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# REGULATION OF POLYPEPTIDE SYNTHESIS DURING EARLY EMBRYOGENESIS IN THE MARINE MUD SNAIL, ILYANASSA OBSOLETA

*City University of New York* Ph.D. 1980

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# REGULATION OF POLYPEPTIDE SYNTHESIS DURING EARLY EMBRYOGENESIS IN THE MARINE MUD SNAIL, ILYANASSA OBSOLETA

by

Mary Ellen McCarthy

A dissertation submitted to the Graduate
Faculty in Biology in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy, The City University
of New York.

1980

Mary Ellen McCarthy

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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# Abstract

## REGULATION OF POLYPEPTIDE SYNTHESIS DURING EARLY EMBRYOGENESIS IN THE MARINE MUD SNAIL, *ILYANASSA OBSOLETA*

by

Mary Ellen McCarthy

Advisor: Dr. Jack Collier

The goal of these studies was to describe the regulation of gene expression during early development in a spiralian embryo through an analysis of the mechanisms by which stage-specific changes in polypeptide synthesis are accomplished.

The approach was a comparison, by two-dimensional electrophoresis, of polypeptides synthesized during early cleavage, mesentoblast and gastrula stages. The relative contribution of maternal and embryogenic transcription to changes in polypeptide synthesis throughout this period was assessed by comparing the polypeptides made by normal and actinomycin D-treated embryos. The mechanisms by which ooplasmic segregation controls polypeptide synthesis were investigated by an analysis of polypeptide synthesis in the isolated cytoplasmic polar lobe and lobeless embryos.

The results presented here show that significant changes in polypeptide synthesis occur as early as the twenty-five cell (mesentoblast) stage that are correlated with the determinative events of this period in development. Further changes in polypeptide synthesis occur at gastrulation.

The analysis of polypeptide synthesis in embryos reared in actinomycin D has demonstrated that (1) all of the polypeptides made during early cleavage and detected by two-dimensional electrophoresis are translated from oogenic mRNAs, (2) stored oogenic mRNP is activated at the mesentoblast and gastrula stages, (3) the translation of most species of maternal mRNA is regulated by transcriptionally independent mechanisms, (4) oogenic mRNA remains a primary source of transcripts for protein synthesis through gastrulation and (5) embryogenic mRNA is first translated at the mesentoblast stage. It is postulated that a group of regulatory genes exist whose transcripts, directly or indirectly, repress the translation of specific oogenic mRNAs.

An analysis of polypeptides made by the isolated polar lobe and the lobeless blastomeres has shown that (1) the polar lobe contains at least 300 species of maternal mRNAs, (2) the polar lobe and lobeless blastomeres translate the same oogenic mRNAs, (3) the polar lobe is able to activate stored oogenic mRNAs and regulates the translation of some maternal mRNAs, (4) the effect of the polar lobe on polypeptide synthesis is delayed until the mesentoblast stage and (5) at the mesentoblast and gastrula stages, it is postulated that factors in the polar lobe cytoplasm (a) regulate the activation of oogenic mRNA, (b) activate the synthesis or translation of embryogenic mRNA and (c) control the expression of regulatory genes whose products repress the translation of specific maternal mRNAs.

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# INTRODUCTION

> "The fundamental problem...which includes all others... is determination."[1]

The egg of the marine mud smail, Ilyanassa obsoleta provided some of the earliest evidence for the role of the ooplasm in determination. This egg forms, at the first and second meiotic divisions and at the first and second cleavages, a series of protrusions of the vegetal cytoplasm called polar lobes (Crampton, 1896; Clement, 1952). By means of the third and fourth polar lobes, the vegetal cytoplasm is segregated at first cleavage into one of the first two blastomeres, the CD, and, at second cleavage, into the D macromere.

The D macromere, because it receives the ooplasm of the polar lobe, is unique; the contents of this cell determine the fates of the first three quartets of micromeres, which form the eyes, velum, shell, statolith, digestive gland, foot and head ectoderm, and the 4d or mesentoblast cell, which forms the heart and intestine (Clement, 1952, 1956, 1962, 1967, 1976). Removal of the third polar lobe results in a larva that lacks, or shows poor organization, of all of these structures (Crampton, 1896; Clement, 1952, 1962; Atkinson, 1971). The mechanism by which the polar lobe ooplasm exerts its determinative effect is unknown.

Ultrastructural studies of the Ilyanassa egg and polar lobe have revealed that the polar lobe cytoplasm is rich in yolk platelets, polyribosomes and membranous vesicles (Crowell, 1964; Pucci-Minafra et al., 1969; Geuskins, 1968). The yolk platelets appear to play no role in determination (Clement, 1968), but the membranous vesicles, derived from an organelle, the polymerosome,

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[1] Edmund B. Wilson, The Cell in Development and Heredity, third edition (New York: The Macmillan Company, 1924), p. 1037

that appears during oogenesis (McCann-Collier, 1977), are similar to the vesicles found in the polar lobe of Dentalium (Reverberi, 1970) and to the basophilic vegetal bodies of the polar lobe of Bithynia (Dohman and Verdonk, 1974), and may play a role in the localization of morphogenetic factors.

Comparisons of nucleic acid synthesis in normal and lobeless embryos have demonstrated that the lobeless embryo synthesizes significantly less RNA at the 25-cell stage and at gastrulation than does the normal embryo (Collier, 1977). Although the Ilyanassa embryo, from the four-cell stage on, is actively engaged in the synthesis of DNA-like RNA (Collier and Yuyama, 1969; Koser and Collier, 1976; Collier, 1976) and poly(A)-RNA (Collier, 1975b; Kidder et al., 1977; Clark and Kidder, 1977), removing the polar lobe does not significantly alter, in pre-gastrula embryos, the accumulation of low molecular weight RNA or high molecular weight RNA (Koser and Collier, 1976), nor does it affect the proportion of poly(A) containing RNA at the 25-cell stage (Collier, 1975b). Thus, the decrease in RNA synthesis in lobeless embryos results in a uniform reduction in the synthesis of all classes of RNA that is not reflected in proportional measurements of RNA accumulation.

Studies of protein synthesis in the Ilyanassa egg have demonstrated that the isolated polar lobe is capable of protein synthesis (Clement and Tyler, 1967; Geuskins, 1968; Teitelman, 1973) and that cleavage stage embryos continue to synthesize protein in the presence of actinomycin D (Collier, 1966; Newrock and Raff, 1976). These observations suggested that the egg and polar lobe contain a store of maternal mRNAs that might have a determinative effect. Donohoo and Kafatos (1973) compared, by single-dimension electrophoresis, the buffer-soluble proteins synthesized by AB and CD half-embryos of Ilyanassa and reported differences in the radioactive profiles obtained from sliced gels. Teitelman (1973), using similar methods, reported no significant differences in the proteins synthesized by normal and lobeless embryos prior to the fourth day of development (post-gastrula stage). With somewhat improved solubilization and electrophoretic separations, Newrock and Raff (1975) and Raff et al. (1976) reported differences in the patterns of proteins synthesized by normal and lobeless embryos as early as the 25-cell stage and that these differences were not eliminated by actinomycin D treatment, suggesting that the polar lobe might exert its determinative effect through the selective distribution of maternal mRNAs or regulators of translation.

The bands obtained after single dimension separations, such as those used in the studies discussed above, contain many individual polypeptides with the same electrophoretic mobility and, therefore, qualitative and quantitative differences in polypeptide synthesis cannot be distinguished by this method. These problems are overcome with the technique of two-dimensional electrophoresis (O'Farrell, 1975), which separates polypeptides on the basis of two distinct properties (isoelectric point and molecular weight); the spots detected in two-dimensional separations represent, for the most part, individual species of polypeptides. While this technique does not resolve polypeptides with isoelectric points over pH 8, such as the histones and some ribosomal proteins (O'Farrell, 1975), it is capable of resolving three hundred or more distinct polypeptides in Ilyanassa embryos and as many as a thousand in sea urchin embryos (Brandhorst, 1976). These polypeptides are probably translated from the moderately prevalent message class, which, in the sea urchin gastrula, comprises 80% of the mass of polysomal RNA (Davidson and Britten, 1979) and has a sequence diversity sufficient to code for approximately 1,000 different polypeptides (McColl and Aronson, 1978). The polypeptides resolved by two-dimensional electrophoresis in this study, therefore, represent 30% or more, of the translation products of this class of mRNA.

Two-dimensional electrophoresis has been used to study the changes in polypeptide synthesis that occur during development in the eggs and embryos of mice (Cullen, et al., 1980; Handyside and Johnson, 1978; Levinson et al., 1978; Dewey et al., 1978 Braude 1979), rabbits (Van Blerkom, 1979; Van Blerkom, and McGaughey, 1978), *Xenopus* (Bravo and Knowland, 1979; Brock and Reeves, 1978) and sea urchins (Brandhorst, 1976; Tufaro & Brandhorst, 1979). Both mammalian embryos studied show changes in polypeptide synthesis very early in development. Van Blerkom (1979) has reported several qualitative differences in the polypeptides made by newly fertilized rabbit eggs and 12 to 16-cell embryos and has shown that some, but not all of these changes also occur in aging, unfertilized oocytes that had undergone spontaneous meiotic maturation. Van Blerkom (1979) suggests that these changes are probably independent of new transcription and a similar suggestion has been made by Cullen et al. (1980) who observed quantitative differences in the polypeptides made by mouse eggs between fertilization and first cleavage. That these changes are not totally dependent on either germinal vesicle breakdown or concurrent transcription, is suggested by the observation (Schultz et al., 1978) that anucleate fragments of germinal vesicle stage mouse oocytes show some of the changes in polypeptide synthesis associated with meiosis in intact eggs.

Braude (1979) has reported that many of the changes in polypeptide synthesis that occur in mouse embryos between the morula and blastocyst stages are unaffected by treatment with $\alpha$-amanitin and Braude and Pelham (1979) have analyzed the cell-free translation products of mouse oocyte RNA to show that it contains RNAs coding for polypeptides that appear only after first cleavage. Some changes in polypeptide synthesis in mouse blastocysts failed to occur in the presence of $\alpha$-amanitin (Braude, 1979).

These observations suggest that in the early mammalian embryo, a combination of both transcriptionally dependent and independent mechanisms are required to regulate stage-specific changes in polypeptide synthesis.

Changes in polypeptide synthesis also occur in _Xenopus_ eggs at fertilization (Bravo and Knowland, 1979) and between cleavage, blastula, gastrula and neurula stages (Brock and Reeves, 1978). The mechanisms that control these changes have not been investigated.

The sea urchin embryo may be an exception, since Brandhorst has reported that very few changes in polypeptide synthesis occur prior to gastrulation; a more complete analysis may, however, reveal that the sea urchin embryo also shows changes in polypeptide synthesis during early development.

