J Plant Growth Regul (2000) 19:265–274
DOI: 10.1007/s003440000039

J P G R
Journal of Plant Growth Regulation

Volvox as a Model System for Studying the Ontogeny and Phylogeny of Multicellularity and Cellular Differentiation

David L. Kirk*

Department of Biology, Washington University, Campus Box 1229, St. Louis, Missouri 63130, USA

ABSTRACT

Volvox carteri, a spherical alga with a complete division of labor between approximately 2000 biflagellate somatic cells and 16 asexual reproductive cells called gonidia, provides a very attractive system for analyzing how a molecular-genetic program for cell-autonomous cellular differentiation may be encoded within a genome. Then, when considered in combination with a group of closely related "volvocine algae" that includes unicellular *Chlamydomonas* plus a series of colonial forms of increasing cell number and complexity, it also provides an attractive model system for analyzing how such a program for multicellularity and cytodifferentiation may have evolved. It is proposed that the following were some of the key steps in this evolutionary pathway: (1) The *Chlamydomonas* cell wall was transformed into an extracellular matrix (ECM) that joined sister cells into a colonial unit. (2) Larger organisms with more abundant ECM were favored because of the role the ECM plays in storing limiting nutrients. (3) In the *V. carteri* lineage the ancestral biphasic "first biflagellate and then reproductive" pathway of development became converted to a dichotomous pathway by introduction of two kinds of cell-type–specific negative regulators: one that blocked growth and reproduction in presumptive somatic cells and one that blocked somatic development in presumptive gonidia. Progress has been made in cloning and characterizing genes that are involved in setting apart the two cell lineages of *V. carteri* and in subsequently controlling their dichotomous differentiation. The strengths and weaknesses of *V. carteri* and its relatives as a model system for studying the evolution of multicellularity are discussed.

Key words: Cell determination; Cell size; *Chlamydomonas*; Cytodifferentiation; Evolution; Extracellular matrix; Germ—soma division of labor; Green algae; Hydroxyproline-rich glycoproteins; Volvocine algae

A major portion of Earth's present biomass and most of its individual organisms are unicellular. Yet multicellular organisms dominate the appearance of our land, seas, and skies. Indeed, if such multicellular organisms had never evolved, our planet would undoubtedly appear as barren to a visitor from outer space as Mars presently does.

What do we know of the origins of these multicellular forms that appear to dominate our biosphere? Both classical and modern evidence indicates that each of the five major groups of conspicu-

Online publication 1 March 2001
*Corresponding author; e-mail: kirk@biology.wustl.edu

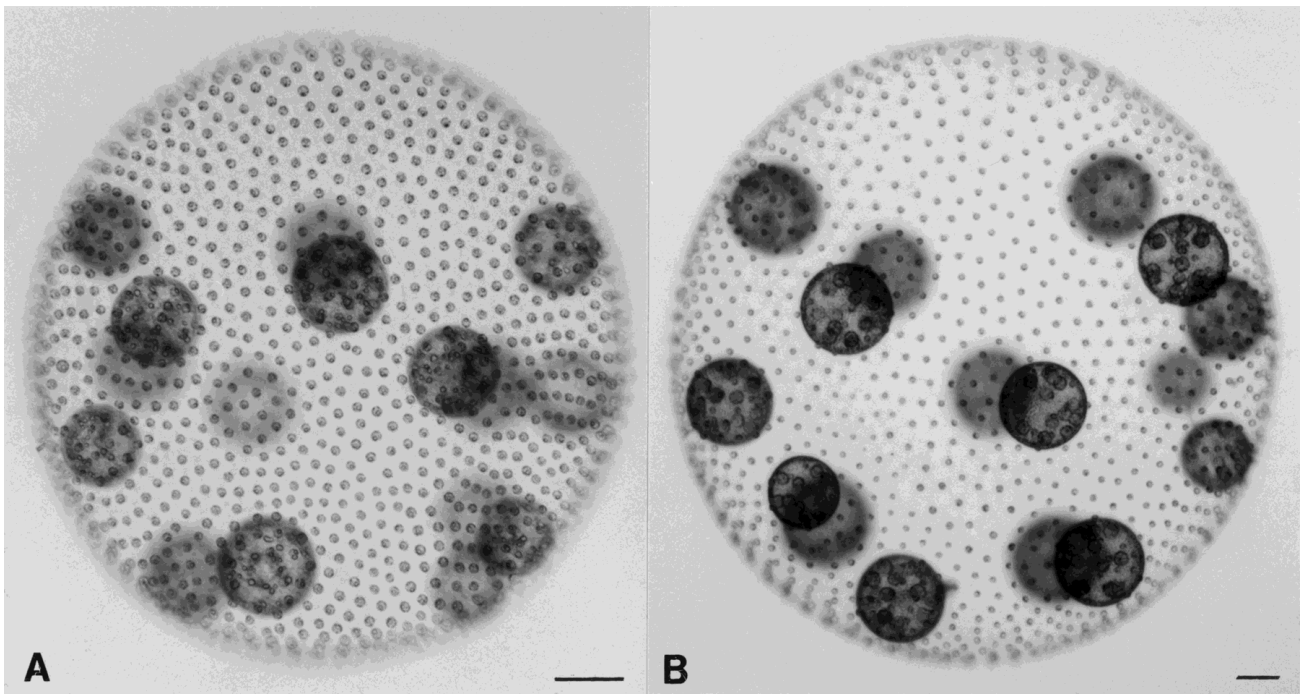

Figure 1. Two stages in the life cycle of *Volvox carteri.* **(A)** A young adult spheroid with approximately 2000 small somatic cells at the surface and 16 gonidia (asexual reproductive cells) just below the surface of a transparent sphere of glycoprotein-rich extracellular matrix (ECM). The gonidia shown here are mature and just about ready to initiate embryogenesis. **(B)** A spheroid about a day older than the one shown in **(A).** By now, a juvenile spheroid containing a new population of somatic cells and gonidia has been produced from each gonidium that was present a day earlier, and both the adult and the juvenile spheroids have enlarged by deposition of ECM. Bars: 50 μm.

