ALTERED PATTERN OF GENE ACTIVITY IN ABNORMAL SEA URCHIN MORPHOGENESIS

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This is to certify that the

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Hiroaka Ozaki

Major professor

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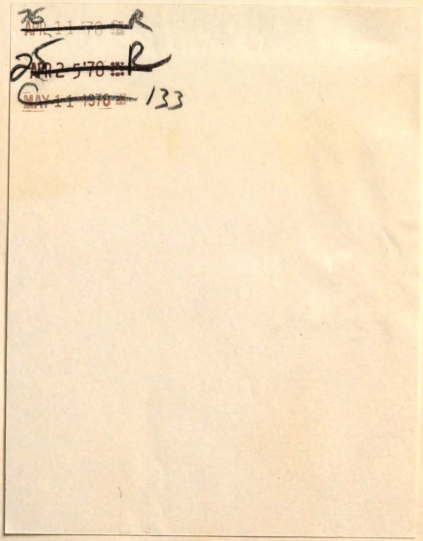
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ABSTRACT

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By

William Rogers Jeffery

Early sea urchin embryos can be experimentally manipulated, by addition of excess Zn^{++} to the sea water, such that abnormal embryos develop with exaggeration of their ectodermal characteristics and suppression of their mesentodermal characteristics. In order to test whether this alteration in development involves changes in the pattern of embryonic gene activity, the rate of transcription and the variety of transcription products in normal and animalized embryos were examined.

As an estimate of the rate of transcription, the kinetics of uridine incorporation into RNA of the embryos was examined. Cleavage stage embryos of both groups incorporate uridine at a low rate which increases after hatching in the normals and has its maximum level off at the prism stage. This acceleration is delayed by about 6 hours in the animalized embryos but the same rate is achieved by these in later development. This difference is not due to any differential permeability to precursor, nor can it be correlated with a difference in cell number between normal and animalized embryos.

Rapidly-labeled RNA was examined by sucrose gradient sedimentation and by RNA/DNA hybridization to DNA excess. Rapidly-labelled RNA from both types of embryos consists of heterogeneous, high molecular weight material, although some differences in size

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Rapidly-labelled RNA was examined by sucrose gradient sedimentation and by RNA/DNA hybridization in DNA excess. Rapidly-labelled RNA from both types of embryos consists of heterogeneous, high molecular weight material, although some differences in size

classes were observed. This RNA hybridized to non-repetitive DNA sequences readily, but to repetitive DNA sequences to a much lower extent.

In order to determine the complexity of the non-repetitive DNA sequences represented in RNA of normal and animalized embryos, hybrid formation between purified radioactive non-repetitive gastrula DNA and excess RNA was determined. RNA was isolated from unfertilized eggs and from normal blastulae and prism larvae and from animalized embryos of comparable ages. The results demonstrate that the complexity of transcription increases during the development of both normal and animalized embryos. Experiments in which RNAs isolated from two stages were combined indicated that extensive homology exists between the populations although some differences were detected between embryos of different ages as well as between normal and animalized embryos of the same age.

The evidence presented above indicates that animalization involves alterations in the pattern of embryonic gene activity and therefore that alterations in embryonic gene activity may be induced in embryos merely by altering the embryos' ionic environment. It is suggested that this effect on gene activity may be a secondary reflection of primary effects of Zn^{++} on the cell surface. The alterations in gene activity may be causal in the alteration of development.

ALTERED PATTERN OF GENE ACTIVITY IN ABNORMAL SEA URCHIN MORPHOGENESIS

By

William Robert Eckberg

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General Introduction

Developmental processes are believed to be controlled by the interaction of the individual's genome with its environment. Differentiation, the selective restriction of a cell's developmental potential, can thus be viewed as the result of such interactions. With such a frame of reference, it may be predicted that (1) alterations in genetic activity may be produced in embryos by altering their environment, and (2) that such alterations would cause alterations in the differentiative patterns of the embryonic cells.

Early development of the sea urchin is believed to be controlled by a coordination of two opposing gradients of morphogenetic influence, one maximal at the animal pole and the other maximal at the vegetal pole (reviewed by Runnström, 1933; Needham, 1942; Lallier, 1964). Disruption of this coordination through chemical or microsurgical manipulation can produce abnormal embryos with exaggeration of their ectodermal characteristics at the expense of their mesentodermal characteristics (called animalized embryos) or with exaggeration of their mesentodermal characteristics at the expense of their ectodermal characteristics (called vegetalized embryos). Strongly animalized embryos develop as a hollow ectodermal sphere with hyperextension of the acronal stereocilia and complete suppression of gut formation; strongly vegetalized embryos develop as a large gut possessing a few small ectoderm-like "blebs" (Hörstadius, 1939; Runnström and Immers, 1970). Such abnormal embryos may be produced by altering the embryos' ionic environment (Needham, 1942; Lallier, 1964). Runnström (1967)

hypothesized that the coordination of the animal and vegetal gradients is mediated through the activity of the embryonic genome.

The present study was undertaken to attempt to correlate experimentally-induced alterations in embryonic development with alterations in embryonic gene activity as a test of the first of the above predictions. Specifically, this study tests directly whether animalization (the production of animalized embryos) involves alterations in the activity of the embryonic genome.

Control Of Development By Variable Gene Activity

The theory of variable gene activity has evolved partially to explain how the fertilized egg is able to differentiate several different types of cells quickly after rapid cleavage. This theory suggests that differentiation is a result of the activity of certain genes or groups of genes and that different groups of genes are active in cells of different states of differentiation. This theory and its relevance to early development have been extensively reviewed by Davidson (1968).

Several important lines of evidence suggest that all cells of an organism contain the same genetic information. Driesch (1892), by exerting pressure on the embryos, was able to distribute early cleavage nuclei of sea urchin embryos to improper regions of the embryo; such embryos developed normally. Spemann (1938) constricted newt eggs so that the two nuclei of the first cleavage remained in one half of the embryo; after a number of cleavages, a daughter nucleus of the cleaving half escaped into

the non-nucleated half, thereby inducing it to cleave. If the constriction was then completed such that the embryo was divided in half, two normal twins resulted with the development of one delayed by the length of time required for the original non-nucleated half to acquire a nucleus. Finally, nuclei from various cells of embryonic and larval tissues of varying differentiated states have been transplanted into enucleated unfertilized amphibian eggs and these have been found capable in some cases of supporting normal differentiation (King and Briggs, 1956; DiBerardino and King, 1967; Gurdon, 1968).

McCarthy and Hoyer (1964) directly tested the equivalence of the genetic materials (DNA molecules) of cells of different differentiated states by molecular hybridization experiments. In their experiments no differences were detected between the DNAs of various adult mouse tissues.

Gene products (RNA molecules) of cells of different differentiated states are not all similar, however, as demonstrated by the same authors and by many others for adult tissues and for normally developing tissues. In sea urchin embryos, for example, differences are detected between RNAs of blastulae and prism larvae (Whiteley et al., 1966, 1970), of unfertilized eggs and blastulae (Glisin et al., 1966), of unfertilized eggs and cleaving embryos (Hynes and Gross, 1970; Mizuno et al., 1974) and between different cells of cleaving embryos (Mizuno et al., 1974). Similarly, the amount of genetic activity varies in cells of different differentiated states. Based on the rate of RNA transcription in vitro by isolated chromatin (Marushige and Ozaki, 1967; Ozaki, 1970) and by molecular hybridization to DNA

of the in vitro transcription products (Chetsanga et al., 1970), the activity of the genome in synthesizing RNA molecules increases during the development of the sea urchin embryo. Thus, while the genetic information is constant in the cells of a given individual, the expression of this information is different between different cells.

Development Of The Gradient Theory Of Sea Urchin Morphogenesis

With the discovery by Driesch (1891) that a normal, but small sea urchin embryo can develop from any blastomere isolated from a cleavage stage embryo up through the 4-cell stage, the idea was advanced by Driesch that the sea urchin embryo is an "harmonious equipotential system", in which any part can give rise to a complete larva. Other embryos, on the other hand, were considered to be "mosaics" in that isolated blastomeres could give rise only to partial embryos (see Wilson, 1925). However, after the third cleavage, isolated blastomeres or groups of blastomeres form normal or abnormal embryos, depending upon the regions of the egg contained in the fragments (Hörstadius, 1939; Berg and Cheng, 1962). Other experiments (Boveri, 1901; Hörstadius, 1939) demonstrated that regional differentiation was present in the unfertilized sea urchin egg along the animal-vegetal axis. By the sixteen-cell stage this regional differentiation is apparent in that three distinct types of blastomeres may be distinguished; tiny "micromeres" at the vegetal pole, a tier of large "macromeres" above them, and another tier of intermediate-sized "mesomeres" at the animal pole. Deletion experiments have shown that each type of blastomere has its own

prospective fate at this stage. Isolated animal half embryos (mesomeres) would develop into a hollow ciliated blastula-like structure with hyperdevelopment of the apical tuft (animalized embryo), whereas isolated vegetal half embryos (micromeres plus macromeres) typically developed into an embryo with an abnormally large and often evaginated gut (vegetalized embryo). These deletion experiments are consistent with the idea that the sea urchin egg, too, is a mosaic, but Hörstadius' (1939) recombination experiments demonstrated that the blastomeres have a broader developmental potential and that the cells of these regions were capable, under the proper influences, of giving rise to other parts of the normal embryo.

Comparable alterations in development have been obtained by adding certain chemicals to the embryos' medium. Herbst (1892) added Li^+ to the sea water in which sea urchin embryos were developing and observed hyperdevelopment of the gut structures at the expense of the ectodermal structures, or vegetalization. Backström and Gustafson (1953) observed that the period of maximum lithium-sensitivity is during early cleavage, the period of embryonic determination. This evidence suggests that lithium-induced vegetalization is a phenomenon of significance to the embryo. MacArthur (1924) suggested that this action was due to a differential suceptability of the animal region to Li^+.

Other chemical agents promote animalization. These include thiocyanate and iodide (Lindahl, 1936), sulfated organic molecules such as Evans Blue (Lallier, 1955b), Zn^{++} ions (Lallier, 1955a), and proteolytic enzymes such as trypsin (Hörstadius, 1949; Moore, 1952; Runnström and Immers, 1966) and pronase

(Lallier, 1969). The sensitivity towards all these agents has been shown to be greatest during the period of embryonic determination (Lallier, 1959, 1964).

Experimental results such as these have been cited in support of the theory that sea urchin development is controlled by the interaction of two opposing gradients of morphogenetic influence, one maximal at the animal pole and the other maximal at the vegetal pole (Runnström, 1933). Normal interaction of the two gradients would produce a normal larva, while suppression of one and/or activation of the other would produce animalization or vegetalization, depending upon which gradient is suppressed or activated. Suppression of the vegetal gradient or activation of the animal gradient would give rise to an animalized embryo, and suppression of the animal gradient or activation of the vegetal gradient would give rise to a vegetalized embryo.

The recent discovery that chemicals with morphogenetic activities may be extracted from unfertilized eggs (Hörstadius et al., 1967; Josefsson and Hörstadius, 1969) and from cleaving embryos (Hörstadius and Josefsson, 1972; Fujiwara and Yasumasu, 1974b) has given support to the theory that the gradients are chemical in nature, although most of the agents have not yet been pruified highly enough to allow the determination of their molecular structures. An antivegetalizing activity has been characterized, however, as 5-methyl cytosine (Fujiwara and Yasumasu, 1974b). This molecule is able to reduce vegetalization of normal embryos without producing typical animalization (cf. Hörstadius, 1972). An analogue of this molecule, 2-thio, 5-methyl cytosine, has been reported to possess an antivegetalizing

and possibly an animalizing activity (Gustafson and Hörstadius, 1956).

Non-Genetic Theories Of Animalization And Vegetalization

The mechanism of the vegetalizing action of Li^+ remains unknown. Lindahl (1936) demonstrated that lithium inhibits some reactions of glycolysis, but Backström (1959) has shown that the embryonic hexose monophosphate shunt activity is not affected by Li^+ treatment during the period of embryonic determination, suggesting that the effect on glycolysis is independent of the morphogenetic effects. Lindahl and Kiessling (1951) observed that Li^+ causes an accumulation of inorganic pyrophosphate in eggs treated during the period of determination and suggested that the formation of ATP is inhibited by Li^+. Runnström and Immers (1971) proposed that the colloidal state of the animal region of the embryo was affected by Li^+, preventing the diffusion of the hypothetical "animalizing substance." Ranzi (1957) reported that vegetalizing agents stabilize proteins in solution against denaturation and suggested that vegetalization results from the stabilization of embryonic proteins.

