 1294. The PCR products were cloned in the pGEM-T vector (Promega, Mannheim, Germany) for amplification and sequencing. The PCR products have a natural *PmeI* cloning site at the 5′ end and a *BglIII* cloning site at the 3′ end with which they could be inserted in the pVcRegA1 plasmid.

A DNA fragment consisting of five copies of a *BamHI* linker sequence was used previously to generate a stable hairpin that blocked ribosomal scanning but not ribosomal shunting (Yueh and Schneider, 1996; Yueh and Schneider, 2000). So we used PCR to construct a fragment containing five copies of the 12 nucleotide *BamHI* linker sequence CGCGGATCCGCG and inserted it ~50 bp upstream of the E3 landing site (Fig. 2) according to Hallmann and Wodniok (Hallmann and Wodniok, 2006). To facilitate cloning, an additional *SalI* site (GTCGAC) was introduced into the central loop of the hairpin (Fig. 6) The first PCR was performed on plasmid pVcRegA1 with the sense primer 5′-TCGTTGCGTTGGAGACCCTTC and the 64-mer antisense primer 5′-AATATATGTCGACTCCGCGCGCGG-ATCCGCGCGCGGATCCGCGACTGTGGTTATCCACAGTGAG. The second PCR was performed on the same template with the 64-mer sense primer 5′-AATATATGTCGACTCCGCGCGCGGATCCGCGCGCGGA-TCCGCGTGGAGCCTTAGTGACGTTTCT and the antisense primer 5′-TGTTGGAGTACTAGGCAGGTC. In both of the 64-mer primers, one third of the bases match the pVcRegA1 sequence at the insertion site, the remaining sequence carries one half of the hairpin sequence (underlined), a *SalI* site (bold and italics), and a short 5′ AT-rich sequence, which disappears upon cloning. The PCR fragments were cloned into the pGEM-T Easy

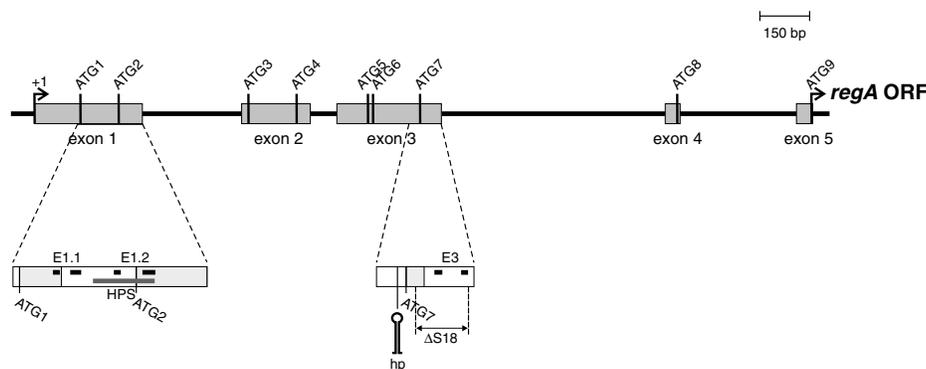


Fig. 2. Map of the genomic 5′ UTR of *regA*. Grey boxes symbolize exons, and the lines between them represent introns. The locations of the upstream ATGs (ATG1–ATG8) and of the functional start codon (ATG9) are indicated. The transcription start (+1) is marked by a bent arrow. The expanded portions below show the ATG1, ATG2 and ATG7 open reading frames (light grey boxes); the black bars indicate the potential bipartite take-off (E1.1 and E1.2) and landing (E3) sites, the location of the triple-hairpin structure (HPS, lower bar), the region deleted in the Δ S18 construct, and the point where the artificial hairpin (hp) shown in Fig. 6 was inserted.

vector (Promega, Mannheim, Germany) and sequenced. The PCR fragments were combined via the *SalI* site and the resulting fragment was introduced into plasmid pVcRegA1 by replacing the corresponding hairpin-free fragment, using the natural *PmeI* and *BglIII* cloning sites (see above). The hairpin-containing plasmid was then propagated in *Escherichia coli* strain SURE (Stratagene, La Jolla, CA).

