CELL SHAPE CHANGES AND THE MECHANISM OF INVERSION
IN VOLVOX

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ABSTRACT

Inversion is a dominant aspect of morphogenesis in Volvox. In this process, the hollow, spheroidal Volvox embryo turns inside-out through a small opening called the phialopore to bring flagella from its inner to its outer surface. Analyses of intact, sectioned, and fragmented embryos by light, scanning electron, and transmission electron microscopy, suggest that shape changes preprogrammed into the cells cause inversion. First, cells throughout the embryo change from pear to spindle shape, which causes the embryo to contract and the phialopore to open. Then cells adjacent to the phialopore become flask-shaped, with long, thin stalks at their outer ends. Simultaneously, the cytoplasmic bridges joining all adjacent cells migrate from the midpoint of the cells to the stalk tips. Together, these changes cause the lips of cells at the phialopore margin to cud outward. Now cells progressively more distal to the phialopore become flask-shaped while the more proximal cells become columnar, causing the lips to curl progressively further over the surface of the embryo until the latter has turned completely inside-out. Fine structural analysis reveals a peripheral cytoskeleton of microtubules that is apparently involved in cellular elongation. Cell clusters isolated before inversion undergo a similar program of shape changes; this suggests that the changes in cellular shape are the cause rather than an effect of the inversion process.

KEY WORDS cell shape changes · cytoskeleton · inversion · microtubules · morphogenesis · Volvox

Embryonic morphogenesis, or the development of form, is frequently accompanied by specific, sequential shape changes in the various cells that participate in the elaboration of the forming structures (2-4, 15). Many of these changes in cell shape can be attributed to the action of cytoskeletal elements such as microtubules and actinlike microfilaments (1, 6, 17, 22, 23). But how are such cytoskeletal changes controlled in a cell- and stage-specific manner? Since the form of an adult individual and its component parts are heritable features, it seems obvious that many aspects of morphogenesis, as well as the cellular processes responsible for them, must be under genetic control. However, we have at present little knowledge of the mechanisms by which specific genes control the spatial and temporal coordination of cytoskeletal elements to generate characteristic form. We have chosen to study morphogenesis in Volvox because it offers unique possibilities for obtaining fresh insights into this problem.

Members of the genus Volvox are simple, multicellular, eukaryotic, photoautotrophic organisms composed of only two cell types (somatic and
the entire process of inversion in wild-type *Volvox carteri f. nagariensis* (the only strain in which inversionless mutants are available). Based on this description, we will postulate our view of the mechanical basis for the inversion process in terms of sequential changes in cell shape. This description will serve as an essential background for future publications detailing the subcellular bases for cell shape changes in wild-type embryos and the subcellular abnormalities that cause mutant embryos to be blocked at specific points in the inversion process.

**MATERIALS AND METHODS**

*Volvox carteri f. nagariensis*, strain HK 10, was obtained from the Indiana University Culture Collection of Algae\(^2\) and used in all studies reported here. Cultures were maintained in synchronous asexual growth and development, using a 48-h light-dark cycle and culture conditions similar to those previously described (8 and footnote 3). Under these conditions, the complete asexual life cycle takes 48 h, and inversion occurs early in the dark period.

Inverting or pre-inversion embryos were released from the parent spheroids by passing the latter through a capillary pipet. Specimens for light microscopy (LM) were fixed in 2.5% glutaraldehyde and 2.5% tannic acid in 0.02 M phosphate buffer, pH 8.0, for 12–16 h at room temperature, washed with buffer, and postfixed 3 h at room temperature in 1% OsO\(_4\) in the same buffer. Specimens for transmission electron microscopy (TEM) were fixed for 2 h at room temperature in a mixture of 2.5% glutaraldehyde and 2% formaldehyde in 0.02 M phosphate buffer, pH 8.0, with 0.002 M CaCl\(_2\). They were then transferred to 2.5% glutaraldehyde with 0.5 to 1% tannic acid in the same buffer, held 12–16 h at 4°C, and postfixed in OsO\(_4\) as described above.

