The program for cellular differentiation in *Volvox carteri* as revealed by molecular analysis of development in a gonidialess/somatic regenerator mutant

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Summary

Development of a ‘gonidialess’/‘somatic regenerator’ double mutant of *Volvox carteri* was analyzed with a number of cell-type-specific cDNA probes that had been identified in a previous study. Whereas in wild-type strains somatic cells and gonidia (asexual reproductive cells) constitute two distinct cell lineages, in this mutant all cells first differentiate as somatic cells and then redifferentiate as gonidia. During the initial period of somatic differentiation, we found that both gonidal and ‘early’ somatic transcripts were accumulated in the mutant, consistent with the idea that it is the *regA* gene product (which is defective in this mutant) that normally acts to suppress gonidial gene expression in somatic cells. Later in development, levels of early somatic transcripts fell abruptly, levels of the late somatic transcripts remained extremely low, and levels of gonidal transcripts rose as the cells redifferentiated. Thus it appears that in the mutant cells the gonidial program of development takes over and somatic differentiation is aborted before the stage at which late somatic genes are normally activated. These results provide molecular genetic support for a model which postulates that three types of genes (including the two that are defective in the strain studied here) are crucial for converting the sequential program of differentiation seen in more primitive volvocalean algae to the dichotomous program of germ-soma differentiation that occurs in wild-type *V. carteri*.

Key words: cell-specific cDNAs, cytodifferentiation, developmental genetic program, germ-soma dichotomy.

Introduction

*Volvox carteri* forma *nagariensis* is a simple multicellular organism that exhibits a complete division of labor between mortal somatic cells and immortal germ cells. In an asexual adult spheroid, somatic cells and reproductive cells (or ‘gonidia’) are present in predictable numbers (about 2000:16) and locations (Starr, 1969, 1970). The generation of these two cell types in a regular pattern can be traced back to distinctive events in embryogenesis (Starr, 1969; Green and Kirk, 1981, 1982): after the first five symmetrical divisions, the 16 anterior blastomeres of the 32-cell embryo divide asymmetrically. The 16 large cells thus produced divide asymmetrically two more times and then withdraw from division. The smaller cells produced by the asymmetric divisions, plus all of the cells produced by symmetric divisions in the posterior half of the embryo, go on to divide a total of about 11 times, giving rise to about 2000 small cells at the end of cleavage. Following embryogenesis, the large cells differentiate as gonidia while the small cells differentiate as somatic cells with characteristic features such as eyespots and flagella. Somatic cells live for several days before they eventually undergo programmed senescence and cell death (Hagen and Kochert, 1980; Pommerville and Kochert, 1981, 1982).

The mechanisms of germ/soma determination and differentiation in *Volvox* have hitherto been analyzed mainly by classical cell-lineage and genetic studies (reviewed in Kirk and Harper, 1986). Recently, we have initiated a molecular approach to these problems by identifying genes that are expressed preferentially in gonidia or somatic cells (Tam and Kirk, 1991). By analyzing the developmental expression of these cell-type-specific genes, a number of distinctive developmental programs have been identified (Tam and Kirk, 1991; Tam *et al.* 1991). The availability of these cell-type-specific probes now makes possible studies of the
underlying molecular changes caused by mutations at various developmentally important regulatory loci, which is the subject of the present report.

One of the most interesting regulatory loci that has been defined in *V. carteri* is the *regA* locus (Starr, 1970; Huskey and Griffin, 1979). Strains with a *regA* mutation exhibit the 'somatic regenerator' (or Reg) phenotype in which somatic cells initially appear to differentiate normally, but later enlarge and redifferentiate as gonidia. These redifferentiated cells exhibit full reproductive potential: they can either cleave to form asexual spheroids (i.e., each can regenerate a complete spheroid), or they can be induced to undergo sexual development (Starr, 1970; Huskey and Griffin, 1979). It has been postulated that the *regA* gene is selectively expressed in somatic cells to suppress all reproductive functions, and is selectively inactivated in gonidia, possibly by mechanisms involving locus-specific rearrangement (Kirk *et al.* 1987).