Volvox strains, cultivation conditions and nuclear transformation

Volvox carteri cultures were maintained in standard *Volvox* medium (SVM) at 30°C under a 16:8-hour light:dark cycle (Kirk and Kirk, 1983; Kirk and Kirk, 1985). SVM lacking the usual urea or ammonium chloride (SVMN) was used to select for ability to reduce nitrate after genetic transformation. This transformation was performed according to Schiedlmeier et al. (Schiedlmeier et al., 1994) with modifications. Cells of the *V. carteri* strain 153-68, which carries non-revertible mutations in both the *nitA* and *regA* loci (Kirk et al., 1999), were co-bombarded with pVcNR15 (Gruber et al., 1996) and various *regA* constructs that were described before. Integration of *regA* constructs after transformation was tested by PCR as described by Stark et al. (Stark et al., 2001).

RESULTS

Biolistic co-bombardment with a selectable marker (the *nitA* gene, encoding nitrate reductase) was used to examine the capacity of different in vitro mutagenized *regA* constructs to achieve phenotypic rescue of a strain that carries non-revertible mutations of both the *regA* and the *nitA* genes (Kirk et al., 1999; Stark et al., 2001). Transforming plasmids integrate into the *Volvox* genome at random locations, frequently in the form of multiple tandem repeats (Babinger et al., 2001). The Nit^+ transformants that were recovered in the experiments reported below exhibited 70 to 80% co-transformation of the unselected marker (in terms of integration of the transgenic DNA), and 25–46 such co-transformants were examined in each case. To simplify comparisons, we have normalized the distribution of phenotypes produced by each of the modified constructs, by setting the frequency with which the control construct (pVcRegA1, shortend A1) restored the WT phenotype equal to 100%.

Mutational ablation of AUG1, AUG2 or ORF2 causes a marked reduction of *regA* expression

Here, we use RNA nomenclature (AUG, etc) when it concerns translation control, and DNA nomenclature (ATG, etc) when it concerns the *regA* gene and the construction of mutant plasmids.

Six mutant versions of the *regA* gene were tested for their ability to cure the Reg phenotype. The constructs tested were all modified versions of the plasmid pVcRegA1 (Kirk et al., 1999), which contains the native *regA* locus (Fig. 2) and which was also used as the positive control for all the transformation experiments reported here. In each mutant construct one of the upstream ATGs was changed to TGG, and if the preceding nucleotide had been an A, it was also changed (to a C). Mutations of ATG4 and ATG8 were not tested, because those two ATGs were followed almost immediately by stop codons (ATGCCATAA and ATGTAA, respectively). (Furthermore, ATG8 is located in exon 4, which can be deleted, together with the non-coding part of exon 5 without any effect; M. Lichtinger, personal communication.) Co-transformation with constructs carrying mutations of ATG3 to ATG7 restored the WT phenotype with frequencies (60–82%) that were not greatly different from the positive control, A1 (100%; Fig. 1A). This finding indicated that those four upstream AUGs play no crucial role in regulating *regA* expression. By contrast, constructs with mutations of ATG1 or ATG2 restored the WT phenotype with substantially lower frequencies (22 and 35%, respectively; Fig. 1A), indicating that those two regions of the UTR are involved in some way in the regulation of *regA* expression.

An open reading frame that we call ORF2 starts at ATG2 and is the longest ORF in the *regA* 5' UTR (Fig. 2). It has 142 codons, and the sequence surrounding the AUG shows the highest similarity of all the upstream ORFs of the *regA* mRNA to the Kozak sequence of *Chlamydomonas reinhardtii*, a close relative of *V. carteri* (Ikeda and Miyasaka, 1998; Rausch et al., 1989). A comparison of this region from three different forms (subspecies) of *V. carteri* revealed 82–88% DNA-sequence conservation, indicating the possible importance of the region. To test for a possible role of ORF2 in controlling *regA* expression, we inserted two in-frame stop codons just downstream of ATG2 (ATG2Stop; ATGGCGTATCCTTGC → ATGGCGTAACTTGA) and tested the resulting construct by co-transformation. The result was dramatic (Fig. 1A): the insertion of the stop codons nearly abolished the ability of the *regA* gene to cure the mutant phenotype. Only two co-transformants with non-Reg phenotypes were found, but they did not show the WT phenotype. Rather, they exhibited the so-called M-Reg phenotype (Fig. 1D), in which some – but not all – somatic cells are restored to the WT condition, and which is thought to be a result of too little RegA protein being produced to maintain the non-reproductive character of all somatic cells (Kirk, 1998).

