Segregation During Ascidian Embryogenesis of Egg Cytoplasmic Information for Tissue-Specific Enzyme Development

(morphogenetic substances/egg localizations/cytochalasin B/acyetylcholinesterase/tyrosinase)

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ABSTRACT  Cleavage-arrested embryos of the ascidian *Ciona intestinalis* were able to differentiate two tissue-specific enzymes—muscle acetylcholinesterase (EC 3.1.1.7) and brain pigment cell tyrosinase (EC 1.10.3.1). Cytochalasin B, colchicine, Colcemid, and podophyllotoxin were used as cleavage inhibitors at early embryonic stages up to the 64-cell stage. Only certain cells in the cleavage-arrested embryos developed these histochemically detectable enzymes, and this ability followed the cell lineage patterns for the two tissues. This result implies the presence of specific positional information in the egg cytoplasm that is differentially segregated during cleavage. There were distinct and separate puromycin and actinomycin D sensitivity periods for the occurrence of each enzyme during development of both normal and cleavage-arrested embryos. The segregated information is apparently neither the enzyme proteins nor RNA templates for enzyme synthesis, but is probably concerned with activation of appropriate genes.

The classical cell lineage studies of ascidian larval development by Conklin and his direct observations on segregation during cleavage of visibly different cytoplasmic regions have strongly suggested the existence in eggs of localized cytoplasmic factors related to later tissue differentiations of the larval organism (1). Many other studies of embryos, both invertebrate and vertebrate, support the hypothesis that cytoplasmic information in eggs is segregated during cleavage into particular tissue areas of embryos (2).

The best modern evidence from nuclear transplantation experiments indicates that neither stable nuclear changes nor an independent nuclear lineage can be responsible for cellular differentiation (3). The same conclusion was reached many years ago from results of studies on pressure-induced changes in the plane of early cleavage (4) and from Spemann’s ligature experiment (5), both of which alter the normal nuclear lineage pattern. Differential activation of certain genetic programs in developing cells is thought, therefore, to result from segregation during cleavage of cytoplasmic determinants that can eventually interact with specific parts of the genome (6, 7).

Studies described here establish a relationship between cytoplasmic segregation and differentiation of tissue-specific proteins. Ascidian larvae have two enzyme localizations that occur relatively early in embryonic development: acetylcholinesterase in muscle cells of the tail (8, 9) and tyrosinase in the brain pigment cells (10, 11). This report takes advantage of Lillie’s well-known observation that some morphological features of development occur in cleavage-arrested annelid eggs (12) and extends his discovery to the demonstration that tissue-specific enzymes are able to develop in certain blastomeres of cleavage-arrested ascidian eggs and embryos (13). Cleavage-arrested ascidian embryos develop acetylcholinesterase and tyrosinase in blastomeres that match the known cell lineage patterns for the two enzyme-containing tissues; this finding implies a differential segregation during cleavage of localized cytoplasmic information related to the development of these enzymes.

MATERIALS AND METHODS

Embryos. Eggs of the ascidian *Ciona intestinalis* (L.) were fertilized artificially and raised in filtered sea water at 18° ± 0.2° (11). The timing of the developmental stages listed in Table 1 was quite reproducible between groups of fertilized eggs. The total period of embryonic development in *Ciona* is very short (18 hr) at 18°. Perhaps as a consequence of this remarkably rapid development, certain drugs that demonstrably affect *Ciona* embryos are neither lethal nor particularly toxic during the time periods applied.