ous modern eukaryotes—the red algae, brown algae, plants, fungi, and animals—has arisen independently from a different unicellular clade (Devereux and others 1990; Sogin 1991; Wainwright and others 1993). Moreover, there are numerous less conspicuous kinds of organisms (including certain *Cyanobacteria,* myxobacteria, actinomycetes, myxomycetes, cellular slime molds, diatoms, ciliates, and others) that arguably are equally deserving of the epithet multicellular, and that all appear to have equally independent evolutionary origins (Bonner 1998; Kirk 1998). In virtually all of these cases, however, the transition from a unicellular ancestor to a multicellular organism with differentiated cell types occurred so far in the distant past—or in such an otherwise obscure group—that we can do little more than guess what kind of unicellular ancestor might have given rise to each of these lineages or the pathway by which it may have done so. *Volvox* clearly provides an exception to this generalization.

The Place of *Volvox* and its Relatives in the Living World

Volvox carteri, which is the species of *Volvox* that has been studied the most, is a spherical green alga with a complete division of labor between two entirely different cell types: approximately 2000 small, biflagellate, *Chlamydomonas*-like somatic cells that are embedded in the surface of a transparent sphere of extracellular matrix (ECM) and about 16 large asexual reproductive cells, called "gonidia," that lie in the ECM just internal to the somatic cells (Figure 1A). The somatic cells are specialized for motility, phototaxis, and chemotaxis; they beat their flagella ceaselessly to propel the spheroid through the water with the distinctive rolling motion that caused Linnaeus to name the genus *Volvox* ("the fierce roller"). But somatic cells are mortal—destined to undergo programmed death when they are only about 4 days old. In marked contrast, the gonidia are nonmotile, potentially immortal cells that are specialized for division and reproduction. Each gonidium divides when only approximately 2 days old to produce a juvenile spheroid that is essentially a miniature adult containing a full complement of new somatic cells and gonidia (Figure 1B). Whereas somatic cells cannot be made to divide by any known means, gonidia cannot be prevented from dividing by any means short of depriving them of energy or killing them.

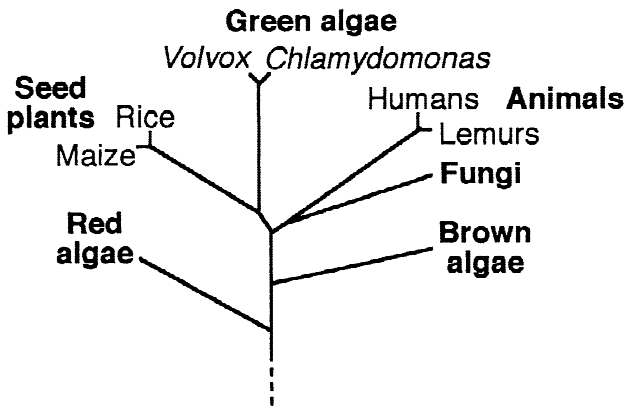

Figure 2. A dendrogram illustrating the temporal branching patterns of selected groups of modern multicellular eukaryotes. Note that the time since unicellular *Chlamydomonas reinhardtii* and *Volvox carteri* last shared a common ancestor is similar to the period during which the grasses and primates have been radiating, and only about 1/20th as long as the period during which the five major groups of multicellular eukaryotes have been evolving. Note also that the green algal and seed-plant lineages have been diverging almost as long as the animal and fungal lineages have.

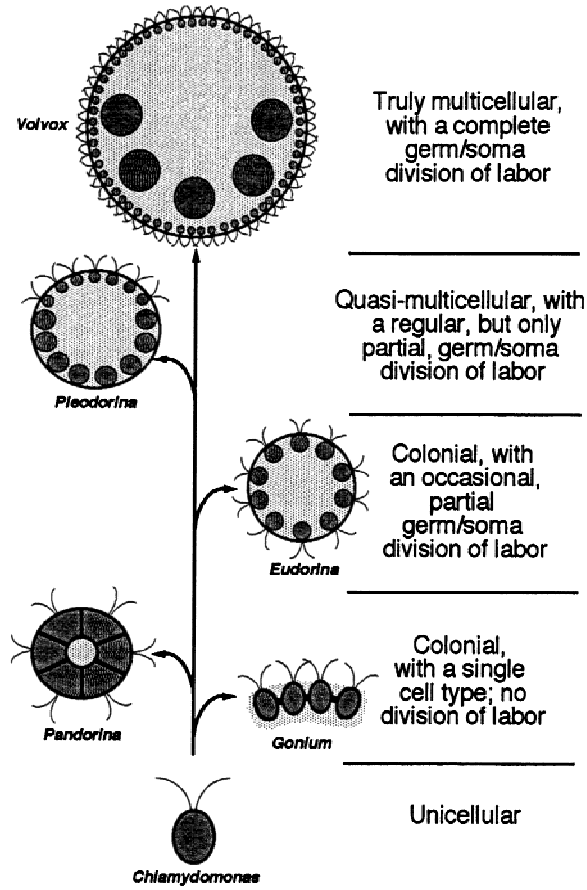

Figure 3. A conceptual scheme indicating some of the major steps that are believed to have occurred during the evolution of the volvocine algae and some of the modern genera exhibiting each level of morphologic and developmental complexity.