For both LM and TEM, specimens were dehydrated in a graded series of ethanol solutions, infiltrated for 12–16 h with Spurr’s low viscosity resin (18) in a rotary shaker, and oriented in flat silicone rubber molds under the dissecting microscope before polymerization of the resin. Both thick sections (for LM) and thin sections (for TEM) were cut with glass knives on an LKB Huxley ultramicrotome (LKB Instruments, Inc., Rockville, Md.). Serial thick (2 μm) sections were observed and photographed with a Zeiss photomicroscope (Carl Zeiss, Inc., New York) with Nomarski interference contrast optics under oil immersion. Thin sections (silver or pale gold) were mounted on Formvar-coated slotted copper grids, stained with a saturated solution of uranyl

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1 R. J. Huskey, personal communication.

2 Through the courtesy of Dr. Richard Starr.

3 Kirk, M. M., and D. L. Kirk. Manuscript submitted for publication.
acetate in methanol for 30 min, poststained for 15 min with Venable and Coggeshall's lead citrate (21), and observed in a Hitachi HU-11c transmission electron microscope.

Specimens to be observed as whole mounts by scanning electron microscopy (SEM) were fixed in the same manner as specimens for LM, but OsO₄ postfixation was omitted since it did not change the specimens' appearance in the SEM but made them extremely brittle. After fixation for 12-16 h, specimens were freed of their vesicles under the dissecting microscope with a pair of finely pointed forceps. Different stages were fixed and processed together as a control to enable us to distinguish stage-specific features from randomly occurring artifacts. After dehydration with graded acetone solutions, the embryos were critical-point dried, transferred to specimen stubs, and coated with a fine layer of gold-palladium in a "Hummer" sputter coater (Technics, Inc., Alexandria, Va.). All SEM was performed with a Cambridge Stereoscan Mark 2A scanning electron microscope.

RESULTS
Gross Aspects of the Inversion Process

The various aspects of the inversion process, already described in part from light microscope studies (19), can be visualized in considerable detail by SEM. At the end of cleavage, the boundaries of individual somatic cells are not clearly distinguishable in a surface view, and gonidia protrude only moderately from the somatic cell layer in which they are embedded (Fig. 1a). At this stage, the phialopore, which has been present from the 16-cell stage, is barely visible as a slight opening at one pole of the spheroid. In the 30- to 60-min interim between the end of cleavage and the beginning of inversion, the embryo undergoes a series of sporadic movements described as "denting" by earlier investigators. As a result of the denting movements, the embryo contracts slightly (resulting in about a 10% reduction in diameter), the boundaries of individual somatic cells become clearly evident, and gonidia project more extensively from the surface (Fig. 1b). Simultaneously, the phialopore widens until its diameter is nearly equal to the diameter of the embryo, and the lips of cells adjacent to the phialopore begin to curl outward (Fig. 1b). These lips continue to curl backward over the surface of the embryo (Fig. 1c), and the region of maximum curvature moves progressively toward the opposite pole of the embryo, involving cells ever further from the phialopore (Fig. 1d, e). By the time the region of maximum curvature reaches the opposite pole, the embryo has turned completely inside-out (Fig. 1f). At the end of inversion, the phialopore lips begin to join once more, restoring the spheroidal configuration and enclosing the gonidia. Individual somatic cells are still clearly distinguishable (Fig. 1g). At this point, flagella (now on the exterior) begin to elongate rapidly and cell boundaries become indistinct as the juvenile spheroid begins to accumulate the extracellular glycoprotein matrix that both separates and binds together the cells of the adult (Fig. 1h). Juvenile spheroids broken open at this stage show extensive cell-surface blebbing, presumably associated with secretion of the matrix material (Fig. 1i). The entire inversion process takes about 45 min to complete. Cleavage, inversion, and the early stages of expansion normally occur inside the parental spheroid, but reproductive cells isolated by mechanical disruption of the parent undergo an identical developmental sequence.

Cell Shape Changes and Movements of the Cytoplasmic Bridges

The gross movements of inversion described above are accompanied by a sequence of specific cell shape changes and by repositioning of the cytoplasmic bridges that join the cells. While some of these changes can be discerned from SEM observation of whole or broken spheroids, the detailed nature of the changes and the manner in which they are temporally and spatially coordinated are best revealed by LM and TEM of sections cut normal to the plane of the phialopore opening.

