NUCLEATING SITES FOR THE
ASSEMBLY OF CYTOPLASMIC MICROTUBULES
IN THE ECTODERMAL CELLS OF BLASTULAE
OF ARBACIA PUNCTULATA

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ABSTRACT

In the ectodermal cells of sea urchin blastulae, the microtubules converge and appear to make contact with three distinct cytoplasmic foci or satellites associated with the basal body of the cilium. Beneath the nucleus, which lies in the apical end of the cell, the microtubules are aligned predominantly parallel to the cell's long axis and could thus make contact with the satellites as is directly suggested by observations on sections at or near the planes of the satellites. After the embryos are treated with low temperature (0°C), the microtubules disassemble; however, the satellites can still be recognized. Upon rewarming, the microtubules reappear. In early stages of reformation, when the tubules in the cell consist of short segments, tubules have only been found in the apical part of the cell. One end of each microtubule appears to make contact with its respective satellite, or is aligned so that it could contact one of the satellites, provided serial sections were cut and collected in order. After longer periods of recovery, the microtubules elongate; as before, one end of each makes contact with a satellite or is aligned so that it could attach to a satellite. Segments of microtubules seen in the basal region of the cell are aligned parallel to the long axis of the cell as in the untreated ectodermal cells and are therefore interpreted as extensions of those tubules making contact with one of the satellites. On the basis of these observations, we suggest that assembly of microtubules is initiated at the satellites. These sites, perhaps best referred to as “nucleating sites,” thereby could exert considerable control over the distribution of microtubules in cells. It is hoped that this preliminary report will be followed up by a more detailed study using serial sections.

It has been shown that microtubules appear to be influential in the production of cell shape (selected examples include Byers and Porter, 1964; Arnold, 1966; Porter, 1966; Renaud and Swift, 1964; Tilney, 1968; Gibbins et al., 1969). Thus, the production of shape changes occurring during cell differentiation can only be understood when the control mechanisms for microtubule assembly and distribution have been elucidated.

One means of controlling the distribution of microtubules in cells would be by regulating their initial assembly from the cytoplasmic pool of subunit protein (Tilney, 1968; Porter, 1966; Gibbins et al., 1969). This contention is supported by the fact that many microtubules appear to be connected at one end to discrete, identifiable foci in the cell. It is not clear, however, whether microtubules actually grow out of these sites or whether
they initiate elsewhere in the cytoplasm and grow toward the sites, eventually making contact. In the growth of cilia or flagella, at least for the outer doublets, it seems that the former holds; but for the cytoplasmic microtubules in general this question is unclear or in dispute. For example, Bajer and Mole-Bajer (1969) have stated that some spindle microtubules may grow toward the kinetochore and attach there in prometaphase, whereas other spindle microtubules may actually be formed and positioned by the kinetochore.

It is also possible that microtubule patterns may be determined by factors other than assembly sites. For example, in the reformation of heliozoan axonemes, the microtubules initially reappear at random in the cell, becoming patterned secondarily (Tilney, 1968). Tilney and Byers (1969) have presented evidence that two species of macromolecular bridges may, in large part, be responsible for the axonemal pattern. Although the possibility of specific initiating sites regulating microtubule assembly has not been ruled out in this system, these foci, if they exist, would most likely be found scattered throughout the cytoplasm, thus making the control of axonemal pattern by these centers unlikely.

In addition, it has been proposed that microtubules may be oriented (during assembly and/or after assembly) by physical and/or chemical factors in the cytoplasm of the cell (Tilney, 1968). For example, microtubules may align in relation to pre-existing structures such as other microtubules, asymmetric gels, or the cell membrane. If the microtubules were to subsequently attach to other tubules or to a membrane, such an orientation might be stable.

To further explain the distribution of microtubules in cells, it is necessary to consider the possibility that the alternative processes just described are not mutually exclusive. Thus, spontaneous assembly from the subunit pool, assembly from randomly dispersed foci, or formation of tubules in a highly oriented fashion from specifically positioned assembly sites, may occur in the same cell at the same or at different times.

On the other hand, it is possible that one or several precisely defined centers could initiate all tubule assembly in a particular cell. Evidence for such a system comes from cells in which microtubules appear to be associated with specific cytoplasmic structures, such as the pericentriolar bodies in ascites tumor cells (see Thé, 1964), the kinetochore region of the chromosome (Brinkley and Nicklas, 1968), and the satellite foci associated with the ciliary basal body of the ectodermal cells of blastulae of Arbacia punctulata (Gibbins et al., 1969).

In this paper we describe experiments designed to determine if foci for microtubule attachment are actually serving as specific microtubule initiation sites. A more rigorous treatment using the same experimental material is currently underway. We chose the ectodermal cells of Arbacia blastulae as an ideal system for study because in every cell, providing it is not undergoing mitosis, the microtubules are oriented so that they could make contact with highly defined cytoplasmic foci (satellites). By disassembling the microtubules with low temperature (Tilney and Porter, 1967) and carefully analyzing their reassembly upon warming, we could study the earliest phases of microtubule reformation. We proposed that if nucleation were to occur directly from these foci, then one end of each microtubule should be connected to one of the satellites at the earliest stage in its reassembly.