Once we had defined the patterns in which cell-type- and stage-specific transcripts are accumulated in the two cell types of wild-type *Volvox*, we were anxious to determine how these patterns are modified in a Reg mutant. Such studies are potentially complicated, however, by the difficulty of synchronizing development in most Reg strains. Because each Reg spheroid produces two cohorts of progeny with different developmental schedules (one cohort derived from *bona fide* gonidia, and one from the regenerating somatic cells), a Reg culture typically contains a complex mixture of progeny of the two types, at widely different developmental stages. In the present study, we have used a *Gls* ('gonidialess')/Reg double mutant to circumvent this technical difficulty. The Gls mutation results in an absence of the asymmetric cleavage divisions that normally set the gonidial lineage apart from the somatic lineage during embryogenesis. On a wild-type background the Gls defect would be lethal. However, in combination with a *regA* mutation, it results in production of a single category of cells, all of which first differentiate as somatic cells and then redifferentiate as gonidia (Kirk *et al.* 1991a). Preliminary observations indicated that the time course of initial differentiation and redifferentiation of Reg somatic cells, and the morphology of these cells at various stages of their development, are essentially identical in the Reg single mutant and in the Gls/Reg double mutant. Therefore, from an operational viewpoint the principal effect of the Gls lesion in the double mutant studied here was to remove the complications imposed by having two cohorts of reproducing cells within a single spheroid, and to permit recovery of large, synchronous populations of Reg cells at various stages throughout the life cycle. From a theoretical viewpoint, however, the combination of these two mutations has an interesting additional consequence: together these mutations convert *Volvox* from an organism with dichotomous differentiation of germ and soma to an organism with a life history similar to that in simpler volvocacean algae (such as *Eudorina*) in which vegetative and reproductive functions are executed sequentially by all cells.

Thus, we hoped that studies of the double mutant might also provide some clues regarding the way in which an ancestral program for sequential differentiation may have become converted into a program for dichotomous differentiation during the evolution of *Volvox*.

In the present study, we first used detailed microscopic examination to define the time course of morphological differentiation in the Gls/Reg double mutant; then we analyzed the underlying program of gene expression in terms of accumulation of various categories of cell-type-specific transcripts at intervals throughout the life cycle. The results indicate that initially the Gls/Reg cells express both gonidial and somatic genes simultaneously, although they only develop the morphological features characteristic of normal somatic cells at this time. However, as development proceeds, the gonidial developmental program of gene expression eventually takes over, somatic gene expression is repressed, and the cells redifferentiate. We discuss these results with respect to a previously developed model that postulated how the *gls* and *regA* genes – plus a third class of regulatory genes – might act to control dichotomous cellular differentiation in *V. carteri*.

### Materials and methods

#### Strains

The strains used in this study were derived from wild-type *Volvox carteri* forma nagariensis cultures obtained from the Culture Collection of Algae at the University of Texas. Eve is a subclone of a female standard strain, HK10, originating from Japan (Starr, 1969). The Gls mutation described in this study arose spontaneously in a Reg progeny from a cross between HB11A (a *regA*~ strain derived from Eve by several rounds of mutagenesis and selection) and PM1 (a wild-type standard male strain originating from Poona, India). (Details of the derivation of these parental strains are reported in Adams *et al.* 1990.) A clonal line of this progeny was used for the present analysis.

#### Synchronous cultures and RNA isolation

Eve and Gls/Reg cultures were maintained under standard culture conditions (Kirk and Kirk, 1983) at 32°C and 30°C, respectively, except that a 16 h light/8 h dark cycle was used to synchronize growth. For developmental analysis of the mutant, spheroids with cleaving embryos were selected manually, and allowed to grow until appropriate times for microscopic examination or RNA isolation.

For RNA isolation, spheroids were concentrated by filtration on 30 μm Nitex mesh, and pelleted by centrifugation at 100 g in a Beckman TJ-6 centrifuge. RNA extraction was performed as previously described (Tam and Kirk, 1991), except that proteinase K at 150 μg ml⁻¹ was included in the lysis buffer for all samples.

For assessing the effect of spheroid disruption on gene expression, spheroids were collected at three different time points and each sample was divided into two equal portions. One portion was lysed for RNA extraction without further treatment, while the second portion was subjected to homogenization in a Dounce homogenizer ( Kontes) to dissociate spheroids into single cells before RNA isolation. At 24 h, cells were still fairly small and somatic-looking, and 16
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strokes with a tight-fitting pestle (B) were used to dissociate spheroids into small fragments or single cells. At 48 h and 72 h, cells had enlarged and regenerated into gonidia, and 10 strokes with a loose-fitting pestle (A) were sufficient for disruption.

Northern blot analysis
To determine the stage-specificity of expression of gonidia- or somatic-cell-specific genes in the Gls/Reg mutant, 8μg samples of total RNA, isolated from cell populations collected at 6h intervals throughout most parts of the life cycle (but at closer intervals during embryogenesis) were used on northern blots, and probed with 32P-labeled DNAs derived from a number of cell-type-specific clones. The derivation of these cell-type-specific clones, and the procedures used for northern blot analysis and determination of the relative poly(A)+ RNA content of samples were previously described (Tam and Kirk; 1991).