Changes in polypeptide synthesis are associated with differentiation in mouse embryos and adult tissues (Klose and Wallenberg-Pachaly, 1976), in amphibian larvae (Bravo and Knowland, 1979; Brock and Reeves, 1978) and in cultured myoblasts (Garrels, 1979). Are similar differences in polypeptide synthesis associated with determination?

Two recent studies indicate that the synthesis of specific polypeptides can be correlated with determination: Gutzeit and Gehring (1979) have shown that the pole cells of _Drosophila_ blastoderm embryos, which are determined to form the primordial germ cells, synthesize several unique proteins and Rodgers and Shearn (1977), in a comparison of polypeptides made by _Drosophila_ imaginal discs determined to form wing, legs or eyes and antennae found that 16-19% of the polypeptides detected were unique to single disc types or were shared by two out of the three disc types examined. While these studies suggest a correlation between the synthesis of specific polypeptides and determination, differences in the prospective potencies of cells may not always be reflected in the polypeptides that they synthesize.

In sea urchins, neither vegetalized embryos (Hutchins and Brandhorst, 1979), nor isolated blastomeres (Tufaro and Brandhorst, 1979) appear to synthesize unique sets of proteins. These observations may reflect the regulative capacities of sea urchin embryos, but they also suggest that many of the polypeptides detected in two-dimensional separations might have little importance in the regulation of early development (see also Dewey et al., 1978).

Waring et al. (1978) have analyzed the proteins present in the polar granules, which appear during oogenesis and are selectively localized in the pole cells of Drosophila embryos and have reported that these organelles contain a unique, highly basic polypeptide that the authors suggest may be causally related to the determination of the germ line. Some polypeptides made during oogenesis are selectively distributed prior to cleavage in axolotl eggs. Jackle and Eagleson (1980) have shown that prior to fertilization, most polypeptides were distributed uniformly along the animal vegetal axis, but some polypeptides were found exclusively in the germinal vesicle. After fertilization and germinal vesicle breakdown, several polypeptides from both the cytoplasm and the germinal vesicle became selectively localized in either the animal or vegetal halfs of the egg. Nothing is known about a mechanism that might permit such translocations of specific polypeptides, nor is the function of these polypeptides understood but studies such as this suggest that the selective distribution of proteins might play a role in the regulation of determinative events.

Despite these recent advances in molecular embryology, the mechanisms that control early development are still largely a matter of conjecture. Almost nothing is known about the way in which determinative factors might control early development and the relationship between maternal and embryogenic information, which must somehow control gene expression during early development, has only begun to be explored.

The objective of this study was to elucidate the mechanisms by which oogenic information and embryogenic information interact to regulate determination and the program of gene expression during the early development of a spiralian embryo. These problems were approached through an analysis of the effect of the polar lobe cytoplasm and of actinomycin D treatment on the changes in two-dimensional patterns of polypeptide synthesis in early cleavage, 25-cell and early gastrula stage *Ilyanassa* embryos.

# MATERIALS

Ampholytes were obtained from LKB. Electrophoresis grade acrylamide for isoelectric focusing was obtained from BioRad Laboratories (Richmond, Calif.). Acrylamide for SDS electrophoresis was obtained from Eastman-Kodak (Rochester, N.Y.) and recrystallized from chloroform and N,N'-methylenebisacrylamide (bis) was obtained from BioRad Laboratories. Tergitol NP-40, Tris base, Tris-HCl, ammonium persulfate, dithiothreitol (DTT) and $\beta$-mercaptoethanol were reagent grade, purchased from Sigma Chemical Co. (St. Louis, Mo.). N,N,N',N'-tetramethylethylenediamine (TEMED) was purchased from Canalco (Bethesda, Md.) and agarose, B-grade, from CalBiochem (San Diego, Calif.). Sodium dodecyl sulfate (SDS) was manufactured by BDH Chemicals Ltd. and purchased through Gallard-Schlesinger Manufacturing Corp. (Carle Place, N.Y.). Ultra pure urea, obtained from Schwarz/Mann (Orangeburg, N.Y.), was deionized on mixed-bed resin columns of AG501x8 (BioRad Laboratories). X-ray film was Kodak X-omat R from Eastman Kodak Co. (Rochester, N.Y.). All other chemicals were reagent grade and obtained from several sources unless specified in the text.

# METHODS

## Animals

Breeding stocks of adult Ilyanassa obsoleta were obtained from Plum Beach, Brooklyn, N.Y. and Beaufort, North Carolina and were maintained in tanks of recirculating sea water at 19°C, as previously described (Collier, 1975).

## Rearing and Staging of Embryos

Eggs were collected and embryos reared in filtered sea water containing antibiotics as previously described (Collier, 1975).

Polar lobes and lobeless embryos were obtained by agitating trefoil stage (two cell embryos prior to third polar lobe resorption) embryos in low calcium and magnesium sea water (Collier, 1975). Normal and lobeless embryos and polar lobes were isolated from the pooled contents of 20-30 egg capsules and normal embryos were exposed to calcium-magnesium low sea water as a control for the effect of ionic imbalance at this stage of development.

When approximately 800 normal and lobeless embryos and polar lobes had been obtained, the polar lobes were placed in pasteurized sea water and the normal and lobeless embryos were each divided into two groups: the first group was placed in pasteurized sea water and the second group in 30 $\mu$g/ml actinomycin D dissolved in sea water with antibiotics added (see below). Normal and lobeless control embryos, normal and lobeless actinomycin D-treated embryos and isolated polar lobes were then placed in the dark, at 19°C and groups of 100 embryos or 200 polar lobes were withdrawn for radioactive labeling as the embryos reached the desired stage of development. Solutions of pasteurized sea water and actinomycin D were changed daily.

All embryos were reared at 19°C and staged by time of development from the two-cell stage. Cleavage stage embryos were preincubated in actinomycin D or sea water for two hours prior to radioactive labeling and consisted of four to eight cells at the start of the labeling period.

I have designated what was previously called a stage 1 embryo as a mesentoblast stage embryo. This is a 24-hour old embryo, reared and staged as described above, that consists of an average of 29 cells and is characterized by having formed the 4d micromere of mesentoblast cell (Collier, 1975).

# TABLE 1

## EFFECT OF ACTINOMYCIN D[1] ON INCORPORATION OF $^3$H-URIDINE INTO ACID PRECIPITABLE MATERIAL

| STAGE OF DEVELOPMENT | PERCENTAGE OF $^3$H-URIDINE INCORPORATED | | PERCENT INHIBITION OF INCORPORATION | ($\bar{X}$) |
|---|---|---|---|---|
| | CONTROL | ACTINOMYCIN D-TREATED | | |
| CLEAVAGE | 1.74 | 0.27 | 84.5 | |
| | 1.13 | 0.16 | 85.8 | (85.2) |
| MESENTOBLAST | 1.56 | 0.28 | 82.1 | |
| | 1.84 | 0.29 | 84.2 | (83.2) |
| GASTRULA | 2.44 | 0.13 | 94.6 | |
| | 3.00 | 0.27 | 91.1 | |
| | 3.46 | 0.09 | 97.4 | (94.4) |

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[1] Concentration of actinomycin D was 30 µg/ml. Experimental embryos were preincubated in actinomycin D for two hours prior to radioactive labeling.

Gastrulation occurs by epiboly approximately 48 hours after first cleavage, 24 hours after the mesentoblast stage.

### Radioactive Labeling of Proteins

Proteins were labeled by incubating embryos or isolated polar lobes of a given stage with $^{35}$S-L-methionine (New England Nuclear, Boston, Mass.) which had specific activities ranging from 600 to 800 Ci/mmole. Four to six $\mu$l of $^{35}$S-methionine were added to 50 $\mu$l of pasteurized sea water containing 60-150 embryos or 200-300 polar lobes. Labeling of actinomycin D-treated embryos was performed in the presence of 30 $\mu$g/ml actinomycin D. The embryos, contained in a drop of sea water on Parafilm, were then placed in a moist chamber, in the dark and incubated for four hours at 19°C. The embryos and polar lobes were washed extensively in fresh sea water at the end of the labeling period, prior to solubilization.

### Actinomycin D Treatment

Actinomycin D (AMD) was obtained from Merk, Sharp and Dohme Research Laboratories (West Point, Pa.). Actinomycin D was dissolved in sea water by shaking overnight and the concentration in solution was determined from its' molar absorbance at 440mu of 24,800 (Gellert *et al.*, 1965). Stock solutions of actinomycin D were prepared fresh every two weeks and were stored in the dark at 4°C.

The effect of different concentrations of actinomycin D on RNA synthesis was determined for gastrula stage embryos by preincubating embryos for two hours in 10, 20, 30 and 40 $\mu$g/ml actinomycin D and then incubating the embryos, in the presence of AMD, in 200 $\mu$Ci/ml $^{3}$H-uridine. Uptake and incorporation of $^{3}$H-uridine into acid precipitable material was determined as described above (see Protein Solubilization). Incorporation of $^{3}$H-uridine into DNA as a percentage of the total incorporation was

## *Table 2*

### EFFECT OF CONTINUOUS INCUBATION IN ACTINOMYCIN D ON UPTAKE AND INCORPORATION OF $^{35}$S-METHIONINE[1]

| STAGE | TOTAL CPM X $10^4$/EMBRYO |  | ACID PPT. CPM X $10^4$ PER EMBRYO |  | PER CENT INCORPORATION |  |
|---|---|---|---|---|---|---|
|  | CONTROL | AMD | CONTROL | AMD | CONTROL | AMD |
| CLEAVAGE | 15.6 | 14.9 | 2.3 | 2.2 | 15.1 | 14.9 |
|  | 13.9* | 15.8* | 2.6* | 2.1* | 18.8* | 13.0* |
|  | 8.9 | 8.8 | 1.2 | 0.8 | 13.3 | 9.3 |
|  | 7.6* | 9.9* | 1.1* | 1.1* | 14.3* | 7.9* |
|  | MEAN |  |  |  | 15.4 | 11.3 |
|  | PER CENT INHIBITION |  |  |  | 26.6 |  |
| MESENTOBLAST | 12.6 | 11.1 | 1.1 | 0.5 | 8.4 | 4.9 |
|  | 14.6* | 18.1 | 1.0* | 0.8* | 6.9* | 4.4* |
|  | 7.5 | 6.5 | 0.6 | 0.2 | 7.5 | 3.6 |
|  | 10.8* | 9.0* | 0.7* | 0.4* | 6.1* | 4.0* |
|  | MEAN |  |  |  | 7.2 | 4.2 |
|  | PER CENT INHIBITION |  |  |  | 41.7 |  |
| GASTRULA | 7.8 | 7.8 | 1.2 | 0.1 | 15.1 | 1.3 |
|  | 5.2* | 7.0* | 0.7* | 0.05* | 12.7* | 0.7* |
|  | 14.4 | 13.9 | 3.2 | 0.2 | 22.5 | 1.6 |
|  | 12.2* | 13.0* | 1.7* | 0.1* | 13.8* | 0.9* |
|  | MEAN |  |  |  | 16.0 | 1.1 |
|  | PER CENT INHIBITION |  |  |  | 93.1 |  |

FOR COMPARISON OF UPTAKE OF METHIONINE BETWEEN CONTROL AND ACTINOMYCIN D-TREATED EMBRYOS $P = 0.40$

1 CONCENTRATION OF ACTINOMYCIN D WAS 30 MICROGRAMS PER ML.

* LOBELESS EMBRYOS

determined by alkaline hydrolysis of RNA in 0.3 N NaOH at 37°C for 18 hours (Crosbie, Smellie and Davidson, 1953). On the basis of these results, 30 $\mu$g/ml actinomycin D was chosen as the standard concentration for these experiments. The effect of this concentration of AMD on $^3$H-uridine incorporation at early cleavage and mesentoblast stages was determined by the same methods (see table 1).