**Materials and Methods**

Eggs and sperm were obtained from Arbacia punctulata by the voltage method (Harvey, 1952). For each experiment, eggs from a single female were fertilized and allowed to develop to the swimming blastula state (11–12 hr after fertilization). Some of these embryos were fixed in 3% glutaraldehyde in sea water (adjusted to pH 7.8–8.0 with dilute NaOH when necessary). Others were placed in an ice-water bath (0°C) for slightly over 1 hr. One sample was fixed in glutaraldehyde while still at 0°C. Others were removed from the cold and fixed after 2, 4, 6, and 8 min of warming. All of the embryos were fixed in glutaraldehyde for 1 hr, washed three times in sea water, and postfixed at 0°C in 1% OsO₄ in sea water for 45 min. After washing in sea water, the specimens were dehydrated and embedded in Epon 812. Thin sections were cut on a Sorvall Porter-Blum MT 2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.), stained with uranyl acetate and lead citrate, and examined with a Philips 300 or Philips 200 electron microscope.

**The Ectoderm: Untreated**

At the stage of embryogenesis reported here, the ectodermal cells of the blastula are columnar in shape. A cilium projects from their free surface or apical end. The basal ends of the cells limit the expanding blastocoel, and at the apicolateral margin the cells are held together by a septate desmosome. At right angles to the basal body of the cilium is a...
centriole, and extending from the basal body is a short ciliary rootlet. The Golgi apparatus is characteristically located between the apical surface and the nucleus (see Fig. 1 a).

As shown by Gibbins et al. (1969), almost without exception the microtubules converge on satellites associated with the basal body (see Fig. 1). Each satellite measures approximately 750 A in diameter and consists of dense material which grades off into the surrounding cytoplasmic matrix. It appears finely fibrous in texture and is difficult to characterize precisely (Figs. 1 b and 1 c). In longitudinal section cut through the basal body, we have never seen more than one satellite. If there were four satellites, each positioned at one “corner” of the basal body (but not necessarily in the same plane), we would generally have to get two foci in a longitudinal section. If the section is cut parallel to but not through the basal body, it is possible to cut through two satellites (Fig. 1 c), although frequently we only cut through one. Of greatest interest is the fact that these satellites are not located on the same plane apicobasally. This explains why we generally cut through only one satellite when the basal body is cut in transverse section (Fig. 1 b). In oblique section, we have nearly cut through three satellites (Fig. 2), thus excluding the possibility of there being only two equally or unequally positioned satellites. From this evidence, we have concluded that there are three equally spaced satellites surrounding the basal body (see our reconstruction in Fig. 3). These satellites do not lie on the same plane apicobasally, a feature we did not include in Fig. 3 for the purposes of clarity. From the satellites the microtubules diverge in all directions, some parallel to the long axis of the cell.

**Blastulae Fixed after 2 Min of Warming**

The rounding of the apical surface remains. In a small percentage of the ectodermal cells a few short segments of microtubules can be found. These are connected to the satellites. In most cells, however, no microtubules can be seen.

**Blastulae Fixed after 4 Min of Warming**

After 4 min of warming, more microtubules have reformed; invariably these are located near the satellites which surround the basal body (Figs. 6 a and 6 b). Considerable variation in the number and even in the presence of microtubules exists in adjacent cells. No microtubules are found in the basal one-third of the cell; spindle microtubules are also reappearing. We found one cell in which several short segments of microtubules could be seen near the nucleus. They were aligned parallel to the long axis of the cell, but we cannot be sure that these segments made contact with a satellite since serial sections were not cut.

**Blastulae Fixed after 6 Min of Warming**

By this stage all the ectodermal cells have microtubules. They appear to be oriented on radii, with their focus on the basal body of the cilia. Furthermore, the microtubular segments are considerably longer than in the previous stages.
FIGURE 2 Oblique section cut through the apical end of an ectodermal cell in the region of the basal body. This section missed the basal body but cut through the centriole (C). Of greatest interest are the three regions indicated by the arrows. These indicate satellites or regions cut very close to a satellite. Since the satellites are at different levels relative to the long axis of the cell (see Fig. 1 c), it is possible in an oblique section to cut very close to all three. × 61,500.

Blastulae Fixed after 8 Min of Warming

By 8 min the microtubules associated with the satellites have elongated appreciably (Fig. 7), and in random sections microtubules can be found lateral to and below the nucleus (Fig. 8). These invariably parallel the long axis of the cell. No microtubules appear to make contact with the limiting membrane at the base of the cell. Abundant 50-A filaments are present in the basal end of the cell and they are oriented parallel to the blastocoel (within 500 A of the limiting membrane); these are generally positioned normal to the tubules. By this stage the apical margin of the ectodermal cells has become considerably flattened, appearing more like that of cells of the untreated ectoderm.