The foregoing results pointed to the importance of the ORF2 region for full expression of *regA*. However, we were never able to detect any ORF2 translation product, even when we attached a hemagglutinin (HA) tag in frame with ORF2 and performed sensitive tests for HA expression with a specific antibody. This led us to suspect that it might be the secondary structure of this region of the mRNA, and neither the translation of ORF2 nor the action of its translation product that renders this region essential for *regA* translation. Accordingly, we attempted to assess the possibility that *regA* translation was controlled by ribosome shunting.

The *regA* RNA has several features that are required for ribosome shunting

In viruses, ribosome shunting requires certain recognizable mRNA features, including stable hairpin structures that interfere with ribosome scanning, and ‘take-off’ and ‘landing’ sites that allow the 40S ribosomal subunit to bypass the hairpins (Ryabova et al., 2002). We therefore analysed the secondary structure of the 5' UTR of *regA* mRNA with the computer program mfold (Zuker, 2003). The thermodynamically most probable structure turned out to include a triple hairpin (Fig. 3) between positions 215 and 277, which is a reminder of the structure of adenovirus that is required for ribosome shunting in that case (Yueh and Schneider, 2000). This is also the region in which two of our introduced mutations – namely, ATG2 and ATG2Stop – were located (Fig. 3). Parallel analyses of secondary structures for the ATG2 and ATG2Stop mutant mRNAs predicted that in both mutants the third hairpin would be lacking. Our calculations of the energy that would be required to open the 62 nucleotide regions of sequence between positions 215 and 277 (Fig. 3, encircled) indicated that opening this region of the mutant 5' UTRs for ribosomal scanning would take 43% less energy than would be required to open the corresponding region of the WT RNA.

Adding to the potential significance of the foregoing observations – and reinforcing the possibility that translation of *regA* mRNA involves ribosome shunting – was the fact that when a parallel structural analysis was performed for the *regA* mRNA of another subspecies of *Volvox carteri* – *V. carteri* forma *kawasakiensis* (Duncan et al., 2006) – we found that the triple-hairpin structure was strongly conserved.