The mechanism of action of the animalizing agents is also unknown. Ranzi (1957) demonstrated that in contrast to the effect of vegetalizing agents, animalizing agents denature proteins in solution and suggested that animalization occurs under conditions of protein denaturation.

Horowitz (1940) showed that the rate of respiration in thiocyanate-treated embryos does not increase above the level found in normal blastulae, however Backström (1955) demonstrated

that the inhibition of respiration by iodosobenzoic acid is minimal during the period of embryonic determination. Backström (1959) also showed that the hexose-monophosphate shunt activity is the same between normal and animalized embryos during the period of embryonic determination.

Recent research has suggested that animalizing agents exert their effects at the cell surface (Lallier, 1968, 1972). It has been shown that Zn^{++} ions increase and Li^{+} ions decrease the animalizing actions of proteases (Lallier, 1969) and that the action of Li^{+} in decreasing the animalizing activity of pronase cannot be accounted for by a direct effect of Li^{+} on the properties of the enzyme. Proteolytic enzymes may act at the cell surface. It is not known whether they are able to enter the cell or whether this would be necessary for them to produce their effect. Recently Lallier (1972) has shown that concanavalin A, a phytohemaglutinin known to interact with the cell surface specifically, is also an animalizing agent and that its effect is enhanced by Zn^{++}. Lallier concluded that Zn^{++}, proteolytic enzymes, and concanavalin A all interfere with the same type of cell surface structures. Timourian (1968) demonstrated that the uptake of $^{65}Zn^{++}$ by sea urchin embryos is low during the period of embryonic determination, when they produce their effects on morphogenesis, further indicating that a primary effect may be at the cell surface level.

None of these possibilities are mutually exclusive, however. It is not unlikely that different animalizing and vegetalizing agents act at different levels to influence complicated control

mechanisms and any one agent may produce effects at more than one level of cellular activity.

Gene Activity And The Gradients

Autoradiographic studies have demonstrated regional differences in the incorporation of ^{14}C-adenine into sea urchin embryos (Markman, 1961a). Markman found that at the early blastula stage, adenine is more strongly incorporated into RNA in the animal region of the embryo, while after the mesenchyme blastula stage incorporation is stronger in the vegetal region. Further studies on isolated animal half embryos (Markman, 1967) showed an activation of incorporation at the blastula stage and a decrease at the gastrula stage of the controls.

The incorporation of precursor into ribosomal RNA (rRNA) is activated at the mesenchyme blastula stage (Giudice and Mutolo, 1967; Sconzo et al., 1970a), the stage at which vegetal differentiation begins to be expressed, but this same activation occurs in disaggregated cells of the embryos, even if the cells are not allowed to reaggregate (Sconzo et al., 1970b; Hynes et al., 1972). Thus, normal cell-to-cell interactions are unnecessary for the activation of rRNA accumulation. On the other hand, rRNA accumulation is inhibited in animalized embryos (Pirrone et al., 1970; O'Melia and Villee, 1972). Such results may be reconciled by two possible interpretations. The first is that rRNA synthesis, stability, and/or processing is specifically inhibited in animalization; the second is that the levels of the animalizing agents used by the investigators were too toxic and the lack of incorporation observed was really a non-specific effect of toxicity.

The transcription inhibitor, actinomycin D, has been used in the study of sea urchin morphogenesis. Gross and Cousineau (1963, 1964) demonstrated that concentrations of actinomycin D sufficient to block nearly all nuclear RNA synthesis would still permit development to occur through cleavage. Giudice et al., (1968) demonstrated that the process of gastrulation is sensitive to actinomycin D treatment at around the time of hatching. Lallier (1963) treated sea urchin embryos during the period of embryonic determination with actinomycin D and found that development was arrested. If the embryos were returned to sea water after the blastula stage had been reached, a great deal of recovery occurred if the concentration of actinomycin D had been low enough. Embryos treated simultaneously with Li^+ and actinomycin D became more strongly vegetalized than those raised in Li^+ alone, while Zn^{++} or Evans Blue-treated embryos were less strongly animalized in actinomycin D, suggesting that animalization and not vegetalization was dependent upon transcription. Markman and Runnström (1963) observed that the animalization of animal half embryos is reduced by treatment with actinomycin D, and also (Markman and Runnström, 1970) that the animalizing effect of trypsin and the "endogenous" animalizing substance of Hörstadius et al. (1967) are reduced by treatment with actinomycin D. However, in contrast to the results of Lallier (1963), the same authors reported that treatment of embryos with actinomycin D reduced the level of vegetalization by Li^+ (Runnström and Markman, 1966), suggesting that Li^+-induced vegalization, too, is gene dependent.

That protein synthesis is altered in animalized and vegetalized embryos may also indicate that gene-level control mechanisms are important in animalization and vegetalization. Berg (1968) concluded that the effect of Li^+ in reducing the rate of protein synthesis in advanced embryos was a secondary reflection of a primary effect on transcription. Carroll et al. (1974) demonstrated that the electrophoretic pattern of newly-synthesized proteins was different between normal and animalized embryos. O'Melia (1972) reported that esterase isozyme activities which appear in normal pluteus larvae do not appear in animalized embryos of the same age. These results, as well, are consistent with an interpretation based on an altered pattern of transcription in the abnormal embryos.

The protein synthesis inhibitor, chloramphenicol, has been found to be a vegetalizing agent (Lallier, 1962; Hörstadius, 1963; Fujiwara and Yasumasu, 1974a). This finding was originally interpreted to indicate that animal differentiation requires protein synthesis. However, another protein synthesis inhibitor, puromycin, did not act as a vegetalizing agent (Fujiwara and Yasumasu, 1974b), suggesting that this activity may not derive from the inhibitory effect on protein synthesis. Inconsistent effects have been obtained when attempts have been made to animalize or vegetalize embryos or isolated animal and vegetal half embryos by amino acids and amino acid analogues (Gustafson and Hörstadius, 1955, 1957; Fudge, 1959; Bosco and Monroy, 1960).

Experiments using inhibitors of nucleic acid and protein synthesis to test for possible gene dependence of animalization and vegetalization have been inconsistent. Experiments using

actinomycin D to test for gene-dependence of animalization have been consistent with such an interpretation, but the results of similar experiments to test the gene-dependence of vegetalization have been inconclusive. It has already been shown that the vegetalizing effect of chloramphenicol probably does not derive from its effect on translation; such results clearly emphasize the major limitation of experiments using metabolic inhibitors: that the experimental results obtained using them do not always reflect specific effects of the inhibitors. Thus these data cannot be said to demonstrate conclusively that embryonic gene activity is involved in determination in the sea urchin embryo.

Gene Activity And Gastrulation

The cell movements involved with gastrulation in the sea urchin embryo have been extensively discussed by Gustafson and Wolpert (1967). Gastrulation begins when the presumptive primary mesenchyme cells, derivatives of the micromeres, detach from the blastula wall, round up, begin characteristic "pulsatory" movements, and migrate into the blastocoel. There they assume a characteristic ring-shapped pattern, fuse into a "cable", and induce an invagination of the presumptive endoderm at the vegetal pole. Cells at the tip of the invaginating archenteron which are destined to become the embryonic mesoderm or "secondary mesenchyme" then send out pseudopodial processes which attach to the blastocoel wall and pull the archenteron in further. Gut formation is completed with the invagination of the ectoderm at the point of contact with the archenteron to form the larval

mouth. At the same time, the cable of primary mesenchyme is differentiating spicules which elongate to give the larva its typical pluteus or "easel" shape. Microtubules have been suggested to be the primary determinants of cell shape in the cable and pseudopods (Gibbins et al., 1969). The ability of the presumptive primary mesenchyme cells to induce gut formation has been suggested to be related to their surface coat of acid mucopolysaccharides (Karp and Solursh, 1974). In addition, it has been shown that serotonin antagonists inhibit the release of primary mesenchyme cells and the onset of invagination and that this inhibition may be reversed by the addition of serotonin (Gustafson and Toneby, 1970, 1971). Treatment with actinomycin D beginning before hatching and extending to the mesenchyme blastula stage also suppresses gastrulation, indicating that gastrulation is gene-dependent (Guidice et al., 1968). At this same time in development, novel gene groups are known to be activated (Whiteley et al., 1966, 1970). These results suggest that gastrulation is under the control of the embryonic genome and that this genomic control may be expressed through the production of serotonin and acid mucopolysaccharides by the presumptive mesenchyme cells.

Control Of Development By Ions

Inorganic ions have been shown to be able to control patterns of differentiation in other systems as well as sea urchin embryos. Barth and Barth (1969, 1972, 1974) have shown that epidermal explants of amphibian gastrulae can be induced to differentiate into nerve and pigment cells under the

influence of Ca^{++}, given the proper ionic environment. Phytohemagglutinin-induced transformation of lymphocytes has been shown to have an absolute requirement for divalent cations (Alford, 1970) and to be accompanied by a temporary uptake of Ca^{++} from the medium (Allwood et al., 1971; Whitney and Sutherland, 1972). Zn^{++} ions were very effective in supporting transformation even in the absence of Ca^{++} (Alford, 1970), and Zn^{++} alone can actually induce transformation (Kirchner and Rühl, 1970).

Direct effects of the ionic environment have been demonstrated on the pattern of puffing of salivary chromosomes (Kroeger and Lezzi, 1966). Such ionic alterations can mimic hormonal effects on the puffing pattern. In addition, ions have been shown to modify the intracellular metabolism of cyclic nucleotides (Rasmussen, 1970). It has been hypothesized that an interaction of intracellular inorganic ions with cyclic nucleotides is responsible for differentiation in many systems, including neural induction, slime mold aggregation, mitosis, and gastrulation in embryos, and chemical teratogeneisis (McMahon, 1974).

STATEMENT OF THE PROBLEM

If variable gene activity is an essential component in the control of developmental processes, then alterations in developmental processes should also involve alterations in the activity of embryonic genes. Animalization involves specific morphological alterations in development, viz. suppression of mesentodermal differentiation and enhancement of ectodermal differentiation. The transcription inhibitor, actinomycin D, interferes with the process of animalization (Lallier, 1963; Markman and Runnström, 1963, 1970), and such experiments led Runnström (1967) to hypothesize that the co-ordination of the animal-vegetal gradients is mediated through the activity of the embryonic genome. In addition, it has been reported that the accumulation of one gene product, rRNA, is inhibited in animalization (Pirrone et al., 1970; O'Melia and Villee, 1972).

A clear demonstration that animalization is gene-dependent must show that specific alterations in gene activity occur in animalization and that these alterations are responsible for the abnormal morphogenesis. The objective of the present research was to determine, experimentally, whether or not animalization actually involves alterations in the pattern of embryonic gene activity. Two experimental approaches have been taken; one to determine the extent of gene activity (RNA synthesis) quantitatively, and the other to examine the diversity of gene products present in normal and animalized embryos.

MATERIALS AND METHODS

Culture of Embryos

Gametes of the sea urchin, Strongylocentrotus purpuratus (Pacific Bio-Marine, Venice, California, or Controlled Environments, Bellvue, Washington) were obtained by the injection of isotonic KCl and fertilized (Tyler and Tyler, 1966), and the embryos were cultured at a concentration of 1% v/v in artificial sea water (Instant Ocean, Aquarium Systems, Eastlake, Ohio) at 15°C either in monolayer cultures in petri dishes or in continuous agitation by a stirrer rotated at 30 rev/min. In addition, cultures contained 250 µg/ml streptomycin sulfate to inhibit bacterial growth.

Chemical Animalization

For animalization, zinc sulfate was added to the sea water to the appropriate concentration. A stock solution of 0.1M $ZnSO_4$ was diluted directly into the sea water. Under these conditions, the $ZnSO_4$ had no effect on the pH of the sea water at the concentrations used. The zinc ion used has been shown to be an effective animalizing agent (Lallier, 1955a, 1959). In any one experiment eggs from the same batch were used for both control and experimental culture.

