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Genetic Control of Development in *Volvox*: Isolation and Characterization of Morphogenetic Mutants

(alga/chemical mutagenesis/temperature sensitive/embryogenesis)

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ABSTRACT **Morphogenetic mutants of the colonial green alga, *Volvox carteri* f. *nagariensis*, were induced by chemical mutagenesis. The 68 independent mutants are classified into 12 readily identifiable phenotypes affecting various stages of asexual development. Nine of the mutants are temperature sensitive with normal development at 25° and mutant development occurring at 35°. Some mutant genes appear to be involved in the regulation of differentiation or the stability of the differentiated state. Other mutations occur in genes apparently responsible for structural components of dividing cells or adult colonies. Two mutants affect different aspects of the posterior–anterior polarization of the mature colony. One mutation affects a gene which acts very early in colonial development, but affects the appearance of the mature colony. The mutants isolated demonstrate the feasibility of using *Volvox* to study the genetic control of early steps in embryogenesis.**

The colonial green alga, *Volvox*, has definite advantages for developmental studies in that it has only two main cell types, simple morphogenesis, an asexual as well as a sexual mode of reproduction, and an easily controlled life cycle (1). These characteristics, in conjunction with its haploid genome, make it especially suitable for a study of the genetic control of development. Although several spontaneous mutants have been isolated (2), chemical mutagenesis has not been reported previously. However, Pall (personal communication) has had some success with chemical mutagenesis in *Volvox*, and Mishra and Threlkeld (3) chemically induced mutations in the related species, *Eudorina*.

Our attention has been confined to the asexual developmental cycle of *Volvox carteri* f. *nagariensis*, which has been described in detail by Starr (1). Asexual development begins with the cleavage of the asexual reproductive cell or gonidium. A spiral cleavage pattern results in the formation of a hollow, spherical embryo with a small pore, the phialopore, at the presumptive posterior end. At the 32-cell stage of division, each of the posterior 16 cells divides unequally to set off the large gonidial initials, which cease to divide. The smaller vegetative initials continue to divide, until a final cell number of 2000–4000 is reached. After the end of cleavage, two bisecting cracks appear in the area of the pore, forming four lips which then fold back over the surface of the colony until the colony inverts completely. As a result of inversion the flagellar ends of the vegetative cells are brought to the outside surface of the colony, and the gonidia to the inside. Each vegetative cell produces extracellular matrix, which increases the diameter of the colony by moving the cells apart after breakage of the cytoplasmic connections which held the cells together during cleavage and inversion. The mature colony is then released from the parent colony to complete the cycle. In this paper we report the chemical induction of mutations that affect various events in the morphogenesis of *Volvox*.

MATERIALS AND METHODS

Maintenance of Cultures. *Volvox carteri* f. *nagariensis* Iyengar, female strain HK-10, obtained from Dr. R. C. Starr, was used in all experiments (Fig. 1). Cultures were maintained axenically in Volvox medium (1) under a light cycle of 16 hr of light and 8 hr of dark at 27°. Stock cultures were grown in screw-cap tubes, and cultures used for experimental purposes were grown in 250-ml bottles or flasks under constant aeration.

Mutagenesis. Asexual female colonies containing young, undivided gonidia were treated with either 0.01 M ethyl methane sulfonate in Volvox medium for 24–30 hr (4) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 50 μg/ml in Volvox medium for 15 min (5). The mutagen was removed by vacuum filtration and the colonies were washed with several changes of sterile Volvox medium before resuspension in fresh medium. The mutagenized culture was distributed into test tubes and grown at 35° until two generations of daughter colonies had been released. Growth at high temperature allowed any temperature-sensitive mutations to be expressed, and the two complete growth cycles allowed mutationally mosaic colonies to segregate. Only one colony of a given phenotype was isolated from any culture tube in order to assure that similar phenotypes resulted from independent mutations. Mutants were subsequently tested for temperature sensitivity by subculturing at 25°.