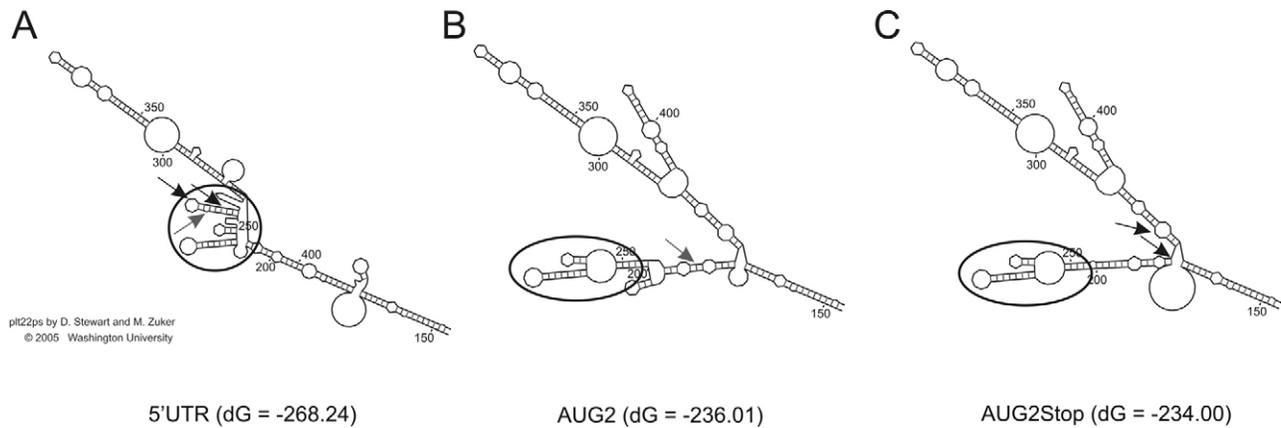


Fig. 3. Secondary structure predictions for homologous portions of the 5' UTRs of WT *regA* mRNA and two mutant mRNAs (ATG2 and ATG2Stop). (A-C) Numbering starts at transcription start (+1; Fig. 2). Grey arrows indicate the location of ATG2; black arrows indicate the locations at which stop codons were introduced. The encircled portion indicates the triple hairpin region between positions 215 and 277 that is discussed in the text. Note the secondary structure in this region changed by mutations. The structures and free enthalpies of the whole 5' UTRs (dG in kcal/mole) were calculated by the program mfold [version 3.1, Zuker (Zuker, 2003)].

In addition to a stable hairpin capable of blocking ribosomal scanning, ribosome shunting also requires 'take-off' and 'landing' sites that permit the 40S ribosomal subunit to bypass the hairpin. In the case of adenovirus, several regions were identified that exhibit complementarity to parts of the 3' end of the 18S rRNA (Fig. 4A), and that are postulated to function as the take-off and landing sites (Yueh and Schneider, 2000). A similar sequence has been found in the human *hsp70-1* gene (Yueh and Schneider, 2000). In the *regA* mRNA of *V. carteri* f. *nagariensis* we detected three such regions (Fig. 2, Fig. 4B): two possible take-off sites, E1.1 and E1.2, upstream of AUG2 and a potential landing site, E3, downstream of AUG7, near the end of exon 3. All those regions of complementarity are fully conserved in *V. carteri* f. *kawasakensis* (Fig. 4B), reinforcing the idea that they are functionally significant.

To evaluate the hypothesis that the putative landing site, E3, plays an important role in *regA* expression, we tested whether a construct with a 51 bp deletion spanning that region (S18, Fig. 2) was functional. The result was unambiguous: this deletion totally abolished *regA* function (Fig. 5), providing strong support for the ribosome-shunting hypothesis.

Another powerful test of the ribosome-shunting hypothesis was provided by previous studies demonstrating that an artificial hairpin that is too stable to be opened by the translation machinery does not impose a barrier to translation of the message, provided that it is inserted upstream of the landing site used for ribosome shunting (Futterer et al., 1993; Yueh and Schneider, 1996). To employ this test, we introduced an especially stable stem-loop structure ~50 bp upstream of the putative E3 landing site (Fig. 2).

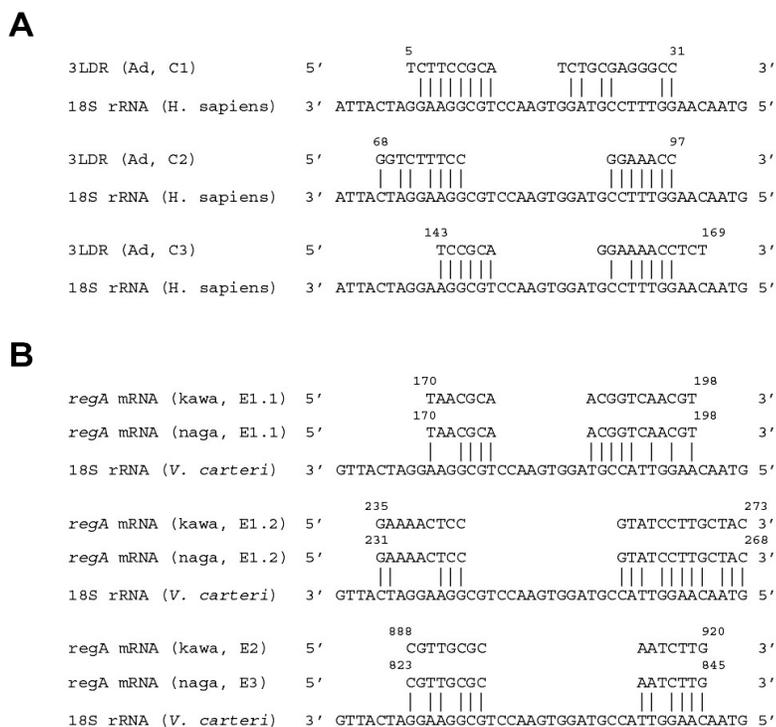


Fig. 4. Bipartite sequence complementarities of the potential ribosome binding sites.