In the pre-cleavage, pre-inversion embryo, somatic cells are approximately pear-shaped, and a lip of cells is curled into the interior of the spheroid in the region of the phialopore. Gonidia are also pear-shaped, and are well recessed into the spheroid wall (Fig. 2a). The flagellar (or nuclear) ends of somatic cells form the inner surface of the spheroid, and their chloroplast ends form its outer surface. As denting occurs (signalling the onset of the inversion process) and the embryo contracts, somatic cells become elongate and spindle-shaped (Figs. 2b and 3a). Most somatic cells develop short, tail-like projections at their chloroplast ends (Fig. 4b). At this stage, all somatic cells remain joined, as they were earlier, by cytoplasmic bridges located approximately midway between flagellar and chloroplast ends (Fig. 4a). As further cellular elongation...
FIGURE 1  Scanning electron micrographs of whole embryos. (a) Pre-inversion embryo. At this stage gonidia (G) protrude only slightly from the somatic cell layer in which they are embedded. Somatic cell boundaries are indistinct and the phialopore (P) is barely distinguishable. (b) As inversion begins, the entire embryo has contracted. Somatic cells have become pointed at their exterior ends and are clearly distinguishable as individual cells. Gonidia (G) protrude more markedly from the surface. The phialopore (P) has opened, and cells at its margin have become joined at their chloroplast ends, forming a distinctive fanlike arrangement at each of four lips (L). (c) As inversion proceeds, the phialopore lips curl back over the surface of the embryo. (d) As the lips progress, they appear to push the gonidia before them, driving them into a single equatorial band. This is known as the “Mexican Hat” stage. Original location of the phialopore is indicated in this and the next picture for orientation purposes. (e) Eventually, the lips pass over the gonidia and enclose them. Pointed chloroplast ends of cells at bottom (B) of embryo indicate that this region has not yet inverted. (f) View into interior of embryo that has fully inverted, but whose lips have not yet come together again. (g) Side view of slightly later stage. Lips (L) approach one another and embryo is top-shaped with its apex at the pole opposite the phialopore lips. Somatic cell boundaries are still distinct. (h) Side view of spheroid after lips have come together. Deposition of matrix has begun and cell boundaries can no longer be distinguished. Note that flagella (F), first visible as short stubs on cells at the phialopore lips in Fig. 1b, have been growing slowly throughout inversion. They now elongate at a rapid rate. (i) Interior view of an embryo intermediate in stage between Fig. 1g and h that has been broken open mechanically. Note that in addition to somatic cells and gonidia (G) there are a number of cells (c) of intermediate size present. Such cells are always observed throughout inversion, but their significance is unknown. Extensive surface blebbing is seen in all cells at this stage as secretion of glycoprotein matrix begins. Bars, 10 μm. (a-h) × 555, (i) × 578.
occurs throughout the embryo and the phialopore continues to open, cells adjacent to the phialopore undergo a second shape change: they convert from a spindle to an elongated tear-drop shape as their flagellar ends expand and their chloroplast ends are drawn out into long "stalks" (Figs. 2c and 3b). These cells are strikingly similar in shape to the "flask cells" seen in the blastoporal region of amphibian gastrulae (3, 4, 14); therefore, we will refer to them by this term. During the transition from spindle to flask shape, the cytoplasmic bridges migrate to the tips of the stalks (Figs. 3b, 3c, 4c, and 4h). The change in cell shape and the movement of the cytoplasmic bridges result in the formation of a fanlike structure by the cells of this area, the apex of the fan being defined by the cytoplasmically continuous stalk tips. The formation of this opening-fan structure results, in turn, in the shifting of the flagellar ends of cells adjacent to the phialopore from the interior of the spheroid to the exterior, forming the four phialopore lips previously described by light microscopy (9, 19) and visible in Fig. 1b. Inversion is thus initiated.

Once cells have swung around the region of maximum curvature, they undergo a third shape change and become columnar. Cytoplasmic bridges remain near the chloroplast ends of the cells. Simultaneously, cells further from the phialopore margin undergo the spindle-to-flask cell transition and the migration of bridges to the stalk tips (Fig. 2d). Thus the spindle-to-flask-to-columnar cell shape sequence (and the accompanying region of maximum curvature) sweeps in a ringlike wave from the phialopore margins toward the opposite pole (Fig. 2e). By the time the cells opposite the phialopore assume the flask cell shape, the embryo has fully inverted and resembles a top, with its apex opposite the phialopore (where gonidia are not found), it is clear that they are not merely underdeveloped gonidia. The significance of these cells is obscure; shortly after inversion they disappear, apparently becoming somatic cells indistinguishable from their neighbors.