The effects of continuous incubation in 30 $\mu$g/ml actinomycin D on uptake and incorporation of $^{35}$S-methionine were determined as described above (see Protein Solubilization) for both normal and lobeless embryos at cleavage, mesentoblast and gastrula stages (see table 2).

Protein Solubilization

Sample preparation was performed essentially as described by Garrels (1979). Washed embryos and polar lobes were homogenized for one minute in a glass tube with a motor-driven Teflon pestle in 50 $\mu$l of a solution containing 50 $\mu$g/ml micrococcal nuclease (Worthington Biochemical Corp., Freehold, N.J.) 2mM $CaCl_2$ and 20mM Tris-HCl, pH 8.8. After a two minute incubation at room temperature, 5 $\mu$l of a solution containing 1 $\mu$g/ml deoxyribonuclease (Sigma Chemical Co., St. Louis, Mo.), 500 $\mu$g/ml bovine pancreatic ribonuclease-A (Sigma Chemical Co., St. Louis, Mo.), 50mM $MgCl_2$ and 0.5 M Tris-HCl, pH 7.0 was added. After an additional two minute incubation, enzymatic reactions were terminated by the addition of 5 $\mu$l of a solution containing 3% SDS and 10% $\beta$-mercaptoethanol and 10 $\mu$l of a solution of 0.05 M ethelenediamine tetraacetic acid (EDTA). The homogenate was heated to 100°C for three minutes, frozen quickly in liquid Freon and lyophilized. Lyophilized samples were dissolved in 50 $\mu$l of a lysis buffer containing 9.5 M urea, 4% tergitol NP-40, 2% pH 5-7 ampholytes and 100 mM dithiothreitol. Samples were stored at -70°C.

Uptake and incorporation of $^{35}$S-methionine were determined for each sample. Uptake was determined by pipetting aliquots of the sample directly into Bray's dioxane-based scintillation cocktail (Bray, 1960) and counting in a scintillation spectrometer. Incorporation of precursor was determined by precipitating aliquots of each sample in 10% trichloroacetic acid, collecting the precipitate on Millipore filters (Millipore Corp., Bedford, Mass.), which were then dissolved in Bray's (1960) solution.

## Electrophoresis

Two-dimensional electrophoresis was performed as described by O'Farrell (1975) with the following modifications. The isoelectric focusing gel mixture contained 2.8% ampholytes composed of a mixture of 1.6% pH 5-7 ampholytes and 1.2% pH 3-10 ampholytes and was polymerized with riboflavin and ammonium persulfate in 10.5 x 0.3 cm lucite tubes. Samples were subjected to non-equilibrium isoelectric focusing for 3000 volt hours in an analytical electrophoresis cell designed for isoelectric focusing (MRA Corp., Clearwater, Fla.) and the focused gels were stored at -20°C. The pH gradient was determined by slicing a focused gel into 1 cm sections and eluting each section overnight in 1 ml distilled water. The pH of the eluents was measured with a glass electrode.

Second dimension electrophoresis of the focused gels was performed on discontinuous SDS slab gels as described by Laemmli (1970) in an apparatus similar to that described by O'Farrell (1975). The running gel, containing 12.5% acrylamide, was poured to a height of 12 cm, polymerized with riboflavin and ammonium persulfate and allowed to stand overnight. A 5 cm stacking gel of 4.75% acrylamide was poured the following day.

Focusing gels were equillibrated with SDS sample buffer (O'Farrell, 1975) containing freshly added $\beta$-mercaptoethanol and dithiothreitol for

1.5 hours and secured to the top of the slab gels with agarose. Electrophoresis was performed at 25 mA per gel, 19°C, until the tracking dye, bromophenol blue, reached the bottom of the running gel. Molecular weight standards were subjected to electrophoresis simultaneously with focused embryo proteins.

After second dimension electrophoresis was completed, the stacking gel was removed and the running gel was prepared for fluorography as described by Garrels (1979). The slab gels were soaked in two changes of dimethyl sulfoxide and overnight in a solution of 10% 2,5-diphenyloxazole (PPO; Scintillation Grade, New England Nuclear Corp., Boston, Mass.) in dimethyl sulfoxide. The fluor-impregnated gels were washed in several changes of water, dried on filter paper and exposed to Kodak X-ray film. Some gels were stained in 50% trichloroacetic acid containing 0.1% Coomassie brilliant blue, R-250 prior to fluorography to reveal molecular weight standards. Multiple exposures were made of each gel. Fluorographs were photographed on a light box with Kodak Plus X panchromatic film and printed at normal contrast on Kodak polycontrast RCII paper.

### Data Analysis

The results presented here are based on the analysis of two replicate electrophoretic separations of proteins from early cleavage and mesentoblast stage embryos, normal and lobeless mesentoblast stage and actinomycin D-treated mesentoblast stage embryos. Three replicate preparations of proteins were compared from normal cleavage stage embryos, isolated polar lobes and cleavage stage actinomycin D-treated embryos. Five replicate comparisons were made of proteins synthesized by normal and lobeless cleavage stage embryos.

For preliminary analysis, fluorographic exposures were chosen such that the number of acid precipitable counts applied to the first dimension gel multiplied by the exposure in days was equal for all gels to be compared.

Fluorograms of second dimension slab gels were divided into ten sectors by drawing, on a transparent overlay, vertical and horizontal lines through sixteen prominent spots that represented polypeptides common to all stages and classes of embryos studied. Since the sectors were delineated by the positions of marker polypeptides and were not fixed in size, they corrected for localized distortions in the gels and for positional variability of polypeptides between runs. Within each sector, spots were identified by their positions relative to the marker polypeptides and to the sector boundaries. For the comparison of any two fluorograms, polypeptides shared by both gels, polypeptides that differed quantitatively and polypeptides unique to one or the other gel were recorded in different colored ink on the transparent overlay.

When all comparisons within replicate sets had been completed, the transparent sheets were compared between replicates and the positions of polypeptides showing the same quantitative or qualitative differences on all replicate comparisons were marked on photographs of the fluorograms.

The spots which differed in these comparisons showed a large range of densities. While those spots with densities in the mid-range (polypeptides 91 and 44 in Fig. 4A) fall well within the linear range of X-ray film response and pose few problems in analysis, spots whose density is either very low (polypeptide 31 in Fig. 4A) or very high (polypeptide 80 in Fig. 4A) may, on an average exposure (500,000 acid precipitable counts exposed for 14 days), fall outside the range of linear film response to exposure (see for discussion Braude, 1979; Laskey and Mills, 1975). For quantitative comparisons, multiple exposures of each gel were examined so that the intensity of the spots to be compared fell within the linear range of film response.

Thus, if a spot was judged as very dense on an average exposure, progressively shorter exposures were examined until the spot was detected as grey rather than black. At these exposures, accurate judgements of quantitative differences within a two-fold range were possible.

Those polypeptides which showed reproducible differences between stages and experimental groups, plus 8 marker polypeptides which did not change were numbered. From an average of 300 polypeptides detected, 98 were numbered and 90 of these showed reproducible differences which were correlated with stage of development or experimental treatment.

# RESULTS

## Objectives and Experimental Design

The goal of these studies was to describe the regulation of gene expression during early development in a spiralian embryo through an analysis of the mechanism(s) by which stage-specific changes in polypeptide synthesis are accomplished.

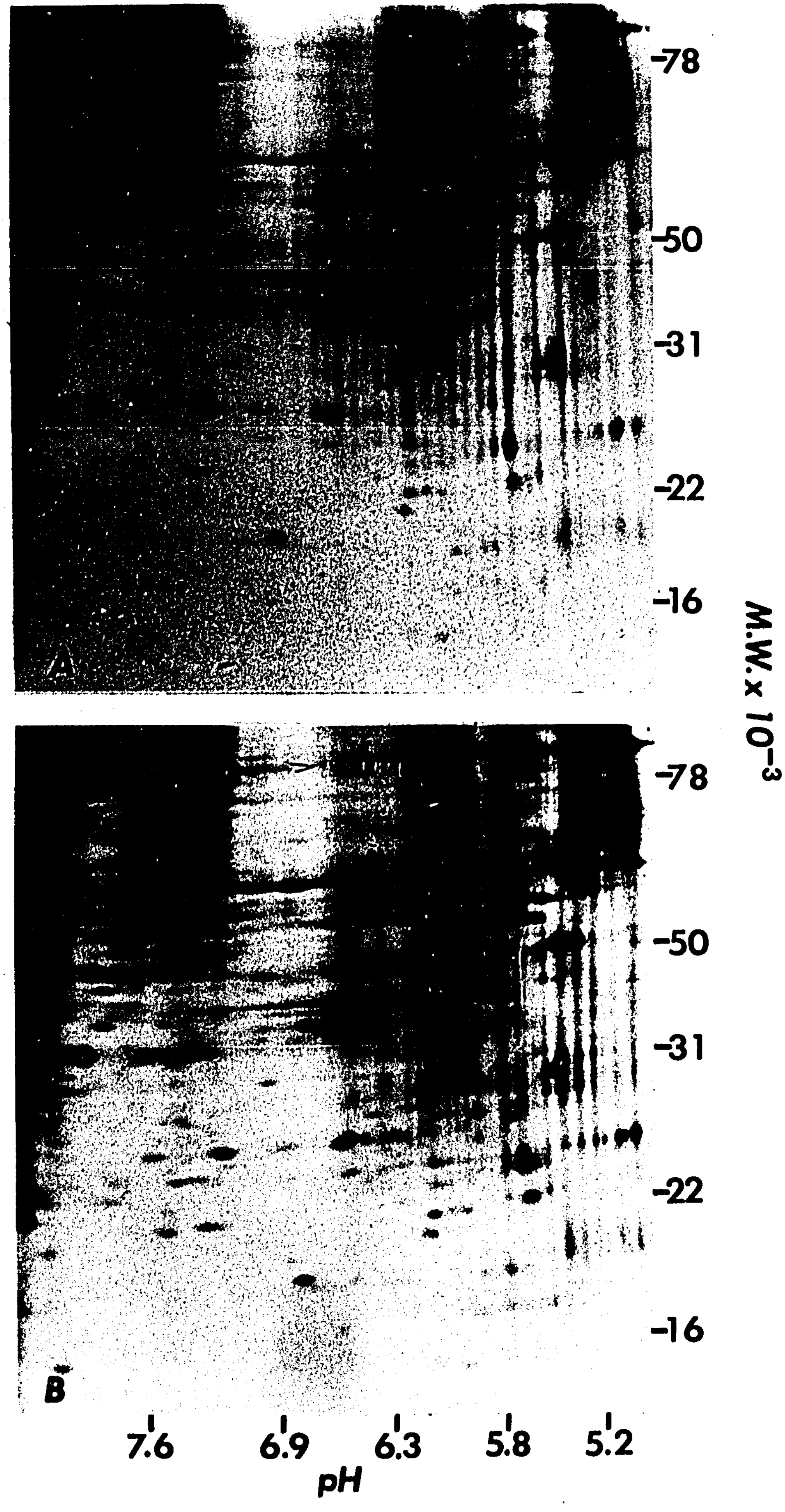
The approach was a comparison, by two-dimensional electrophoresis, of polypeptides synthesized during early cleavage, mesentoblast and gastrula stages. The relative contribution of maternal and embryogenic transcription to changes in polypeptide synthesis throughout this period was assessed by comparing the polypeptides made by normal and actinomycin D-treated embryos. The mechanism(s) by which ooplasmic segregation controls stage-specific changes in polypeptide synthesis were investigated by (1) an analysis of polypeptide synthesis in the isolated polar lobe, (2) a comparison of polypeptide synthesis in normal and lobeless embryos and (3) a comparison of the effects of actinomycin D on polypeptide synthesis in normal and lobeless embryos.