Determination of the Number of Cells per Embryo

During early cleavage stages, whole cells were counted in living embryos. For later stages, the embryos were fixed in

neutral 1% formalin made in artificial sea water, and squash preparations were made with the aceto-orcein fast green stain (Kurnick and Ris, 1948). The nuclei were then counted using an ocular grid (American Optical 1409A) for reference. The counts were taken to represent the number of cells per embryo. Standard deviations of the nuclear counts from different embryos from the same population were always less than 10% of the mean number of cells for individuals in the population.

Uptake and Incorporation of Radioactive Uridine

Equal aliquots containing approximately 10^4 embryos were obtained from the cultures at the times specified, washed, suspended to 0.25 ml in sea water containing 250 µg/ml streptomycin sulfate, and incubated with 2 µCi/ml ^{3}H-5-uridine (specific activity 20 Ci/mmole) Schwartz-Mann, Orangeburg, N.J.) for the length of time specified for each experiment. Incubation was stopped by the addition of ice-cold sea water containing a 10^4-fold excess of non-radioactive uridine. The embryos were collected on Whatman 3MM paper discs by filtration, washed and dried. For the determination of uptake, the discs were placed directly in a toluene based scintillation fluid containing 4 gm/l PPO and 0.25 gm/l dimethyl POPOP without further treatment and their radioactivity was measured by a Packard Tri-Carb 3320 Spectrometer. For the determination of incorporation into RNA, the dried discs were washed for 15 min successively in each of two changes of ice cold 5% trichloroacetic acid (TCA), one of 95% ethanol and one of ethyl ether. The discs were then dried and their radioactivity was measured as above. Filter discs of control and animalized embryos were always processed

together in any given experiment. The radioactivity was expressed as the net counts after subtraction of background. The relative net standard error of the counts was always less than a 5% of the total counts.

Preparation of Radioactive RNA

Prism stage embryos (48 hr) and animalized embryos of the same age were labelled at a concentration of 10% v/v in artificial sea water containing 250 µg/ml streptomycin sulfate and 3.3 µCi/ml uridine 5-^{3}H for 30 min. RNA was extracted by a modification of the hot phenol-SDS method of Girard (1967). Embryos were homogenized in 5 vol acetate-EDTA (0.01M Na acetate, 0.01M Na EDTA, pH 5.1) containing 0.1% SDS and 0.1% bentonite, and RNA was extracted at 60° by shaking with an equal volume of phenol. The aqueous phase was reextracted twice with phenol at 0° and the RNA precipitated by ethanol.

Sucrose Gradient Centrifugation Analysis of Radioactive RNA

Radioactive RNA was layered over a 28 ml linear 2.5-15% sucrose gradient made up in acetate-EDTA and centrifuged in a Spinco SW25.1 rotor at 25,000 rev/min for 18 hr at 4°. Fractions were collected dropwise after bottom puncture, and radioactive RNA coprecipitated by 10% TCA with 100 g/ml yeast RNA. Precipitates were collected on glass fiber filters (Reeve-Angel # 984H), rinsed with 5% TCA, dried at 60°, and their radioactivity determined by liquid scintillation counting.

Extraction of Sea Urchin Sperm DNA

DNA was extracted from sperm by the method of Whiteley et al. (1970). One gm of washed sperm was suspended in 125 ml EDTA-Tris (0.1M EDTA, 0.04M Tris-HCl, pH 8.2) and an equal volume of EDTA-Tris containing 2% SDS was added and the lysate heated to 60° for 10 min. The lysate was then treated at 37° with 50 g/ml pronase for 8 hr. Pronase had been preincubated at 1 mg/ml for 2 hr to destroy nuclease activity. DNA was extracted with an equal volume of phenol and reextracted with an equal volume of chloroform-iso-amyl alcohol (24:1) and precipitated by ethanol.

Shearing and Alkaline Sedimentation Analysis of DNA

DNA was dissolved at a concentration of 1-2 mg/ml in 0.1 x SSC (0.15M NaCl, 0.015M Na citrate, pH 7.0), sheared in an omnimixer at maximum speed for 5 min, further sheared by sonication (20 sec at maximum noise in 2 ml or less volume), dialyzed against distilled water, and lyophilized. DNA was than dissolved in 0.12M PB (equimolar mixture of mono- and disodium phosphate) to the desired concentration (4-8 mg/ml). Sometimes shearing by sonication was performed after lyophilization. Neither the sedimentation nor the reassociation properties of the DNA was affected by delaying sonication.

Alkaline sedimentation was performed according to the procedure of Abelson and Thomas (1966). 50-100 g DNA was alkalai-denatured (0.33N NaOH, 10 min at room temperature) and layered over a 4.8 ml linear 5-20% sucrose gradient made up in 0.9M NaCl, 0.1M NaOH. Gradients were centrifuged at 20°C and 50,000 rev/min for 6 hr in an SW50L rotor. Fractions were collected

dropwise after bottom puncture and the A_{260} of each fraction was determined. Radioactive samples were coprecipitated with 100 µg/ml sea urchin sperm DNA by 10% TCA and processed as above for RNA. The $S_{20,w}$ was calculated for the peak according to the equation of Abelson and Thomas (1966) and the single-stranded nucleotide length was determined according to Wetmur and Davidson (1968). Samples longer than 400-500 nucleotides were resonicated to that length, except in one experiment in which the reassociation kinetics of 900 nucleotide-long fragments were determined.

Reassociation of Sea Urchin Sperm DNA

Sheared DNA (100 µg) was heat denatured (10 min at 100°) and allowed to reassociate at 60° to various Cots (moles nucleotide x sec x liter^{-1}, Britten and Kohne, 1968) in either sealed capillary tubes or in MicroFlex tubes (Kontes Glass Company). Reassociation kinetics were measured in two ways:

1. Single and double-stranded components were separated by chromatography on a 1.2 x 1 cm hydroxy-apatite (HAP) column at 60°. Single-stranded DNA was eluted with 10 vol 0.12M PB; double-stranded DNA was eluted with 10 vol 0.5M PB. The percent reassociated was calculated from the absorbance readings (at 260 nm) of the material recovered from the column. The 0.5M PB fraction absorbance was multiplied by a factor of 1.28 to compensate for its hypochromicity (Melli and Bishop, 1969).
2. Single-stranded DNA was degraded by S1 nuclease. S1 nuclease was prepared from Aspergillus oryzae α-amylase (Sigma) through step 4 of the method of Vogt (1973), and concentrated by

chromatography on a small column of DEAE-cellulose. Reassociated DNA was diluted to 150 µg/ml with 0.1M NaCl, 0.001M $ZnSO_4$, 5% glycerol, 0.03M Na acetate, pH 4.6 and treated with 20-50 units/ml S1 nuclease at 45° for 30 min. For non-radioactive DNA, samples were chilled, precipitated by 5% perchloric acid, filtered through a Millipore filter, and the absorbance (at 260 nm) of the filtrate (single-stranded material) was read. Radioactive samples were precipitated by TCA with 100 µg/ml bovine serum albumin (BSA) and the radioactivity in double-stranded material was determined by liquid scintillation counting as described above for RNA.

RNA/DNA Hybridization in DNA Excess

Before hybridization, radioactive RNA was digested at 37° for 1 hr with 50 µg/ml DNase (RNase-free, Worthington). After additional deproteinization with pronase (50 µg/ml for 1 hr at 37°) and phenol, the RNA was precipitated by ethanol. The RNA was then dissolved in 0.12M PB and sheared to approximately 6s by sonication. Final preparations had specific activities of approximately 1000 dpm/µg and the radioactivity was greater than 99.5% alkalai-labile. RNA specific activities from normal and animalized embryos were identical.

Sheared radioactive RNA (1 µg) was added to a 100-fold excess of sperm DNA in 0.12M PB containing 0.1% SDS. After heat denaturation (5 min at 100°) the mixtures were incubated at 60° to the desired DNA Cot, diluted to 5 ml in 0.24M PB and divided in half. One half was incubated with 20 µg/ml RNase (bovine pancreas, Worthington) for 20 min at 37°. RNase had

been heated previously to 80° for 10 min in 0.24M PB to destroy DNase activity. The other half was treated similarly but without RNase. Mixtures were precipitated by TCA, and the radioactivity was determined as above.

Preparation of Radioactive Non-repetitive DNA

A 1% suspension of embryos was labelled from fertilization to the early gastrula stage (36 hr) with 2 μCi/ml of thymidine methyl-^{3}H (16.7 Ci/mmole, Schwartz-Mann). DNA was purified by the method of Marmur (1961) from nuclei prepared by the procedure of Loeb (1969). Embryos were washed twice in 0.53M NaCl, 0.53M KCl (19:1), once in 1M dextrose, and once in SSC. The final pellet was suspended in 30 vol SSC and homogenized by two passages through a #20 gauge hypodermic needle. The homogenate was mixed with an equal volume of 2M sucrose and centrifuged at 15,000 x g for 30 min. The nuclear pellet was suspended in 5 ml EDTA-Tris, heated to 60° for 10 min, made 1% in SDS and 1M in $NaClO_4$, shaken with an equal volume of chloroform-isoamyl alcohol and precipitated by ethanol. DNA was dissolved in 5 ml 0.1 x SSC and incubated with 50 μg/ml heat-treated RNase at 37° for 30 min. DNA was made to 0.1M Tris, pH 9.0, 0.1M NaCl, and 1% SDS, shaken with an equal volume of phenol, and precipitated by ethanol. Final preparations had specific activities of about 120,000 dpm/μg.

Purified DNA was then dissolved in 0.12M PB and sheared. An aliquot was denatured and allowed to reassociate in a large excess of non-radioactive sperm DNA. The kinetics of reassociation of radioactive and non-radioactive DNA were the same.

Radioactive DNA was then heat-denatured and incubated to Cot 30. Single-stranded material was purified from HAP, heat-denatured, reincubated to Cot 60, and repurified from HAP. The purified non-repetitive DNA (single-stranded after both incubations) was dialyzed against distilled water, lyophilized, and dissolved in a small volume of 0.12M PB. That this purified non-repetitive DNA is essentially free from repetitive DNA sequences was shown by the reassociation kinetics of the radioactively labelled non-repetitive DNA in the presence of a large excess of non-radioactive total sperm DNA (fig. 11).

RNA/DNA Hybridization in RNA Excess

Non-radioactive RNA was extracted from unfertilized eggs and from normal blastulae (24 hr) and prism larvae (48 hr) and from animalized embryos of comparable ages as described above. The isolated RNA was further pruified by DNase digestion and cetyltrimethyl ammonium bromide precipitation (Bellamy and Ralph, 1968), sheared, dialyzed against distilled water, lyophilized, and dissolved in 0.5M NaCl, 0.001M EDTA, 0.02M Tris-HCl, pH 7.4, containing 0.1% SDS (after Leong et al., 1972).

Heat-denatured radioactive non-repetitive DNA was incubated with an excess of RNA in the above buffer at 60°. After incubation the mixtures were diluted to 150 µg/ml RNA in the S1 nuclease buffer and divided in half. Half was treated with S1 nuclease as described above, and the other half was treated similarly but without S1 nuclease. Table I summarizes the conditions of incubation and gives the RNA/DNA ratios used. All incubations were to the same equivalent Cot with respect

Table I

Incubation conditions for saturation of radioactive non-repetitive DNA with RNA

RNA input (mg/ml)	DNA input (μg)	volume* (ml)	incubation time (hr)	RNA/DNA	equivalent DNA Cot
10	0.3	0.3	48	10,000	2.14
10	0.3	0.15	24	5,000	2.14
10	0.6	0.15	12	2,500	2.14
5	0.6	0.15	12	1,250	2.14
5	0.6	0.1	9	833	2.14
2.5	0.6	0.1	9	417	2.14
1.25	0.6	0.1	9	209	2.14

*incubation buffer: 0.5M NaCl, 0.001M EDTA, 0.02M tris, pH 7.4. Incubation temperature: 60°.

to DNA (2.14) to eliminate variation in the data due to DNA renaturation.

In order to determine the extent of DNA renaturation and the possible contamination of the RNA preparations with DNA, RNA which had been hydrolyzed (0.3N NaOH, 18 hr at 37°) was incubated with the DNA as above. Less than 1.1% of the input radioactivity was S1 nuclease-insensitive and this value was subtracted from the observed percents reassociated. Since this value was not greater than the value observed at that Cot in the excess of total sperm DNA (fig. 10), the RNA preparations were not contaminated with DNA.