Because of its unusual combination of plant-like traits (such as photoautotrophy) and animal-like traits (such as motility and a germ–soma division of labor), many late 19th century biologists imagined *Volvox* to be the evolutionary "missing link" between plants and animals. Contemporary analysis indicates that even at the level of individual genes, *Volvox* is, indeed, a curious mixture of plantlike and animal-like features (Schmitt and others 1992). But it is now clear that this does not reflect the ancestral position of *Volvox* in the living world; rather it reflects the fact that the green algal lineage diverged from the lineage leading to the land plants not long after their common ancestor had diverged from the animal-fungal lineage (Figure 2). Indeed, molecular phylogenetic analysis reveals that *V. carteri* is an evolutionary newcomer, having last shared a common ancestor with unicellular *Chlamydomonas reinhardtii* only about 50 Mya (Rausch and others 1989). In contrast, the lineages leading to the land plants and the metazoa are believed to have made their separate transitions to multicellularity about 20 times that long ago, and by the time that the green flagellates were beginning to explore the pathway that would eventually lead from *Chlamydomonas* to *Volvox,* plants and animals as specialized as the grasses and primates were already undergoing diversification (Figure 2).

The existence of a coherent group of living green flagellates that are intermediate between *Chlamydomonas* and *Volvox* in size and complexity constitutes another powerful feature recommending *Volvox* as a model for studying the origins of multicellularity and cytodifferentiation. Certain of these "volvocine algae" (colonial organisms traditionally grouped in the family Volvocaceae) can be arranged in order of increasing cell number, increasing ratio of extracellular matrix (ECM) to cellular volume, increasing organismic size, and increasing tendency to divide life's labors between mortal somatic cells and immortal germ cells—all of which peak with *Volvox* (Figure 3). Then, of course, it scarcely requires a leap of creative imagination to speculate that such a sequence might resemble the pathway by which *Volvox* evolved from *Chlamydomonas.* This appealing scheme has made its way into countless textbooks over the decades. But is it valid?

Molecular evidence indicates that although the

volvocine algae (including *C. reinhardtii;* Coleman and Mai 1997) do constitute a closely related, monophyletic group; the actual phylogenetic history of the group is considerably more complicated than Figure 3 suggests. As more and more volvocine isolates and molecular markers are analyzed, it becomes increasingly clear that many (if not most) of the genus and species names that have traditionally been used for these flagellates identify grades of organizational complexity and *not* monophyletic clades (Coleman 1999; Kirk and others unpublished; Larson and others 1992; Nozaki and others 1995, 1997, 1999, 2000). Although such studies are still incomplete and although seldom do any pair of these studies analyze the same set of taxa, the picture that is emerging from all of them combined is emphatically *not* a linear tree of the sort depicted in Figure 3. Rather, the picture that emerges is of a highly branched volvocacean family tree, on which morphologically similar genera and/or species may be found on quite different branches. That is to say, molecular phylogenetic analysis now suggests that most traditionally recognized volvocacean genera and species are either paraphyelitic, polyphyletic, or both! Of particular significance in the present context is the growing evidence that different species of *Volvox* have arisen independently on at least two (Larson and others 1992; Nozaki and others 1995, 1999, 2000)—and possibly several—different branches of the volvocacean family tree (Coleman 1999; Kirk 1998).

The fact that multicellular organisms with the size and germ–soma division of labor that distinguish the genus *Volvox* have evolved at least twice in such a geologically brief span of time leads to a pair of interesting conjectures: (1) Relatively strong selective forces must have acted in the environments that are inhabited by the volvocaceans (primarily shallow, temporary ponds, but sometimes permanent lakes; Kirk 1998) to foster individuals of larger size and increased propensity for cellular differentiation. (Field studies revealing the probable nature of these selective forces have been reviewed in Kirk 1998.) (2) It must not require many genetic changes to go from a unicell-like *Chlamydomonas* to a colonial organism with a single cell type, such as *Pandorina* (Figure 3), and then to a multicellular organism with differentiated cell types, such as *Volvox*. This, in turn, leads one to anticipate that it might ultimately be possible to trace out in some detail the pathway by which the genetic program for cellular differentiation that is used by *Volvox* evolved—provided, of course, that the genetic program for *Volvox* cytodifferentiation is itself accessible to developmental-genetic analysis.

The Strengths of *Volvox carteri* as a Developmental Genetic Model

For the first 260 years after Antoni van Leeuwenhoek (1700) first described *Volvox,* many biologists recognized its potential usefulness as a model system for studying one important biologic issue after another (reviewed in Kirk 1998), but no one succeeded in maintaining *Volvox* cultures in the laboratory for more than a few weeks. This changed dramatically in the 1960s, when Bill Darden, then a graduate student in Richard Starr's laboratory, found a medium in which *V. aureus* (one of the most widely distributed species of *Volvox*) flourished indefinitely, permitting him to generate the first detailed descriptions of the complete asexual and sexual reproductive cycles of any species of *Volvox* (Darden 1966). Darden's success so fired Richard Starr's interest that Starr set aside his studies of other volvocacean algae and launched a campaign to collect, bring into culture, and study representatives of all 18 known (and several previously unknown!) species of *Volvox* from ponds, puddles, and lakes around the globe. As a result of these peripatetic studies, he soon identified a male/female pair of strains of *V. carteri* forma *nagariensis* that he had isolated from a pond in Japan as the members of the genus that were most amenable to genetic and developmental analysis (Starr 1970). His judgment proved correct, and these particular isolates and their progeny (hereafter simply called *V. carteri*) soon became the laboratory standards for the small cadre of experimentalists studying *Volvox.* Among the strengths of *V. carteri* as a developmental-genetic model (including those identified by Starr, plus several that have been discovered more recently) are the following:

- *V. carteri* normally reproduces asexually, increasing about 16-fold every 2 days. Thus, large isogenic clones can be established quickly.
- Asexual development can be synchronized with a light–dark cycle (Figure 4), so that large, uniform populations of spheroids can be obtained at any desired developmental stage.
- The asexual life cycle is simple (Starr 1969 and Figure 4): The approximately 16 gonidia present in each spheroid become mature at about 2 days of age, whereupon each initiates a sequence of 11 to 12 rounds of rapid cleavage divisions that are

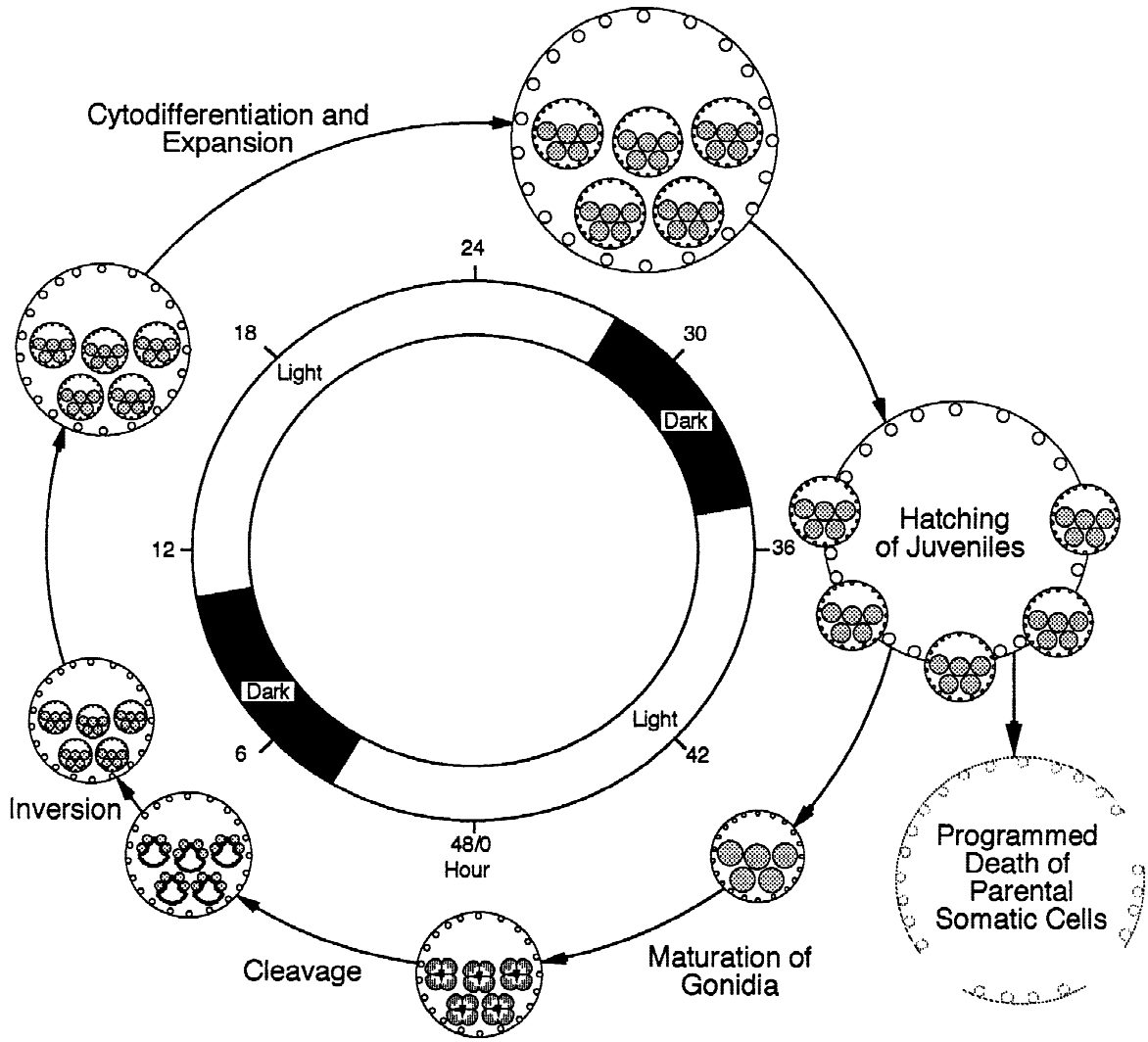

Figure 4. The asexual life cycle of *V. carteri,* as synchronized by a light-dark cycle. See the text for a brief description of the major events occurring in each of the major phases of this 2-day cycle.

completed in about 7 h and that produce all the cells that will be present in an adult of the next generation. A predictable subset of these divisions are asymmetric and set apart large and small sister cells that give rise to the gonidial and somatic lineages, respectively. The embryo produced by the end of cleavage has its gonidia on the outside and the flagellar ends of its somatic cells pointing inward but quickly corrects this awkward condition by turning itself right-side-out in a morphogenetic process called "inversion." During the remaining 40 h of the 2-day life cycle, the resulting juvenile spheroids enlarge by means of deposition of a complex, transparent, glycoprotein-rich ECM, whereas their gonidia grow and mature. Part way through this "expansion" phase, the juveniles hatch out of the parental spheroid, whereupon the somatic cells of the parental spheroid undergo programmed death (Figure 4).

