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The midblastula transition, the YSL transition and the onset of gastrulation in *Fundulus*

J. P. TRINKAUS

Department of Biology, Yale University, New Haven, Connecticut 06511, USA

Summary

The first signs of cell motility appear in *Fundulus* toward the end of cleavage, after cleavages 11 and 12. When blastomeres cease cleaving, their surfaces undulate and form blebs. At first, these blebbing cells remain in place. Gradually thereafter they begin movement, with blebs and filolamellipodia serving as organs of locomotion. Non-motile cleaving blastomeres have thus differentiated into motile blastula cells. This transformation corresponds to the midblastula transition of amphibian embryos.

Gastrulation in *Fundulus* begins with vegetalward contraction of the external yolk syncytial layer. This causes narrowing of the E-YSL and initiates the epibolic expansion of the blastoderm. Convergent movements of deep cells within the blastoderm begin toward the end of this contraction. The YSL forms as a result of invasion of the yolk cell cytoplasm by nuclei from open marginal blastomeres during cleavage. These YSL nuclei then undergo five metachronous divisions. After this, they divide no more. YSL contraction begins approximately 1.5 hours after cessation of these divisions (21-22°C). This cessation of nuclear divisions is preceded by a gradual decrease in rate. (1) The duration of each succeeding mitosis increases steadily and often some nuclei do not divide at mitosis V. (2) The duration of interphases between succeeding mitoses also increases, but to a much greater degree, and the longest interphase by far is the last one, I-IV, between mitoses IV and V. (3) The mitotic waves responsible for mitosis V move much more slowly than those for the first four mitoses and invariably decelerate. This gradual cessation of YSL nuclear divisions clearly sets the stage for the contraction of YSL cytoplasm and thus the beginning of gastrulation. We call this the YSL transition. It is not to be confused with the midblastula transition, which occurs 3-4 hours earlier. The MBT commences cytodifferentiation; the YSL transition commences morphogenesis.

Key words: *Fundulus*, midblastula transition, yolk syncytial layer, YSL transition, gastrulation.

Introduction

Gastrulation in teleost fishes has two major aspects: movement of so-called deep cells within the blastoderm to congregate and form the embryo and, concomitantly, spectacular epiboly of the blastoderm and its underlying yolk syncytial layer to encompass eventually a large sphere of viscous, fluid yolk. Although these processes take place together, they are conveniently separable for analysis. Deep cells are confined to the space between the monolayered cell surface layer of the blastoderm, the enveloping layer (EVL), and the underlying yolk syncytial layer (YSL), which separates them from the yolk. Their movements are coordinated with the epiboly of the EVL and YSL, but do not contribute to its mechanism (Trinkaus, 1984b). Epiboly proceeds independently of the activities of the deep cells and depends on expansion of the EVL, which in turn depends on expansion of the YSL, with the cooperation of the diminishing yolk cytoplasmic layer (YCL) (Trinkaus, 1984a,b; Betchaku and Trinkaus, 1986).

A fascinating feature of the gastrulation movements of deep cells and the vast epibolic expansion of the EVL and YSL is that they both seem to proceed at about the same rate in different teleost species, willy-nilly, regardless of the size of the egg. In small eggs, like those of the zebrafish or *Serranus* (Wilson, 1889), epiboly is quickly finished and, at closure of the yolk plug, gastrulation of the deep cells is far from completion. In large eggs, like those of the trout (*Salmo*), epiboly is a long process and gastrulation by deep cells is complete and embryo formation is well underway long before closure of the yolk plug. In medium-size eggs, like those of *Fundulus* and *Oryzias*, gastrulation movements of deep cells are essentially complete and embryo formation has just begun at the end of epiboly. If one therefore takes into account specific differences in the size of the yolk sphere, the gastrulation movements of superficially very different teleost eggs are really quite comparable.