Measurement of cell size
Diameters of cells were measured with an ocular micrometer, at a magnification of 250–400×. At each time point, cells in 5–10 different spheroids were chosen randomly for measurement.

Results
The mutant used for this study possesses two mutations: a mutation at the regA locus, which causes somatic cells to redifferentiate into gonidia; and a Gls mutation, which abolishes asymmetric divisions and normal gonidial specification. Below, we will describe and compare the phenotype of this double mutant, in both morphological and molecular terms, with that of a wild-type (WT) strain, Eve, that was used in two other related studies (Tam and Kirk, 1991; Tam et al. 1991).

Cellular phenotypes through the asexual life cycle
The life cycle (Fig. 1) of the Gls/Reg mutant was synchronized by the same 16 h light/8 h dark cycle as was used for the WT strain. Whereas one life cycle of the WT strain is completed in 48 h (for detail, see Tam and Kirk, 1991), that of the mutant takes 72 h. For presentation purposes, the life cycle of this mutant is divided into three major phases (Fig. 1): first is an embryonic (E) phase (4–13 h), in which new individuals are formed from redifferentiated cells by a series of symmetric divisions (Fig. 2A,B); second is a somatic (S) phase (13–37 h), during which all cells develop and continue to exhibit morphological features characteristic of somatic cells (e.g. two flagella, one eyespot and one cup-shaped chloroplast per cell, Fig. 2C); third is a gonidal (G) phase (37 h to 4 h of the next life cycle), in which cells gradually lose their somatic features, redifferentiate as gonidia, and gain the potential for asexual reproduction (Fig. 2D,E). Unlike the original Reg strain from which this mutant was derived, regenerated ‘gonidia’ in this double mutant are resistant to sexual induction (K. Stamer, personal communication). Hence genetic analysis of the Gls/Reg mutant is not yet available.

In this mutant, cell size appears to correlate with the differentiated state of the cells. Diameters of cells measured at various times during the life cycle are shown in Table 1. Fully regenerated ‘gonidia’ at a few hours before the onset of embryogenesis were about half the diameter (thus 1/8 of the volume) of WT gonidia at a comparable stage. In most cases, these cells divided only about 7–8 times, and gave rise to progeny containing 128 or 256 cells. Apart from the absence of asymmetric cleavages, all other aspects of embryogenesis, including the orientations of successive cleavage planes, cell shape changes and inversion (by which embryos turn inside out), appeared to be normal. Therefore, during embryogenesis a regular sphere of cells of similar sizes was derived from each redifferentiated ‘gonidium’. During the additional few hours that these newly formed spheroids remained in the dark, little additional development occurred. The average diameter of cells before cytodifferentiation began was about 4μm, which was very similar to that of WT

Fig. 1. Life cycle of the Gls/Reg mutant. The 72-h life cycle is synchronized by a 16 h light/8 h dark cycle, and can be divided into three phases of development: (E) Embryonic phase (4–13 h). Cells enter embryogenesis at about 4 h. A rapid series of 7–8 equal divisions occurs, followed by inversion of embryonic spheroids at around 9 h. Little additional development of embryonic spheroids takes place after inversion, until the next phase of development. (S) Somatic phase (13–37 h). Cytodifferentiation begins when the lights come back on at 13 h. All cells differentiate as somatic cells, and they retain somatic features such as eyespots and flagella throughout this period of development. Juveniles are released and become free-swimming shortly after differentiation. (G) Gonidal phase (37–4 h). Cells resorb their somatic features soon after 37 h, and they gradually enlarge and assume the vacuolated morphology of gonidia. Finally they become mature and are ready for a new round of embryogenesis.
Fig. 2. Gls/Reg cells at different stages of development. (A,B) Embryos at 32- and 64-cell stage. Asymmetric cleavages do not take place in this mutant, as they do in wild-type embryos at the transition from 32-cell to 64-cell stage. Other than the loss of asymmetric cleavages, the embryos appear to be very regular, indicating that all other aspects of embryogenesis are almost normal. (C) A free-swimming juvenile spheroid at 24 h. Initially all cells develop into somatic cells, with characteristic features such as flagella (not visible here) and prominent eyespots. (D) A spheroid at 48 h. Cells have lost their somatic features and have begun to enlarge, but they have not assumed the vacuolated morphology of gonidia yet. Cellular redifferentiation is somewhat asynchronous, usually being more advanced in cells at the posterior than at the anterior end of the spheroid. (E) A fully regenerated spheroid at 72 h. Cells have enlarged to about 1/2 the diameter of normal gonidia, and developed features typical of gonidia, such as vacuolated cytoplasm and multiple pyrenoids. Scale bar is 25 μm. Magnifications for A and B are the same.