- Somatic cells can be separated from gonidia at most stages of the life cycle, facilitating biochemical and molecular analysis of the developmental programs of the two cell types.
- Like all related green algae, *V. carteri* is haploid in all active phases of the life cycle, so that most mutations reveal themselves at once.
- For reasons that are not entirely clear (but may relate to the transposons discussed later) both "spontaneous" and induced mutants appear with greater frequency in the strains that Starr isolated from Japan than in any other *Volvox* isolates that

have been studied, including other formas (subspecies) of *V. carteri.*

- Differentiation of the two cell types is largely—if not entirely—cell autonomous. Therefore, as long as they produce cells capable of asexual reproduction, mutants with profound morphologic abnormalities are fully viable. Consequently, mutants with abnormalities in virtually all visible aspects of development can be recovered.
- Sexual reproduction can be induced at will, so formal genetic analysis is possible (Huskey and others 1979), and a preliminary linkage map of the genome containing more than 150 markers of various types is now available (Kirk 1998).
- Stable nuclear transformation with exogenous DNA is possible, and cotransformation of nonselectable markers with selectable markers occurs with relatively high (30–80%) efficiency (Schiedlmeier and others 1994).
- Several families of transposons have been identified, but the most important of these is an inducible transposon that jumps so well that it was named *Jordan* (Miller and others 1993); it has proven to be useful for tagging and recovery of genes regulating development (Kirk and others 1999; Miller and Kirk 1999).
- Although heterologous transgenes usually fail to be expressed in *Volvox,* this deficiency was overcome in the case of a bacterial antibiotic resistance gene by combining it with a *Volvox* promoter and introns, generating a dominant selectable marker (Hallmann and Rappel 1999).
- An inducible *Volvox* gene can be used to engineer constructs carrying either an inducible promoter or a reporter gene (Hallmann and Sumper 1994).
- It has been possible to introduce a gene from *Chlorella,* a rather distantly related green alga, into *V. carteri,* thereby changing its physiologic properties (Hallmann and Sumper 1996).

The Cytologic, Genetic, and Molecular Bases of *V. carteri* Ontogeny and Phylogeny

By exploiting various of the features listed previously, it has been possible to make significant progress toward explaining the genetic program underlying *Volvox* development and to begin exploring the way in which this program may have evolved. Because detailed and well-documented discussions of these topics are available to interested readers in other recent reviews (Kirk 1997, 1998, 1999; Sumper and Hallmann 1998), they will be discussed here only briefly, and with minimal additional documentation.

The first step toward volvocacean multicellularity undoubtedly was the conversion of the Chlamydomonas cell wall into a colonial ECM. A key step in early volvocacean evolution clearly was the elaboration of an ECM that would bind a set of sister cells into an organized and functional unit—a colony. Two features of *Chlamydomonas* biology appear to have served as preadaptations that facilitated this important evolutionary step: (1) each *Chlamydomonas* cell is enclosed in a noncellulosic cell wall composed primarily of a complex set of hydroxyproline-rich glycoproteins (HRGPs) that exhibit a clear propensity for self-assembly and rapid evolutionary diversification; (2) *Chlamydomonas,* like all other green flagellates with a coherent cell wall, exhibits an unusual form of cell division known as "multiple fission," in which each cell grows 2-fold, and then divides rapidly n times to produce 2^n daughter cells that are all enclosed within the cell wall of the mother cell. This division pattern results in 2^n young sister cells being held in intimate contact while they elaborate their cell walls. Thus, it presumably took only a modest change in HRGP biosynthesis to convert walls that separate cells from one another into a shared ECM that unifies them. Indeed, the principal difference between *Chlamydomonas* and those members of the genus *Gonium* that are the smallest colonial volvocaceans is that whereas *Chlamydomonas* sister cells produce completely separate walls, the walls of *Gonium* sister cells become "spot-welded" to one another by localized specializations while the cells are still tightly packed within the mother-cell wall (Nozaki 1990). In larger volvocaceans, the conversion of the cell wall into an ECM is carried further by means of additional modifications that result in the equivalent of the outermost, quasi-crystalline layer of the *Chlamydomonas* cell wall surrounding the entire colony while the individual cells are suspended in a more-or-less complex assortment of HRGPs that are thought to represent an evolutionary transformation of the relatively amorphous and inconspicuous inner layer of the *Chlamydomonas* wall (reviewed in Kirk 1998, 1999; Sumper and Hallmann 1998). Then as cell number increased in the volvocine lineage, organismic size increased even more rapidly, as a consequence of a progressive increase in the ratio of ECM to cellular volume (Figure 3). The selective force driving this exponential evolutionary increase in volvocacean size is postulated to have been the advantage that the ECM provided as a storage site for the nutrients—particularly phosphorous—for which all green algae compete (reviewed extensively in Kirk 1998).

Dichotomous differentiation of V. carteri cells is mediated by two types of negative regulators acting on the ancestral program of development. In *Chlamydomonas* and all colonial volvocine algae that contain only a single cell type, all cells exhibit a biphasic pathway of development that we can characterize as "first biflagellate and then reproductive." That is to say, all cells of these organisms develop initially as biflagellate cells that are actively motile throughout the growth phase, and then each cell transforms into a relatively (or completely) nonmotile reproductive cell that undergoes a rapid sequence of cleavage divisions. In *V. carteri* this ancestral biphasic pathway of development has become converted into a dichotomous one in which all of the motile functions of the organism are executed by the somatic cells while all of the reproductive functions are executed by the gonidia. Mutational analysis indicates that the genetic basis for this complete division of labor resides in two types of negative regulatory genes that are differentially activated in the two cell types: the *regA* gene acts selectively in the small cells destined to become somatic cells to repress growth and reproductive development, whereas the *lag* genes are acting in the gonidial initials to repress flagellar biogenesis and other aspects of somatic development. When *regA* is mutant, the small cells follow the ancestral "first biflagellate and then reproductive" pathway of development, and when one or more of the *lag* genes is mutant, the large cells follow this default pathway.