In this essay, I shall deal first with the differentiation of motility of the deep cells during and after the cessation of cleavage and then with the events leading to gastrulation: the onset of epiboly of the YSL and EVL and directional movements of the deep cells. The material for all of our work on these subjects has been the embryo of *Fundulus heteroclitus*. For illustrations of the cleavage, blastula and

gastrula stages of *Fundulus*, see Armstrong and Child (1965). The YSL and the blastoderm in a late blastula stage are shown in Fig. 1. See also Fig. 2 in Trinkaus (1993).

The differentiation of deep cell motility

The first signs of deep cell motility appear in *Fundulus* when cleavage is nearing its end, during and after cleavages 11 and 12. Although cleavages 11 and 12 differ little if at all from previous cleavages in their duration, they do differ from all preceding cleavages in two other important respects. Cleavage 10 appears to be the last complete cleavage in *Fundulus*, i.e., all blastomeres divide. Cleavage 11 is sometimes incomplete and frequently asynchronous and cleavage 12, the last cleavage, is invariably highly incomplete and highly asynchronous and occasionally does not occur at all. The military precision of the first ten cleavages gradually degenerates to the disarray of the last. Cleavage in *Fundulus* clearly does not cease abruptly, with a bang; it seems rather to grind slowly to a halt.

As cleavage is winding down, the surfaces of many non-cleaving blastomeres begin to bleb. This motile activity, which is totally absent during the first ten cleavages, first appears on the surface of some non-cleaving deep blastomeres during cleavage 11, is frequent just after that, before and during the highly variable 12th cleavage, and seems to involve almost all deep blastomeres soon after. At this point, when speeded up with time-lapse, the blastoderm seems a seething mass of jostling cells. However, when blebbing is observed in detail, it appears gradually. The first blebs are preceded by gentle, slow undulations of the cell surface (Trinkaus, 1973, 1985). Then, some minutes later, a sector of the cell surface explodes to form a hemispheric bleb. As these blebs form, each blebbing cell remains in place. There is no cell movement. Then, gradually, one cell after another begins to move. The prior blebbing activity is a prelude to this movement (Trinkaus, 1973, 1985). With this translocation of the deep cells of the blastula, a new phase of development has begun. Non-motile cleavage blastomeres have differentiated into motile, moving post-cleavage cells. (It is important to note, incidentally, that EVL cells have not been observed to bleb.) The time of the onset of deep cell surface deformation and blebbing is significant for two reasons. (1) It occurs only after the regular, rapid cleavage phase of development has ceased, as is true of many embryos. (2) It often commences within minutes after the cessation of cleavage of a blastomere, whether at cleavage 11 or 12. There is little apparent lag. It seems as if, once a cell's motile system is no longer tied up in rapid cytokinesis, it is quickly available for motile activity, in this case for blebbing (Trinkaus, 1980). This phenomenon corresponds to the so-called midblastula transition that has been intensively studied in amphibian embryos (Signoret and Lefresne, 1971; Johnson, 1976; Gerhart, 1980; Newport and Kirschner, 1982a, b).

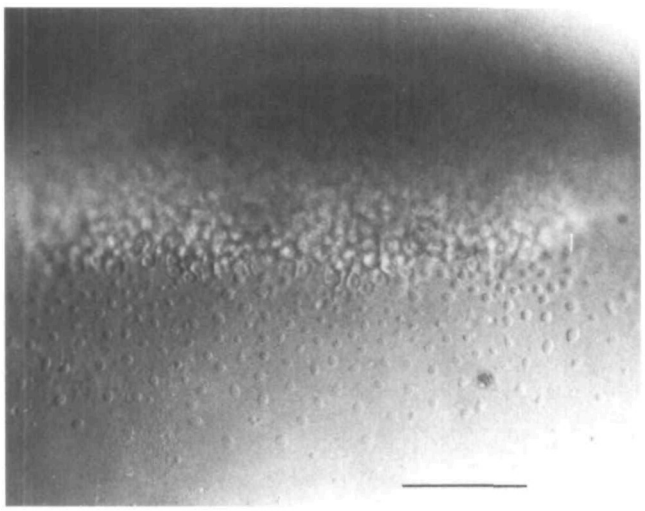

Fig. 1. A definitive yolk syncytial layer (lower part of the micrograph) after the last division of its nuclei. The blastoderm, in a late blastula stage (stage 12), occupies the top half of the micrograph, but only some of its marginal deep cells are in focus. Note the width of the YSL and the approximate spacing of its nuclei. Contraction of the YSL (Fig. 2) begins about an hour and a half after this stage (at 21-22°C). Scale bar equals 200 μm.

