Review

Volvox carteri: Molecular Genetics of Cell differentiation

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Evolving multicellularity and differentiation

Two remarkable properties render *Volvox*, the spheroidal green alga, an outstanding model for investigating cellular differentiation and its molecular-genetic origins: First, *Volvox* is a multicellular organism showing the simplest kind of cytodifferentiation, the division of labor between only two types of cells, somatic and germ cells (gonidia). Second, *Volvox* and its simpler unicellular and colonial relatives, the volvocine algae *Chlamydomonas*, *Gonium*, *Pandorina*, *Eudorina* and *Pleodorina*, form an extant, coherent family of organisms with excellent promise for studies of the molecular evolution of multicellularity and cellular differentiation (Kirk, 1998; Schmitt et al. 1992).

Sequence comparisons for 18S rRNA gene loci suggest that, although the green algae in the order Volvocales shared a common ancestor with higher plants, they constitute a separate lineage - with *Volvox* at its apex - that has long been separated from the lines leading to vascular plants and animals (Rausch et al. 1989). This is illustrated by a schematic phylogenetic tree shown in Fig. 1. On the basis of cytochrome sequence comparisons, Amati et al. (1988) estimated that lines leading to the modern green algae and to the higher plants diverged about 700-750 million years ago, about 500 million years after plants and animals had last shared a common ancestor. 'Molecular clock' methods applied to the rRNA data, and the number of silent exchanges observed in tubulin genes, led to an estimate that the evolution of *Volvox carteri*, a multicellular organism with complete differentiation of soma and germ line, from a *Chlamydomonas*-like unicellular ancestor may have occurred as recently as 35 million years ago (Rausch et al. 1989). This relatively late emergence of *Volvox* as a "modern" organism raises the possibility of tracing

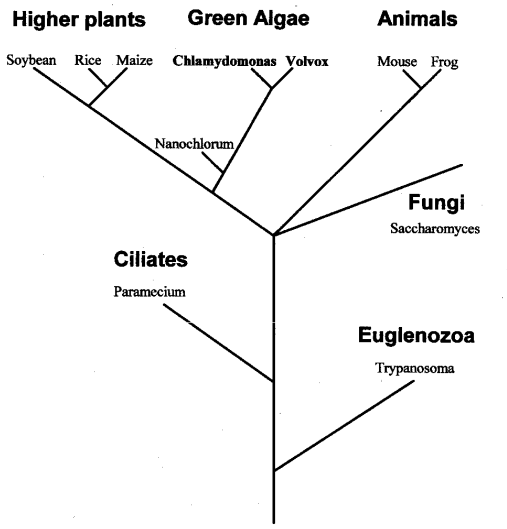

Fig. 1 Eukaryotic phylogenetic tree (not to scale) based on 18S rRNA sequence comparisons of selected organisms (modified from Rausch et al. 1989). The volvocine algae, *Chlamydomonas* and *Volvox*, appear at the tip of the green algal branch (Chlorophyta) that diverged long ago from the higher plant lineage.

the molecular-genetic pathway in the volvocine lineage by which that program of cell differentiation evolved.

Embryogenesis: asymmetric cell division and germ-soma dichotomy

A *V. carteri* gonidium may be viewed as immortal stem cell that divides mitotically to produce more gonidia and a multitude of mortal somatic cells (Starr, 1969). Under a 16-hour light/8-hour dark regime and at optimum conditions the morphogenetic program of the *V.carteri* life cycle is regular and reliably yields predictable numbers of the two entirely different cell types. Each cell division requires ca. 35min, the entire cleavage period lasting six to seven hours. Prior to each cell division, the nuclear genome undergoes one round of replication (Kobl et al. 1998). Within individual embryos, divisions are completely synchronous; between different embryos in one parental spheroid, cleavages may deviate from synchrony by one to three minutes. The first five divisions are symmetric and divide the embryo into 32 equal-sized daughter cells. The sixth division is symmetric in the two posterior tiers of cells, but it is asymmetric in the 16 anterior cells, which leads to 16 pairs of large and small sister cells. The large cells are gonidial initials (for the next generation), whereas the small equivalents and the remaining cells (after another 5 to 6 cell divisions) will produce the somatic cells. After the final cleavage division and a pause of up to one hour, inversion commences with a wave of contraction. The phialopore widens and the entire embryo turns "inside out". This moves the 16 large gonidia to the interior and the flagellar ends of somatic cells to the exterior. About 45 minutes after the onset of inversion, the juvenile spheroid is completed. Cytodifferentiation begins and extracellular matrix deposited between the cells causes an expansion of the spheroid. Four hours after embryogenesis somatic cells and gonidia are fully separated, but it takes another 20 hours, until the mother spheres release 16 juvenile spheroids, who upon further maturation start a new cleavage division cycle. The "old" somatic cells embedded in the matrix of the former mother spheroid have turned light green, they senesce and die after another 30 to 40 hours.