The *regA* gene has now been cloned and shown to encode a putative transcriptional repressor (Kirk and others 1999). Many putative *regA* target genes have also been characterized, and, somewhat surprisingly, they were found to be nuclear genes encoding chloroplast proteins (Meissner and others 1999). This has led to the working hypothesis that *regA* prevents reproductive development in somatic cells by blocking chloroplast biogenesis, thereby preventing the growth that would be necessary for the development of any reproductive potential. The nature and mechanism of action of the *lag* genes remains to be explained.

Asymmetric division is a centrally important feature of V. carteri development, because in this species cell size determines cell fate. As noted previously, the presumptive gonidial and somatic lineages of a *V. carteri* embryo are set apart by a stereotyped set of visibly asymmetric divisions. Owing to three successive rounds of such asymmetric divisions, combined with a difference in the total number of divisions completed, by the end of cleavage the volume of a gonidial initial is approximately 30-fold that of a somatic initial. This is critically important, because a variety of different genetic and experimental studies have all led to the conclusion that it is the difference in cell size at the end of cleavage—rather than any difference in cytoplasmic quality—that determines which cells will activate the somatic versus the gonidial program of differentiation (Kirk and others 1993). The mechanism by which embryonic cells measure their size at the end of cleavage and transduce the results into selective activation of one of the two alternative programs of gene expression remains obscure. However, insights into the genetic control of the asymmetric divisions that are required to establish large and small cells have begun to accumulate: Mutations at any of the (at least two, and probably more) *gls* loci abolish asymmetric cell division without affecting symmetric cell division, indicating that the products of the *gls* genes are required to shift the cell division plane from the center of a dividing cell to one side. The *glsA* gene has been cloned and found to encode a protein related to the Hsp40 class of chaperones (Miller and Kirk 1999). Both the GlsA protein and a developmentally regulated *V. carteri* Hsp70 (which is a potential binding partner of GlsA) associate with the mitotic spindle—as do their homologues in mammalian cells. Detailed immunocytologic studies aimed at explaining how these spindle-associated chaperones may participate in shifting the cell division plane in asymmetrically dividing *Volvox* cells are now underway, as are efforts to clone and characterize other *gls* genes and their products (SM Miller, personal communication).

Naturally, as quickly as developmentally important genes of *V.* carteri (such as *regA* and *glsA*) are cloned and characterized, studies aimed at explaining their evolutionary histories are being initiated. A priori, one can imagine at least three classes of genes that might be involved in programming evolutionary novelties: (1) genes that have been cobbled together from unrelated bits of DNA relatively recently and that encode proteins of truly novel structure and function, (2) members of ancient gene families that have become modified in more-or-less subtle ways to generate products with novel properties, and (3) strongly conserved genes whose products have taken on novel functions only as a result of changes that have occurred in other gene products with which they interact. It should be interesting to learn which of the genes that now play critical roles in *V. carteri* germ–soma specification may fall into each of these categories. Ultimately, however, it should be of even greater interest to determine how similar or how different the genetic pathways may have been that led to the evolution of different species of *Volvox* on different branches of the volvo-

cacean family tree. How many ways may there be to skin this particular evolutionary cat?

Some Shortcomings of *Volvox carteri* as a Model System Appear

Organisms evolve to exploit particular niches in the natural world, of course, not to satisfy the yearnings of experimental biologists searching for ideal model systems for their laboratories. As the articles in this issue surely illustrate, no model system ever comes with all features that some experimentalist might find desirable. It is often possible, of course (particularly in this present transgenic era), to circumvent certain apparent shortcomings of a model organism through cleverness and/or hard work. Examples abound. However, in some cases the undesirable traits that complicate or preclude various studies are so intrinsic to the biology of the organism under consideration that efforts to circumvent them are quite unlikely to succeed. Sometimes this is readily apparent a priori. As just one example, I cannot imagine anyone thinking it would be worth trying to eliminate, or even ameliorate, two features of the *V. carteri* genome that complicate cloning and analysis of genes of interest: the genome is very GC-rich, and introns are unusually abundant (Schmitt and others 1992). In other cases, however, the apparent futility of trying to change certain less-than-desirable attributes is recognized only after repeated attempts fail. A few examples based on experiences in our laboratory and others include the following:

- *Complementation analysis.* Like other green algae, *V. carteri* is normally haploid in all active phases of the life cycle; therefore complementation analysis is currently impossible, and loci are defined solely by recombination. In *Chlamydomonas* the numbers of "vegetative diploids" arising in standard crosses is small, but nevertheless it is adequate for assessing complementation. Vegetative diploids of *V. carteri* have been recovered only twice (both times inadvertently). In both cases the diploids exhibited morphologic abnormalities, had low vigor, and were of very limited use (Adams and others 1990; Meredith and Starr 1975). Repeated efforts to force diploidy by crossing strains with linked conditional lethals have failed uniformly. It now appears that circumventing this shortcoming would require much more effort than most people would believe could be justified.
- *Mendelian analysis of severe morphologic aberrations.* In contrast to asexual reproduction (which is largely cell autonomous and can therefore occur efficiently in strains with extreme morphologic aberrations), sexual reproduction in *V. carteri* requires a specific set of morphologic relationships within the female spheroid. To find and fertilize an egg, a sperm must first contact and be activated by the flagella of a somatic cell on the surface of a fertile female, then digest its way into the adjacent ECM, and then wiggle about randomly in the ECM until it bumps into an egg (reviewed in Kirk 1998). Mutations that significantly perturb any of the normal morphologic relationships within a female spheroid (as many of the most interesting mutations do) make fertilization difficult-to-impossible for the sperm to achieve. Attempts to circumvent this difficulty by, for example, combining sperm, flagella from sexual wild-type females, and eggs from the mutant of interest within a small volume of semisolid medium have met with zero success. As a result, Mendelian analysis of many of the morphologic mutants of greatest developmental interest has not yet been accomplished, and it is not likely to be accomplished without extraordinary effort.
- *Auxotrophs.* The only available auxotrophic strains of *V. carteri* are those that require reduced nitrogen because they lack a functional nitrate reductase. Over the years, both the reasons for seeking other types of auxotrophic mutants of *V. carteri* and the methods of seeking them have changed, but the success rate of such searches remains firmly fixed at zero. Several considerations indicate that further efforts in this regard may be futile: *V. carteri* is an obligate photoautotroph that is unable to use any exogenous reduced carbon source for energy or growth. Even after the organism was transformed with the gene encoding the hexose transporter of *Chlorella* (which made it capable of incorporating exogenous glucose into glycoproteins), it remained incapable of using glucose for heterotrophic growth (Hallmann and Sumper 1996). The only amino acid transport system that *V. carteri* appears to have is for arginine, and although this transporter permits the cells to accumulate arginine and incorporate it into proteins efficiently (Kirk and Kirk 1978), repeated efforts to isolate arginine auxotrophs have all failed. Correspondingly, although arginine auxotrophs of the related unicell *C. reinhardtii* have been available for decades, repeated efforts in several laboratories to isolate other categories of amino acid auxotrophs have met with uniform failure. It is often suggested that genetic redundancy of biosynthetic pathways in these algae may be the reason for such failures, but direct evidence for this is lacking.

- *Cryopreservation.* The only stage of the *V. carteri* life cycle that can be maintained in a dormant state in either nature or the laboratory is the diploid zygospore. This fact, combined with the difficulty of producing zygotes with many of the most interesting kinds of mutants, means that many mutants can only be maintained in liquid medium under slow-growth conditions, where they require regular care. Some years ago we established a collaboration with expert cryobiologists in an effort to circumvent this shortcoming, but we never recovered a single viable cell after any combination of freezing and thawing protocols. The magnitude of the hurdle to be overcome in this project became starkly apparent when we found that we could recover no viable cells after refrigerating a culture at +4°C for only an hour.

Potentially Surmountable Shortcomings of *Volvox carteri*

The foregoing list not withstanding, there are a number of technical improvements that would substantially improve the usefulness of *V. carteri* as a model system and that we presently have no reason to believe are out of reach. Important targets for future improvement efforts include (in no particular order) the following:

- *Improved transformation efficiency.* At present, the efficiency of nuclear transformation in *V. carteri* is adequate to test the ability of cloned genes to rescue appropriate mutants, but it is not nearly high enough to make cloning genes either by complementation with genomic libraries or by insertional mutagenesis with a selectable transgene a realistic possibility. Even a 10-fold increase in transformation efficiency would have a salutary effect, and a larger increase would be even better, of course.

- *Homologous recombination.* Exogenous DNA is usually incorporated into the *V. carteri* genome in random locations by nonhomologous recombination. However, evidence that a system for homologous recombination exists was provided when a fragment of a wild-type *nitA* (nitrate reductase-encoding) gene was used to repair the mutational defect in the corresponding allele of a mutant (Hallmann and others 1998). A method for capitalizing on this homologous recombination system to make targeted gene modifications a realistic possibility would be exceptionally valuable.

- *Antisense/iRNA technology.* An efficient method of inhibiting intrinsic gene activity with antisense vectors or double-stranded RNA also would be enormously valuable.

- *GFP technology.* A gene encoding green fluorescent protein (GFP) has been synthesized with the *C. reinhardtii* codon bias and used to label both a *Chlamydomonas* protein and the product of a bacterial transgene (Fuhrmann and others 1999). Adaptation of this GFP technology to *V. carteri* would make a very important contribution to many kinds of studies.

- *Improved inducible promoters and reporter constructs.* The promoter and reporter constructs that were based on the *V. carteri* aryl sulfatase (AS) gene (Hallmann and Sumper 1994) demonstrated the feasibility of using such technology in *Volvox,* but the AS promoter lacks the strength and the AS reporter lacks the sensitivity and temporal resolution required for many types of studies. More vigorous promoters and reporters should be sought.

- *Cell-type–specific promoters.* As soon as a more sensitive reporter cassette is available, a promoter trap should be performed to identify strong cell-type–specific promoters that could then be used to express transgenes of various types in one cell type or the other.

And while we are at it, why not ask our Fairy Godmother for a complete genome sequence?

ACKNOWLEDGMENTS

Research in my laboratory has been supported in recent years by the National Science Foundation (MCB-9304447 and IBN-9904739) and the U.S. Department of Agriculture (95-37304-2365 and 97-35304-4626).

REFERENCES

Adams CR, Stamer KA, Miller JK, McNally JG, Kirk MM, Kirk DL. 1990. Patterns of organellar and nuclear inheritance among progeny of two geographically isolated strains of *Volvox carteri.* Curr Genet 18:141–153.

Bonner JT. 1998. The origins of multicellularity. Integrative Biol 1:27–36.

Coleman AW. 1999. Phylogenetic analysis of "Volvocaceae" for comparative genetic studies. Proc Natl Acad Sci USA 96:13892–13897.

Coleman AW, Mai JC. 1997. Ribosomal DNA ITS-1 and ITS-2 sequence comparisons as a tool for predicting genetic relatedness. J Mol Evol 45:168–177.

Darden WH. 1966. Sexual differentiation in *Volvox aureus.* J Protozool 13:239–255.

Devereux R, Loeblich III AR, Fox GE. 1990. Higher plant origins and the phylogeny of green algae. J Mol Evol 31:18–24.