Table 1. Changes in cell diametera during the asexual life cycle

<table>
<thead>
<tr>
<th>Hour</th>
<th>Gls/Reg cells</th>
<th>Wild-type cells</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>33.7±3.2 (41)b</td>
<td>3.6±0.7 (115)c</td>
</tr>
<tr>
<td>12</td>
<td>3.7±0.6 (62)</td>
<td>7.1±0.6 (67)c</td>
</tr>
<tr>
<td>24</td>
<td>6.7±0.8 (82)</td>
<td>7.4±0.7 (66)c</td>
</tr>
<tr>
<td>30</td>
<td>8.4±1.0 (42)</td>
<td>7.4±0.7 (66)c</td>
</tr>
<tr>
<td>36</td>
<td>8.7±0.9 (18)</td>
<td>7.6±0.6 (59)c</td>
</tr>
<tr>
<td>48</td>
<td>27.0±4.2 (44)</td>
<td>67.4±3.4 (48)d</td>
</tr>
<tr>
<td>54</td>
<td>27.2±4.5 (66)</td>
<td></td>
</tr>
<tr>
<td>72/0</td>
<td>36.0±5.8 (75)e</td>
<td></td>
</tr>
</tbody>
</table>

a Values are mean diameter in μm±standard deviation (sample size in parenthesis).
bEmbryos at or just before the onset of cleavage.
cSomatic cells.
dMature gonidia, 2 h before cleavage.
eRedifferentiated cells, 4 h before cleavage.

somatic initials at the equivalent stage (Table 1, 12 h). All cells of the mutant rapidly developed flagella and eyespots after the lights came back on at 13 h; then, shortly after they became motile, the young spheroids broke out of the parental extracellular matrix that had held them together and became free-swimming. By 24 h, spheroids had expanded considerably by the deposition of extracellular matrix, but the individual cells had only enlarged slightly, to about 7 μm. WT somatic cells at this time were about the same size, suggesting that the differentiation of mutant cells up to 24 h was similar to that of WT somatic cells. In the next 6 h, however, the mutant cells enlarged somewhat more rapidly, so that by 30 h their average diameter exceeded that of WT somatic cells by about 1 μm, indicating that redifferentiation into gonidia had begun. Nevertheless, these cells still possessed flagella and eyespots, as they did throughout the following dark period (29–37 h). While little visible change occurred in the dark, rapid enlargement and dedifferentiation of the mutant cells began soon after the light period resumed at 37 h. In the next 11 h, these cells enlarged about 3-fold in diameter, lost their eyespots and flagella, and slowed down their deposition of extracellular matrix. In the last 24 h of the life cycle, the dedifferentiated cells continued to enlarge somewhat, and they gradually assumed the characteristic vacuolated morphology of mature gonidia. Finally, at an average diameter of about 36 μm, they initiated a new round of embryogenesis.

Northern blot analysis

In two recent studies (Tam and Kirk, 1991; Tam et al. 1991), we identified 31 cDNA clones that correspond to genes that are expressed differentially in WT gonidia and somatic cells. To understand how these programs of differential gene expression are modified in the Gls/Reg mutant, we used northern blot hybridization to measure the abundance of transcripts corresponding to a number of the cell-type-specific genes in the mutant. 8 μg samples of total RNA isolated from the Gls/Reg strain at intervals throughout its 72 h life cycle were applied to northern blots. Equal amounts of two total RNA samples isolated from WT gonidia and somatic cells (at 42 h and 24 h, respectively) were also included on each blot as positive controls. The same set of blots were then probed successively with labeled DNAs from nine gonidia-specific and 12 somatic-cell-specific cDNA clones, as well as two previously defined Volvox genes (α-tubulin and an extracellular matrix gene, SSG 185) that exhibit somatic-cell-specific expression in WT (Tam and Kirk, 1991). As an additional
control, these RNA blots were probed with a cDNA, C38, that hybridizes to an RNA that is present at a fairly uniform level in WT gonidia and somatic cells at all stages. Representatives of the resulting autoradiograms are shown in Fig. 3. The band intensities on all autoradiograms were quantitated by scanning densitometry. Densitometer readings were then normalized with respect to the relative poly(A)+ RNA contents of the various RNA samples, and the resulting data were expressed as percentages of the normalized value for the hybridization signal produced by the appropriate WT-RNA control. The results are presented graphically in Figs 4 and 5.