Thermal Stability of Hybrids

The thermal stability of sheared native DNA and of reassociated DNA was determined by HAP chromatography. Duplex structures were absorbed to the column in 0.12M PB at 60°, and the temperature of the column was raised to 100° in increments of 5°. After equilibration for 5 min at each 5° increment the column was washed with 5 vol of 0.12M PB. The A_{260} or radioactivity was determined in each fraction, and the percent released was calculated based on the total released A_{260} or radioactivity. The cumulative percent released was then plotted as a function of temperature of elution.

The thermal stability of radioactive non-repetitive DNA/total sperm DNA hybrids and non-repetitive DNA/RNA hybrids was determined by S1 nuclease digestion. Hybrids were diluted to 0.1M NaCl and their thermal stability was determined by raising the temperature of the solution from 60° to 100° in increments

of 5° as above. At each 5° increment an aliquot was removed and digested with S1 nuclease, and the S1 nuclease-insensitive radioactivity was determined.

RESULTS

Animalization of Sea Urchin Embryos by Treatment with the Zinc Ion

In order to determine the concentration of zinc ions which exerted the most specific effect and produced an homogeneous population of morphologically well definable animalized embryos, the embryos were cultured beginning 1 hour after insemination in the continuous presence of three different added concentrations of zinc sulfate: 10^{-4}M, 5 x 10^{-4}M, and 10^{-3}M. The highest concentration was too toxic and the embryos tended to degenerate. The lowest concentration, on the other hand, appeared to exert a marginal effect, and consistent effects were not obtained. Many of the embryos thus treated developed atypical spicules and some formed gastral plates, although none ever actually invaginated a gut. The intermediate concentration gave very uniform and reproducible results. The embryos developed into large thin-walled motile blastulae covered with stereocilia and endowed with a small aggregate of cells in the blastocoel near the vegetal pole (figure 1). Primary mesenchyme migration occurred at the same time as in the controls, but hatching was delayed by about 12 hours and was less synchronous in the animalized embryos. Such embryos differentiated larval pigmentation, but only after 5-7 days. Normal embryos develop such pigmentation within 36-48 hr. Animalized embryos survived as long as non-fed control embryos. The zinc concentration of

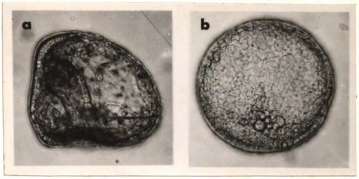

Figure 1

a. Normal prism larva of Strongylocentrotus purpuratus. Regional differentiation of the gut and the presence of the skeletal spicules are apparent. (x 250)

b. Animalized embryo of the same age and from the same batch as the embryo shown in (a) but raised in the presence of 5×10^{-4}M $ZnSO_4$ added to the sea water. The outer diameter of the animalized embryo is greater due to the fact that the presumptive endodermal and mesodermal cells are a part of the ectoderm. Aggregated descendents of the primary mesenchyme cells are in the blastocoel and skeletal spicules are lacking (x 250)

5×10^{-4}M appeared to exert the most specific effects on sea urchin development; therefore this concentration was used in all subsequent experiments.

Rate of Cleavage

Animalization did not alter the rate of cleavage. The number of cells per embryo showed no detectable difference between animalized and normal embryos at any time during development (figure 2).

Rates of Uridine Uptake and Incorporation

Under the present conditions of labelling, the uptake of uridine continued at a nearly constant rate for at least the first 60 min of exposure; however the rate of incorporation of uridine into TCA-precipitable material became reduced after 20-30 min (figure 3). This reduced incorporation rate reflects the turnover of newly synthesized RNA (Kijima and Wilt, 1969). Thus for the following experiments, a 30 min pulse of radioactive uridine was used.

In the course of normal development the rate of uridine uptake increased by a factor of 6 from the level of the pre-hatching stages (before 25 hr of development) to that of the mid-gastrula stage (after 35 hr). The animalized embryos followed essentially the same pattern (figure 4).

In the course of normal development a relatively low level of incorporation prevailed before hatching, after which the incorporation rate increased rapidly through gastrulation and continued to rise at a reduced rate through the prism stage. The increase is approximately 18-fold by 61 hr of development. In the animalized embryos, the onset of the rise in the incorporation rate was delayed by about 6 hr, but after 45 hr of development the rates of incorporation were identical in control and animalized cultures (figure 5).

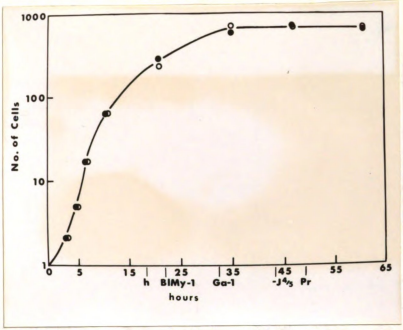

Figure 2

Number of cells during the development of normal (○) and animalized (●) sea urchin embryos and major morphological events occurring in normal development. h, hatching blastula; BlMy-1, early mesenchyme blastula; Ga-1, first evidence of invagination; -J4/5, gut invaginated 4/5 of body length; Pr, prism larva (Whiteley and Baltzer, 1958).

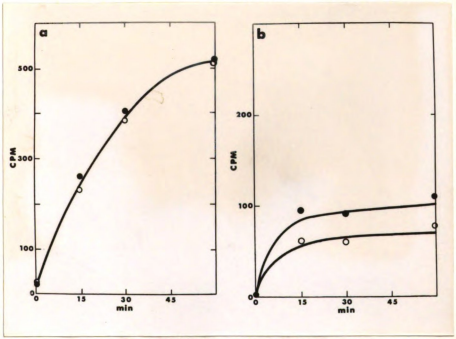

Figure 3

a. Uptake of ^{3}H-uridine during a 60 min pulse by normal (●) and animalized (0) embryos.
b. Incorporation of ^{3}H-uridine into TCA-insoluble material during a 60 min pulse by normal (●) and animalized (0) embryos.

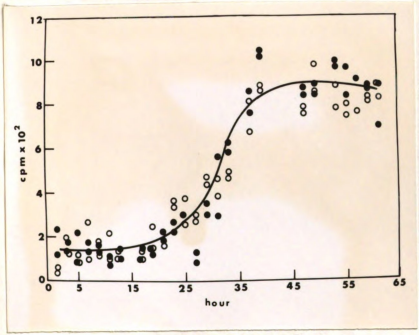

Figure 4

Uptake of ^{3}H-uridine during a 30 min pulse by normal (0) and animalized (●) sea urchin embryos during development.

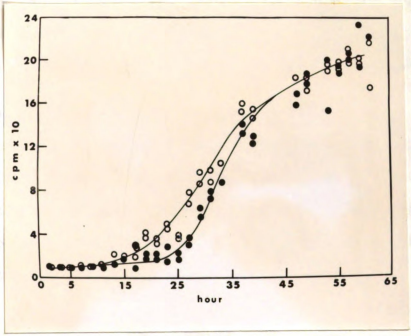

Figure 5

Incorporation of ^{3}H-uridine into TCA-insoluble material by normal (0) and animalized (●) embryos during development.

Molecular Size of Rapidly-Labelled RNA

The molecular size characteristics of rapidly-labelled RNA have been determined by sucrose gradient analysis (figure 6). Such RNA from both normal and animalized embryos consists primarily of heterogeneously-sedimenting, high molecular weight material. It was reproducibly observed that the radioactive label tended to be in larger molecular weight material in the animalized embryos than in the normal embryos.

Molecular Size of Sheared DNA Fragments

The alkaline sedimentation characteristics of sheared radioactive gastrula DNA is shown in figure 7. The $S_{20,w}$ at the center of the peak has been calculated (Abelson and Thomas, 1966) to be 6.3, corresponding to a single-stranded nucleotide length of 450 (Wetmur and Davidson, 1968).

Genomic Structure of the Sea Urchin

The reassociation kinetics of sea urchin sperm DNA fragments 450 and 900 nucleotides long have been determined by HAP chromatography (Table II, figure 8). 450 nucleotide-long fragments appear to consist of three distinct components; one reassociating with a $\frac{1}{2}$Cot of less than 0.01 and comprising about 8% of the genome, one reassociating with a $\frac{1}{2}$Cot of approximately 0.3 and comprising approximately 30% of the genome, and a third component reassociating with a $\frac{1}{2}$Cot of approximately 250 and comprising approximately 60% of the genome. Based on the reassociation kinetics, the first two components are considered to consist of repetitive sequences and the third is considered to consist of non-repetitive sequences (Britten and Kohne, 1968).

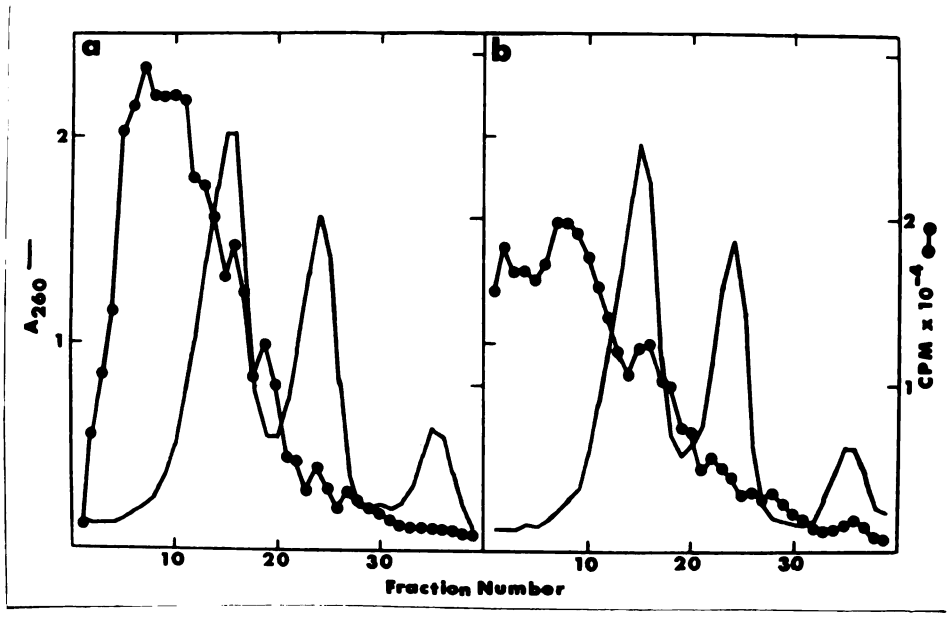

Figure 6

Sucrose gradient centrifugation analysis of newly-synthesized RNA from normal (a) and animalized (b) sea urchin embryos labelled for 30 min with ^{3}H-uridine beginning 48 hr post-fertilization.

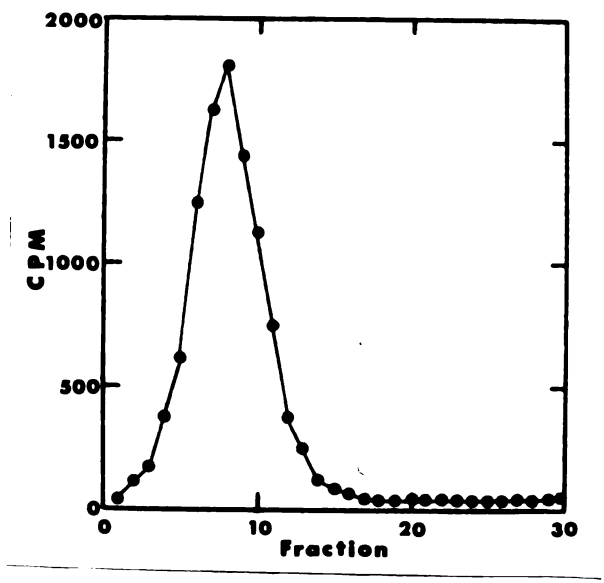

Figure 7

Alkaline sucrose gradient sedimentation analysis ^{3}H-thymidine-labelled gastrula DNA. The peak (fraction 8) corresponds to an $S_{20,w}$ equal to 6.3 (Abelson and Thomas, 1966) and a single-stranded nucleotide length of 450 (Wetmur and Davidson, 1968).