Although the MBT of *Fundulus* resembles that of *Xenopus* in so far as the appearance of motility is concerned, as yet we lack the critical biochemical information that research on *Xenopus* has provided (Newport and Kirschner, 1982a, b). The cessation of rapid mitosis no doubt leads to the establishment of the G_1 phase in *Fundulus*, with accompanying commencement or augmentation of transcription, as in *Xenopus*, but study of this in *Fundulus* is for the future.

In the meantime, however, we possess considerable information on the subsequent motile activity of these blebbing cells (Trinkaus, 1973, 1985). About an hour or more after cells begin blebbing, they begin to move, at first occasionally and soon more and more. The blebs become organs of locomotion. Instead of retracting immediately, a bleb persists, shows circus movement and cytoplasm pours into it. The bleb then protrudes more and the rest of the cell follows. We have termed this 'blebbing movement' (see also Trinkaus and Erickson, 1983; Fink and Trinkaus, 1988). Soon after this, some of these blebs spread on the substratum, usually the internal YSL, to form broad, thick lamellipodia and then these lead the cells in movement. Thick filolamellipodia (Trinkaus and Erickson, 1983) soon appear as well. By means of these varied protrusive activities, deep cells eventually move actively within the segmentation cavity. Two aspects of these early movements are noteworthy. (1) The cell movements are apparently random. No directional bias has been observed. (2) Cells begin movement in full sway in a so-called 'blastula' stage, long before the official beginning of gastrulation. The deep cells of *Fundulus* are obviously well prepared to participate in the directional movements of gastrulation well before that stage is reached. The MBT occurs in *Fundulus* 3-4 hours before contraction of the YSL and the beginning of epiboly (see below) and even longer before the gastrulation movements of deep cells. Another interesting feature of the MBT in *Fundulus* is its stability. Once cells differentiate into a motile state and begin blebbing, their adhesive affinity for other cells develops independently of their normal associations in the blastoderm. They do the same when isolated in vitro (Trinkaus, 1963).

Slowing and cessation of nuclear divisions in the YSL set the stage for the onset of gastrulation

During teleost epiboly, the EVL and the YSL spread together to encompass the large, spherical yolk sphere (Wilson, 1889; Stockard, 1915). Once formed, the EVL adheres firmly to the underlying YSL solely by its marginal cells by means of tight junctions, which become more and more extended as tension within the EVL increases with its steady expansion in epiboly (Betchaku and Trinkaus, 1978). In spite of this tension and the extensive junctional complex joining them to each other (Lentz and Trinkaus, 1971) and their high resistance barrier (Bennett and Trinkaus, 1970), individual EVL cells continually rearrange in the plane of the monolayer during epiboly, adjusting to the geometrical problems imposed by the expansion of an originally rather flat monolayered cell sheet over a sphere (Keller and Trinkaus, 1987). Indeed, the meticulous coordination of these rearrangements of the EVL cells, as they actively participate in epiboly, constitutes one of the most precise of all morphogenetic cell movements. But crucial though these beautiful EVL cell rearrangements are for the integrated progress of epiboly, they do not provide the motive force. All evidence points to the yolk syncytial layer. The blastoderm will not undergo epiboly unless it is attached to the YSL. The YSL, in contrast, undergoes complete epiboly in the complete absence of the blastoderm (Trinkaus, 1951). Moreover, it does so faster when relieved of the burden of the clinging blastoderm (Betchaku and Trinkaus, 1978) and will even surge ahead locally, if one small region is surgically freed of the restraint of the EVL (Trinkaus, 1971). Perhaps the most impressive evidence of the epibolic force of the YSL comes at the very beginning of epiboly. Soon after the external YSL (E-YSL) has formed a wide nucleated belt around the perimeter of the blastoderm (Fig. 1 and Trinkaus, 1951), it undergoes a spectacular narrowing (Fig. 2). This is caused by active cytoplasmic contraction (for evidence, see Trinkaus, 1984b). With this contractile narrowing of the E-YSL, the margin of the EVL and the rest of the blastoderm (Fig. 2) and the