Cleavage Inhibition. Several drugs were used as cleavage (cytokinesis) inhibitors on *Ciona* eggs and embryos. The following concentrations of the drugs inhibited both early and later cleavages and are slightly higher than the minimum concentrations necessary to prevent cleavage: cytochalasin B (Imperial Chemical Industries) at 2 μg/ml, colchicine (Sigma Chemical Co.) at 200 μg/ml, Colcemid (Ciba Pharmaceutical Co.) at 20 μg/ml, and podophyllotoxin (S. B. Pennick and Co.) at 10 μg/ml. Cytochalasin B interferes with microfilaments (14) and possibly other structural components of the cell. The interference prevents cytokinesis in *Ciona* embryos but does not inhibit nuclear divisions. Colchicine, Colcemid (N-methyl-N-desacetylcolchicine), and podophyllotoxin are all inhibitors of microtubule formation (15), which prevent both mitotic activity and cytokinesis in cells exposed to them.

Enzyme Histochemistry. Embryos for acetylcholinesterase (EC 3.1.1.7) reactions were fixed for 2 min in cold (5°) 80% ethanol (8) and reacted for 2–4 hr at 37° by the direct-coloring thiocholine method (16). The cholinesterase activity in *Ciona* embryos has been shown to be an acetylcholinesterase (9).

Tyrosinase (EC 1.10.3.1) was demonstrated by a dopa oxidase reaction. Embryos were first fixed for 1–2 hr in cold 70% ethanol and then incubated for 4 hr (37°) in two changes of the reaction medium (11). The histochemical reaction in *Ciona* pigment cells could be prevented by inhibitors of tyrosinase (11).

Enzyme activities in normal embryos were tested at various development times. Embryos that were reared in cleavage
Figs. 1-3. Acetylcholinesterase activity in normal embryos at 8 hr (Fig. 1), 9 hr (Fig. 2), and 10 hr (Fig. 3).

Figs. 4-10. Acetylcholinesterase activity at 12-14 hr in cytochalasin B-arrested cleavage stages: 1-cell (Fig. 4), 2-cell (Fig. 5), 4-cell (Fig. 6), 8-cell (Fig. 7), 16-cell (Fig. 8), 32-cell (Fig. 9), and 64-cell (Fig. 10).

Fig. 11. Dopa oxidase activity at 12 hr in a cytochalasin-arrested 32-cell stage.

Fig. 12. Melanin and acetylcholinesterase activity in a cytochalasin-arrested 5-hr embryo. Acetylcholinesterase reaction was done at 22 hr.

All figures are at the same magnification: ×235.
inhibitors were examined for acetylcholinesterase activity at 12–14 hr after fertilization and tested for dopa oxidase activity at 10–12 hr. Since Ciona embryos are small (about 80 μm in diameter), the reaction products were examined in permanent whole mounts of the embryos that were dehydrated in ethanol after the reactions, cleared in xylene, and mounted in dammar resin.

**Inhibition of Protein and RNA Synthesis.** Puromycin di-HCl (Nutritional Biochemicals Corp.) was completely effective at 200 μg/ml in blocking the appearance of histochemically detectable enzyme. Ciona embryos that were treated for 1 hr with puromycin and subsequently reared during hr 5–9 of development in sea water containing both 200 μg/ml of puromycin and 0.4 mM L-[14C]valine, specific activity 3.3 Ci/mol (New England Nuclear Corp.), had 99% of isotope incorporation into the acid-insoluble fraction inhibited by the drug. The experimental (11) and assay (17) procedures used are described elsewhere.

Actinomycin D (Sigma) at a concentration of 20 μg/ml differentially inhibits the occurrence of histochemically detectable enzymes in Ciona embryos, depending on the times at which it is administered. A concentration of 15 μg/ml will only partially inhibit enzyme occurrence. Actinomycin at 20 μg/ml causes maximal (70%) inhibition of labeled uridine incorporation in another ascidian species (18); the uninhibited fraction was found to be low molecular weight RNA. This concentration also causes maximal inhibition of RNA synthesis in Ciona embryos (19).