## Early Cleavage Stage

In designing the experiments described here, I asked whether (1) the polypeptides made at this stage are translated from maternal mRNA or newly transcribed RNA, (2) mRNAs are selectively localized in the polar lobe or the lobeless blastomeres and (3) the polar lobe or the lobeless embryo contain factors that regulate transcription or translation during early cleavage.

Fig. 1A. Fluorogram of polypeptides made by normal two to eight-cell stage embryos. Embryos were incubated for 4 hours in $^{35}$S-methionine, polypeptides were extracted and separated by two-dimensional electrophoresis (non-equilibrium isoelectric focusing followed by SDS polyacrylamide gel electrophoresis) as previously described. The pH gradient of the first dimension gel is shown at the bottom of Fig. 2B and the molecular weights of polypeptides separated by SDS gel electrophoresis are shown at the right margins of Figs. 1A and 1B.

Fig. 1B. Fluorogram of polypeptides made by actinomycin D-treated two to eight-cell stage embryos. Embryos were pre-incubated in 30 μg/ml actinomycin D from first polar body formation to the two cell stage, followed by a four hour incubation in $^{35}$S-methionine in the presence of actinomycin D.


-78
-50
-31
-22
-16
A
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-22
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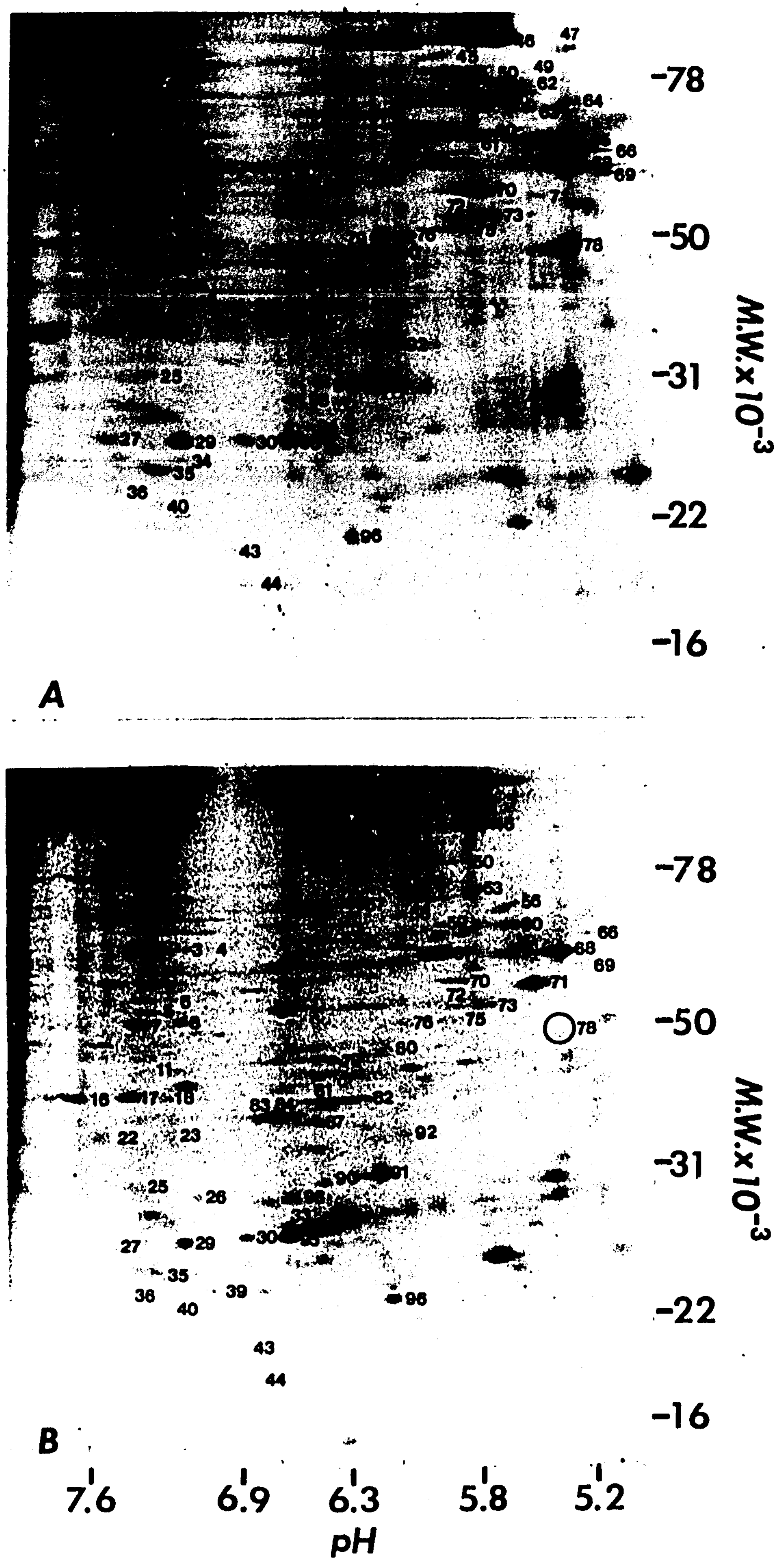
B
M.W.x $10^{-3}$
7.6
6.9
6.3
5.8
5.2
pH

Fig. 2A. Fluorogram of $^{35}$S-methionine labeled polypeptides synthesized by freshly isolated polar lobes. Small numerals in this and subsequent figures, designate polypeptides that change during development or as a result of polar lobe removal or actinomycin D treatment.

Fig. 2B. Fluorogram of polypeptides made by isolated polar lobes cultured in sea water for 24 hours prior to a 4 hour incubation in $^{35}$S-methionine.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72
73
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34
35
36
40
43
44
96
-78
-50
-31
-22
-16
M.W.x10⁻³
A
50
53
56
60
66
68
69
70
71
72
73
75
76
78
3
4
5
80
11
16
17
18
81
82
83
86
87
92
22
23
25
26
90
91
27
29
30
35
36
39
40
96
43
44
-78
-50
-31
-22
-16
M.W.x10⁻³
B
7.6
6.9
6.3
5.8
5.2
pH

## Polypeptides Made during Early Cleavage Are Translated from Oogenic mRNAs

To establish which of the polypeptides made during early cleavage are the translation products of maternal mRNA, two-dimensional electropherographs of $^{35}$S-methionine labeled polypeptides extracted from normal and actinomycin D-treated embryos were compared.