Ultrastructural Features of Cells at Inversion

A cytoskeleton of peripheral microtubules is apparent in somatic cells from the time they assume the spindle shape early in inversion through the end of the morphogenetic process (Fig. 4d, e, f, and g). As might be expected, cellular elongation is accompanied by elongation of the cytoskeleton; in the stalks of flask cells located in the regions of maximum curvature, the cytoskeleton consists of a prominent ring of microtubules just below the plasmalemma. The tail-like projections seen at the chloroplast ends of spindle cells distal to the region of maximum curvature resemble stalks in their morphology, except that they are considerably shorter and, of course, they are not joined to one another at the tips by cytoplasmic bridges (Fig. 4b).

Nuclei of inverting cells undergo rather minor shape changes during inversion to conform to changes in overall cell shape. Chloroplasts, however, undergo very extensive changes in shape. In spindle-shaped cells the chloroplasts are conical, but in flask cells the chloroplasts are extremely elongate; often a tubular arrangement of chloro-
plast membranes can be seen to extend virtually the entire length of the stalk (Fig. 4d and g). Although starch granules are numerous in chloroplasts of inverting cells, pyrenoid bodies are not seen until after the conclusion of inversion. The beginnings of eyespot formation (in association with chloroplast membranes) are seen in some cells late in inversion. Golgi bodies, which presumably play an active role in the secretion of matrix materials after inversion, are seen between nucleus and chloroplast throughout inversion (Fig. 4a).

Curiously, basal bodies of the paired flagella are oriented at right angles to one another throughout inversion, whereas they come to be parallel shortly after inversion and maintain that orientation in the adult. Although flagella appear to beat actively during inversion, they do not appear to play an essential motile role in the inversion process. Through most of inversion, flagella are extremely short and stublike and their beating has little propulsive effectiveness in cells isolated from inverting embryos. Furthermore, flagellateless mutants are known which invert normally.

**Shape Changes in Isolated Cells**

Cells and cell clusters isolated by mechanical
dissociation of cleaved, but unverted embryos undergo the same sequence of shape changes that are observed in cells of intact, inverting embryos (reference 7 and Fig. 5). Singly or in clusters, the cells first elongate to become spindle-shaped with a rather thick projection at the chloroplast end. Then this projection becomes drawn out into a stalk equivalent to those found in flask cells at regions of maximum curvature in intact embryos. In some cases, if cells draw apart at this stage, it is obvious that such flask cells are joined at the stalk tips. Finally, the stalks are withdrawn and the cells assume a columnar and later, a cuboidal shape. The time required for this entire series of shape changes in isolated cells is comparable to that required for inversion (~45 min).

**DISCUSSION**

The fact that cells isolated from pre-inversion *Volvox* embryos undergo shape changes similar in type and timing to those seen in the cells of intact embryos suggests that these shape changes are preprogrammed in the cells and are the cause of the inversion process rather than a result of it. The observations reported here can thus be fitted together to provide the following coherent model of the mechanical basis of inversion.

In the first event of inversion, all somatic cells change from a pear shape to a spindle shape, due to elongation along their flagellar-chloroplast axes.
and contraction in the plane of the spheroid wall. Since the cells remain tightly linked at their midpoints by cytoplasmic bridges, the reduction in cell diameter in the plane of the wall necessarily leads to a reduction in the area covered by the spherical syncytium (by analogy, if one reduces the dimensions of polygonal subunits used to tile a surface without increasing the number of subunits, one cannot tile as large a surface). This reduction in area expresses itself in two ways: by a modest decrease in the overall diameter of the spheroid and by the opening of the phialopore—the only point at which tension can be relieved.