**RESULTS**

**Acetylcholinesterase Development in Cytochalasin B.** Acetylcholinesterase activity is first detected histochemically in the presumptive muscle cells of the early neurula; localized staining is seen at 8 hr of development but not at 7 hr. Staining intensity of the cells increases progressively with development time. The histochemical method used here is at least as sensitive as the commonly used Eillman quantitative technique, since a comparable time of new enzyme occurrence in Ciona was demonstrated by this quantitative assay (19). Figs. 1–3 illustrate the location and relative staining intensity of developing muscle cells of the tail at three different embryonic stages. There is unusual cellular specificity of this reaction, although a light background staining occurs that undoubtedly results from the small amount of acetylcholinesterase present in the unfertilized egg (19). As noted previously by others (8, 20), there is no histochemically detectable localization of acetylcholinesterase activity in the Ciona nervous system.

Embryos that were placed in cytochalasin B at various cleavage stages and examined histochemically at 12–14 hr of development time had developed acetylcholinesterase activity in some of the blastomeres. Uncleaved eggs treated with cytochalasin immediately after fertilization usually develop a strong enzyme reaction in 5–10% of the eggs (Fig. 4). In some batches of eggs the reacting percentage was as high as 50–80%. Cleavage-arrested 2-cell stages developed enzyme in one or both blastomeres (Fig. 5), and arrested 4-cell stages produced enzyme in one or two blastomeres (Fig. 6). The number of embryos developing enzyme in arrested 2- and 4-cell stages was slightly more than for 1-cell stages. 8-Cell stages developed enzyme in one or two blastomeres (Fig. 7), and arrested 16-cell stages formed enzyme in up to four blastomeres (Fig. 8). From the 8-cell stage onward, almost all of the cleavage-arrested embryos developed enzyme in some blastomeres. In cytochalasin-arrested 32-cell stages, as many as six blastomeres could be found differentiating acetylcholinesterase (Fig. 9), and in cleavage-arrested 64-cell stages as many as eight blastomeres produced enzyme (Fig. 10). In the early cleavage-arrested stages, there was a range in the number of enzyme-producing blastomeres, but at the 64-cell stage usually most of the reacting embryos produced eight enzyme-containing cells. The number of reacting embryos varied from one batch of eggs to another. The reasons for this variation are not known.

The maximum numbers of acetylcholinesterase-containing blastomeres at each cleavage-arrested stage as illustrated in Figs. 4–10 (1 at 1-cell, 2 at 2-cell, 2 at 8-cell, 4 at 16-cell, 6 at 32-cell, and 8 at 64-cell) follow precisely the known pattern of cell lineage for larval muscle-cell development in ascidian embryos (1, 21). Since the relative positions of the blastomeres in cytochalasin-arrested embryos remain fixed, the blastomeres containing acetylcholinesterase are readily seen to be those of the presumptive muscle cells.

**Tyrosinase Development in Cytochalasin B.** Tyrosinase (dopa oxidase) activity normally begins to develop in Ciona at 9 hr of development time (11). In cytochalasin-arrested 32-cell stages, enzyme activity likewise develops at 9 hr. At this cleavage-arrested stage, tyrosinase activity is found in the two bilaterally placed cells (Fig. 11) known from marking experiments to be the lineage cells for the two larval pigmented cells (22). By placing normal embryos in cytochalasin at 1-hr intervals up to the time of first melanization (12 hr), it is possible to observe, on the basis of size change and cell position, that the pigment lineage cells of Ciona divide only three more times after the 32-cell stage, and that the two pigment cells of the larval brain (otolith and ocellus pigment cell) are direct descendants of the two bilateral cells at the 32-cell stage. At each division only one daughter cell retains the ability to form enzyme.

After the pigment lineage cells have passed through their 6th cleavage, cleavage-arrested embryos will develop melanin pigment in both lineage cells if the embryos are simply left in cytochalasin past the time of normal pigmentation (12–15 hr; Table 1). Melanin is the natural product of tyrosinase activity.
By doing an acetylcholinesterase reaction on such embryos after the melanin has formed, it is possible to see segregation for both enzymes in the same embryos (Fig. 12).