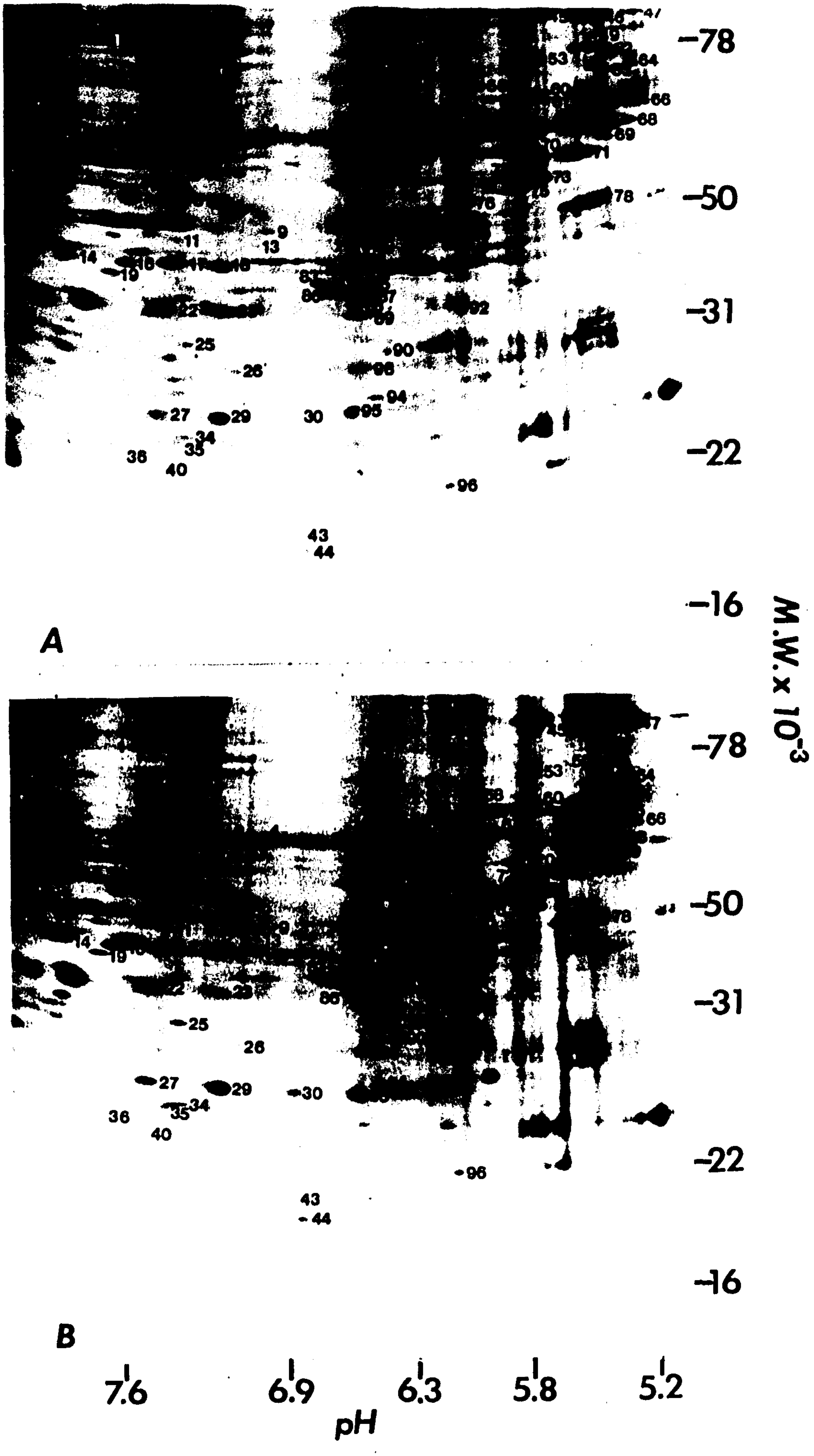
Figure 1A shows a fluorogram of polypeptides synthesized by two to four-cell embryos and separated by two-dimensional electrophoresis. Figure 1B illustrates the pattern of polypeptides synthesized by embryos reared from the time of first polar body formation in 30 $\mu$g/ml actinomycin D. In three replicate comparisons, no reproducible differences were detected between the two groups of embryos in the relative rates of accumulation of any of the three hundred polypeptides made at this stage of development.

These results demonstrate that the polypeptides made during early cleavage and resolved by two-dimensional electrophoresis are insensitive to actinomycin D and are probably translated from oogenic mRNAs and that the regulation of their translation is, at this stage, independent of new transcription.

While actinomycin D does have side effects, as shown by the twenty-six percent inhibition of $^{35}$S-methionine incorporation in actinomycin D-treated cleavage stage embryos (table 2), the finding that the relative rates of accumulation of three hundred polypeptides made at this stage are unaltered by actinomycin D, demonstrates that the repression of protein synthesis by actinomycin D does not selectively alter the translation of specific mRNAs. Further, the concentration of actinomycin D used in these experiments does not affect the uptake of $^{35}$S-methionine (table 2) or retard cleavage, although it does repress $^{3}$H-uridine incorporation

Fig. 3A. Fluorogram of polypeptides made by normal four to twelve-cell stage embryos. Embryos were incubated for 4 hours in $^{35}$S-methionine, $4 \times 10^5$ acid precipitable CPM were applied to the first dimension gel and the second dimension gel was exposed for 6 days.

Fig. 3B. Fluorogram of polypeptides made by four to twelve-cell stage lobeless embryos. Radioactive labeling, number of acid precipitable CPM and exposure time as in 3A.

M.W. x 10^-3
-78
-50
-31
-22
-16
A
B
7.6
6.9
6.3
5.8
5.2
pH
47
53
64
66
68
69
71
73
78
9
11
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36
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83
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92
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98

into acid precipitable material by 85% (table 1).

## Normal and Lobeless Embryos Translate the Same Maternal Messenger RNAs

The polypeptides synthesized by normal and lobeless cleavage stage embryos were compared to establish whether the determinative events of this period in development are associated with the selective localization and/or translation of maternal mRNAs in the polar lobe or the lobeless blastomeres.

Figure 3A shows a fluorogram of 300 polypeptides synthesized by normal embryos between the four and twelve-cell stages and figure 3B, the corresponding pattern obtained from lobeless embryos. In five replicate comparisons, no reproducible qualitative or quantitative differences in polypeptide synthesis were found between normal and lobeless embryos at this stage of development.

These data indicate that there is no selective localization of mRNAs or of factors affecting translation during early cleavage, in either the lobeless blastomeres or, by inclusion of the polar lobe cytoplasm, in the normal embryo.

## The Isolated Polar Lobe Translates the Same mRNAs as the Lobeless Embryo

The definitive test of the hypothesis that mRNAs are selectively localized during polar lobe formation was the comparison of the polypeptides made by the isolated polar lobe with those made by the lobeless blastomeres during cleavage.

Freshly isolated polar lobes were incubated in $^{35}$S-methionine for four hours and the polypeptides extracted from this cytoplasmic egg fragment are illustrated in the electropherograph in figure 2A. A comparison of

## TABLE 3

## POLYPEPTIDE SYNTHESIS IN ISOLATED POLAR LOBES

| | NATURE OF CHANGE IN POLAR LOBE | POLYPEPTIDES INVOLVED IN CHANGE[1] | NORMAL STAGE SPECIFIC CHANGE | PROBABLE MODE OF REGULATION |
|---|---|---|---|---|
| (A) | Absent in 24 Hour Polar Lobe | 62 63 64 65 | Absent in Mesentoblast State | Operation of Translational Repressor in the Polar Lobe |
| (B) | Absent in all Polar Lobes | 10 12 15 21 24 28 31 32 37 38 39 41 42 48 51 52 54 55 74 77 93 97 | Unique to Mesentoblast Stage | Absence of mRNP and/or mRNP Activator in Polar Lobe |
| (C) | Decrease in Rate of Accumulation | 2 3 4 23 78 | Decrease in Rate of Accumulation | Presence of Translational Repressor in the Polar Lobe |
| (D) | Decrease in Rate of Accumulation | 22 47 53 11 | No Change in Rate of Accumulation | Abnormal Operation of Repressor in the Polar Lobe |
| (E) | Increase in Rate of Accumulation | 16 17 26 | Increase in Rate of Accumulation | mRNP Activator or Translational Activator in the Polar Lobe |
| (F) | Increase in Rate of Accumulation | 71 94 | Decrease in Rate of Accumulation | Absence of Translational Repressor in the Polar Lobe |
| (G) | Unique to 24 Hour Polar Lobe | 33 56 | Unique to Mesentoblast Stage | mRNP Activator in the Polar Lobe |
| (H) | Present but do not Increase in 24 Hour Polar Lobe | 5 6 8 18 20 40 61 72 73 76 | Increase in Rate of Accumulation | Absence of Translational Activator or mRNP Activator in the Polar Lobe |
| (I) | Present in 24 Hour Polar Lobe | 25 66 83 | Absent in Mesentoblast Stage | Absence of Translational Repressor for these Polypeptides in the Polar Lobe (see also (A) above) |

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[1] Numbers refer to individual polypeptides illustrated in Figures 1A and 1B.

polypeptides synthesized by normal cleavage stage embryos with those made by isolated polar lobes revealed that the polar lobe synthesized all three hundred species of polypeptides characteristic of this stage of development and, in three independent comparisons of polypeptides made by polar lobes and lobeless blastomeres, no polypeptides were found to be unique to either group.

These findings demonstrate that there is no selective localization of the mRNAs translated during early cleavage in the polar lobe or the lobeless blastomeres. In addition, because this egg, prior to polar lobe formation, is arrested in meiotic diakinesis, a stage at which the chromosomes are highly condensed and are probably transcriptionally inactive (McCann-Collier, 1979), the ability of the isolated, cytoplasmic polar lobe to synthesize all of the polypeptides characteristic of intact cleavage stage embryos, is further evidence that these polypeptides are translated from oogenic mRNAs.

## Mesentoblast Stage

In the normal embryo, the mesentoblast cell is formed from the D macromere twenty-four hours after first cleavage, some three hours in advance of the other members of the fourth quartet of micromeres. It is determined, by the presence of the polar lobe cytoplasm, to give rise to the mesoblast and enteroblast cells of the embryo. During this critical 24-hour period, the polar lobe cytoplasm also exerts an effect on micromeres not in the D quadrant by induction. In addition, the fates of several micromeres formed during this period are determined independently of the polar lobe cytoplasm (Clement, 1976, 1967, 1962).

In the following series of experiments, I ask whether the determinative events that occur between early cleavage and the mesentoblast stage are correlated with changes in polypeptide synthesis, in the source(s) of mRNA or in the mechanism(s) by which protein synthesis is regulated.

### Extensive Changes in Polypeptide Synthesis Occur between Cleavage and Mesentoblast Stages

The first step in the analysis of polypeptide synthesis in mesentoblast stage embryos was to identify the polypeptides which changed, either qualitatively or quantitatively, between cleavage and mesentoblast stages.

Figure 4A shows a fluorogram of $^{35}$S-methionine labeled polypeptides synthesized by normal mesentoblast stage embryos. Two independent comparisons of polypeptides synthesized by mesentoblast stage embryos and early cleavage stage embryos (Fig. 3A), revealed both qualitative and quantitative stage-specific differences. All of the polypeptides that changed reproducibly between cleavage and mesentoblast stages were numbered and the differences were summarized in column 1 of table 4.