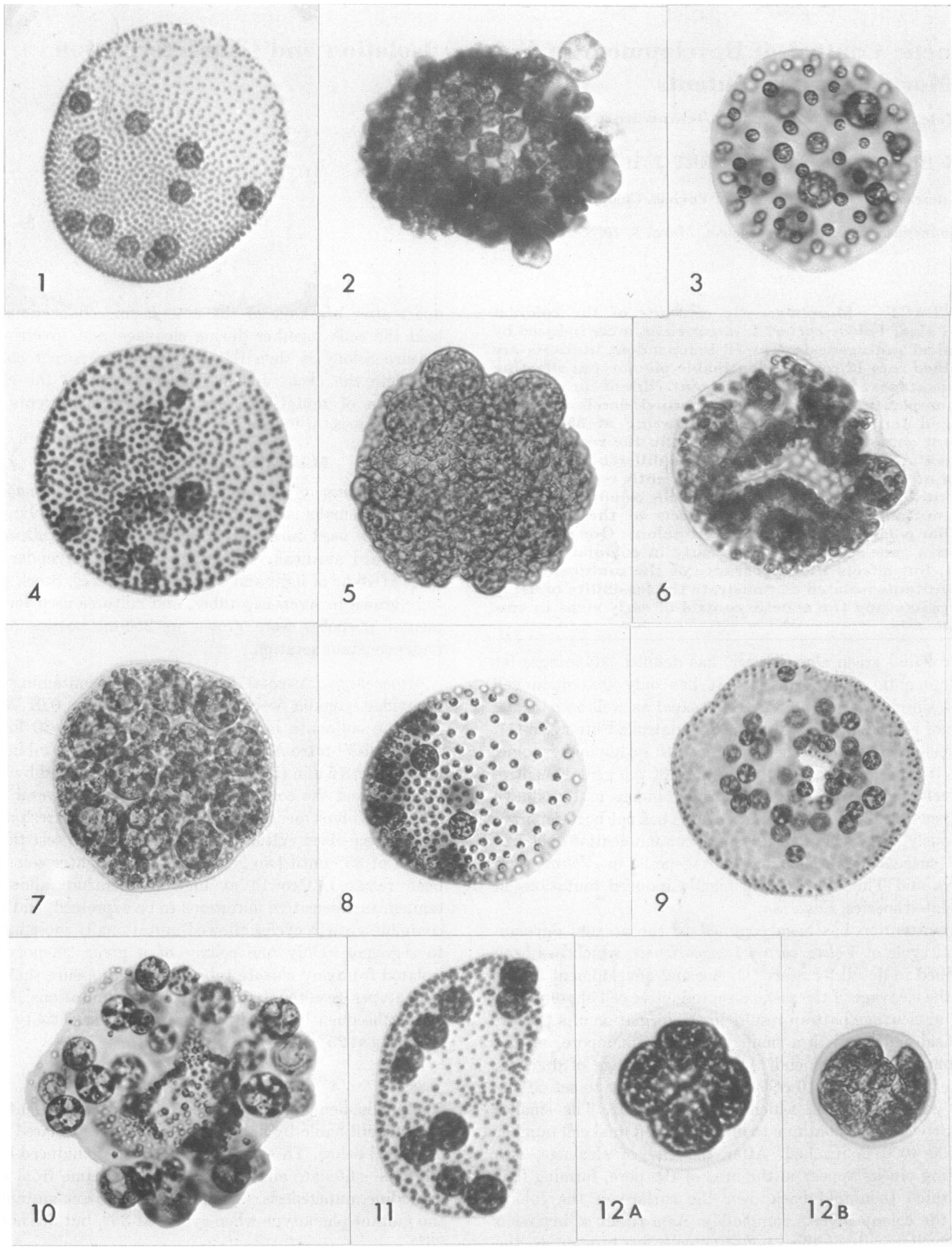
RESULTS

68 Independent mutations representing 12 different phenotypes, identifiable by light microscopy, were isolated and are described below. Three of the mutants were induced by ethyl methane sulfonate and the remaining 65 came from nitrosoguanidine mutagenesis. (Temperature-sensitive mutants have the mutant phenotype when grown at 35°, but are normal at 25°).

(*1*) Somatic regenerator (*reg*-1 through 16). Fig. 2. Post-inversion colonies of this mutant appear normal at first, but later the somatic cells begin to enlarge and lose their flagella

and eventually develop into functional gonidia. *Reg*-1 was induced by ethyl methane sulfonate and the other 25 *reg* mutants by nitrosoguanidine.

(*2*) Small somatic regenerator (*s-reg*-1 through 8). Fig. 3. The somatic cells of these mutants undergo regeneration as described above for *reg*, but the mutant colonies contain



FIGS. 1–12. *Volvox carteri* f. *nagariensis* Iyengar, female strain HK-10, wild type and mutants described in text. (*1*) wild type ×56. (*2*) *reg*-9 ×39. (*3*) *s-reg*-1 ×161. (*4*) *mul*-1 ×63. (*5*) *inv*-1 ×210. (*6*) *q-inv*-2 ×280. (*7*) *exp*-2 ×168. (*8*) *u-exp* ×168. (*9*) *do* ×105. (*10*) *post* ×105. (*11*) *fr* ×77. (*12A*) wild type at four-cell stage ×280. (*12B*) *fr* at four-cell stage ×280.

about 125 cells instead of the normal cell number of 2000–4000. Colonies developing in fresh medium contain about 250 cells, and the somatic cells do not undergo regeneration. Unlike *reg* mutants, the *s-reg* daughter colonies resulting from regenerated vegetative cells are not released from the mother colony. *S-reg*-1 was induced with ethyl methane sulfonate; the others by nitrosoguanidine.