The *regA* gene: key to cell differentiation

A typical asexual spheroid of *V.carteri* contains 16 large reproductive cells (gonidia) and a surface layer of about 2000 small, biflagellate somatic cells. Gonidia are immotile and specialized for reproduction. Somatic cells, by contrast, are specialized for motility but have no reproductive potential. Once differentiated, they never divide, and they eventually undergo programmed death (Pommerville and Kochert, 1982). The *regA* gene plays a central role in establishing this germ-soma dichotomy by acting as a negative regulator of reproductive development in somatic cells. Whereas *regA*$^{+}$ mature somatic cells have ceased to divide, *regA*$^{-}$ somatic cells regain the full reproductive potential of gonidia including the ability to divide (Huskey and Griffin, 1979; Kirk et al. 1987; Starr, 1970). Mutations in *regA* have no effect on "true" gonidia indicating that the gene is not expressed in gonidia. The expression pattern of *regA* in the *V. carteri* life cycle is schematically viewed in Fig. 2.

By using the *Volvox* transposon *Jordan* (Miller et al. 1993), we have successfully tagged and cloned the *regA* gene locus (Kirk et al. 1999). Rescue of a *regA*$^{-}$ mutant via biolistic co-transformation with a selectable marker (Schiedlmeier et al., 1994) was used to test the identity and integrity of the cloned *regA* gene. The 12.5-kb *regA* transcription unit generates a 6.725-nucleotide mRNA that emerges at the beginning of somatic cell differentiation. It encodes a 111-kDa RegA protein with all features of a transcription factor that is localized to the nucleus. The HA-tagged protein accumulates progressively for several hours, and then it remains moderately abundant

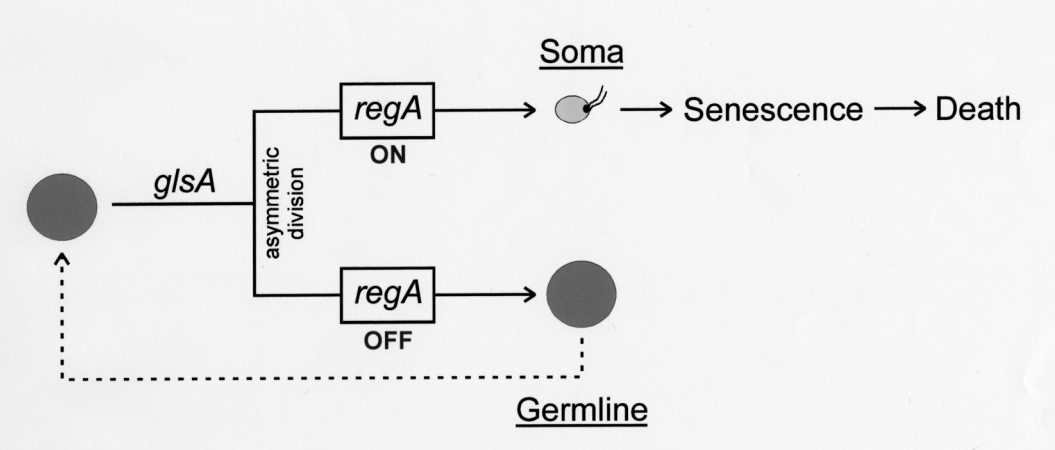

Fig. 2 The postulated roles two types of genes in establishing and maintaining the germ-soma dichotomy of *V.carteri*. After a series of symmetric divisions, the *gls* gene acts to permit asymmetric division and the formation of large and small cells (Miller and Kirk 1999). After the termination of embryogenesis, the *regA* gene is expressed selectively in the small cells, which leads to repression of reproductive genes, notably those encoding essential chloroplast proteins, and causes these cells to undergo terminal differentiation (Kirk et al. 1999).

throughout the life of the somatic cells, but it is never detected in gonidia. These features of RegA are consistent with its role as transcriptional repressor in somatic cells of functions required for germ cell development.

What are the targets of RegA?

Previously, Tam and Kirk (1991) used cell-type and stage-specific cDNAs to define a set of 18 "maturation-abundant gonidial genes", whose transcripts accumulate in developing gonidia (and in *regA*$^{-}$ somatic cells), but not in wild-type (*regA*$^{+}$) somatic cells. This expression pattern was highly suggestive of genes that are negatively regulated by RegA. All 18 cDNAs have been sequenced and characterized (Choi et al. 1996; Meissner et al. 1999). Of these, 14 DNA sequences encode chloroplast proteins of known function, two encode putative chloroplast proteins of unknown function, and another two encode polypeptides with no recognizable features. The 14 assigned nuclear genes encode proteins involved in the most important aspects of chloroplast biology: light harvesting, photolysis of water, transfer of reducing power from photosystem II to photosystem I and from photosystem I to the dark reactions, starch biosynthesis, ATP generation and chloroplast gene expression.