Table II

Sea urchin sperm DNA reassociation kinetics

Cot	A_{260}/ml	hr	%binding[1]	corrected binding[2]	% recovered[3]
0.01	0.202	0.1	7.0	0.0	108
	0.404	0.05	8.5	1.5	111
0.1	0.404	0.5	12.9	6.2	104
	0.505	0.4	8.5	1.5	109
0.3	2.02	0.33	19.3	13.1	98
1.0	2.02	1.0	25.0	19.2	106
	4.04	0.5	34.7	29.7	106
3.0	2.02	3.0	35.4	30.4	107
	4.04	1.5	36.5	31.6	100
	4.04	1.5	37.0	32.2	86
10.	10.1	2.	41.0	36.5	98
	10.1	2.	42.0	37.6	145
	20.2	1.	40.7	36.2	107
30.	10.1	6.	50.5	46.7	116
	20.2	3.	52.0	48.3	101
	20.2	3.	48.7	44.8	78
100.	101	2.	64.5	61.8	126
	101	2.	65.0	62.3	111
	101	2.	69.0	66.6	105
	202	1.	61.0	58.0	85
300.	101	6.	73.5	71.5	96
	101	6.	73.5	71.5	103
	202	3.	72.0	69.9	85
1000.	101	20.	93.0	92.5	84
	101	20.	92.0	91.4	118
	202	10.	93.0	92.5	84
3000.	101	60.	96.5	96.2	88
	202	30.	97.0	96.8	103
10000.	101	200.	99.0	98.9	80

values were obtained in three separate experiments using two independent preparations of DNA from Strongylocentrotus purpuratus sheared to 450 nucleotides. All incubations carried out in 0.12M PB at 60°C; single- and double-stranded fractions separated by HAP.

[1]= 1.28 x A_{260} in 0.5M PB/total recovery.

[2]= (observed fraction bound - zero time binding)/(1 - zero time binding) x 100. (zerotime binding was 7.1%).

[3]= (A_{260} in 0.12M PB + 1.28 x A_{260} in 0.5M PB/input A_{260}) x 100

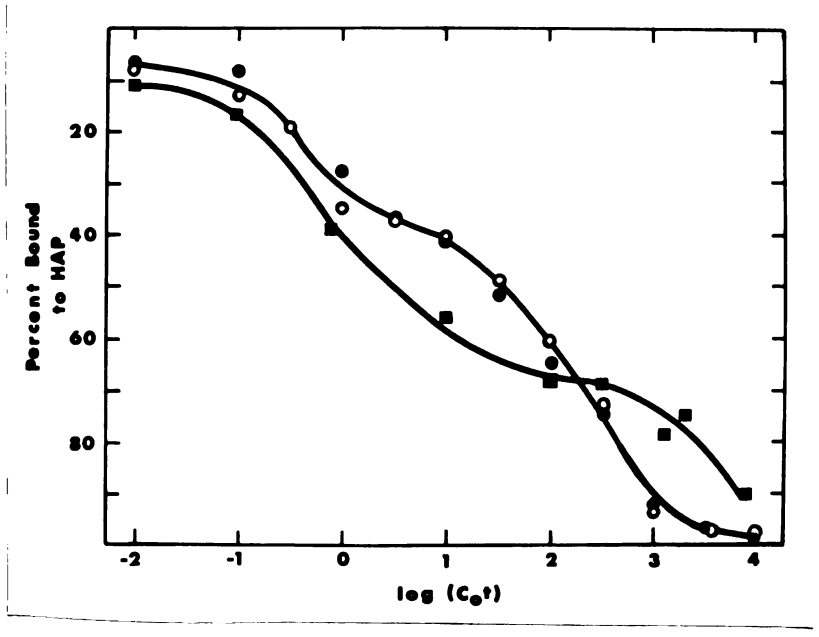

Figure 8

Reassociation kinetics of 900 and 450 nucleotide long fragments of sea urchin sperm DNA assayed by hydroxyapatite (HAP) chromatography. The open and filled circles represent data obtained in different experiments using independent preparations of 450 nucleotide long DNA fragments; the filled squares represent data from the 900 nucleotide long fragments.

900 nucleotide-long fragments also show components with characteristic ½Cot values. 11% of the genome reassociates before a Cot of 0.01, 40% of the genome reassociates broadly over Cots of 0.1 to 100, and 20-30% of the genome reassociates with a ½Cot of at least 1000.

The thermal stability of reassociated sea urchin sperm DNA as assayed by HAP chromatography is given in figure 9. The Tm is 82.5°C, almost identical to that of sheared native DNA (83.5°C), indicating that reassociation has been specific, and thus that the reassociation conditions have been stringent.

The reassociation kinetics of 450 nucleotide-long fragments of sea urchin sperm DNA have been determined by S1 nuclease digestion. Assayed under these conditions 30% of the DNA has reassociated by a Cot of 10, and the slowly-reassociating component reassociates with a ½Cot of approximately 800 (figure 10).

Templates for Rapidly-Labelled Heterogeneous RNA

In order to determine whether rapidly-labelled RNA is transcribed from repetitive or non-repetitive DNA sequences and whether the relative transcription from repetitive and non-repetitive DNA sequences is different between normal and animalized embryos, RNA/DNA hybridization was carried out in DNA excess. RNAs from normal prism larvae and from animalized embryos of the same age both reassociated primarily with non-repetitive DNA (figure 11). At a Cot of 30, 7-10% of the material was RNase-insensitive, but since 3-4% was RNase-insensitive at zero-time, the actual amount hybridized is probably on the order of 5% at

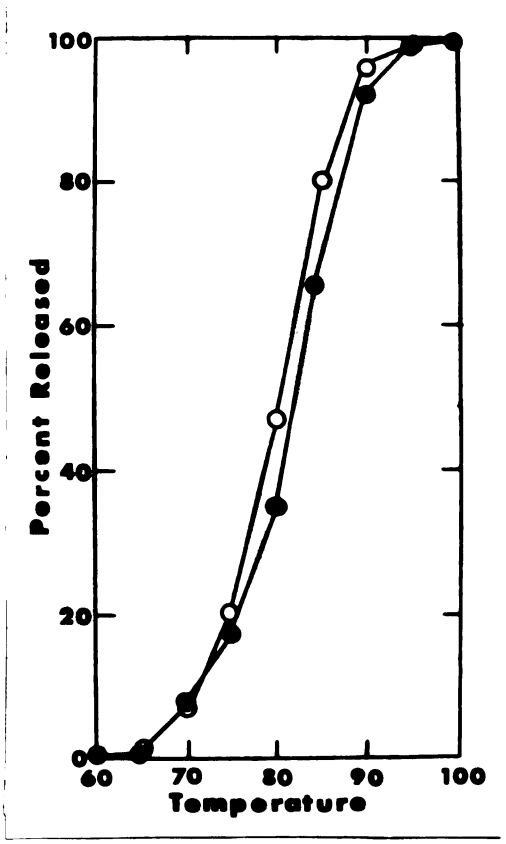

Figure 9

Thermal stability of reassociated sperm DNA/DNA hybrids (●) and ^{3}H-gastrula DNA/sperm DNA hybrids (0). The Tm's are 82.5^{o} and 80.0^{o}, respectively.

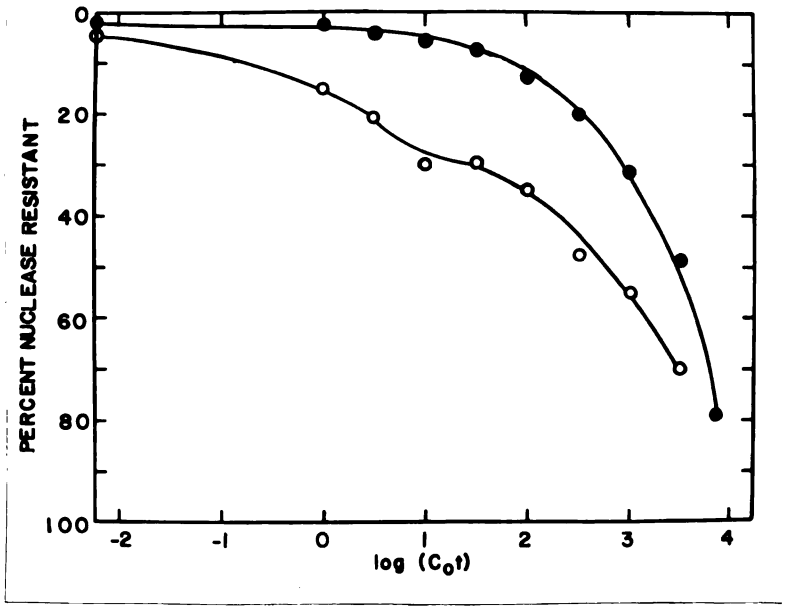

Figure 10

Reassociation kinetics of sea urchin sperm DNA assayed by S1 nuclease (0). Reassociation kinetics of radioactive non-repetitive gastrula DNA in the presence of a 100-fold excess of total sperm DNA assayed by S1 nuclease (●).

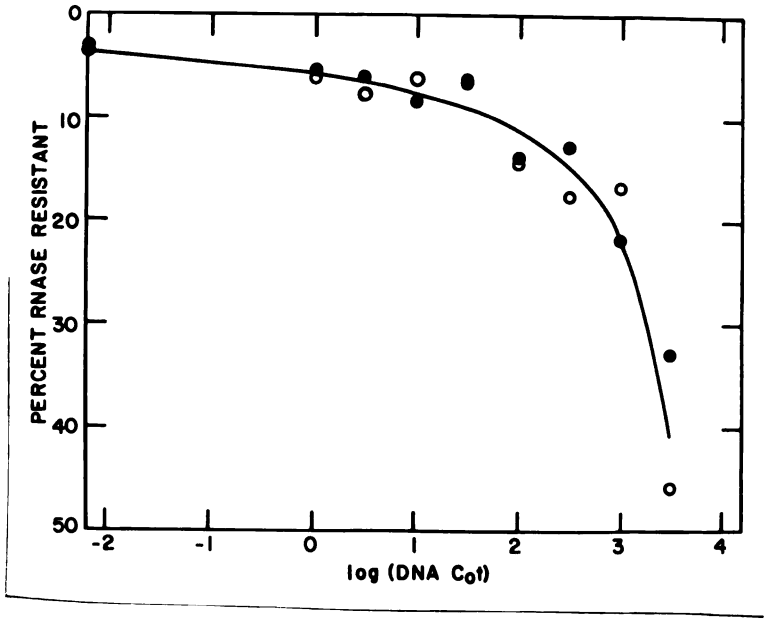

Figure 11

Kinetics of hybridization of 30 min pulse-labelled RNA from normal prism larvae (0) and from animalized embryos of the same age (●) incubated in the presence of a 100-fold excess of total sperm DNA.

this Cot. The ½Cot for the reaction of RNA with non-repetitive DNA is within a factor of 2 of that for the DNA reassociation. The scatter of the data precludes a more precise determination of the hybridization kinetics.

Genomic Representation in RNA of Normal and Animalized Embryos

In order to assay directly for the amount of genomic information expressed in RNA during the development of normal and animalized embryos, RNA/DNA hybridization was carried out using purified radioactive non-repetitive DNA.

The technique employed for saturation hybridization involved the incubation of labelled DNA of high specific activity with a large excess of non-radioactive RNA. At saturation, doubling of the RNA concentration or time of incubation results in no further increase in the percent hybridization. A typical saturation curve is presented in figure 12 and shows that, in the case of normal prism larvae, saturation is approached when 7.7% of the DNA is hybridized. Assuming asymmetric transcription, this corresponds to approximately 15.4% of the non-repetitive DNA. A similar experiment with RNA isolated from animalized embryos of the same age (figure 13) shows that, in this case, saturation is approached when approximately 8.3% of the DNA is hybridized. It should be pointed out that since saturation is never actually reached, the data represent minimal estimates of the genomic information present.