(*3*) Multiple gonidia (*mul*-1 and 2) nitrosoguanidine. Fig. 4. More than the normal 16 gonidia are present in each colony of these mutants, as a result of the occurrence of pairs of gonidia in many of the positions normally occupied by single gonidia. These paired gonidia are smaller than usual.

(*4*) Nonexpander (*exp*-1 through 8) nitrosoguanidine. Fig. 5. The colonies in these mutants do not expand appreciably after inversion. Although extracellular matrix is synthesized, the cells remain close together and the colony increases in diameter only slightly. *Exp*-4 and *exp*-8 are temperature sensitive.

(*5*) Unequal expander (*u-exp*-1) nitrosoguanidine. Fig. 6. Cells in the anterior half of mutant colonies are greatly separated, with a more than normal amount of matrix between cells. The posterior portion of the colony expands very little, and the cells remain close together. This mutant is temperature sensitive.

(*6*) Dissolver (*dis*-1 and 2) nitrosoguanidine. Soon after inversion of these mutants, the colony falls apart, resulting in free-swimming vegetative cells and gonidia that fall to the bottom of the culture vessel. The gonidia develop normally through inversion, but the resulting colony again falls apart. *Dis*-1 is temperature sensitive.

(*7*) Delayed dissolver (*d-dis*-1) nitrosoguanidine. The colonies of this mutant invert and expand normally. However, by the time the gonidia have begun cleavage the mother colony has fallen apart. This mutant is also temperature sensitive.

(*8*) Noninverter (*inv*-1 through 15) nitrosoguanidine. Fig. 7. These mutants do not go through the inverison process after the cessation of cell division. Since the gonidia remain on the exterior of the colony, the daughter colonies develop around the periphery of the parent colony. These daughter colonies remain close to the mother colony, and if the culture is not disturbed, large aggregates of colonies will be formed. *Inv*-13 is temperature sensitive.

(*9*) Quasi-inverter (*q-inv*-1 and 2) nitrosoguanidine. Fig. 8. When the pore opens at the beginning of inversion of this mutant, one of the two bisecting cracks extends almost around the entire circumference of the colony. Since the phialopore lips encompass essentially the entire colony, they cannot bend back over the rest of the colony as they would normally do. Instead, the extensive free edges bend back, and inversion occurs simultaneously over most of the colony. The anterior end of the colony never fully inverts and a deep fold is left in the resulting colony. Both of these mutants are temperature sensitive.

(*10*) Double posterior (*post*-1) nitrosoguanidine. Fig. 9. Twice the normal number of gonidia are present in this mutant, and they are distributed over the whole colony. The eyespots in the vegetative cells are small or lacking in all parts of the colony, a property that is characteristic of posterior cells in wild-type colonies. Inversion does not proceed beyond the initial steps.

(*11*) Dougnut (*do*-1) ethyl methane sulfonate. Fig. 10. This mutant also contains twice the normal number of 16 gonidia, but it differs from *post* in two respects. The distribution of eyespots is apparently normal, large in the anterior end and small in the posterior end, and the colonies invert. Inversion initially appears normal, but towards the end of the process a second pore opens up in the presumed anterior end and inversion takes place at both ends simultaneously. The two inverting ends of the colony meet and fuse, resulting in a colony shaped like a torus, or doughnut. Under poor growth conditions the colonies contain fewer gonidia and sometimes invert normally. The gonidia are usually smaller than normal and often occur in pairs, as in the *mul* mutant.

(*12*) Fruity (*fr*-1) nitrosoguanidine. Fig. 11. The adult colony of this mutant is elongated and bent as a result of an alteration of the early cleavage divisions. Normally, after two cleavage divisions the cells adhere to each other along a total length that is less than the diameter of the original gonidium (Fig. 12*A*). Thus the periphery of the 4-cell embryo is lobed. These 4 cells then divide obliquely to form an 8-cell embryo consisting of two tiers of cells (1). In the *fr*-1 mutant the first four cells remain closely attached along the entire diameter of the original gonidium, and the embryo does not have a lobed appearance (Fig. 12*B*). Before the next cleavage, the connections between two of the cells break; at the 8- and 16-cell stages, the colony consists of a single curved layer of cells, rather than 2 or 4 tiers of cells. This mutant is temperature sensitive.