DISCUSSION

In the introduction to this paper we hypothesized the presence of nucleating sites for microtubule assembly in differentiating cells. From our experiments with Arbacia blastulae we conclude (a) that three precisely positioned foci do exist in the ectodermal cells, (b) that microtubule assembly initiates at these sites, and (c) that concomitant spontaneous assembly from the subunit pool does not seem to be occurring to any appreciable degree. Our conclusions are based upon the observations that when the microtubules begin to reform, between 2 and 4 min of warming, one end of each microtubule segment actually appears to make contact with its respective satellite. With longer periods of warming, the tubules increase in length and after 8 min microtubules can be found in the basal region of the cell. When seen in the basal region of the cell, these segments of microtubules parallel the long axis of the cell, and thus can be interpreted as extensions of the tubules which make

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contact with the satellites. This conclusion is strengthened by the fact that, even in untreated cells, near the basal body it is extremely uncommon to find any tubules which could not, in an adjacent section, make contact with one of the satellites. Because it is most plausible that the few segments of microtubules seen in the basal region of the cell are products of elongation from the sites, we suggest that spontaneous cytoplasmic reassembly of microtubules is not occurring concomitant to assembly from the nucleating sites. Of course, we recognize the possibility that tubules, if so formed, may not have been seen in the micrographs examined if they happened to have been cut obliquely during thin sectioning. Nevertheless, even if that were the case, we would expect to see more randomly positioned tubules at the base of the cell and elsewhere. It is also possible that our sample was not sufficiently large, for we encountered a high degree of variation in the rapidity of recovery. Thus, although the majority of tubules seen below the nucleus can be interpreted as products of elongation from the satellites, we cannot say unequivocally that spontaneous assembly is not occurring at all, nor can we eliminate all possibility of break-off and dispersal of tubules from the sites. These points can only be answered unequivocally if serial sections are cut through the whole cell at various stages in rewarming. At present such a study would be exceedingly laborious. We are currently investigating the possibility of using high voltage electron microscopy with stereo pairs to settle these issues definitively.

_Nucleation and Orientation: Two Concepts_

Microtubule nucleating centers, while instrumental in ordering the assembly of microtubules in certain cells such as the ectodermal cells, do not necessarily account solely for the direction of microtubule growth and their ultimate pattern in cells. Rather, the orientation of the tubules might in part be controlled by other physical and chemical factors.

A case where nucleation of microtubules and their orientation might be distinguished can be seen in the growth of spindle fibers (bundles of microtubules) from the kinetochore. Nicklas (1967) has demonstrated that, when a chromosome is positioned at right angles to the metaphase plate, there is an equal possibility that the spindle fiber will grow towards either pole. Thus, the spindle fiber seems to recognize the direction in which it is heading. Unfortunately, in the literature to date the two concepts have been somewhat confused, or at least not clarified. For example, Porter (1966) and Inoué and Sato (1967) have used different words, "initiating sites" and "orienting sites," to describe the function of the kinetochore. Both are correct for some systems, for if a nucleating site is accurately positioned it may also give direction to growth (as in ciliary growth). In the ectoderm cells of _Arbacia_, however, the tubules diverge in all directions from spots or satellites. Here the direction of growth could be largely taken over by other factors. Likewise, in the just mentioned experiments of Nicklas (1967), nucleation and orientation are quite different.

Acknowledging such situations, we suggest that initiation of microtubule assembly be termed "nucleation," and that positioning, directioning and relocation of microtubules be termed "orientation," also that respective sites of assembly be termed "nucleating sites," and mechanisms of orientation be described in terms of their particular mode of operation (for example, orienting forces, processes, or structures).
Perhaps such a conceptual clarification will simplify the task of experimental design and thus more conclusively elucidate one process or the other in a chosen system. Certainly such knowledge cannot help but increase our understanding of development.

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FIGURE 4  Longitudinal section of apical region of ectodermal cell after treatment with low temperature. The apical surface becomes prominently rounded during low temperature application. Although the cilium appears intact, and a satellite (S) can be distinguished, there is a conspicuous lack of microtubules. X 68,000.
Figure 5  Transverse section through the apical end of an ectodermal cell fixed during treatment with low temperature. The basal body is cut in transverse section. No microtubules can be seen. X 55,000.
By this time the microtubules, although still associated with the satellite (S), have elongated considerably. $\times 55,000$.

Figure 6 Longitudinal section of the apical portion of ectodermal cells after 4 min of warming following low temperature application. The section is cut parallel to the basal body but not through it. a. Association of microtubules with the satellite (S) is evident. Also present are a centriole (C) and a portion of the ciliary rootlet (R). b. The microtubules (mt) make contact with a satellite. Fig. 6 a, $\times 68,000$; Fig. 6 b, $\times 68,000$. 
**Figure 8 a.** Low magnification micrograph of two ectodermal cells fixed after 8 min of warming. The blastocoel (B) is indicated. **b.** Higher magnification of the region depicted in a. Note the microtubules (arrows) running parallel to the long axis of the cell. They are situated near the basal surface. Fig. 8 a, × 6000; Fig. 8 b, × 55,000.

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