Expression of gonidial genes

Expression of nine gonidial mRNAs was examined (Figs 3A, 4). As anticipated, all nine of these mRNAs were found to be present in abundance during the G phase, when cells were redifferentiating as gonidia. It is important to note that the temporal patterns of abundance of these gonidial mRNAs during the G and E phases of development in the mutant closely resemble those observed during corresponding stages in the development of WT gonidia: As in WT embryos, G167 mRNA was most abundant in early embryogenesis (6h), and it dropped to a minimum level at the time of inversion (9h). In contrast, all of the other gonidial mRNAs reached maximum abundance in both mutant and WT organisms during gonidial maturation, and they then declined to very low levels during embryogenesis.

Interestingly, however, during the S period, when the mutant cells exhibited all the morphological features of somatic cells, both categories of gonidial transcripts were much more abundant than they ever are in WT somatic cells. In WT, measurable quantities of ‘maturation-abundant’ gonidial transcripts were detected in somatic cells only transiently during the first few hours of cytodifferentiation; they had completely disappeared by 18h of development (Tam and Kirk, 1991). In the mutant, in contrast, really substantial amounts of these gonidial transcripts were present as the cells were initially differentiating as somatic cells. Moreover, although a transient decrease in the levels of these gonidial transcripts did occur after 18h, they did not completely disappear from the mutant cells as they did from WT somatic cells. The expression pattern of G167 (the ‘embryogenesis-abundant’ gonidial gene) in the mutant cells is even more noteworthy. Transcripts of this gene were, at most, barely detectable in WT somatic cells at any stage of development. But in the mutant the G167 transcript was present at really substantial levels during the entire S phase of the life cycle. These results indicate clearly that young Gls/Reg cells fail to shut off gonidial gene expression as young WT somatic cells do – even though the mutant cells possess all of the morphological features of normal somatic cells at this stage of their development.

Somatic genes

In the WT strain, transcripts corresponding to SSG 185...
and five 'early' somatic genes (S25, S27, S28, S34, S153) began to accumulate at high levels in the somatic lineage at the end of embryogenesis, and then remained at moderate levels for at least 60 h (Tam and Kirk, 1991; Tam et al. 1991). In the Gls/Reg mutant, these early somatic transcripts were also present in significant levels in the 12–18 h period that spans the late-E to mid-S phase; however, in contrast to the WT pattern, levels of all these transcripts fell to low or even undetectable levels in the late S phase, and remained low through the entire G phase (Figs 3B, 5A). Similarly, a high level of the α-tubulin transcript was observed in the mutant during the E and S phases (Fig. 5A). But then, instead of remaining elevated as it does in WT somatic cells, the tubulin mRNA level fell to low levels in the late S phase and remained so throughout the G phase. In short, it appears that the expression of early somatic genes is initially activated in the mutant as it is in normal somatic-cell development, but then expression of these genes is thoroughly repressed during the later two-thirds of the mutant life cycle.

Seven other somatic genes are first expressed in the WT strain much later than the early somatic genes that have just been discussed; transcripts of these 'late' genes were not detected in somatic cells by in situ hybridization until the cells were about 42-h old (Tam et al. 1991). Strikingly, transcripts of these seven 'late' somatic genes were virtually undetectable in the mutant at all time points (Figs 3B, 5B), suggesting that the
expression of the late somatic genes is not activated at all during development of the mutant.

Effects of spheroid disruption on gene expression
In our initial study, in order to determine the cell-type-specificity of transcript accumulation by northern blot analysis, we had to mechanically disrupt WT spheroids into single cells to obtain purified populations of somatic cells and gonidia. Having once established the cell-type-specificity of expression of these genes, we were then able to compare transcript levels in intact and disrupted spheroids. Such comparisons indicated that accumulation of several of the somatic transcripts was stimulated substantially by the disruption (Tam and Kirk, 1991). In the present study, because there is only one type of cell in the Gls/Reg mutant, cell purification was unnecessary, and therefore mechanical disruption of spheroids was omitted in the routine RNA isolation procedure. However, in order to rule out the possibility that the differences that we observed in gene expression patterns of the WT and mutant strains might be a result of the different cell treatment procedures used in the two different studies, the following control experiment was conducted. Mutant spheroids were collected at three different time points (24, 48, 72 h) of the life cycle, and each culture was then divided into two equal samples. At each time point one sample was used for RNA isolation without further treatment, while the second was disrupted in a Dounce homogenizer to break it into small fragments or single cells before RNA extraction. Equal amounts (8 μg) of these six total RNA samples were then used on northern blots and hybridized with the same set of probes used above. The ratios of hybridizing-RNA band intensities observed in the samples from intact and dissociated spheroids of the same time point were then computed.