Cleavage-arrested 1-cell stages do not produce tyrosinase, and only small numbers of the cytochalasin-arrested embryos between the 2- and 16-cell cleavage stages produce enzyme. Usually just one of the bilateral lineage cells at these early stages develops tyrosinase; occasionally both do. At the arrested 32-cell stage (5th cleavage) up to 50-70% of the embryos produce dopa oxidase, and the majority of these have enzyme in both blastomeres. Embryos arrested after the pigment cell lineage has completed 6th cleavage (about 5 hr) will develop tyrosinase and even melanin in both lineage blastomeres of essentially all the embryos. Reasons for the low reactivity in the arrested early stages are not known; the proportion of reacting embryos in the early stages also varies greatly among groups of embryos.

Effect of Mitotic Inhibitors. Embryos that have been arrested with colchicine in the 2-cell stage and later cleavage stages illustrate exactly the same segregation patterns for development of the two enzymes as were seen with cytochalasin B. Complete series of colchicine-arrested embryos were examined. Selected cleavage stages reared in Colcemid and podophyllotoxin give rise to the expected numbers of enzyme-containing cells. The cells of embryos in these drugs do not remain rounded up and stationary as they do in cytochalasin, but tend to become distorted in shape and often to follow their normal migratory patterns. Interpretation of results is correspondingly more difficult with mitotic inhibitors.

Puromycin Inhibition of Enzyme Development. Puromycin prevents the occurrence of acetylcholinesterase activity in embryos treated continuously with it from 7 hr onwards. Embryos treated continuously from 8 hr onwards develop slight traces of enzyme activity. Similarly, embryos treated with puromycin from 8 hr onwards do not develop dopa oxidase activity, but slight localized activity occurs in embryos treated at 9 hr. The later puromycin treatment is begun, the more histochemical staining develops for either enzyme. Since these puromycin sensitivity periods coincide with the times that these enzymes are first detected, the enzymes are undoubtedly synthesized at these times.

The time of first acetylcholinesterase occurrence, as well as of puromycin sensitivity, is also between 7 and 8 hr in cleavage-arrested 8-cell stages. Likewise, the times of tyrosinase occurrence and of puromycin sensitivity are between 8 and 9 hr in cytochalasin-arrested 32-cell stages. This evidence strongly suggests that the enzymes arise de novo in later development and are not present in the fertilized egg.

Actinomycin D Inhibition of Enzyme Development. Actinomycin D prevents development of acetylcholinesterase in normal embryos reared in the drug from the 5th hr of development onwards. Embryos treated at 6 hr eventually develop low amounts of the enzyme activity in some embryos, and those treated at 7 hr develop a large amount of activity in most of the embryos. Similarly, actinomycin treatment at 6 hr prevents the appearance of dopa oxidase activity, but treatment at 7 hr and 8 hr does not. In either case, the later actinomycin treatment is begun, the more histochemically detectable enzyme activity develops. The differential effect of actinomycin D on the two enzymes rules out simple toxicity as an explanation of the inhibitory effect. In addition, embryos can be reared in actinomycin for 4 hr periods (from 4-8 hr for acetylcholinesterase or 5-9 hr for dopa oxidase) and many embryos still develop enzyme activity after removal from the drug. Such embryos develop abnormally, but actinomycin inhibition of enzyme occurrence is reversible.

The same actinomycin sensitivity periods are found for acetylcholinesterase occurrence in cytochalasin-arrested 8-cell stages and for dopa oxidase development in arrested 32-cell stages. Apparently, new RNA synthesis that begins between 5 and 6 hr is necessary for acetylcholinesterase development, and RNA synthesis that begins between 6 and 7 hr is needed for tyrosinase synthesis. These results suggest that mRNA templates for the enzymes are not present in the egg and are unlikely to be the materials segregated during cleavage.

Possibly mRNA synthesis is necessary to activate a preformed mRNA. However, there is no evidence of such activation for other examples of preformed mRNA in eggs (23). Also, since activation of a mRNA is likely to be an all-or-nothing process, one would not expect to see the graded effect of actinomycin observed above.