(A) Complementarities of the 3' hairpin of human 18S rRNA and the human adenovirus tripartite leader (3LDR, Ad, C1–C3). Nucleotide positions relative to the +1 start site of transcription are indicated (Yueh and Schneider, 2000). (B) Complementarities of the 3' hairpin of *V. carteri* 18S rRNA (Rausch et al., 1989) and three regions of the 5' UTR of *regA* from *V. carteri* f. *nagariensis* and *V. carteri* f. *kawasakensis* (Duncan et al., 2006). E1.1 and E1.2 are located in the first exon; E2 is in the second exon (Intron 2 will not be spliced in comparison to *nagariensis*); and E3 in the third exon. Nucleotide positions relative to the +1 start site of transcription are indicated (Fig. 2).

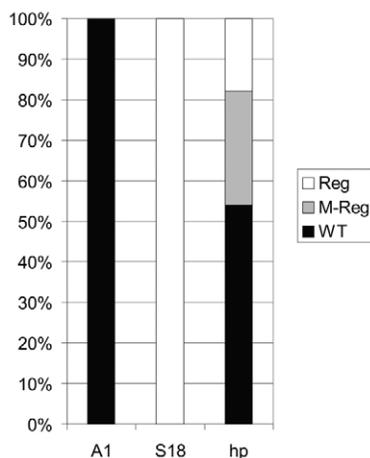


Fig. 5. The normalized abundance of different phenotypes.

Histogram showing the normalized abundance of different phenotypes among organisms co-transformed with the WT *regA* construct (A1=100%), the mutant construct (S18) lacking the potential landing site, that is complementary to S18 rRNA, and the mutant construct containing a stable hairpin structure (hp; Fig. 6) introduced upstream of the landing site (Fig. 2).

With a calculated ΔG of -87 kcal/mole, this stem-loop structure (Fig. 6) is even more stable than those employed in previous studies (-61 kcal/mole or -80 kcal/mole, respectively) (Kozak, 1989; Yueh and Schneider, 1996). When we tested this construct by transformation, 82% of the co-transformants exhibited phenotypic rescue. (However, 28% of the co-transformants exhibited the M-Reg, rather than the WT, phenotype, indicating that in them the level of *regA* expression was adequate to repress reproductive activity in most, but not all, somatic cells.) These results make it clear that *regA* can be expressed in spite of the presence of a very stable hairpin in the 5' UTR, and thus they provide additional strong support for the hypothesis that ribosome shunting is the major mechanism controlling the translation of the *regA* gene in *V. carteri*.

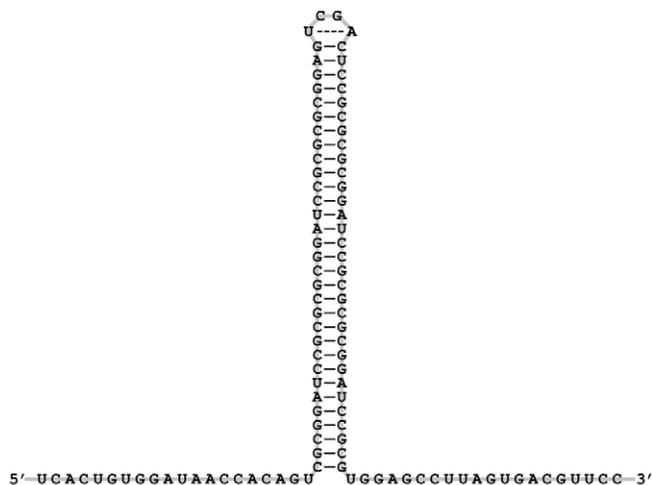


Fig. 6. Secondary RNA structure. Sequence-derived RNA secondary structure (ΔG of -87 kcal/mole) transcribed from the synthetic hairpin introduced into the 5' UTR of *regA* (Fig. 2).