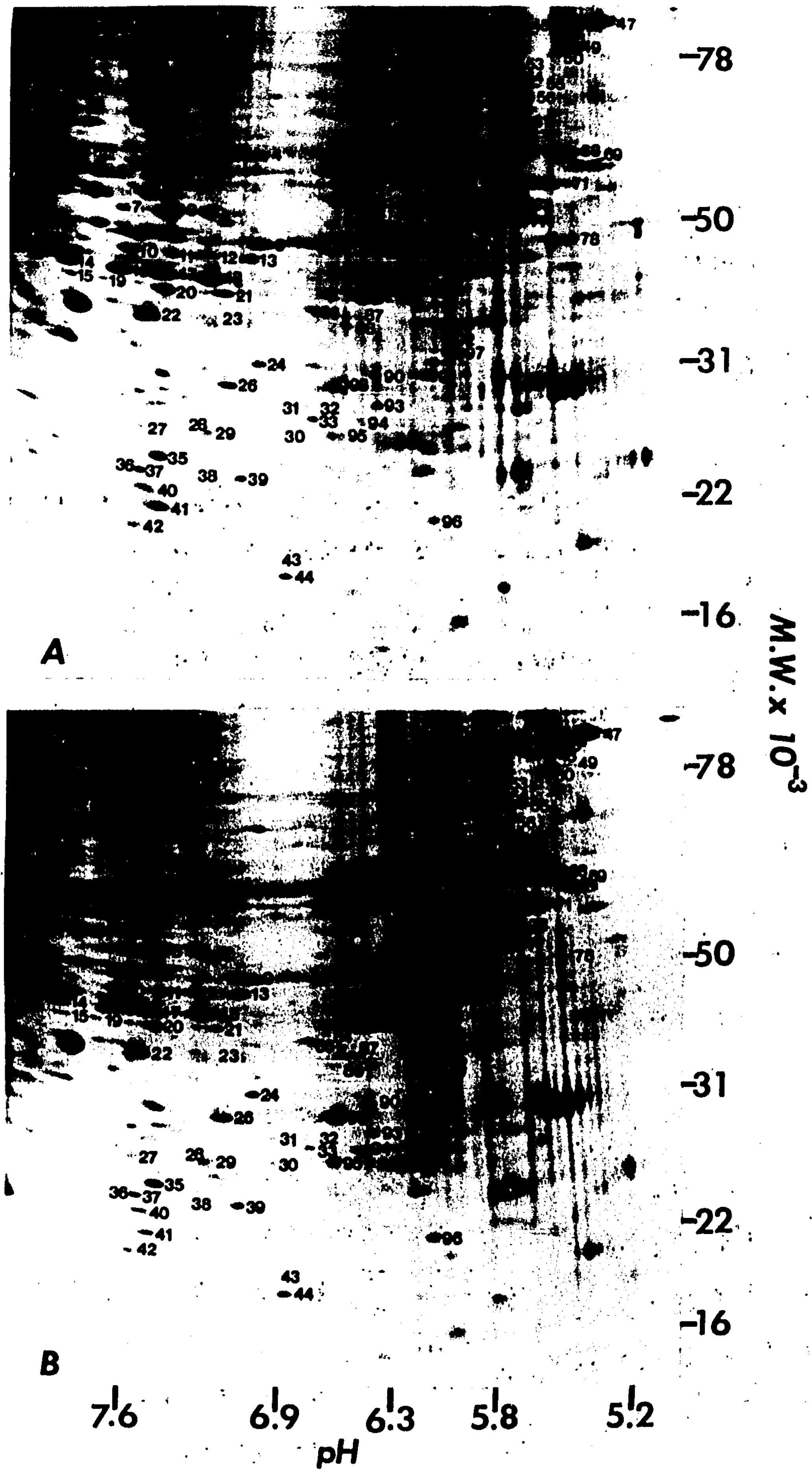
Twelve polypeptides (4%) were found to be unique to cleavage stage embryos, disappearing in the 24-hour old normal embryo. Twenty-four polypeptides (8%) were unique to mesentoblast stage embryos, appearing for the first time 24 hours after first cleavage.

A larger number of polypeptides showed changes in relative rates of accumulation between cleavage and mesentoblast stages; twenty (6.7%) increased in the 24-hour old embryo, while eighteen (6%) showed a decrease in relative accumulation between cleavage and mesentoblast stages. A group of twenty-four polypeptides that did not change between cleavage and mesentoblast stages, but that were affected by actinomycin D treatment or polar lobe removal are listed in line 2 of table 4.

The period of development from early cleavage to mesentoblast cell formation is marked by extensive changes in polypeptide synthesis; of

Fig. 4A. Fluorogram of polypeptides made by normal mesentoblast stage embryos. Embryos were incubated for 4 hours with $^{35}S$-methionine, $3 \times 10^5$ acid precipitable CPM were applied to the first dimension gel and the second dimension gel was exposed for 8 days.

Fig. 4B. Fluorogram of polypeptides made by lobeless mesentoblast stage embryos. Radioactive labeling, number of acid precipitable CPM and exposure time as in 4A.


A
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# Table 4

## Polypeptide Synthesis by Cleavage and Mesentoblast Embryos

| | | Normal | | Actinomycin D-Treated Embryos |
|---|---|---|---|---|
| Polypeptides Unique to Cleavage<br>25 34 60 62 63 64<br>65 66 83 84 85 89 | | absent in normal mesentoblast stage | | 84 89 |
| Polypeptides Constant between Cleavage and Mesentoblast Stages | | 1 7 22 27 30 36<br>43 44 47 49 50 53<br>59 67 75 79 81 82<br>86 88 90 92 96 98 | same | 7 22 27 30 36 43<br>47 49 50 59 67 79<br>86 88 92 98 |
| | | | increase | 44 81 82 90 96 |
| | | | decrease | 1 53 75 |
| Polypeptides Unique to Mesentoblast Stage | | 10 12 15 21 24 28<br>31 32 33 37 38 39<br>41 42 48 51 52 54<br>55 56 74 77 93 97 | same | 33 38 42 48 52 55<br>56 74 93 |
| | | | increase | 39 |
| | | | decrease | 12 21 24 41 51 54<br>77 97 |
| | | | absent | 10 15 28 31 32 37 |
| Polypeptides which Change between Cleavage and Mesentoblast Stages | increase | 5 6 8 9 11 13<br>14 16 17 18 20 26<br>35 40 61 69 72 73<br>76 80 | same | 5 6 8 14 16 17<br>18 40 61 72 73 76 |
| | | | increase | 26 35 |
| | | | decrease | 9 11 13 20 69 80 |
| | decrease | 2 3 4 19 23 29<br>45 46 57 58 68 70<br>71 78 87 91 94 95 | same | 2 4 19 23 45 46<br>57 58 70 78 87 |
| | | | increase | 29 68 71 91 94 95 |
| | | | decrease | 3 |

three hundred polypeptides resolved, twenty-five percent change qualitatively or quantitatively within this critical 24-hour period.

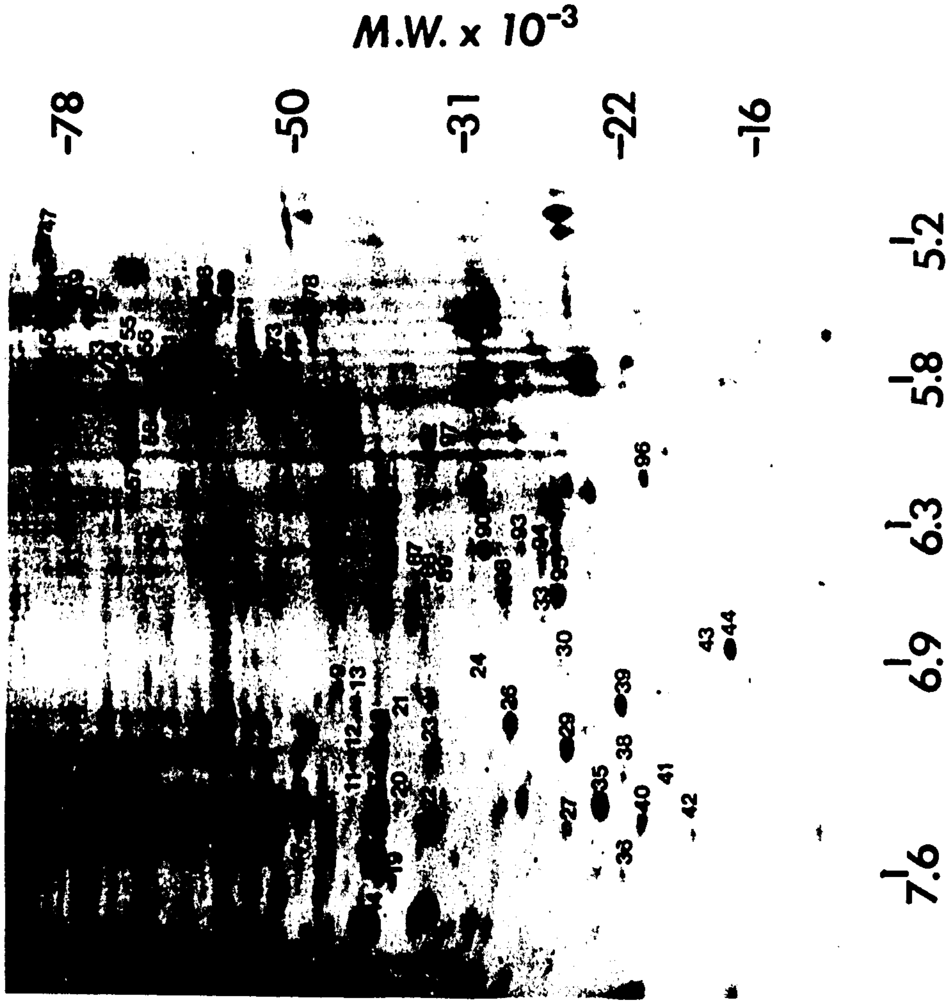
## Several Sources of mRNA and New Modes of Regulation Are Used at the Mesentoblast Stage

The polypeptides made by normal mesentoblast stage embryos were compared with those made by mesentoblast stage embryos reared in actinomycin D, to establish which of the polypeptides synthesized at this stage were coded for by maternal or embryogenic mRNAs and to determine whether the changes in polypeptide synthesis that occur between cleavage and mesentoblast stages were regulated at the transcriptional or the translational level.

The effect of continuous incubation in 30 µg/ml actinomycin D from first cleavage on the pattern of polypeptide synthesis in mesentoblast stage embryos is illustrated by the electropherogram in figure 5. At this stage, continuous incubation in actinomycin D resulted in a 41.7% inhibition of protein synthesis (table 2), therefore, fluorographic exposures were increased to compensate for the lower number of acid precipitable counts applied to the first dimension gels. The results of two replicate comparisons of polypeptides extracted from control and actinomycin D-treated mesentoblast stage embryos are summarized in column 2 of table 4. When polypeptides synthesized by actinomycin D-treated embryos were compared with those synthesized by controls, four classes of response to the inhibition of RNA synthesis were observed: 83% of the polypeptides resolved did not change as a result of actinomycin D treatment, 2% were absent, 6% showed a decrease in relative accumulation and 5% increased in the presence of actinomycin D.

The majority of the polypeptides resolved at this stage were unaffected by the application of actinomycin D; none of the unnumbered polypeptides changed reproducibly as a result of transcriptional inhibition and

Fig. 5. Fluorogram of polypeptides made by actinomycin D-treated normal mesentoblast stage embryos. Embryos were reared from first cleavage in 30 μg/ml actinomycin D, incubated at the mesentoblast stage in $^{35}$S-methionine for 4 hours, $1.4 \times 10^5$ acid precipitable CPM were applied to the first dimension gel and the second dimension gel was exposed for 16 days.