Fuhrmann M, Oertel W, Hegemann P. 1999. A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii.* Plant J 19:353–361.

Hallmann A, Rappel A. 1999. Genetic engineering of the multicellular green alga *Volvox:* A modified and multiplied bacterial antibiotic resistance gene as a dominant selectable marker. Plant J 17:99–109.

Hallmann A, Rappel A, Sumper M. 1998. Gene replacement by homologous recombination in the multicellular green alga *Volvox carteri.* Proc Natl Acad Sci USA 94:7469–7474.

Hallmann A, Sumper M. 1994. Reporter genes and highly regulated promoters as tools for transformation experiments in *Volvox carteri.* Proc Natl Acad Sci USA 91:11562–11566.

Hallmann A, Sumper M. 1996. A transgenic *Volvox* expressing the *Chlorella* hexose/H^+ symporter gene is highly useful for biochemical work. Proc Natl Acad Sci USA 93:669–673.

Huskey RJ, Griffin BE, Cecil PO, Callahan AM. 1979. A preliminary genetic investigation of *Volvox carteri.* Genetics 91:229–244.

Kirk DL. 1997. The genetic program for germ–soma differentiation in *Volvox.* Annu Rev Genet 31:359–380.

Kirk DL. 1998. *Volvox:* Molecular genetic origins of multicellularity and cellular differentiation. New York: Cambridge University Press. 381 p.

Kirk DL. 1999. Evolution of multicellularity in the volvocine algae. Curr Opin Plant Biol 2:496–501.

Kirk MM, Kirk DL. 1978. Carrier-mediated uptake of arginine and urea by *Volvox carteri* f. *nagariensis.* Plant Physiol 61:549–555.

Kirk MM, Ransick, McRae SE, Kirk DL. 1993. The relationship between cell size and cell fate in *Volvox carteri.* J Cell Biol 123:191–208.

Kirk MM, Stark K, Miller SM, Müller W, Taillon BE, Gruber H, Schmitt R, Kirk DL. 1999. *regA,* a *Volvox* gene that plays a central role in germ-soma differentiation encodes a novel regulatory protein. Development 126:639–647.

Larson A, Kirk MM, Kirk DL. 1992. Molecular phylogeny of the volvocine flagellates. Mol Biol Evol 9:85–105.

Meissner M, Stark K, Cresnar B, Kirk DL, Schmitt R. 1999. *Volvox* germline-specific genes that are putative targets of RegA repression encode chloroplast proteins. Curr Genet 36:363–370.

Meredith RF, Starr RC. 1975. The genetic basis of male potency in *Volvox carteri* f. *nagariensis* (Chlorophyceae). J Phycol 11:265–272.

Miller SM, Kirk DL. 1999. *glsA,* a *Volvox* gene required for asymmetric division and germ cell specification, encodes a chaperone-like protein. Development 126:649–658.

Miller SM, Schmitt R, Kirk DL. 1993. *Jordan,* an active *Volvox* transposable element similar to higher plant transposons. Plant Cell 5:1125–1138.

Nozaki H. 1990. Ultrastructure of the extracellular matrix of *Gonium* (Volvocales, Chlorophyta). Phycologia 19:1–8.

Nozaki H, Itoh M, Sano R, Uchida H, Watanabe MM, Kuroiwa T. 1995. Phylogenetic relationships within the colonial Volvocales (Chlorophyta) inferred from *rbc*L gene sequence data. J Phycol 31:970–979.

Nozaki H, Ito M, Uchida H, Watanabe MM, Kuroiwa T. 1997. Phylogenetic analysis of *Eudorina* species (Volvocaceae, Chlorophyta) based on *rbcL* gene sequences. J Phycol 33:859–863.

Nozaki H, Misawa K, Kajita T, Kato M, Nohara S, Watanabe MM. 2000. Origin and evolution of the colonial Volvocales (Chlorophyceae) as inferred from multiple, chloroplast sequences. Mol Phylogenet Evol 17:256–268.

Nozaki H, Ohta N, Takano H, Watanabe MM. 1999. Reexamination of phylogenetic relationships within the colonial Volvocales (Chlorophyta): An analysis of *atpB* and *rbc*L gene sequences. J Phycol 35:104–112.

Rausch H, Larsen N, Schmitt R. 1989. Phylogenetic relationships of the green alga *Volvox carteri* deduced from small-subunit ribosomal RNA comparisons. J Mol Evol 29:255–265.

Schiedlmeier B, Schmitt R, Müller W, Kirk MM, Gruber H, Mages W, Kirk DL. 1994. Nuclear transformation of *Volvox carteri.* Proc Natl Acad Sci USA 91:5080–5084.

Schmitt R, Fabry S, Kirk DL. 1992. In search of the molecular origins of cellular differentiation in *Volvox* and its relatives. Int Rev Cytol 139:189–265.

Sogin ML. 1991. Early evolution and the origin of eukaryotes. Curr Biol 1:457–463.

Starr RC. 1969. Structure, reproduction, and differentiation in *Volvox carteri* f. *nagariensis* Iyengar, strains HK 9 and 10. Arch Protistenkd 111:204–222.

Starr RC. 1970. Control of differentiation in *Volvox.* Dev Biol 4(suppl):59–100.

Sumper M, Hallmann A. 1998. Biochemistry of the extracellular matrix of *Volvox.* Int Rev Cytol 180:51–85.

van Leeuwenhoek A. 1700. Concerning the worms in sheep, livers, gnats and animalicula in the excrement of frogs. Phil Trans R Soc Lond 22:509–518.

Wainwright PO, Hinkle G, Sogin ML, Sticke SK. 1993. Monophyletic origins of the Metazoa: An evolutionary link with fungi. Science 260:340–342.