In the second event of inversion, the cells in the four phialopore lips become transformed into flask cells with long and slender stalks; simultaneously, the phialopore bridges migrate to the stalk tips. This transition results in a marked reduction of center-to-center distance at one end of the cells and an increase in center-to-center distance at the opposite end, which produces stress. Given that the bridges are now located at the outer (chloroplast) ends of the cells, there is only one way the stress can be relieved: by an outward curling of the lips. And curl out they do, like an opening fan. Next, this transition from spindle-to-flask shape occurs in cells even more distal to the phialopore, while at the same time more proximal

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**FIGURE 3** Scanning electron micrographs of portions of inverting embryos. (a) Exterior view of a region of an inverting embryo distal to the region of maximum curvature. Note pointed chloroplast ends of somatic cells with cellular processes. In addition to typical somatic cells and gonidia (G), some cells of intermediate size (C) are seen. (b) Magnified view of phialopore lip of embryo shown in Fig. 1b. Note that stalks (S) at the chloroplast ends of flask cells are all joined at their tips (T). (c) View of the region of maximum curvature in a midinversion embryo that has been broken mechanically. Note the fusion of all the stalks at their tips to form a syncytial strand. Note also that gonidia bear flagella during inversion. Bars, 10 μm. (a) × 1,147; (b) × 2,294; (c) × 1,376.

**FIGURE 4** Transmission electron micrographs of selected portions of somatic cells from inverting embryos. (a) Spindle-shaped cells from a region distal to the ring of maximum curvature. Note that at this stage cells are joined by cytoplasmic bridges (arrow) at the level of the Golgi apparatus (G), which lies between the chloroplast (C) and nucleus (N). (b) Section through the projection of a spindle-shaped cell distal to the region of maximum curvature. Note funnel-shaped chloroplast (C) and peripheral microtubules (arrows). (c) Chloroplast ends of cells about to become part of the region of maximum curvature. Stalk formation has begun and cytoplasmic bridges (arrows) are now located at the tips of the cells. (d) Cross section through the stalk of a flask cell in the region of maximum curvature. Note peripheral microtubules (arrows) and chloroplast membranes (C). (e) Cross section through the nuclear region of a columnar cell that has just passed the region of maximum curvature. Note nucleus (N) and peripheral microtubules (arrows). (f) Grazing section through the perinuclear region of a cell in the area of maximum curvature. Note parallel array of microtubules (arrows). (g) Photomontage of longitudinal section through the stalk of a flask cell in the region of maximum curvature. Note continuous peripheral microtubules (arrows) and chloroplast membranes (C) in the tip at the level of the cytoplasmic bridge (B). (h) Tips of an array of flask cells showing cytoplasmic bridges (arrows). Bars (a–c, e, and h), 1 μm; (f and g) 0.5 μm; (d) 0.25 μm. (a) × 8,355; (b) × 13,177; (c) × 13,518; (d) × 103,376; (e) × 22,393; (f) × 26,128; (g) × 42,600; (h) × 12,269.
FIGURE 5  A cluster of cells released mechanically from a pre-inversion embryo and photographed at intervals over a 45-min period with Nomarski optics. The time sequence runs from left to right, top to bottom. Note that stalks are relatively thick when first extended and then diminish in diameter. Ultimately, stalks are withdrawn and cells assume the cuboidal shape characteristic of postinversion spheroids. Bar, 10 μm. x 1,176.

cells assume a columnar shape. Thus the region of maximum tension moves in ringlike fashion from the phialopore region toward the opposite pole of the embryo, and is expressed as a progressive movement of the region of maximum curvature. By the time this wave of cell shape change has moved to cells farthest from the phialopore, the embryo has turned inside-out, and inversion is essentially complete.

Previous authors (7, 19) have described the formation of projections at the chloroplast ends of somatic cells during inversion. Both Kelland (7) and Pickett-Heaps (11, 12) suggested that tapering of cells at the chloroplast ends was required for bending of the syncytium at the point of maximum curvature. Published photographs (11, 12) indicate such "tapering" actually results in the formation of flask cells (rather similar to those seen in *V. carteri*) at the point of maximum curvature in inverting embryos of other *Volvox* species. However, previous authors have not emphasized the central importance of these flask cells in the inversion process. In fact, when Kelland observed a phenomenon similar to flask cell formation in isolated pieces of inverting embryos, he concluded that it was an abnormality resulting from his dissection because he had not observed such cell shapes in intact embryos (7). His failure to visualize the flask cells in intact embryos is not surprising: the stalks of these cells are obscured from view in intact embryos except at the instant the phialopore lips are first curling outward (Fig. 1b). Further examination of other species is required, but we suspect that flask cell formation will be found to play a substantial role in inversion in all members of the genus.