These findings suggest that germ-soma differentiation and chloroplast biogenesis must be intimately connected in *V.carteri*. It has been suggested that the huge chloroplast of a pre-cleavage gonidium becomes progressively subdivided during cleavages, such that a small portion of it (< 0.05%) ends up in each of the somatic cells of the progeny embryo. If these somatic cells cease to synthesize new chloroplast proteins after the end of embryogenesis due to repression by RegA, they would be unable to grow to any significant extent and, hence, they could not reproduce. The contribution of RegA to the establishment of germ-soma differentiation thus resides in the repression of chloroplast biogenesis in somatic cells, thereby preventing those cells to grow sufficiently to be able to reproduce (Fig. 2). This suggestion does not necessarily imply that the nuclear-encoded chloroplast proteins are

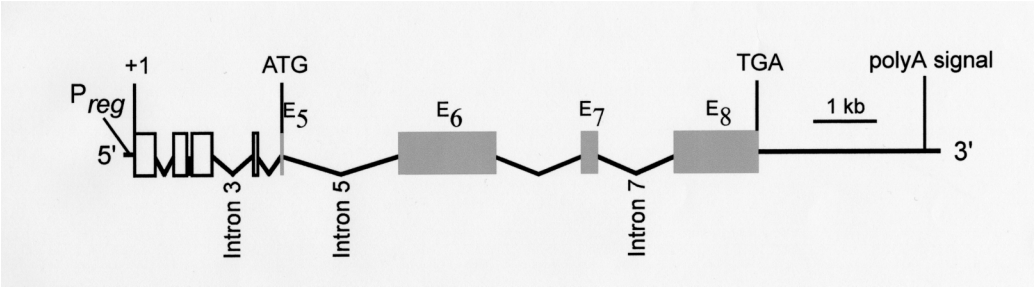

Fig. 3. Organisation of the *regA* gene locus of *V.carteri* exhibiting eight exons, only of them (E5-E8) being translated, and seven introns. Transcription from the promoter, *Preg*, starts at position +1, ATG and TGA mark translation start and stop signals. Mutants containing deletions of either intron 3 or intron 5 fail to express the *regA* gene in somatic cells and result in the RegA$^-$ phenotype: somatic cells regain the full reproductive potential of gonidia. If intron 7 is deleted, the *regA* gene is expressed in gonidia and the gene product, RegA, suppresses reproductive functions: gonidia cease to divide and lose their photosynthetic potential, thus causing the entire spheroid to die after two or three cycles ("*fruitless*" phenotype; Stark et al., 2001).

direct targets of RegA. In a network of feedback relationships, RegA would only need to repress one or a few key nuclear genes in order to suppress chloroplast biogenesis completely. One of our major challenges for the immediate future, therefore, is to determine which (if any) of the genes defined here and which of the "upstream" genes may be direct targets of RegA regulation.

What regulates the RegA regulator?

The 12.5-kb *regA* gene consists of eight exons, the first four of them untranslated, seven introns and 5' and 3' untranslated regions (Fig. 3; Stark et al. 2001). The gene is "switched on" in differentiating somatic cells, but it remains silent in the germ cells (Fig. 2). If *regA* is inactivated by mutation, somatic cells dedifferentiate to reproductive germ cells. Reversely, if *regA* is being forced into expression by placing it under the control of the strong constitutive promotor of a b-tubulin gene (Mages et al., 1995), gonidia cease to divide, remain small and appear blackish, wheras somatic cells differentiate quite normally. These "*fruitless*" mutants die after one or two cycles, clearly indicating that the (artificial) expression of *regA* in gonidia represses the reproductive potential and thus renders such mutants lethal. It is therefore a crucial requirement of *Volvox* differentiation that *regA* remains silent in gonidial, but that it becomes active in differentiating somatic cells (Kirk et al. 1999).

We have approached this central issue of differential *regA* gene expression by a systematic deletion analysis including the upstream portion and the seven introns of this gene (Fig. 3). The minimal promoter is limited to 42 bp upstream of transcription and contains putative TATA and initiator sequences, but no regulatory elements. However, introns 3 and 5 both contain enhancers absolutely needed for *regA* gene expression in somatic cells. On the other hand, intron 7 is essential for silencing *regA* in gonidia. If deleted, it leads to the *fruitless* phenotype described above indicating that intron-seven less mutants express *regA* in germline cells. Intron 7 bears an open reading frame contigous with those of exons 7 and 8; the introduction of nonsense mutations into this frame did not affect normal development suggesting that intron 7 is spliced and eliminated before translation. The most simple interpretation of these results suggests that introns 3 and 5 contain enhancer elements that are activated in differentiating somatic cells and that intron 7 contains a "silencer" active in gonidia throughout

the life cylce (Stark et al., 2001). Cell-specific factors interacting with these elements are under current investigation.

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