The haploid genome of the sea urchin, S. purpuratus, contains 0.77×10^{-9}mg DNA (Tyler and Tyler, 1966), corresponding to 7×10^{8} nucleotide pairs. If we assume 60% non-repetitive

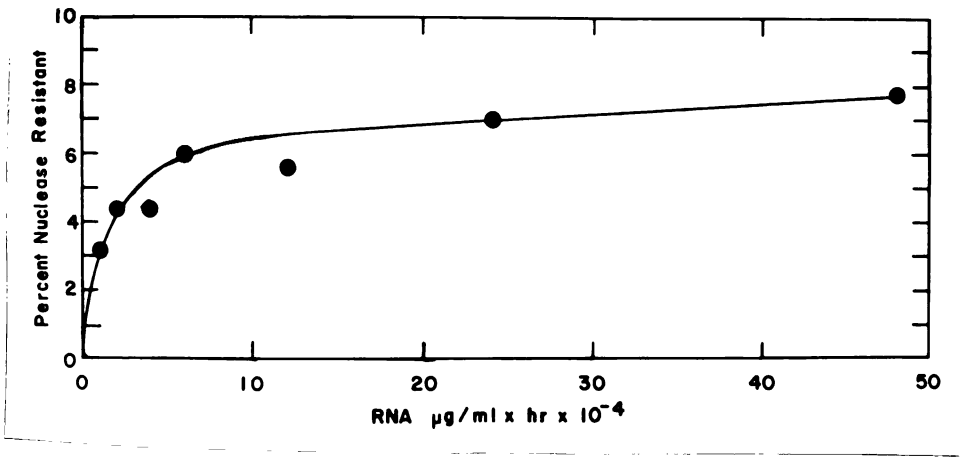

Figure 12

Hybridization of radioactive non-repetitive gastrula DNA with RNA from normal prism larvae. Conditions are as given in Table I and in Materials and Methods.

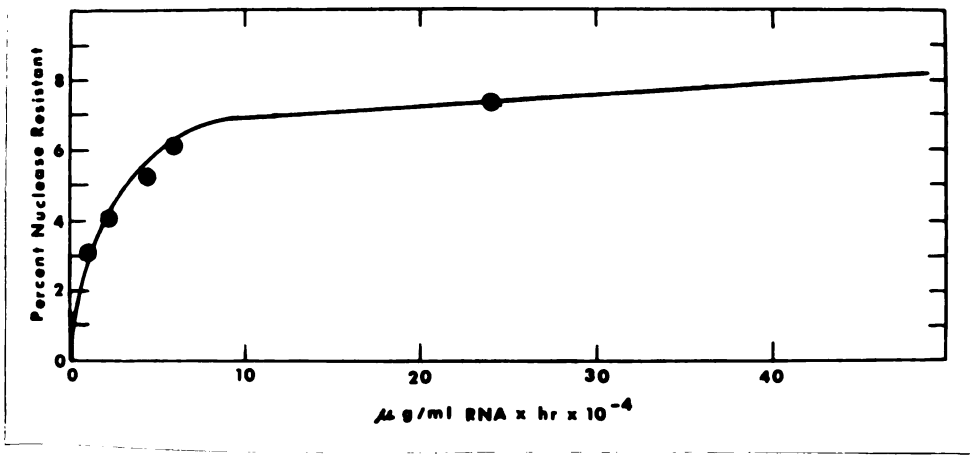

Figure 13

Hybridization of radioactive non-repetitive gastrula DNA with RNA from animalized embryos of the same age as the prism larvae shown in Figure 12. Conditions are as given in Table I and in Materials and Methods.

(figure 8), the complexity of this portion of the genome is 4.2×10^8 nucleotide pairs. Since at least 15.4% of this information is represented in the RNA of a normal prism larva, this information corresponds to at least 6.5×10^7 nucleotide pairs and the complexity of the RNA is at least 6.5×10^7 nucleotides.

Figure 14 and table III summarize the apparent saturation values obtained at all stages tested. Calculations similar to the above demonstrate that the base sequence diversity of the RNA present increases during development from 3.8×10^7 nucleotides in the unfertilized egg, to 6.0×10^7 nucleotides in the blastula, and 6.6×10^7 nucleotides in the prism larva and that at both stages tested the animalized embryos contain genomic information equivalent to an additional 0.4×10^7 nucleotides.

To compare the transcripts present at different stages of the development of normal and animalized embryos, additive experiments were performed in which the DNA was hybridized with RNAs from two different stages simultaneously. The difference between the arithmetic sum of the saturation values of each RNA preparation separately and that obtained in the additive experiments represents the extent of homology between the RNA populations. The results of these experiments are summarized in Table III and figure 14. Since the concentration x time factor for each RNA reactant was 24×10^4 µg/ml x hr, the total concentration x time factor was 48×10^4 µg/ml x hr for RNA species common to the two preparations. Thus an exact quantitative estimate of the homology between the two populations cannot be made. However, by comparing the value from the additive experiment with those from the longer and shorter incubations, upper and

Table III

Percent of non-repetitive DNA hybridized to RNA from various developmental stages of normal and animalized embryos.

Stage	RNA µg/ml x hr x 10^{-4}	
	24	48
unfertilized egg	4.4	4.5
blastula (24 hr)	6.4	7.3
prism (48 hr)	6.9	7.7
animalized (24 hr)	6.6	7.6
animalized (48 hr)	7.4	8.1
blastula + animalized (24 hr)*	8.0	
prism + animalized (48 hr)*	8.9	
prism + blastula*	7.9	
animalized (24 hr) + animalized (48 hr)*	7.1	

*in additive experiments the concentration x time factor for each RNA reactant was 24 x 10^4 µg/ml x hr.

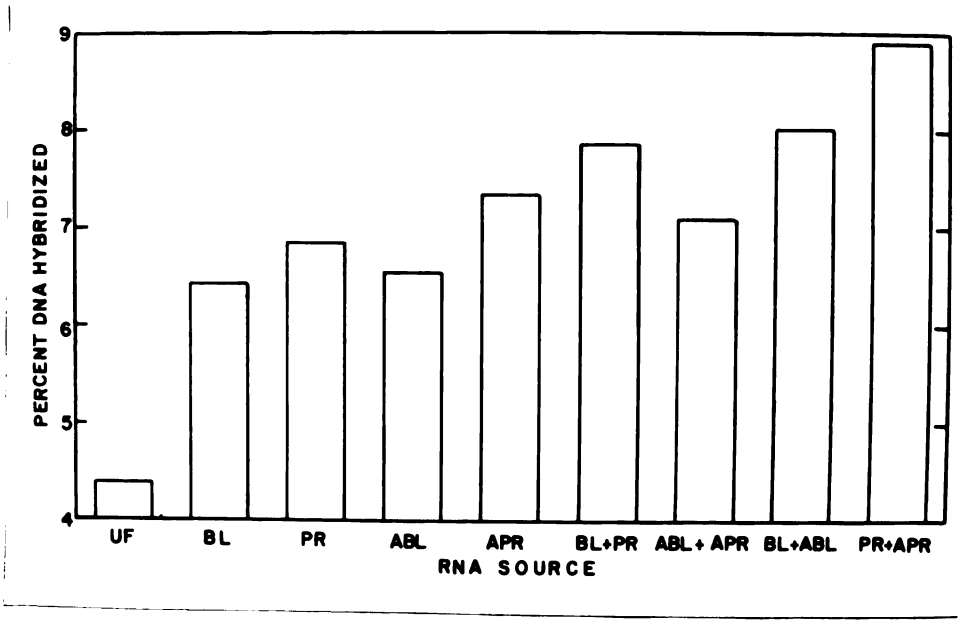

Figure 14

Summary of the hybridization data obtained at all stages tested after incubation for 24 x 10^4 µg/ml x hr as given in Table I. For the combination experiments, the concentration x time factor for <u>each</u> RNA reactant was 24 x 10^4 µg/ml x hr.

lower limits can be placed on the extent of homology. For the prism + blastula experiment, 6.4 + 6.9 - 7.9 = 5.4, the lower limit of the percent of the genome represented in both the normal blastula and prism. The upper limit is 7.3 + 7.7 - 7.9 = 7.1. Since $\frac{5.4}{6.9} = 0.79$ and $\frac{7.1}{7.7} = 0.92$ we conclude that between 79 and 92% of the sequences present in the prism are also present in the blastula. By similar calculations, between 78 and 95% of the non-repetitive DNA transcripts of 24 hr blastulae are also present in 24 hr animalized embryos, between 78 and 90% of the non-repetitive DNA transcripts of the 48 hr prism larvae are also present in 48 hr animalized embryos, and essentially 100% of the non-repetitive transcripts in 24 hr animalized embryos are retained in 48 hr animalized embryos. Thus the difference between normal and animalized embryos of the same age is of the same magnitude as the difference between normal blastulae and prism larvae.

Thermal Stability of RNA/DNA Hybrids

The thermal stability of radioactive non-repetitive DNA/total sperm DNA hybrids and that of radioactive non-repetitive DNA/RNA hybrids have been determined by S1 nuclease digestion (figure 15). The DNA/DNA hybrids melt very sharply with a Tm of 78°, whereas the RNA/DNA hybrids melt sharply with a Tm of 71°. The sharp melting profile, indicating that all hybrids are of approximately equal stability, is characteristic of non-repetitive DNA/RNA hybrids and not of repetitive DNA/RNA hybrids. The observed depression is due, at least in

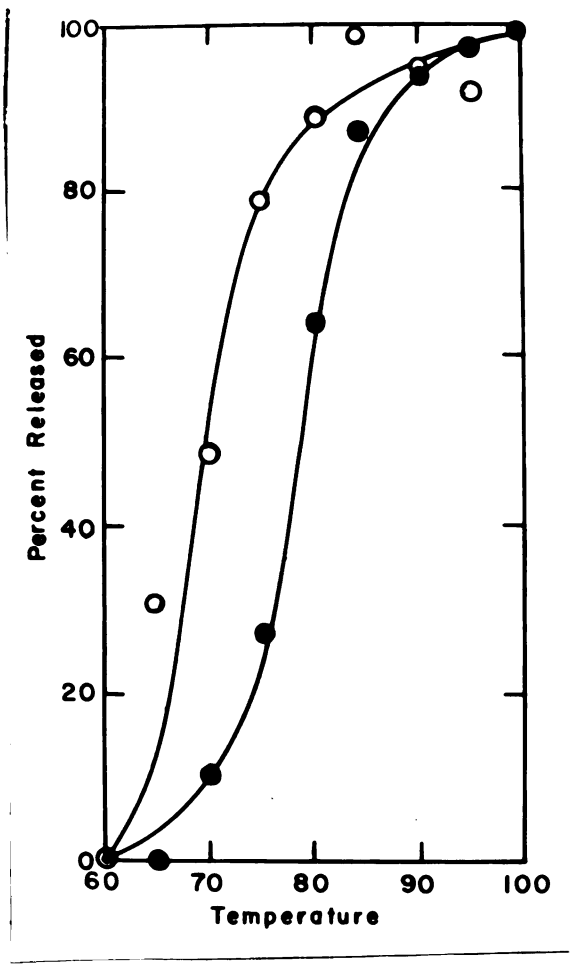

Figure 15

Thermal denaturation profile of radioactive non-repetitive gastrula DNA/total sperm DNA hybrids (●) and radioactive non-repetitive gastrula DNA/RNA hybrids (0) in 0.1M Na^+, assayed by S1 nuclease. The Tm of the DNA/DNA hybrids is 78^o; the Tm of the RNA/DNA hybrids is 71^o.

part, to short hybrid regions (Hayes et al., 1970; Smith et al., 1974). The maximum length of the hybrids is about 200 nucleotide pairs as limited by the size of the RNA molecules hybridized. With the above considerations and the fact that well-matched bacterial RNA/DNA hybrids can melt with approximately a 5^{o} drop in Tm (Bolton and McCarthy, 1964), it can be concluded that very little missmatch is present, and thus that near locus specificity has been achieved.

DISCUSSION

Animalization by Treatment With Excess Zinc Ion

Treatment of sea urchin embryos with excess Zn^{++} at the concentration used here results in specific effects of developmental significance. Acronal stereocilia are hyperextended around the embryo, invagination of the gut fails to occur, and the primary mesenchyme cells aggregate after migration into the blastocoel and fail to differentiate spicules. No identifiable endoderm or mesoderm is formed. Such differentiation, interpreted as hyperdevelopment of derivatives of the animal polar regions at the expense of the derivatives the vegetal polar regions, is called "animalization." That the phenomena observed do not indicate general deterioration of the embryo is indicated by the following: (1) viability equal to that of non-fed control embryos; (2) rate of cleavage equal to that of control embryos; (3) rate of uridine uptake equal to that of control embryos throughout development; (4) synthesis of heterogeneous high molecular weight RNA at a similar rate to control embryos during later development; (5) increase in the complexity of non-repetitive DNA transcripts with development. This system provided a means to study the involvement of the genome in experimentally altered development.