Spontaneous mutants similar to the *reg* and *mul* mutants described above have been described by Starr (2). Pall (personal communication) has reported isolating mutants similar to *reg*, *inv*, and *dis*.

DISCUSSION

The various mutations described above affect both cellular differentiation and different aspects of *Volvox* morphogenesis. The somatic regenerators exhibit modified cell differentiation. Normally, in the mature asexual colony, cell division is restricted to the 16 gonidia. However, in the regenerators all of the cells of the colony retain the potential to divide and produce daughter colonies. Since the development of the additional reproductive cells appears to differ in the two types of regenerators, the asexual reproductive pathway may be controlled by more than one regulatory gene. The large number of independent regenerator mutants isolated could be taken as additional evidence that several genes may be involved in cell differentiation. More reasonable explanations for their frequent isolation are that they are very easy to recognize and that they have much increased reproductive capacity.

The *mul* mutants appear to affect the division pattern of the gonidial initials. Occasionally in normal colonies, these cells may divide asymmetrically once or twice after their formation to produce small somatic cells before ceasing division (2). The paired gonidia of the mutant could arise by a symmetrical division of the gonidial initial, resulting in two small gonidia rather than a gonidium and a vegetative cell.

The nonexpander and dissolver mutants would appear to have altered matrix synthesis or deposition. The nonexpanders could produce a smaller amount of matrix or an altered matrix material; alternatively, the characteristics of the cells themselves—such as failure of the protoplasmic connections to stretch and/or to break—could prevent the cells from be-

coming widely separated. Preliminary observations on two nonexpanders suggest that one has an altered matrix structure and that in the other the protoplasmic connections appear to be affected. Evidently, only the posterior cells are affected in the unequal-expander.

The dissolver mutants produce no detectable matrix, but whether any precursors are made is not known. The delayed-dissolver may produce an unstable matrix material, or the dissolution could result from a premature and more extensive manifestation of the normal daughter colony release mechanism, which may involve enzymatic breakdown of the matrix.

Any of the events associated with normal inversion could be affected in the various noninverting mutants, such as opening of the phialopore, cell shape changes (6, 7), or restriction of the protoplasmic connections to the chloroplast ends of the cells, where they act as "hinges" during inversion (8). In addition, premature deposition of matrix may prevent inversion. Since the mechanics of the inversion process are poorly understood, there are doubtless other possibilities.

Although the quasi-inverter never takes on the appearance of a typical inverting colony, the mechanics of inversion are probably normal. The only difference is that the process occurs over a large area of the colony simultaneously along the edges of the elongated cracks, instead of only at the bend region; therefore, inversion is completed more rapidly. The failure of the anterior end of the colony to complete inversion is most likely the result of mechanical restriction by the vesicle in which the colony develops. In Kelland's (7) series of operations on *Volvox* colonies, a preinversion colony of *V. globator* received two long lateral tears in the process of being removed from its vesicle. In this case the anterior end completed inversion. This result suggests that removal of a *q-inv* colony from its vesicle would allow complete inversion. The formation of the long cracks in the quasi-inverter could be the result of weak protoplasmic connections between the cells. In some *Volvox* species with thick protoplasmic connections, cracks at the pore do not form at all (7).

The doughnut and double-posterior mutants both have twice the normal number of gonidia. The basis for this increased number appears to differ in the two mutants. The vegetative cells of the *post* mutant are entirely of the type characteristic of the posterior half of a normal colony; that is, they lack eyespots. Both ends of the colony are morphologically posterior and, therefore, the gonidia develop in normal positions. The doughnut mutant has a normal distribution of anterior and posterior cells as evidenced by eyespots, and the extra gonidia develop in the anterior end where they are normally absent. Thus, one mutant is a polarity mutant, having lost anterior–posterior differentiation; in the other mutant, the asymmetric divisions are no longer restricted to the posterior half of the colony. From the foregoing explanation, the *post* mutant would be expected to invert from both ends rather than not invert, and the doughnut mutant would be expected to invert normally rather than from both ends. Why the two mutants behave differently both from each other and from their expected behavior during inversion remains to be explained.

The fruity mutant illustrates the dependence of the final shape of the colony on the pattern or initial cleavage divisions, as well as the importance to final colony morphology of proper cell to cell adhesions.

Microscopical and biochemical observations on the morphogenetic mutants described in this paper should allow a more detailed description of development in *Volvox*. Temperature-shift experiments similar to those performed with other organisms (9, 10) are being done on some of the temperature-sensitive mutants. Preliminary results indicate that some of the gene functions are required long before their phenotype is apparent. We are also in the process of a genetic analysis of the mutants in order to determine the number of genes controlling various phenotypes and their linkage patterns.

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