**DISCUSSION**

Two tissue-specific enzymes of *Ciona* embryos were observed to develop in cleavage-arrested embryonic cells; the enzymes differentiated in precursor cells of the appropriate lineage for the particular larval tissues in which they would normally occur. Since the occurrence of the enzymes is sensitive to puromycin inhibition, it seems unlikely that some form of the enzyme is being segregated. This finding implies the existence of enzyme-related cytoplasmic information localized in the egg and differentially segregated during cleavage. Since normal development occurs in at least some centrifugally stratified *Ciona* eggs (24), as well as in some of those from which up to half of the egg cytoplasm has been removed (25), the segregated information is undoubtedly not located in the free cytoplasm of the egg. To be so faithfully segregated by cleavage, this information is probably positioned in a "two-dimensional" map on the egg surface, perhaps in either the plasma membrane or the cortical gel layer. The very strictly determinate cleavage pattern of the ascidian egg would then apportion these surface materials to the appropriate blastomeres and cells.

These conclusions raise important unanswered questions about where such information originates, how it becomes positioned on the egg surface during oogenesis, and what activates it or causes it to leave the cell surface later in development. If the morphogenetic substances originate from the activity of the egg nucleus, as seems reasonable, then there must be specific sites on the developing egg surface where particular kinds of information become attached. Possibly these attachment sites are created by follicle cell activity (26).

Since enzyme-related RNA synthesis and subsequent enzyme synthesis occur at about the same times in cytochalasin-arrested as in normal embryos, a "clock" or counting mechanism of some kind must be determining the time at which cytoplasmic information can interact with the genome. This mechanism might possibly involve release of the information from cell surfaces. The clock is not apparently regulated by the events of cytokinesis nor, judging from similar results with colchicine-arrested cells, does it seem to be controlled by the mitotic cycle of the nucleus.

Many earlier investigators have concluded that morphogenetic substances in egg cytoplasm were being differentially...
segregated during early embryonic development (2), but the exact nature of the segregated materials has never been established. Most speculation focuses on the possibility that these are informational macromolecules that are able to initiate specific gene activities (27). Since small diffusible molecules could probably not exert the cell-specific control of enzyme expression seen clearly in cleavage-arrested embryos, the information is presumably in the form of larger molecules. However, no evidence excludes the intriguing possibility that such morphogenetic information might occur as stable conformations of ordinary cell-surface components, and not be informational macromolecules in any orthodox sense.

Preformed mRNA might conceivably be at least one kind of morphogenetic substance localized in determinate eggs, such as those of ascidians. Claims that no RNA synthesis occurs during ascidian larval development lend support to this view (28). However, there is equally persuasive evidence that RNA synthesis does occur (18, 19, 29), and it seems prudent for the moment to accept the positive demonstrations. Actinomycin experiments reported here demonstrate that enzyme differentiation is dependent on new RNA synthesis that occurs during gastrulation and early neurulation. This new RNA is likely to be mRNA for the tissue-specific enzymes that are synthesized several hours later.

This report indicates a relationship between preformed cytoplasmic information in ascidian eggs and the subsequent differentiation of histospecific enzymes of the embryo. Present evidence suggests that segregated information related to the occurrence of acetylcholinesterase and tyrosinase is neither the enzymes themselves nor mRNA for the enzymes. The unknown substances presumably function to initiate transcriptions of enzyme mRNA, and perhaps do so by establishing stable regulatory pathways for activating genes or groups of genes (6). Possibly the segregating materials are either proteins or RNA, which have been postulated as parts of these regulatory mechanisms (7). In any event, enzyme differentiation in cleavage-arrested eggs and embryos is an interesting phenomenon. It should prove useful for further exploration of the properties of egg cytoplasmic information and the relationship of this information to gene activation and the determination of embryonic parts.

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