Uptake and Incorporation of Radioactive Uridine

The uptake and incorporation of radioactive uridine provided a means to study the effect of animalization on the rate of transcription since the rate of uridine uptake was not affected by the concentration of Zn^{++} used to animalize the embryos in the present experiment. This is in sharp contrast with the reported inhibition of phosphate uptake by Zn^{++} -animalized embryos (Pirrone et al., 1970), giving the use of uridine as an RNA precursor a distinct advantage in these studies. The normal rate of uridine uptake also contrasts with an inhibition by Zn^{++} of valine uptake (see Appendix).

The results on the incorporation of uridine during normal development confirm and expand the results of Markman (1961b) and of Kijima and Wilt (1969). More recently, similar results have also been presented by Mizuno et al. (1973). Under the conditions used, uridine taken up by these embryos is incorporated into RNA (Kijima and Wilt, 1969; Nemer, 1962; Mizuno et al., 1973). If constancy of precursor pool specific activity and little change in catabolic processes can be assumed, the acceleration of the rate of incorporation during development is due to an actual increase in RNA synthesis. Given these assumptions, the data indicate that the actual rate of RNA synthesis increases by a factor of 2-3 during the developmental period studied. This value corresponds well with data obtained by direct measurement of precursor pool specific activity (Kijima and Wilt, 1969) and chromatin template activity (Marushige and Ozaki, 1967).

Since the rate of uptake is the same for both the normal and animalized embryos, the differences in the incorporation rates are not due to permeability change. Furthermore, if the precursor pool size and catabolic processes are the same in the normal and abnormal embryos, the incorporation should reflect real differences in their RNA synthetic activities. The delay in the acceleration of RNA incorporation corresponds in time to the period of development during which novel gene groups begin to be activated (Whiteley et al., 1970), some of which are necessary for gut formation (Barros et al., 1966; Giudice et al., 1968). It is thus possible that animalization results from a failure of transcription of genes specifying the vegetal regions of the embryo and their derivatives.

When the rate of incorporation is expressed as a function of nuclear number, the incorporation rate decreases from a very high level during early cleavage, reaching a minimum, and then increases again, reaching a plateau level at the early prism stage (figure 16; Table IV). During early cleavage, cytoplasmic RNA incorporation predominates (Nemer, 1963; Chamberlain, 1970; Craig, 1970; Selvig et al., 1970; Hartman et al., 1971) and this may explain the apparently high rate of synthesis during cleavage. Nuclear RNA synthesis predominates during later development. Then the rate increase indicates actual activation of RNA synthesis per nucleus, contrary to other reports (Kijima and Wilt, 1969). The discrepancy may be resolved by the fact that by their smaller number of data points, Kijima and Wilt may have missed the minimum rate obtained.

Table VI

Incorporation of ^{3}H-uridine/nucleus and incorporation/uptake/nucleus during a 30 min pulse during the development of normal and animalized sea urchin embryos.

hours[1]	number of cells[2]	normal I/cell[3]	normal %I/cell[4]	animalized I/cell[3]	animalized %I/cell[4]
1	1	11.7	0.278	9.9	0.054
5	4	2.98	0.038	2.5	0.016
9	24	0.565	0.038	0.400	0.024
13	100	0.165	0.0229	0.172	0.0182
17	205	0.1825	0.0065	0.130	0.00745
21	325	0.227	0.00262	0.1155	0.00520
25	420	0.178	0.00595	0.108	0.00131
29	525	0.186	0.00401	0.110	0.00352
33	610	0.165	0.00357	0.137	0.00235
37	675	0.218	0.00245	0.179	0.00163
47	750	0.246	0.00302	0.213	0.00247
49	760	0.226	0.00248	0.237	0.00272
53	765	0.251	0.00309	0.228	0.00231
57	770	0.261	0.00320	0.270	0.00253
61	775	0.238	0.00320	0.270	0.00336

[1]= hours after fertilization.

[2]= from Figure 2.

[3]= counts/min incorporated into TCA-insoluble material + cell number.

[4]= counts/min TCA-insoluble/counts/min TCA-insoluble + -soluble ÷ number of cells x 100.

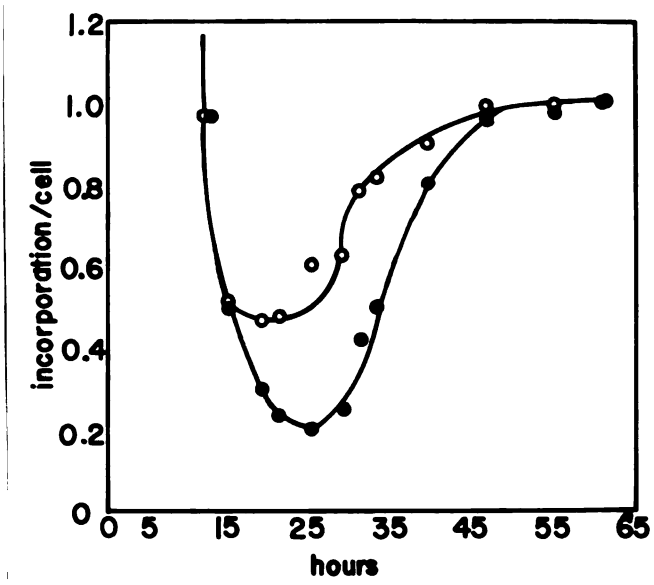

Figure 16

Incorporation of ^{3}H-uridine per nucleus by normal (0) and animalized (●) sea urchin embryos during development. The units on the ordinate represent cpm/nucleus/embryo multiplied by a constant in order to give the rate of incorporation per nucleus an arbitrarily assigned value of 1.0 at the prism larva stage.

In animalized embryos, the acceleration is delayed, but the same plateau level is reached. Since both the control and animalized embryos are cleaving at the same rate, the difference in the timing of activation of RNA synthesis suggests that cellular multiplication and RNA synthesis activation in development can be dissociated.

Characteristics of Rapidly-labelled RNA Molecules

The acceleration in the rate of incorporation during development has been demonstrated predominantly to involve an increase in the synthesis of high molecular weight RNA (Nemer, 1963; Timofeeva et al., 1968; Emerson and Humphreys, 1970). Sucrose gradient analysis demonstrates that most of the radioactivity incorporated by prism larvae and by animalized embryos during a short pulse label is incorporated into heterogeneously-sedimenting high molecular weight material, presumably of nuclear origin (Aronson and Wilt, 1969; Kijima and Wilt, 1969; Hogan and Gross, 1972; Aronson et al., 1972). Such rapidly-labelled heterogeneous RNA molecules turn over with a ½-life of less than 30 min (Kijima and Wilt, 1969; Brandhorst and Humphreys, 1972). Heterogeneous nuclear RNA (hnRNA) is characteristic of the eukaryotic nucleus, makes up a large fraction of the nuclear RNA, turns over rapidly, and contains the precursors of cytoplasmic messenger RNA (mRNA) (Soeiro et al., 1966; Imaizumi et al., 1973).

The data of figure 6 also indicate that the hnRNA of animalized embryos tends to be of larger molecular weight than that of normal embryos. This effect was quite reproducible. Three possible explanations may be offered for this behavior.

First, differential aggregation or degradation of the hnRNA may make the molecules appear to be larger or smaller than the actual transcripts. Such aggregation and degradation were not observed under conditions which minimize nucleolytic digestion of the RNA during extraction (Holmes and Bonner, 1973), however, and EDTA, SDS and bentonite present during extraction all inhibit nuclease activity. Therefore, it is tentatively concluded that the larger size of the hnRNA from animalized embryos is not due to differential aggregation or degradation during extraction. Such a conclusion must, however, be verified by performing the sedimentation analysis under denaturing conditions (Boedtker, 1968; Strauss et al., 1968). Second, chain termination may be suppressed, resulting in excessively long RNA molecules being transcribed from the same DNA sequences. Third, different DNA sequences may be transcribed. The present experiments do not attempt to distinguish between the latter two possibilities.

Structure of the Sea Urchin Genome

The fact that separated complementary strands of DNA (denatured DNA) are able to recognize each other and recombine in a specific way based on nucleotide sequence (renature) provides a powerful tool for the analysis of genomic structure. From the kinetics of renaturation a large amount of information can be obtained: for example the complexity or number of different sequences of the genome, the presence or absence of multiple copies of genes or closely-related sequences

(repetitive sequences), and the frequency of a given gene in the genome. The amount of renaturation (reassociation) observed is a function of the complexity of the sequences present, the length of the fragments used, the initial concentration of denatured reactants (C_o), the time of incubation (t), and the ionic strength of the medium. The amount of reassociation may thus be plotted, holding other parameters constant, versus the product of the initial concentration of reactants and the time of incubation, the Cot.

Among the parameters which affect the extent of reaction at any given Cot value is the nucleotide fragment length. From figure 8, it can be seen that increasing the mean fragment length from 450 nucleotides to 900 nucleotides causes the repetitive components to appear to comprise a larger fraction of the genome. This effect is due to the interspersion of repetitive and non-repetitive sequences in the genome (Graham et al., 1974). The observation that the slowly-reassociating fraction of the longer segments reassociates more slowly than that of the shorter segments may be due to the increase in viscosity of the medium caused by the longer DNA fragments.

Another of the parameters known to affect the apparent reassociation kinetics of DNA is the method used to assay for reassociation (Britten and Kohne, 1968). Comparing figures 8 and 10, it is evident that the reaction of 450 nucleotide-long fragments appears to proceed approximately 3 times slower when assayed by S1 nuclease than when assayed by HAP. Because the shearing of nucleic acids is random, hybrids will possess non-hybridized ends which will nevertheless be retained by

HAP and thus scored as having been hybridized. With S1 nuclease, non-hybridized ends are degraded and thus scored as not having been hybridized. Thus S1 nuclease would give a more stringent criterion for hybridization, and the kinetics would be comparable to those obtained by optical methods.

Figures 8 and 10 also show a small but significant fraction of apparent DNA/DNA duplex formation even at very low Cot values. When duplex formation was assayed immediately after denaturation ("zero-time duplex") by S1 nuclease, about 3% of the genome appeared to be in duplex form; by HAP assay about 7% appeared to be in duplex form. This material may be: (1) intrastrand duplex formed by "foldback" of regions of a strand containing internal homology and therefore with potential secondary structure, (2) interstrand duplex from very highly repetitive sequences, or (3) single-stranded DNA which is observed in the duplex fraction as an artifact. If this fraction is an artifact, the value may be subtracted from each data point and a "corrected" Cot curve may be plotted (Davidson et al., 1973). Such manipulation may also be valid for the HAP data presented here based on the following evidence: (1) the lack of agreement on the amount of zero-time binding measured by the two methods, and (2) the unexpectedly large low-Cot binding to HAP observed for purified radioactive non-repetitive DNA (Table V). Corrected binding values are given for comparison in Table I.

Table V

Percent of purified non-repetitive DNA recovered from HAP as apparent DNA/DNA duplex at low Cot values.

number of times fractionated at high Cot	observed percent duplex at low Cot
1	18
2	12
3	14

Radioactive gastrula DNA was heat-denatured (100°, 5 min) and incubated to Cot 30 and fractionated on HAP. An aliquot of the single-stranded material was incubated with a large excess of non-radioactive sperm DNA to Cot 0.01 and fractionated on HAP. Unadsorbed radioactive DNA was again heat-denatured, incubated to Cot 60 and fractionated on HAP. An aliquot was examined as above for low-Cot binding to HAP. The process was repeated at Cot 60 for the unadsorbed fraction. An aliquot of this fraction was then examined for low-Cot binding to HAP. Less than 0.9% of this material was resistant to S1 nuclease (cf. Figure 10) and therefore less than 0.9% of this material was actual DNA/DNA duplex.

RNA Transcripts of Normal and Animalized Embryos

When RNA is present in trace quantities in a DNA/DNA reassociation mixture, the RNA will hybridize to the DNA and the kinetics of hybrid formation will reflect the degree of reiteration of the homologous DNA in the genome. For the interpretation of such experiments, the DNA excess must be large; at least 70-100-fold (Bishop, 1972). In the experiments reported here, the DNA excess was 100-fold, but it should be mentioned that over 90% of the cellular RNA in the reaction mixture is ribosomal and transfer RNA, in which very little label is detected (figure 6). Thus it is likely that the DNA is in at least a 1000-fold excess over hybridizable radioactive RNA. Under these conditions, the reaction should give an accurate picture of the reiteration frequency of the genes transcribing hnRNA in normal and animalized sea urchin embryos. From these data, we conclude that the representation in hnRNA of the repetitive sequences of the sea urchin genome is small. Smith et al. (1974), using purified gastrula hnRNA and a DNA excess of over 100,000-fold obtained similar results. The nature of the zero-time RNase-insensitive material was not further studied, but this fraction may correspond to the fraction of hnRNA known to be double-stranded (Kronenberg and Humphreys, 1972; Molloy et al., 1974). This material could be rendered RNase-insensitive either through intramolecular base pairing or through its rapid hybridization to DNA (Jelinek and Darnell, 1972).