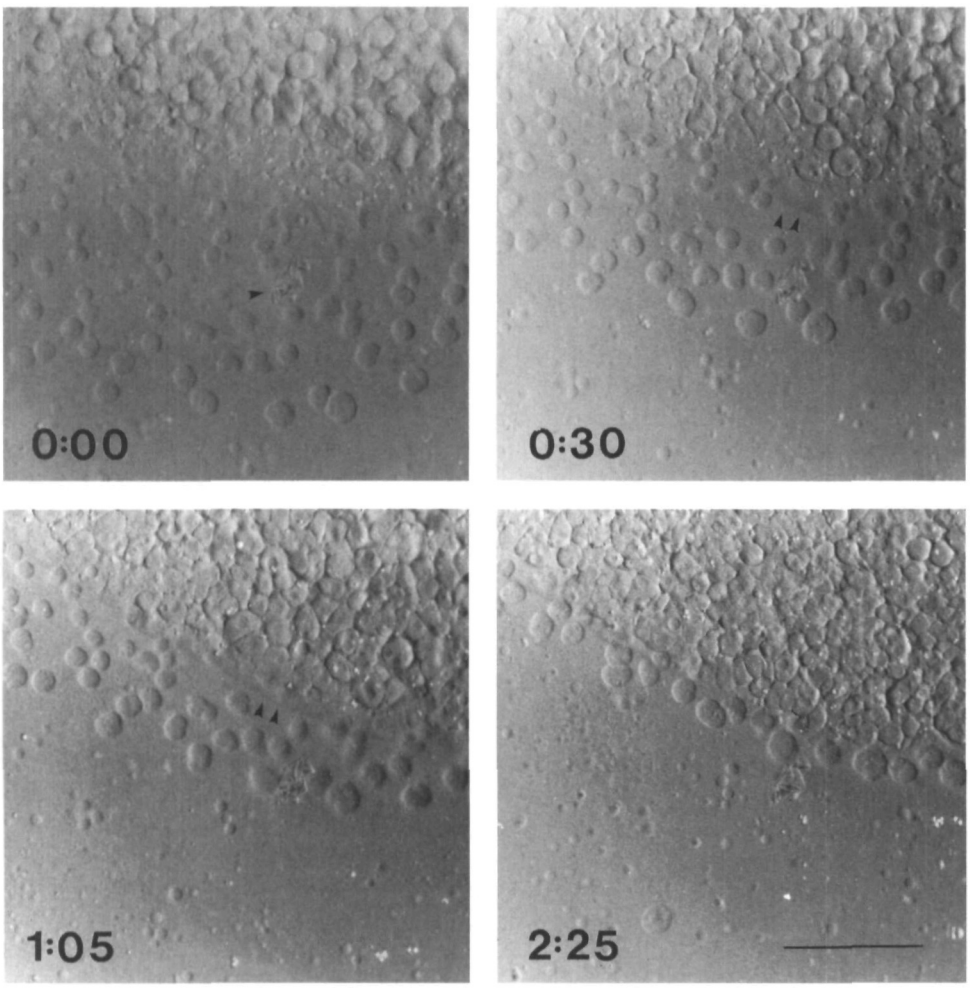

Fig. 2. Photomicrographs illustrating the contraction of the E-YSL, epiboly of the blastoderm, the crowding of the YSL nuclei and the disappearance of the nuclei beneath the blastoderm. 28°C. Time in hours and minutes. Scale bar equals 100 µm. 0:00 The E-YSL has contracted approximately one-third. Some nuclei are now in the I-YSL underneath the blastoderm in the cytoplasm of the I-YSL. Those that remain peripheral to the blastoderm are already crowded and several are in contact with each other. A dust fragment lodged between the egg and the coverslip serves as a stable reference point (arrowhead). The blastoderm is now in stage 13 and epiboly has just begun. 0:30 In this short period, the E-YSL has narrowed a great deal and many nuclei have disappeared beneath the blastoderm. The E-YSL is in its most rapid phase of contraction. Blastoderm epiboly has advanced considerably. The margin of the enveloping layer is evident (arrowheads). Many of the deep cells in the blastoderm are motile. 1:05 Contraction of the E-YSL has continued but its rate now is slower than during the first 30 minutes. Blastoderm epiboly is also moving more slowly. The border of the EVL is visible (arrowheads). The E-YSL nuclei are now densely packed and more have disapeared beneath the blastoderm. The embryo is now in stage 14½ and the germ ring is just evident. Convergence of deep cells has begun. 2:25 The contraction and narrowing of the E-YSL is almost complete. Only an irregular row of YSL nuclei is now visible. The blastoderm has continued to expand, but at a lower rate than previously. The border of the EVL is not visible in this micrograph. A number of motile deep cells engaged in convergent movements of gastrulation (Trinkaus et al., 1992) are now in focus. The embryo is in stage 15.

internal YSL (I-YSL) are pulled vegetalward in a sweeping movement of epiboly (Trinkaus, 1984b, Figs 3 and 4).

This represents the beginning of gastrulation, for not only does EVL and I-YSL epiboly begin but, soon after, the highly motile deep cells undergo involution (Thorogood and Wood, 1987; Wood and Timmermans, 1988; Warga and Kimmel, 1990) and convergence (Sumner, 1904; Oppenheimer, 1936; Pasteels, 1936; Ballard, 1966, 1973; Trinkaus et al., 1992). Deep cells converging in the germ ring are illustrated in Fig. 2 (at 2:25). As the converging deep cells join the embryonic shield, they participate in its anteroposterior extension. The result is a fully formed embryo. Teleost gastrulation thus offers a classic example of convergent extension, a hallmark of vertebrate gastrulation.