The results (Table 2) indicate that in the mutant, as in the WT strain, spheroid disruption caused no significant enhancement of transcript accumulation for most of the genes, including all of the gonidial and early somatic genes, and two of the late somatic genes. Indeed, levels of three of the gonidial transcripts (G5, G12 and G46) actually declined substantially following disruption at the 24 h stage. Therefore, even if mechanical disruption had been used routinely in the present study to prepare the mutant spheroids for RNA isolation, qualitative features of the expression patterns of all these genes would have been similar. Even with the reduction in transcript abundance observed for three gonidial transcripts following disruption, the levels of these transcripts were significantly higher in the mutant at 24 h than they were in WT somatic cells at that time point.

Transcripts of the remaining five late somatic genes, namely S5, S9, S12, S23 and S33, were previously found to be accumulated to markedly higher levels in the WT strain following mechanical disruption of the spheroids (Tam and Kirk, 1991). Equal or greater effects were seen for these five transcript families following disruption of mutant spheroids – but only at the 24 h time point, when cells had the morphology of somatic cells.

Effect of light on somatic regeneration
By 30 h, the levels of most early somatic transcripts in the mutant had begun to decline markedly from earlier values and were already well below the levels seen in WT somatic cells at 30 h. In addition, the increase in cell size that was detected at 30 h indicated that the cells probably had begun their redifferentiation process by then. However, visible dedifferentiation of cells did not become apparent until 38–40 h. What caused this delay in visible dedifferentiation of somatic cells? Since this
delay coincided with the second dark period of the life cycle, we considered it possible that dedifferentiation might have been arrested in the dark. To test this possibility, two equivalent cultures were allowed to develop under the usual conditions until 28 h. One culture was then transferred to continuous light, while the other culture was allowed to go into the dark at the usual time (29 h). These two cultures were examined at 34 h. By this time, most cells under continuous illumination had lost their eyespots and flagella, and had enlarged to an average of 13 μm in diameter, which represented a 4-fold increase in volume. In contrast, those grown in the dark still retained somatic features and remained at about 8 μm in diameter, as at 30 h. These observations indicate that although transcriptional changes associated with regeneration had already begun to take place, further progress in somatic regeneration was inhibited in the dark.

Discussion

In the present study, by using a number of cell-type-specific mRNAs as markers, we were able to delineate the underlying molecular changes leading to a modified differentiation program in the Gls/Reg double mutant. We found that as Gls/Reg cells first differentiated after embryogenesis, they developed features typical of somatic cells and accumulated transcripts of both gonidial and early somatic genes. Later on, at around 36 h, these cells began to lose their somatic features and started to acquire the morphology of gonidia. Before this obvious cell redifferentiation began, marked reduction in the levels of the early somatic transcripts had already taken place. It also appeared that late somatic gene expression was not activated at any stage of development in the mutant. These observations provide important insights as to how dichotomous cellular differentiation in V. carteri may be controlled, and will be the focus of most of this discussion. However, before taking up that topic, we will first discuss a secondary (but interesting) finding, namely, photoregulation of the somatic regeneration processes.

Photoregulation of somatic regeneration

In Volvox, many processes such as cytodifferentiation (Kirk and Kirk, 1985), flagellar development (Coggin and Kochert, 1986), and programmed senescence of somatic cells (Pommerville and Kochert, 1982) have been shown to be photoregulated. All of these processes are suspended when organisms are placed in the dark, but are rapidly reinitiated in the light. In the present study, we found that dedifferentiation of somatic features and redifferentiation of Gls/Reg cells into gonidia were also arrested in the dark, but occurred rapidly in organisms left in, or returned to, the light. Previous studies on the mechanisms of photoregulation of cytodifferentiation show that whereas the protein synthetic patterns of cells change dramatically at the dark-to-light transition, translatable RNA contents remain largely the same, suggesting that the onset of differentiation is controlled at the translational level (Kirk and Kirk, 1985). We believe that the dark arrest of somatic regeneration observed in the present study might also be mediated at the protein synthetic level, since obvious changes in transcript accumulation patterns had already been observed before the cells entered the dark period in which the delay of regeneration occurred. Unlike cytodifferentiation, de-differentiation of somatic structures is expected to involve degradative processes. However, it is possible that the mechanism for photoregulation of these dedifferentiation processes may be similar to that involved in the programmed senescence of somatic cells. As somatic cells enter the senescence phase, the complexity of polypeptide patterns is drastically reduced, presumably through protein degradation (Hagen and Kochert, 1980). Yet, it has been demonstrated that senescence can be delayed by either putting the cells in the dark, or by inhibiting protein synthesis with cycloheximide (Pommerville and Kochert, 1982). This not only indicates that new protein synthesis is required for the degradative events that lead to senescence, but also that such protein synthesis may be under photoregulatory control. In any case, it appears that both differentiation and dedifferentiation processes in V. carteri are influenced by the light. Further studies are warranted to determine whether in all cases these effects are mediated at the translational level.