M.W. x 10$^{-3}$
-78
-50
-31
-22
-16
5.2
5.8
6.3
6.9
7.6
pH
47
55
56
78
96
90
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8

forty-eight of the numbered polypeptides showed the same relative rates of accumulation in actinomycin D-treated embryos as in controls. That the inhibition of RNA synthesis from first cleavage failed to alter, either qualititatively or quantitatively, the synthesis of these polypeptides, suggests that they are translated from oogenic mRNAs. Of special significance were those polypeptides (33, 38, 42, 48, 52, 55, 56, 74 and 93) that appeared for the first time at the mesentoblast stage, whether or not transcription was inhibited by actinomycin D, suggesting that they are coded for by oogenic mRNAs, stored in inactive form during early cleavage and activated, by a transcriptionally independent mechanism, at the mesentoblast stage.

Only six polypeptides, present in control mesentoblast stage embryos, were absent from actinomycin D-treated mesentoblast stage embryos; these polypeptides would, in control embryos, have appeared for the first time at the mesentoblast stage. Their absence in actinomycin D-treated embryos suggests that they may be translated from embryogenic mRNAs.

Eighteen polypeptides showed a decreased rate of relative accumulation in actinomycin D-treated embryos when compared with controls; most of the decreases that occurred as a result of actinomycin D treatment affected those polypeptides that were unique to mesentoblast stage embryos or that, in controls, showed increased relative rates of accumulation between cleavage and mesentoblast stages. Two alternative explanations of the decrease in accumulation of these polypeptides in actinomycin D can be advanced: either these polypeptides are translated from two sources of mRNA--oogenic and embryogenic--or they are translated from maternal mRNA but require transcriptionally dependent activation to achieve normal levels of accumulation. Our experimental design does not allow us to distinguish between these alternatives.

An unexpected finding was that fourteen polypeptides showed increased relative rates of accumulation in actinomycin D-treated embryos in comparison with controls and two polypeptides that normally disappeared between cleavage and mesentoblast stages were present in actinomycin D-treated mesentoblast stage embryos. These two responses to actinomycin D appear to be analogous and may be controlled by the same or similar mechanism(s). That these polypeptides continue to be synthesized despite the inhibition of RNA synthesis indicates that they are translated from oogenic mRNAs. The increase observed in the relative rates of accumulation of these polypeptides when transcription is inhibited suggests that the translation of the oogenic mRNAs coding for these polypeptides is normally repressed by a transcriptionally dependent mechanism(s). On the basis of these data, the existence of a group of repressor genes is postulated; the transcripts made from these genes, directly or indirectly, repress the translation of mRNAs and their action can be eliminated by actinomycin D (Tompkins, et al. 1972).

These data show that, between cleavage and mesentoblast stages, new sources of mRNA and new control mechanisms are used: transcription of embryogenic mRNA is activated, oogenic mRNA is released, and the translation of oogenic mRNA is regulated by new transcription. Nevertheless, the majority of changes that occur between cleavage and mesentoblast stages take place normally in actinomycin D, indicating that the primary mechanism by which the program of stage-specific changes in polypeptide synthesis is accomplished is the transcriptionally independent, translational regulation of maternal mRNAs. The term, translational regulation, is used here in its broadest sense to describe a variety of mechanisms operating at the level of the ribosome and mRNA to affect the stability of mRNAs and/or the rate of initiation of polypeptide synthesis.

An analysis of polypeptide synthesis in isolated 24-hour old polar lobes, lobeless mesentoblast stage embryos and actinomycin D-treated lobeless embryos was undertaken, to establish whether ooplasmic segregation results in the localization of factors that affect the utilization of new sources of mRNA or the activation of new control mechanisms at the mesentoblast stage.

## Oogenic mRNA Translation is Regulated in the Isolated 24-Hour Old Polar Lobe

The polypeptides synthesized by freshly isolated and 24-hour old polar lobes were compared, to determine whether the mRNAs present in the lobe are stable during this period and to establish whether factors that regulate the translation of mRNA are present in this cytoplasmic egg fragment.

Isolated polar lobes were placed in pasteurized sea water for 24 hours, incubated in $^{35}$S-methionine and the labeled proteins were extracted and separated by two-dimensional electrophoreses. An electropherogram of polypeptides synthesized by 24-hour old polar lobes is shown in figure 2B. In three replicate comparisons, it was found that the majority of the polypeptides synthesized by freshly isolated polar lobes (fig. 2A) continued to be synthesized by polar lobes that had been allowed to stand in sea water for 24 hours prior to incubation in $^{35}$S-methionine.

Table 3 summarizes the differences in polypeptides synthesized by freshly isolated and 24-hour old polar lobes and relates these differences to the normal pattern of changes in polypeptide synthesis observed in intact embryos between cleavage and mesentoblast stages.

Four polypeptides (line A, table 3) were present in freshly isolated polar lobes, but disappeared in 24-hour old polar lobes; these same polypeptides were also absent from normal mesentoblast stage embryos, but not from preparations of polypeptides synthesized by intact cleavage stage embryos. Twenty-two polypeptides unique to normal mesentoblast stage embryos were absent from both freshly isolated and 24-hour old polar lobes (line B). A decrease in the rate of accumulation of nine polypeptides occurred in day-old polar lobes (lines C and D); five of these polypeptides showed corresponding decreases in normal mesentoblast stage embryos (line C).

Three polypeptides (16, 17 and 26) were found to increase at 24 hours in both normal embryos and polar lobes (line E). An increase in accumulation of polypeptides 71 and 94 was observed in 24-hour old polar lobes (line F), but not in intact embryos between cleavage and mesentoblast stages. Polypeptides 33 and 56 were not present in freshly isolated polar lobes, but, as in normal embryos, appeared for the first time in 24-hour old polar lobes.

While some of the stage-specific changes in polypeptide synthesis that were observed in intact embryos occurred in aging polar lobes (lines A, C, E, G), a large number of stage-specific changes failed to occur normally (lines B, H, I) and some of the differences detected between freshly isolated and 24-hour old polar lobes were not characteristic of normal embryos (lines D, F). Several conclusions can be drawn from these observations: (1) the cytoplasmic polar lobe contains at least three hundred species of maternally transcribed mRNAs, many with half-lifes of at least 12 hours, (2) the polar lobe cytoplasm is capable of regulating the translation of some of these oogenic mRNAs and (3) the interaction of both animal and vegetal halves of the egg is required for the normal regulation of some of the stage-specific changes in polypeptide synthesis.

In the next sections, this last conclusion will be tested further by an examination of polypeptide synthesis in lobeless mesentoblast stage embryos.

### The First Differences in Polypeptide Synthesis in Normal and Lobeless Embryos Occur at the Mesentoblast Stage

Figure 4B is a fluorogram of polypeptides synthesized by 24-hour old

# Table 5

## Polypeptide Synthesis by Lobeless Embryos

| Normal | Lobeless | | Lobeless Actinomycin D-Treated | |
|---|---|---|---|---|
| Polypeptides Unique to Cleavage Stage<br>25 34 60 62 63 64<br>65 66 83 84 85 89<br>absent in normal mesentoblast stage | | 84 89 | increase | 84* 89* |
| Polypeptides Constant between Cleavage and Mesentoblast Stages<br>1 7 22 27 30 36<br>43 44 47 49 50 53<br>59 67 75 79 81 82<br>86 88 90 92 96 98 | increase | 81 82 | same | 7 22 27 36 43 47<br>49 50 67 79 81* 82*<br>86 88 92 98 30 |
| | same | 1 7 22 27 36 43<br>44 47 49 50 53 59<br>67 75 79 86 88 90<br>92 96 98 30 | increase | 44 90 96 |
| | | | decrease | 1 59* 75 |
| | | | absent | 53* |
| Polypeptides Unique to Mesentoblast Stage<br>10 12 15 21 24 28<br>31 32 33 37 38 39<br>41 42 48 51 52 54<br>55 56 74 77 93 97 | same | 10 12 15 21 24 28<br>31 32 33 37 38 39<br>41 42 48 51 52 54<br>55 56 74 77 93 97 | same | 33 38 42 48 52 55<br>56 74 93 |
| | | | increase | 39 |
| | | | decrease | 12 21 24 41 77 |
| | | | absent | 10 15 28 31 32 37<br>51* 54* 97* |
| Polypeptides which Change between Cleavage and Mesentoblast Stages | | | | |
| increase<br>5 6 8 9 11 13<br>14 16 17 18 20 26<br>35 40 61 69 72 73<br>76 80 | same | 5 6 8 9 11 13<br>18 26 35 40 61 69<br>72 73 76 80 | same | 5 6 8 14 18 40<br>61 72 73 76 |
| | | | increase | 16* 17* 26 35 |
| | decrease | 14 16 17 20 | decrease | 9 11 13 20 69 80 |
| decrease<br>2 3 4 19 23 29<br>45 46 57 58 68 70<br>71 78 87 91 94 95 | same | 2 3 4 19 23 29<br>45 46 57 58 68 70<br>87 91 95 | same | 2 3* 4 19 23 45<br>46 57 58 70 71<br>78 87 94 |
| | increase | 71 94 | | |
| | decrease | 78 | increase | 29 68 91 95 |