Because of the crucial importance of the contraction of the YSL in the onset of *Fundulus* gastrulation, I have recently investigated the complete development of the YSL in detail (Trinkaus, 1990, 1993). Very briefly, the nuclei of the YSL are derived from open, marginal blastomeres of late cleavage stages, as in other teleosts, and then divide five times to form the definitive, wide syncytial layer encircling the blastoderm (Fig. 1). About 1.5 hours after completion of the last (fifth) mitosis, this broad E-YSL begins its contraction, which progresses slowly over a 4-5 hour period and has a number of dramatic results. (1) The E-YSL narrows (Fig. 2). (2) This contractile narrowing causes the initially smooth surface of the E-YSL to buckle, throwing it into complex folds (Betchaku and Trinkaus, 1978). (3) At the margin of the E-YSL, the surface folds become the sites of highly localized programmed endocytosis (Betchaku and Trinkaus, 1986). (4) The cytoplasm of the E-YSL thickens, as it is compressed by its contraction (see Fig. 19 of Betchaku and Trinkaus, 1978). (5) Nuclei of the E-YSL become increasingly crowded as their cytoplasmic environment narrows along with its thickening (Fig. 2). (6) Epiboly of the I-YSL and the blastoderm commences, as their margins are pulled by the contracting E-YSL closer and closer to the E-YSL margin (Fig. 2). (7) YSL nuclei disappear into the I-YSL cytoplasm beneath the advancing blastoderm to form the nucleated I-YSL (Trinkaus, 1993). About two-thirds the way through this YSL contraction, deep cells within the blastoderm abandon their casual, undirected locomotory activities and begin the more disciplined movements of gastrulation.

Unfortunately, we have no information as yet at the molecular level of the activities of the cytoplasmic contractile proteins in the E-YSL during this contractile period. But we do have some useful morphological data. The cortical cytoplasm of the E-YSL, and especially its surface folds, is packed with microfilaments. Since they are the 4-6 nm variety, we presume that they are actin-containing (Betchaku and Trinkaus, 1978).