In order to assay for the complexity of RNA transcripts present at different stages of development, RNA/DNA hybridization was carried out using purified non-repetitive DNA in a large excess of RNA. Under such conditions, the contribution of the DNA to the rate of hybridization may be ignored and the reaction is pseudo first-order, dependent upon the initial concentration of hybridizable RNA sequences and the time of incubation (Firtel, 1972, Kennell, 1971). In experiments using total cellular RNA the actual concentration of hybridizable RNA sequences is unknown since most of the cellular RNA is ribosomal and transfer RNA which do not bind to non-repetitive DNA (Brown and Weber, 1968; Mutolo & Giudice, 1967; Sy and McCarty, 1970). Nevertheless, saturation plateaus can be determined and the extend of homology between two populations of RNA can be estimated. Although purified non-repetitive DNA fractions will contain in the limit one copy of each repetitive DNA sequence, all or nearly all the observed hybridization must be to non-repetitive sequences since this DNA is vastly enriched for non-repetitive sequences, and nearly all rapidly-labelled heterogeneous RNA (Smith et al., 1974; figure 11) and all messenger RNA (Goldberg et al., 1973) hybridize to non-repetitive DNA.

The data of Table III demonstrate that during the development of both normal and animalized embryos the complexity of transcription increases and that stage-specific patterns are observed. Transcripts are present at each stage tested which are unique to that particular stage. It is of particular interest that relatively large differences are observed between

the unique DNA sequence transcripts of normal and animalized embryos of the same age. These data demonstrate that animalization involves alterations in the pattern of embryonic gene transcription. These data also demonstrate that the homology between the RNAs derived from non-repetitive DNA of different stages of development is similar to that of RNAs derived from repetitive DNA sequences (Whiteley et al., 1970).

In addition it may be noted that the saturation levels obtained at different stages of development correspond roughly to the complexity of transcription at analogous stages in the development of other organisms. Davidson and Hough (1971) saturated 0.9% of the non-repetitive portion of the Xenopus laevis genome unfertilized egg RNA. Since the Xenopus genome is about 4 times as large as that of the sea urchin, the complexity of the stored RNA messages in the unfertilized Xenopus egg is similar to that in the unfertilized sea urchin egg. Davis and Wilt (1973) have reported that the unfertilized egg of the marine worm, Urechis caupo, which has a genome slightly larger than that of the sea urchin, contains RNA complementary to 4.3% of its genome. Thus the RNA complexity in the Urechis unfertilized egg is nearly the same as that in the sea urchin unfertilized egg. Schultz et al. (1973) have shown that 1.8% of the non-repetitive portion of the rabbit genome is represented in RNA in preimplantation blastocysts and that 2.5% is represented in post-implantation blastocysts which have begun to differentiate. Since the rabbit genome is about 4 times as large as that of the sea urchin, the actual amount of information in these stages of rabbit embryos is very close to that present in sea urchin

embryos at the analogous stages. Similar values have been reported for early mouse development (Church and Brown, 1972). Such results are not surprising as the embryos of all these organisms are carrying out similar activities at analogous stages; eg. mitosis, primary germ layer formation, maintenance of metabolism, etc.

The high degree of homology between the RNAs of different stages of development may reflect great stability of messages. The unfertilized egg contains messages sufficient to support development through the blastula stage (Tyler, 1967). It is possible that some of these messages are retained for long periods during development. It is believed that during development messages are transcribed long before they are to be used by the embryo to support differentiation (Tyler, 1967; Giudice et al., 1968; Whiteley et al., 1970). The presence of such long-lived messages would lead to the observed high degree of homology between the RNAs present at different stages of development. The ½-life of newly-synthesized mRNA in sea urchin embryos has been reported to be about 75 minutes (Brandhorst and Humphreys, 1972). Recent research has indicated that in cultured cells the ½-life of mRNA is much longer, however (Murphy and Attardi, 1973). The existence of even a small fraction of very long-lived mRNA in sea urchin embryos might provide the results obtained. Alternatively, most of the messages in RNA in the early sea urchin embryo may not be concerned with differentiation, but rather with activities common to all cells. These possibilities are not mutually exclusive.

Alteration of Sea Urchin Development by Zinc

It has been proposed that the coordination of cells in a developing system is accomplished through a system of intercellular communication based on the mobilization of and response to inorganic ions (Rasmussen, 1970; Barth and Barth, 1974). In sea urchin development, excess Zn^{++} might act directly within the cells, interacting with enzymes or chromatin to produce alterations in the expression of embryonic genes. Alternatively, excess Zn^{++} might affect the concentration and/or distribution of intracellular ions merely by altering the external ionic environment of the cell (Barth and Barth, 1974).

While Zn^{++} might exert its effects either internally or externally or both, there is indirect evidence that the primary effects of Zn^{++} in controlling morphogenesis are produced at the cell surface level. It has not been demonstrated that Zn^{++} has any specific effect on embryonic chromatin or on any intracellular enzyme systems, and it has been observed that the uptake of radioactive Zn^{++} by embryonic cells is minimal during cleavage (Timourian, 1968), the period of embryonic determination when Zn^{++} has been shown to produce its specific morphogenetic effects (Lallier, 1959). Other animalizing agents such as proteolytic enzymes (Hörstadius, 1949; Lallier, 1969) and the plant lectin, concanavalin A (Lallier, 1972), which presumably act at the cell surface (Lallier, 1972) act as synergists with Zn^{++} in producing their effects. In addition, it has been shown that Zn^{++} produces at least two biochemical alterations in the cell surface, namely in inhibition of the transport

mechanisms for phosphate (Pirrone et al., 1970) and valine (see Appendix).

It is known that Ca^{++} interacts with cyclic nucleotides within the cell to stimulate protein kinase activities which, in turn, control cellular activities (Rasmussen, 1970). If the primary effects of Zn^{++} are at the cell surface, alterations in gene activity might be produced indirectly, either through the effects of some of the intracellular protein kinases on chromatin-associated proteins (McMahon, 1974) or through the binding of cyclic nucleotides directly to transcriptional units (Zubay et al., 1970; Emmer et al., 1970). It has been shown that the Ca^{++}- and Mg^{++}-dependent ATPase of sarcoplasmic reticulum possesses a Ca^{++}-dependent and -selective ionophore as an integral part of its structure and that this ionophore is inhibited in its Ca^{++} conductance by Zn^{++} (Shamoo and MacLennan, 1974). Such a Ca^{++} pump has been shown to exist in the cortex of the sea urchin egg (Mabuchi, 1973). Thus Zn^{++} may exert its effects by preventing Ca^{++} efflux from or influx into embryonic cells and these effects may be mediated through a direct influence on the Ca^{++} pump.

Conclusions

Processes of differentiation may be viewed as resulting from the interaction of the organism's genome with its environment. Such a view predicts that (1) alterations in gene activity may be produced in embryos by altering their environment, and (2) that such alterations would cause alterations in the differentiative patterns of the embryonic cells. The present

study tested the first of these predictions. It was demonstrated that animalization of sea urchin embryos, induced by excess Zn^{++} in the sea water, involves quantitative and qualitative alterations in embryonic gene activity. The data are consistant with, but do not prove the hypothesis that Zn^{++}-induced animalization results from such alterations in embryonic gene activity. These alterations may be mediated through the intracellular metabolism of Ca^{++}.

APPENDIX

APPENDIX

Effect of Zinc on the Uptake and Incorporation of Valine

A preliminary experiment was performed to study the uptake and incorporation of valine during the development of normal and animalized sea urchin embryos. Equal aliquots containing approximately 10^4 embryos were obtained from control and animalized cultures when the controls were hatched blastulae and prism larvae, washed, suspended to 0.25 ml in sea water containing 250 μg/ml streptomycin sulfate, and incubated with 1.32 μCi/ml ^{14}C-valine (specific activity 275 mCi/mmole, Schwartz-Mann). Incubation was stopped by the addition of ice-cold sea water containing a 10^4-fold excess of non-radioactive valine. The embryos were collected on Whatman 3MM paper discs by filtration, washed and dried. For the determination of uptake, the discs were placed directly in a toluene-based scintillation fluid and their radioactivity was determined by liquid scintillation counting. For the determination of incorporation into protein, the dried discs were washed for 15 min successively in ice cold 5% TCA, 95° 5% TCA, ice cold 5% TCA, 95% ethanol, and ethyl ether. The discs were then dried and their radioactivity was determined.

Figure 17a shows that the uptake of ^{14}C-valine is inhibited approximately 85-90% in animalized embryos. Figure 17b shows that incorporation is inhibited to a similar extent. When the

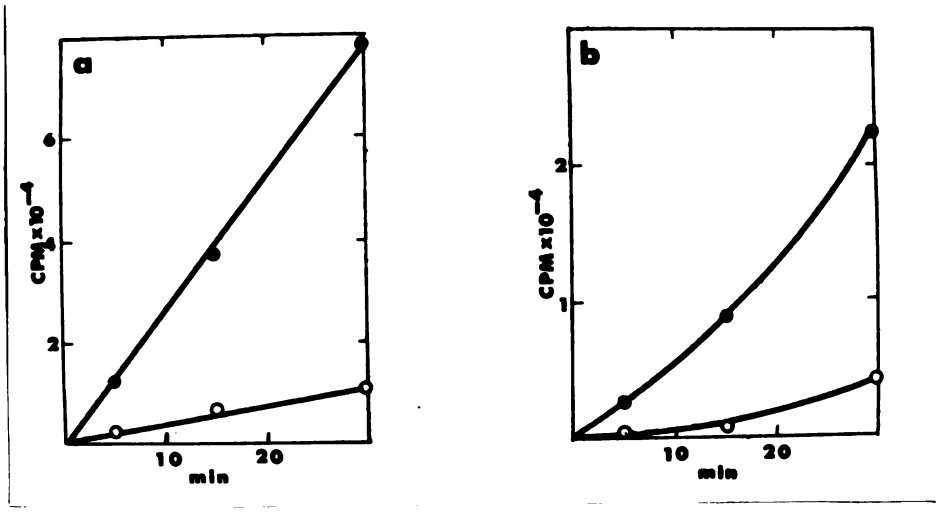

Figure 17

a. Uptake of ^{14}C-valine during a 30 min pulse by normal (●) and animalized (0) embryos.
b. Incorporation of ^{14}C-valine into TCA-insoluble material during a 30 min pulse by normal (●) and animlaized (0) embryos.

percent incorporation is calculated, it can be seen that all the inhibition of incorporation is explained on the basis of the inhibition of uptake (Table VI). Since the animalized embryos had been washed with normal sea water, this inhibition was not due to a direct effect of exogenous Zn^{++} in the sea water on the valine.

In order to test whether this effect on valine transport was directly involved with animalization or was due to some effect of Zn^{++} on the transport mechanism, normal prism larvae were incubated with 5×10^{-4}M $ZnSO_4$ added to the sea water for 30 min, washed with sea water 4 times over a ½-hour period, and incubated with ^{14}C-valine as above. These embryos showed an 85-90% inhibition of uptake, just as the animalized embryos did. Thus the inhibition of uptake is due to a direct irreversable effect of Zn^{++} on the cell surface transport mechanism and not to an effect of animalization on uptake. A similar inhibition of phosphate uptake has been reported (Pirrone et al., 1970). Thus Zn^{++} inhibits the uptake of phosphate and valine in the sea urchin, but not the uptake of uridine. Since the percent incorporation is the same in normal and animalized embryos, it can be concluded that animalization does not affect the protein synthetic machinery directly, even though there are differences between the newly-synthesized proteins of normal and animalized (Carroll et al., 1974).

Table VI

Percent incorporation into TCA-insoluble material of ^{14}C-valine by normal and animalized sea urchin embryos during a 30-min pulse label.[1]

minutes incubation	normal percent incorporated	animalized percent incorporated
5	21.9	21.6
15	23.6	9.3
30	27.3	31.6

[1]= data from Figure 17.

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