Gls/Reg cells express somatic and gonidial genes simultaneously

Cells in a Gls/Reg spheroid are formed by a series of symmetric divisions of a somatic cell that has redifferentiated to become a 'gonidium'. In the absence of asymmetric divisions, no 'true' gonidial initials are produced in embryogenesis; all cells initially differentiate as somatic cells when cytodifferentiation begins at 13 h. This has been interpreted to mean that the gls gene product is essential for setting up asymmetric divisions, and that a close link exists between asymmetric divisions and the establishment of the gonidal lineage (Kirk, 1990; Kirk et al. 1991a, b). Gls/Reg cells were similar in size and morphology to WT somatic cells for the first 12–18 h of differentiation. During this period, they also accumulated high levels of the early somatic transcripts that are expressed in WT somatic cells shortly after embryogenesis. These early somatic transcripts included α-tubulin, which is a major component of flagella, and SSG 185, which encodes a polypeptide in the CZ3 layer of extracellular matrix (Ertl et al. 1989). These molecular results were consistent with our visual observations which indicated that the mutant cells were assembling flagella and extracellular matrix at this stage.

Although Gls/Reg cells and WT somatic cells were indistinguishable from each other during the first 18 h of differentiation in terms of morphology and accumulation of early somatic transcripts, they differed markedly in accumulation of gonidial transcripts: In the WT strain, transcripts of G167 (the 'embryogenesis-abundant' gonidial gene) were virtually undetectable at
any stage of somatic-cell development, and transcripts of the 'maturation-abundant' genes could only be found transiently, and at very low levels, in juvenile somatic cells during the first few hours of cytodifferentiation (Tam and Kirk, 1991). In contrast, substantial levels of the transcripts of both classes of gonidial genes were present in Gls/Reg cells throughout the entire S phase of the life cycle. Clearly, gonidial gene expression is not suppressed in the mutant cells as it normally is in WT somatic cells. Based on several lines of evidence that indicate that regA suppresses gonidial development in WT somatic cells (Huskey and Griffin, 1979; Kirk et al. 1987), and our observation that gonidial transcripts disappeared at a time when the regA gene product is believed to exert its action (Huskey and Griffin, 1979), we have previously postulated that the suppression of gonidial gene expression that is observed in early differentiating WT somatic cells may be caused by the action of the regA gene product (Tam and Kirk, 1991). This concept is supported by the present finding that a mutation at the regA locus is accompanied by a failure to suppress gonidial gene expression after the initial few hours of cytodifferentiation.

Although the lack of the regA function allows continuous expression of gonidial genes, this is inadequate to overrule the original cell-fate specification that has apparently occurred, because Gls/Reg cells initially expressed somatic-cell-specific gene transcripts and differentiated morphologically as somatic cells. Therefore, during the early differentiation, Gls/Reg cells appeared to be expressing the gonidial and the somatic program simultaneously. However, these two developmental programs did not remain turned on together for a prolonged period of time, as we will discuss next.

Repression of somatic genes in regenerating cells
After an initial period of high abundance, the early somatic transcripts declined markedly in abundance by 30h. Levels of these early somatic transcripts then remained low, while the abundance of gonidial transcripts increased, and the cells gradually acquired gonidial morphology during the G phase of the life cycle. In addition to turning down the expression of early somatic genes, the mutant cells failed to ever accumulate transcripts of the late somatic genes to levels anywhere near as high as the levels that characterize WT somatic cells after 42h. Together, these results indicate that the somatic gene expression program is completely shut off in the last half of the Gls/Reg life cycle. It is tempting to conclude that such repression of somatic gene expression after the early S phase might be mediated by certain gonidial gene products that have accumulated by that time. Even if this is the case, however, such a mechanism is inadequate to account for the suppression of somatic transcripts that occurs in the development of normal WT gonidia, since such transcripts are scarcely detectable in normal gonidia, even at very early stages of development (Tam and Kirk, 1991).