As indicated, the contraction of the E-YSL commences soon after the cessation of mitotic activity in the YSL. In consequence, details of this cessation deserve close attention. In the first place, the arrest of nuclear divisions after the last mitosis (M-V) is definitive and occurs simultaneously throughout the YSL. Nuclei enter a kind of permanent interphase after M-V. Contraction of the fully nucleated YSL commences about 1.5 hours after M-V and occurs

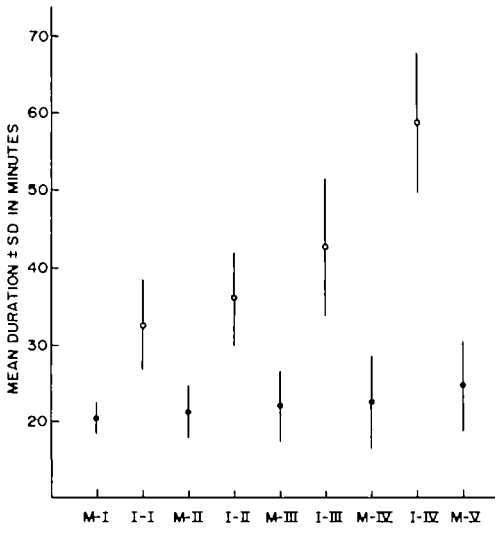

Fig. 3. A graph depicting the duration of the mitoses of the YSL and the interphases between them, as measured in continuous time-lapse video tapes. The duration of the mitoses are represented by solid circles and those of the interphases by open circles. Along the abscissa, each successive mitosis is represented by M-I, M-II, etc. Each successive interphase after each mitosis is represented by I-I (after M-I), I-II (after M-II), etc. M-V is the last mitosis. M-V is often incomplete (not shown on this graph).

essentially simultaneously around the periphery of the blastoderm. There is obviously a close temporal and circumferential relationship between the cessation of YSL nuclear divisions and the contraction of YSL cytoplasm. But the relationship begins prior to this. Although the cessation of mitosis in the YSL is abrupt, the events leading up to it are not. The duration (and variability) of each succeeding YSL mitosis increases gradually from the first one (M-I), the mean of which is 20.3 ± 2.0 minutes, to the last (M-V), the mean of which is 24.7 ± 6.1 minutes (Fig. 3). Moreover, M-V is often incomplete; some nuclei do not divide. Also, and probably more significant in the present context, the duration of the interphases between succeeding mitoses increases, and to a much greater degree (from a mean of 32.4 ± 5.7 minutes for interphase I (I-I) to a mean of 58.6 ± 9.1 minutes for I-IV) (Fig. 3). I-IV occurs between M-IV, the penultimate mitosis, and M-V, the last mitosis. (For quantitative details, see Trinkaus, 1993.) It should be emphasized that I-IV is not only the longest interphase of the series; it is by far the longest.

Variation in another aspect of the YSL mitoses is also relevant. These YSL mitoses are metachronous. Mitotic wave fronts progress through the YSL cytoplasm and stimulate nuclei to enter mitosis. These waves vary greatly in speed and regularity. Some waves move at a uniform rate across the field of observation; others accelerate; still others decelerate. Significantly, the wave fronts for M-V are the only ones that are consistent. They almost always move most slowly and they always decelerate as they move (Trinkaus, 1993). It seems likely that this deficient wave front is part of the cause of the frequent incompleteness of M-V (see Trinkaus, 1993).

There is clearly an attenuation of the mitotic forces of

the YSL with successive mitoses. The causes of this attenuation are of much interest and could well be the vast increase in the nucleocytoplasmic ratio of the YSL, as the number of its nuclei progressively increases (Edgar et al., 1986; see discussion in Trinkaus, 1993). But consideration of this fascinating subject is outside the province of this paper. On the contrary, the results of this attenuation (longer mitoses, longer interphases and fading mitotic waves) are very relevant to the subject at hand - the events leading to contraction of the YSL cytoplasm. In the absence of further analysis, there seem to be two possible reasons for assuming that this slowing of mitotic activity in the YSL forms the basis for its contraction: a diminution of the antagonism between mitotic division and cytoplasmic contractility and the appearance of the G_1 phase of the mitotic cycle, during which transcription and the synthesis of new contractile proteins might occur. These possibilities are not mutually exclusive.