DISCUSSION

regA is one of the principal genes controlling cell differentiation in *V. carteri*. It is expressed only in the somatic cells, where it suppresses growth and reproduction by preventing chloroplast biogenesis (Kirk et al., 1999; Meissner et al., 1999). The *regA* transcript first appears by 12 hours after the start of the embryonic development, but the RegA protein can be detected only 4 hours later (Kirk et al., 1999). That fact, combined with the observations that the level of RegA protein is much lower than would be expected from the mRNA level, and that eight upstream start codons must be bypassed before *regA* translation can be initiated, led to our assumption that *regA* expression is regulated at the translational as well as the transcriptional level.

In many eukaryotic mRNAs, AUGs and small ORFs in the 5' UTR act as potential ribosome traps that must be bypassed in order for the initiation complex to reach the true initiation codon and begin translating the real message (Kozak, 2002). Four mechanisms for bypassing such potential ribosome traps are known. We will consider the evidence for and against the involvement of each of those four mechanisms in the case of *regA* translation.

Leaky scanning and reinitiation

In leaky scanning, the initiation complex sometimes fails to recognize a potential start codon on the mRNA, because the surrounding sequence deviates from the ideal, so-called Kozak sequence (Kozak, 2002), so (with some finite frequency) it bypasses that codon. Reinitiation occurs when a 40S ribosomal subunit remains attached to the mRNA after the translation of a short upstream ORF has been completed, and it then continues scanning until it reaches another start codon and a new initiation complex has been assembled (Kozak, 2002). Although ribosomes can get beyond upstream AUGs and ORFs by either of these methods, in both cases these upstream elements retard the ribosomes in their journey to the real initiation codon. Therefore, removal of upstream AUGs and ORFs that would otherwise need to be bypassed by one of these mechanisms always causes an increased rate of translation of the main message (Wang and Rothnagel, 2004). In the case of *regA*, however, the opposite effect was observed: although mutational ablation of AUG3-AUG8 had little effect, mutations of AUG1, AUG2 and ORF2 all caused marked decreases in *regA* expression (Fig. 1A). This led us to exclude both leaky scanning and reinitiation as potential mechanisms regulating the rate of *regA* translation.

Internal ribosome entry site

Many viral mRNAs and some long, eukaryotic cellular mRNAs possess an IRES at which an initiation complex is assembled, rather than at the 5' cap (Hellen and Sarnow, 2001). Three observations reported here speak to the issue of whether *regA* translation depends on initiation at an IRES. (1) Point mutations at different positions in the 5' UTR should not all lead to reduced expression (Hellen and Sarnow, 2001; Pelletier et al., 1988), except a putative IRES would span the whole 779 nucleotides from AUG1 to the end of exon 3 [but we are not aware of any IRES longer than 395 nucleotides (Bernstein et al., 1997)]. (2) A stable hairpin at a site downstream of an IRES should block progression of any initiation complex that was formed at that IRES, but we have found that a stable hairpin inserted into exon 3 does not prevent *regA* expression (Fig. 5). This appears to rule out the possibility that *regA* translation is dependent on an IRES upstream of exon 3. (3) One of our colleagues has established that no significant reduction of *regA* expression occurs when both exon 4 and the untranslated portion of exon 5 are deleted (M. Lichtinger, personal communication). This appears to rule out the

possibility that an IRES downstream of exon 3 is required for *regA* expression. These observations, taken together, make it extremely unlikely that translation of *regA* mRNA relies to any significant extent on an IRES.

Ribosome shunting

Ribosome shunting is a mechanism in which a ribosome bypasses a region of stable secondary structure in the mRNA by first dissociating from the RNA at a ‘take-off’ element upstream of the blocking structure, and then reassociating with the message downstream of the structure, at a ‘landing site’ element. Here we have found evidence that the *regA* mRNA contains all three types of elements that are required for shunting: namely, a region of stable secondary structure, two potential take-off sites and one potential landing site.