Model for the control of cellular differentiation in V. carteri
Based in part on the observations just discussed, we have developed a working hypothesis for the control of cell differentiation in V. carteri that includes the concept that two alternative mechanisms exist for repressing somatic gene expression in cells destined to become gonidia. In WT embryos, the larger cells produced by asymmetric division never develop the morphological features of somatic cells (Starr, 1969), nor do they express somatic genes at any significant level (Tam and Kirk, 1991). This suggests that in the large cells that are produced in the presence of a functional gls gene product, a mechanism must exist for completely suppressing the somatic program of development, so that these cells develop into gonidia directly. This mechanism is not activated in the uniformly small cells that are produced in a Gls mutant, and hence all Gls cells express the early portion of the somatic cell program. Presumably, if these cells did not also have a mutation of the regA locus, they would repress gonidial gene expression, and would then go on to express the late portion of the somatic program, become terminally differentiated, and eventually die. But in the Gls/Reg double mutant, gonidial gene expression is not suppressed. Therefore, gonidial gene products accumulate, and they suppress somatic gene expression by a different mechanism than that normally seen in the 'true' gonidia of WT individuals.

In all of the unicellular and colonial members of the order Volvocales -- and, indeed, in many other species of Volvox -- reproductive cells are normally produced from cells that first produce (and then resorb) flagella, eyespots and other morphological features that normally characterize the terminally differentiated somatic cells of V. carteri. This makes it appear that the mechanism of suppression of somatic gene expression seen in the Gls/Reg double mutant of V. carteri may well be ancestral, whereas the mechanism observed in WT V. carteri gonidia is derived. If so, additional genes must have been added that permit early and complete suppression of the somatic cell program in the cells set apart by asymmetric division as presumptive gonidia. Candidates for such genes have been identified.

In V. carteri strains that have a mutation at any one of the several lag ('late gonidia') loci, asymmetric division appears to occur relatively normally, but the larger cells produced first differentiate morphologically as 'large somatic cells' before redifferentiating as gonidia (Kirk, 1990; Kirk et al. 1991b). This is the phenotype to be predicted, under the working hypothesis outlined above, for individuals having defects in the genes that are normally activated in presumptive gonidia to suppress all aspects of somatic gene expression. Thus, we postulate that it is the lag genes that are normally activated in the larger cells of WT embryos and that then act to suppress somatic development in these cells. We further postulate that because of the differences produced through asymmetric division, the large cells produced in a Lag mutant (like the large cells of WT embryos) fail to activate the regA gene, and therefore...
they do not suppress gonidial gene expression. But they also cannot suppress somatic gene expression because of their lag{sup}−{sub} mutation. This would account for their having the same pathway of morphogenetic differentiation as all cells of a Gls/Reg mutant. This working hypothesis predicts that the presumptive gonidia of a lag{sup}−{sub} mutant should exhibit the same pattern of accumulation of somatic and gonidial transcripts as is seen in cells of the Gls/Reg mutant studied in the present report. This prediction is subject to test via {it in situ} hybridization.

In summary, our working hypothesis presently is as follows. (a) The ancestral volvocacean program for cell differentiation permits simultaneous expression of what we have defined as somatic and gonidial genes. This permits the cells to initially develop the morphological features of somatic cells. But the accumulating gonidial gene products eventually suppress expression of the somatic genes, causing the cells to redifferentiate as reproductive cells. (b) In {it V. carteri} three categories of genes act to convert this sequential program of development to a dichotomous one: (1) the function that is defective in Gls mutants permits the asymmetric divisions that produce large and small sister cells in WT embryos; (2) in the smaller cells so produced, the regA locus is activated to suppress expression of gonidial genes, so that the cells go on to express the late somatic genes (which may have also been relatively recent additions to the genome); (3) in the larger cells produced by asymmetric division, the lag loci are activated, leading to early suppression of somatic genes and to direct development as gonidia, without first passing through the intermediate vegetative phase characteristic of more primitive volvocaceans. (It is interesting to note that the differential activation of lag and regA loci that we postulate occurs in the large versus small cells produced by asymmetric division in WT {it Volvox} bears at least a formal similarity to the differential activation of genes involved in mating-type switching that occurs in the mother versus daughter cells that are produced by asymmetric budding in homothallic yeast strains; reviewed in Herskowitz, 1989.)

Key elements of the working hypothesis outlined above were proposed some years ago on the basis of morphological analysis of mutants only (Kirk and Harper, 1986). The present study is the first to provide molecular genetic support for such a hypothesis.

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