Repositioning of the cytoplasmic bridges during inversion has also not been clearly described by previous authors. Pickett-Heaps (11) stated that "By the time the colonies were ready for inversion..."
... protoplasmic connections ... were concentrated almost exclusively at the chloroplast end of the cells; the other ends (occupied by the nucleus) were not interconnected by this stage although they had been in younger coenobii. This description left it unclear whether the connections moved or whether cells that were earlier joined at two or more points lost some of their connections and retained only those located at the chloroplast ends. Our observations remove all ambiguity on this point (at least with respect to *V. carteri*): even in embryos well into the inversion process there is no sign of cytoplasmic connections at the chloroplast ends of cells distal to the point of maximum curvature (see Figs. 1b, d, e, and 3a). At this stage, cells are joined only at their midpoints (Fig. 4a) although moments later they will be seen to be joined only at their chloroplast tips. We are driven to the conclusion that the bridges move (at least in a relative sense). Thus we visualize a much more active role for the bridges in inversion than Pickett-Heaps did: He suggested (11) that they act rather passively as "hinges" permitting inversion. We believe the bridges are responsible (along with the change in cell shape) for generating an appreciable portion of the motile force of inversion as they migrate from the region of maximum cell diameter to the region of minimum cell diameter.

Having postulated that changes in cell shape and migration of cytoplasmic bridges are responsible for inversion, the next two questions that arise are: (a) How are these changes brought about at the subcellular level and (b) how are they controlled and coordinated in the embryo as a whole?

The studies reported here provide some insights into the first question. At all stages of inversion, from the time of "denting" to the closing of the phialopore, we find an extensive cytoskeleton of microtubules running below the plasma membrane, parallel to the long axes of the cells. It is reasonable to assume that this microtubular girdle is responsible, at least in part, for the observed cellular elongations. But microtubules may not be the only cytoskeletal elements involved. The very preliminary fine structural studies of inversion reported here have failed as yet to provide structural evidence for a contractile system in cells undergoing shape changes; however, at least two lines of indirect evidence suggest that such a contractile system might play a key role in the inversion process. First, we have observed that, whereas colchicine blocks spindle and flask cell formation and, hence, the outward curling of the phialopore lips, cytochalasin A blocks inversion at an even earlier step: it prevents the reduction in somatic cell diameter that characterizes the beginning of inversion. Second, it appears that in both intact inverting embryos and in cell clusters isolated before inversion, cells undergoing stalk formation first develop a relatively thick projection at their chloroplast ends which only later contracts radially in such a manner that a simultaneous decrease in the diameter of the stalks and an increase in the diameter of the flagellar ends of the cells is achieved (Fig. 5). Based on such observations, we have developed the working hypothesis that a contractile system, probably actinlike in nature and closely associated with the membrane, is very likely involved in conjunction with the microtubular system in effecting changes in cellular shape. Visualization of such a contractile system, if it exists, will undoubtedly require more specific cytological methods than we have employed to date. (We have thus far not visualized in *V. carteri* the whorls and striations seen by Pickett-Heaps in *V. tertius* near the cytoplasmic bridges.) Thus, while many precedents for the coordinated activity of microtubular and microfilamentous cytoskeletal elements exist (1, 6, 17), further analysis will be required to determine whether both are involved in this system.

The question of how cytoskeletal elements are controlled at a subcellular level and coordinated at the supercellular level to generate specific, predictable forms has proven refractory in many cases. But it is for precisely this kind of question that we anticipate *Volvox* may ultimately provide answers. A variety of Mendelian mutations have been identified which block inversion at specific points (17 and footnote 6). We anticipate that future analysis of the characteristic defects of these various mutant strains may provide fresh insights into the nature of the genetic control of morphogenesis in the wild-type organism. Furthermore, as Holtfreter (3) has said, "Invagination is everywhere associated with a temporary transformation of the constituent cells into flask shapes." Current research with numerous systems...
has borne this out. Hence, it appears that elucidation of the genetic control of cell shape in inverting Volvox may be of considerable general significance in furthering our understanding of morphogenesis in more complex organisms.

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