The potential block to scanning that we discovered by secondary structure analysis of WT *regA* mRNA (Fig. 3) is a region of stable secondary structure that lies between nucleotides 215 and 277 and shows a triple hairpin structure that appears to be a reminder of the hairpin structure of adenovirus that is required for ribosome shunting in that case (Yueh and Schneider, 2000). Further secondary structure analysis revealed that the mutations in AUG2 and ORF2 that caused such marked decreases in *regA* expression also changed the nature of the most probable secondary structure in the nucleotide 215–277 region, removing the third hairpin. Furthermore, the structural changes resulting from these simple mutational changes reduced by nearly half the amount of energy that would be required to open up this region and permit passage of a scanning ribosome! Once past this region of the mutant RNAs, of course, the scanning ribosome would then encounter six additional AUGs before it could reach the true start codon, AUG9, explaining why these mutations caused such a marked decline in *regA* expression.

The putative ribosomal take-off and landing sites that we located in *regA* mRNA, like those found in human adenovirus mRNA and other messages exhibiting ribosomal shunting, are short regions of complementarity to the 3′ end of the 18S rRNA (Fig. 4). To test whether the putative landing site, E3, really had any important role to play in controlling *regA* expression, we used transformation to test how effective a *regA* construct lacking this region of the sequence would be in rescuing a Reg mutant. The result (Fig. 5) was unambiguous: such a construct was totally inactive – wholly incapable of rescuing the Reg-mutant phenotype – just as our ribosome-shunting hypothesis predicted it would be.

Further strong support for our ribosome-shunting hypothesis came from the experiment in which an extremely stable synthetic hairpin was inserted just upstream of the putative ribosome-landing site (Fig. 2). Insertion of a stable hairpin in this location had no discernible effect on the ability of the *regA* transgene to rescue a Reg mutant (Fig. 5), which was once again fully consistent with our ribosome-shunting hypothesis.

The only alternative to the ribosome-shunting hypothesis that we can think of to explain the fact that a stable hairpin in exon 3 does not diminish *regA* expression would be an assumption that there is an IRES located somewhere downstream of exon 3. However, that possibility can be ruled out, because it has previously been demonstrated that deletion of all of exon 4, plus the untranslated part of exon 5, has no discernible effect on *regA* expression (M. Lichtinger, personal communication).

In short, the most viable hypothesis at the moment clearly is that translation of the *Volvox carterii regA* gene involves ribosome shunting. As far as we are aware, this is the first case in which ribosome shunting has been invoked with respect to translational

control in a green eukaryote, and only the second time it has been invoked in print with respect to any eukaryotic cellular mRNA. [The only precedent we are aware of involves the human *hsp70* mRNA (Yueh and Schneider, 2000).] But it would be truly unusual if a control mechanism that is used by many viruses, a green alga and humans were not used elsewhere in the Eukaryota. Hence we would strongly encourage colleagues to consider ribosome shunting seriously as a possible mechanism employed in other cases of eukaryotic translational regulation.

How do we imagine that ribosome shunting occurs on the *regA* mRNA?

The translation initiation complex would be assembled at the 5′ cap and would then scan along the *regA* mRNA until it recognized the first start codon, AUG1, and translated the adjacent short ORF1. This ORF has only 14 codons, resulting in a brief translation event that, in other cases that have been studied, favours continued scanning and reinitiation at the next AUG (Kozak, 2002; Wang and Rothnagel, 2004). After leaving ORF1, however, the 40S subunit would be stopped by the triple-hairpin structure before it could reach AUG2. It would then be shunted from the take-off site, E1.1, to the landing site at the end of exon 3, E3, thereby bypassing AUG2 to AUG7. Because AUG8 is followed immediately by a UAA stop codon, it is not likely to be mistaken for an initiation site (Pooggin et al., 2000), so translational initiation would start at AUG9, causing the RegA protein to be produced.

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