It is well-established that there is an antagonism between mitosis and cell movement (e.g., Trinkaus, 1980; Trinkaus et al., 1992). Since cell movement depends in part on cytoplasmic contractility, there also must be an antagonism between mitosis and the cytoplasmic contractility associated with cell motility. It is not unreasonable, therefore, to propose that when the contractile mitotic machinery of the YSL is released, it should be available for other contractile activities, namely the contraction of the YSL cytoplasm.

It seems certain that the longer interphases between successive mitoses of the YSL, in particular, the last, longest interphase (I-IV) and the permanent interphase after M-V, make possible the establishment and augmentation of G_1 phase. This, of course, would allow the activation and augmentation of transcription and the consequent expression of new gene products, such as the molecular machinery for massive cytoplasmic contraction. Although, unfortunately, there has as yet been no investigation of mRNA during the development of the YSL of *Fundulus*, this matter has been investigated with great care in *Drosophila*, where certain aspects of early syncytial development are remarkably similar to the development of the YSL in teleosts. As in *Fundulus*, the interphases of the last nuclear divisions of *Drosophila* gradually increase in duration, in particular the last one (nuclear cycle 14), whose interphase is much longer than the preceding ones (Foe and Alberts, 1983). This last nuclear division is then followed immediately by an important new cytodifferentiation - cellularization, which in turn is quickly followed by a new morphogenesis - gastrulation. In *Drosophila*, several studies have shown that newly synthesized mRNA is first detectable at nuclear cycle 11 and that transcription increases substantially with each cycle thereafter (for references, see Edgar et al., 1986). This sequence certainly suggests that cellularization is triggered by this mRNA synthesis, especially that which occurs during the long, last nuclear cycle. Since the cellular form changes that lie at the basis of the next event, gastrulation, depend heavily on cytoplasmic contraction (Trinkaus, 1984a), the analogy with *Fundulus* is not completely farfetched. Obviously, studies like these would be desirable in *Fundulus*.

A puzzling feature of the YSL morphogenetic transition in *Fundulus* is the long lag between the last mitosis of the YSL and the contraction of the YSL cytoplasm - about 1.5 hours at 21-22°C. It could be that the E-YSL cytoplasm is ready to contract much sooner but is restrained to do so by the firmly adherent blastoderm. To test this hypothesis, I removed the blastoderm as quickly as possible after the cessation of the last YSL mitosis. The result was conclusive. The E-YSL quickly contracted and the I-YSL quickly expanded (Trinkaus, 1984b). The result was precocious epiboly of the I-YSL. This experiment indicates that the normal slow delay in the contraction of the E-YSL after cessation of nuclear division is due to inhibitory restraint imposed by the adhering blastoderm (actually the EVL). It would seem, therefore, that the E-YSL actually becomes contractile very soon after cessation of its nuclear divisions, but is normally restrained for an hour or so by the attached blastoderm. In view of this, I suggest that when the E-YSL eventually contracts, after its normal delay, it does so either because of an eventual weakening of the restraint imposed by the blastoderm or because of a sudden increase in its contractile force.

This YSL transition that brings on gastrulation in *Fundulus* naturally reminds one of the famous midblastula transition. However, they are really separate processes. The MBT occurs much earlier in development and involves the cessation of nuclear divisions and cytokinesis in cleaving blastomeres, not the much later cessation of nuclear divisions in a syncytium. In addition, and more importantly, the MBT results in the onset of motility, G_1 and transcription only in individual deep blastomeres, whereas the YSLT results in the onset of global morphogenetic movements of the whole embryonic system. To express it succinctly, the midblastula transition commences cytodifferentiation; the YSL transition commences morphogenesis.

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