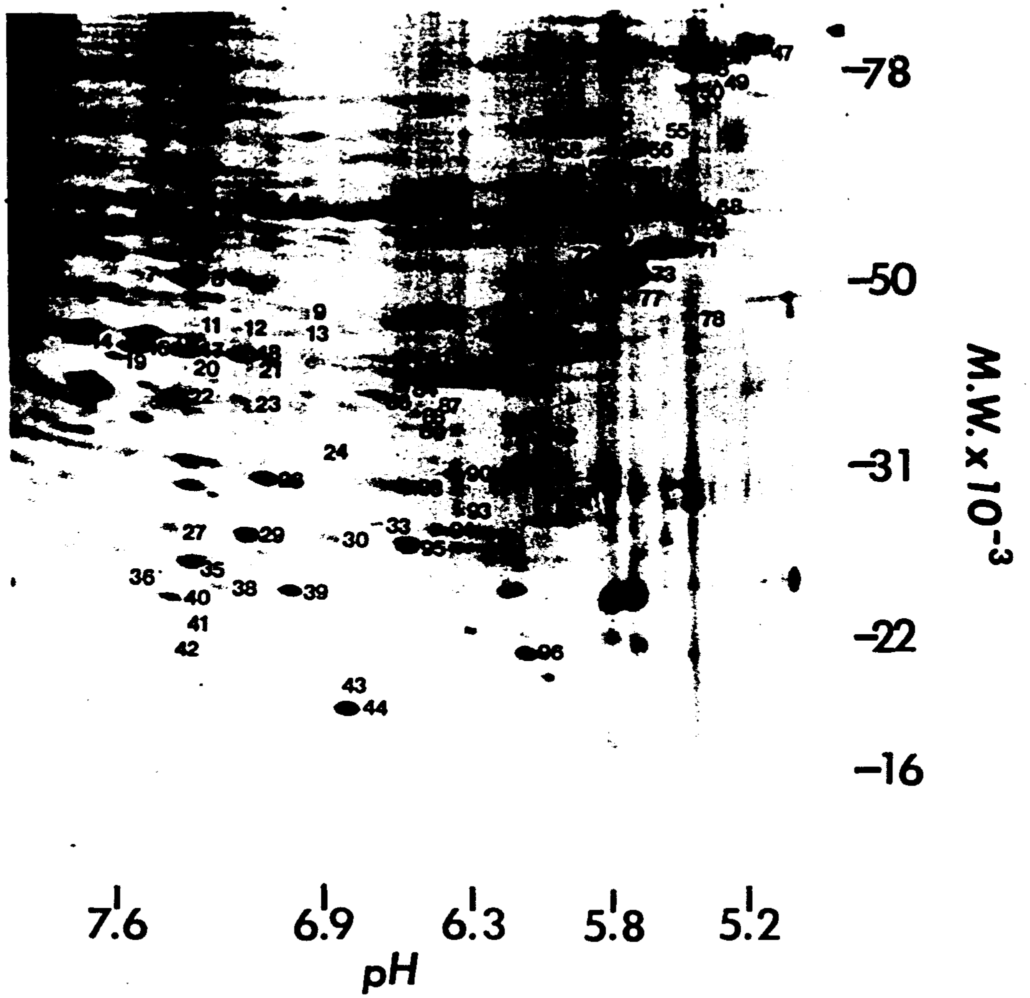
embryos from which the polar lobe had been removed at first cleavage. The results of two replicate comparisons of polypeptide synthesis in normal and lobeless mesentoblast stage embryos are summarized in column 2 of table 5. Of an average of three hundred polypeptides resolved in these experiments, fourteen polypeptides (4.7%) differed between normal and lobeless embryos at this stage of development.

Polypeptides 14, 16, 17 and 20 normally increased between cleavage and mesentoblast stages, but in the lobeless embryo, the relative rates of accumulation of these polypeptides did not change and therefore, the relative accumulation of these polypeptides in the lobeless embryo was substantially less than in normal mesentoblast stage embryos. Polypeptide 78 also showed a decreased rate of accumulation in lobeless mesentoblast stage embryos in comparison with normal embryos of the same stage; this polypeptide normally decreases between cleavage and mesentoblast stages and, therefore, the relative rate of accumulation of this polypeptide in the lobeless embryo is abnormal in degree but not in direction.

The relative rates of accumulation of polypeptides 81, 82, 71 and 94 increased in lobeless mesentoblast stage embryos in comparison with normal embryos and two polypeptides, 84 and 89, that were unique to cleavage stage embryos, failed to disappear in 24-hour old lobeless embryos. These polypeptides were similarly affected by the application of actinomycin D, suggesting that removal of the polar lobe might result in the failure of a transcriptionally dependent, translational repressor to be synthesized or activated.

Additional clues to the nature of the lobeless embryo's defects were found by an analysis of polypeptide synthesis in actinomycin D-treated lobeless mesentoblast stage embryos.

Fig. 6. Fluorogram of polypeptides made by actinomycin D-treated lobeless mesentoblast stage embryos. Embryos were reared in actinomycin D (30 μg/ml) from first cleavage until the mesentoblast stage, incubated in $^{35}$S-methionine for 4 hours, 2.1 x $10^5$ acid precipitable CPM were applied to the first dimension gel and the second dimension gel was exposed for 12 days.


M.W. x 10$^{-3}$
78
50
31
22
16
pH
7.6
6.9
6.3
5.8
5.2

## Translational Regulation of Oogenic mRNAs is Abnormal in Lobeless Embryos

To determine whether the defects in polypeptide synthesis seen in lobeless embryos were transcriptionally dependent, polypeptides synthesized by actinomycin D-treated lobeless mesentoblast stage embryos were compared with those made by actinomycin D-treated normal and untreated lobeless mesentoblast stage embryos.

A fluorogram of polypeptides synthesized by lobeless mesentoblast stage embryos reared from first cleavage in actinomycin D is shown in figure 6. In two independent experiments, polypeptides made by actinomycin D-treated lobeless embryos were compared with those made by actinomycin D-treated normal embryos (Fig. 5). Only four polypeptides differed reproducibly between the two groups. Polypeptides 53, 51, 54 and 97 were reduced in actinomycin D-treated normal embryos, but were completely absent in actinomycin D-treated lobeless embryos. The absence of these polypeptides in lobeless embryos when they are only reduced in normal embryos in the presence of actinomycin D, suggests that either (1) the oogenic mRNAs that code for these polypeptides are localized in the polar lobe, and thus, are absent from the lobeless embryo or (2) the lobeless embryo lacks factors needed to activate the translation of stored oogenic mRNAs. That the levels of accumulation of these polypeptides in untreated lobeless embryos was the same as that of untreated normal embryos, despite the reduction of polysomal oogenic mRNA in the lobeless embryo, suggests that, in the absence of actinomycin D, transcription of the genes for these polypeptides proceeds at normal, or above normal, rates in the lobeless embryo.

Two replicate comparisons of polypeptide synthesis in lobeless actinomycin D-treated embryos with that of control lobeless mesentoblast stage embryos, revealed several differences in the response of normal and lobeless embryos to inhibition of RNA synthesis. The effect of actinomycin D on the relative accumulation of specific polypeptides in lobeless embryos is summarized in column 3 of table 5. All polypeptides that are affected by actinomycin D treatment differently in lobeless than in normal embryos are indicated on this table with an asterisk.

When the relative rates of accumulation of polypeptides 81, 82, 71 and 94 were compared in actinomycin D-treated and untreated lobeless embryos, no differences were detected. A similar pattern was observed for polypeptides 84 and 89 that were absent from normal mesentoblast stage embryos but present in normal and lobeless actinomycin D-treated embryos and in lobeless mesentoblast stage embryos. Thus, in comparison with normal mesentoblast stage embryos, three experimental classes (normal actinomycin D-treated, lobeless actinomycin D-treated and lobeless control embryos) showed the same increase in the relative accumulation of these polypeptides.

One interpretation of these observations is illustrated in panel 3B of figure 9. The increase in accumulation of these polypeptides in actinomycin D-treated normal embryos indicates that the translation of the mRNAs coding for these polypeptides is normally repressed by a transcriptionally dependent mechanism; therefore we postulate the existence of a gene(s) whose transcripts act, directly or indirectly, as translational repressors of specific mRNAs. That these polypeptides also increase in lobeless embryos suggests that these embryos fail to activate transcription of the putative repressor gene and leads us to postulate that the transcription of this gene(s) requires the presence of an inducer that is localized in the polar lobe and thus, absent from the lobeless embryo. In the absence of the polar lobe-localized inducer, transcription of the repressor gene is never initiated and, therefore, treatment of lobeless embryos with actinomycin D has no further effect on the accumulation of these polypeptides.

An alternative explanation, which we cannot rule out, is that the transcription of the repressor gene is constitutive, but that the presence of a factor localized in the polar lobe is required to activate the product of the repressor gene.

Polypeptides 14 and 78 were decreased in lobeless control embryos in comparison with normal embryos, but not in comparison with lobeless actinomycin D-treated embryos. The synthesis of these polypeptides in normal embryos is also unaffected by actinomycin D and, therefore, it would seem that in both normal and lobeless embryos, these polypeptides are translated from oogenic mRNAs and their synthesis is regulated by a transcriptionally independent control mechanism. In the lobeless embryo, however, the decrease in the accumulation of these polypeptides suggests that the oogenic mRNAs coding for these polypeptides are translated less efficiently or are decreased in concentration at the mesentoblast stage.

Polypeptide 20 showed a decreased relative rate of accumulation in lobeless embryos in comparison with normal control embryos. A further decrease resulted, in lobeless embryos, from transcriptional inhibition, indicating that the synthesis of this polypeptide is partially dependent on new transcription. The level of accumulation of polypeptide 20 was the same in actinomycin D-treated normal and lobeless embryos, suggesting that the translation of oogenic mRNA coding for polypeptide 20 proceeds normally in both classes of embryos. The difference in the level of accumulation of this polypeptide in lobeless and normal control embryos may therefore be attributed to a partial reduction, in the lobeless embryo, of transcription of either embryogenic mRNA coding for polypeptide 20 or a factor that enhances the translation of oogenic mRNA coding for polypeptide 20.

Polypeptides 16 and 17 showed increases in relative rates of accumulation in lobeless actinomycin D-treated embryos when compared with untreated lobeless embryos. In the normal embryo, the mesentoblast stage-specific increase in these two polypeptides is unaffected by actinomycin D treatment, indicating that they are translated from oogenic mRNAs and that their translation is regulated by transcriptionally independent mechanism(s). It is significant that these polypeptides also increase in the isolated 24-hour old polar lobe, suggesting that the factors that regulate this stage-specific increase may be localized in the polar lobe. In the lobeless embryo, the mesentoblast stage-specific increase in these polypeptides fails to occur and, in addition, the increased accumulation in actinomycin D-treated lobeless embryos suggests that the translation of the oogenic mRNAs coding for these polypeptides is repressed by a transcriptionally dependent mechanism that does not operate in the normal embryo.

The lobeless mesentoblast stage embryo thus, fails to (1) correctly regulate the activation of oogenic mRNAs (polypeptides 53, 54, 51, 97, 16, 17, 14 and 78), (2) synthesize or activate transcriptionally dependent translational repressors (polypeptides 81, 82, 71, 94, 84, 89) and supplement or activate oogenic mRNA by new transcription (polypeptide 20). With this one exception, all of the polypeptides whose synthesis is abnormal in the mesentoblast stage lobeless embryo are translated from oogenic mRNAs and the primary effect of the polar lobe on new transcription appears to be on the transcription of regulatory genes whose products control the translation of specific maternal mRNAs.

## Gastrula Stage

After the formation of the mesentoblast cell, the next major event in the development of the mud snail embryo is gastrulation, which occurs by epiboly and is judged complete, by closure of the blastopore, at 54 hours post-fertilization at 19°C (Collier, 1976). We have compared the polypeptides synthesized by normal mesentoblast stage embryos with those made by gastrula stage embryos to determine whether changes in polypeptide synthesis are associated with this period of morphogenetic movements and segregation of the mesoblast and enteroblast lineages. Because this embryo, when reared from early cleavage in actinomycin D, arrests at gastrulation (Feigenbaum and Goldberg, 1965; Collier, 1966; Newrock and Raff, 1975), we have analyzed polypeptide synthesis in actinomycin D-treated gastrulae to determine whether there is an increase in the number of polypeptides that are translated from embryogenic mRNAs or that are regulated by transcriptionally dependent mechanisms.

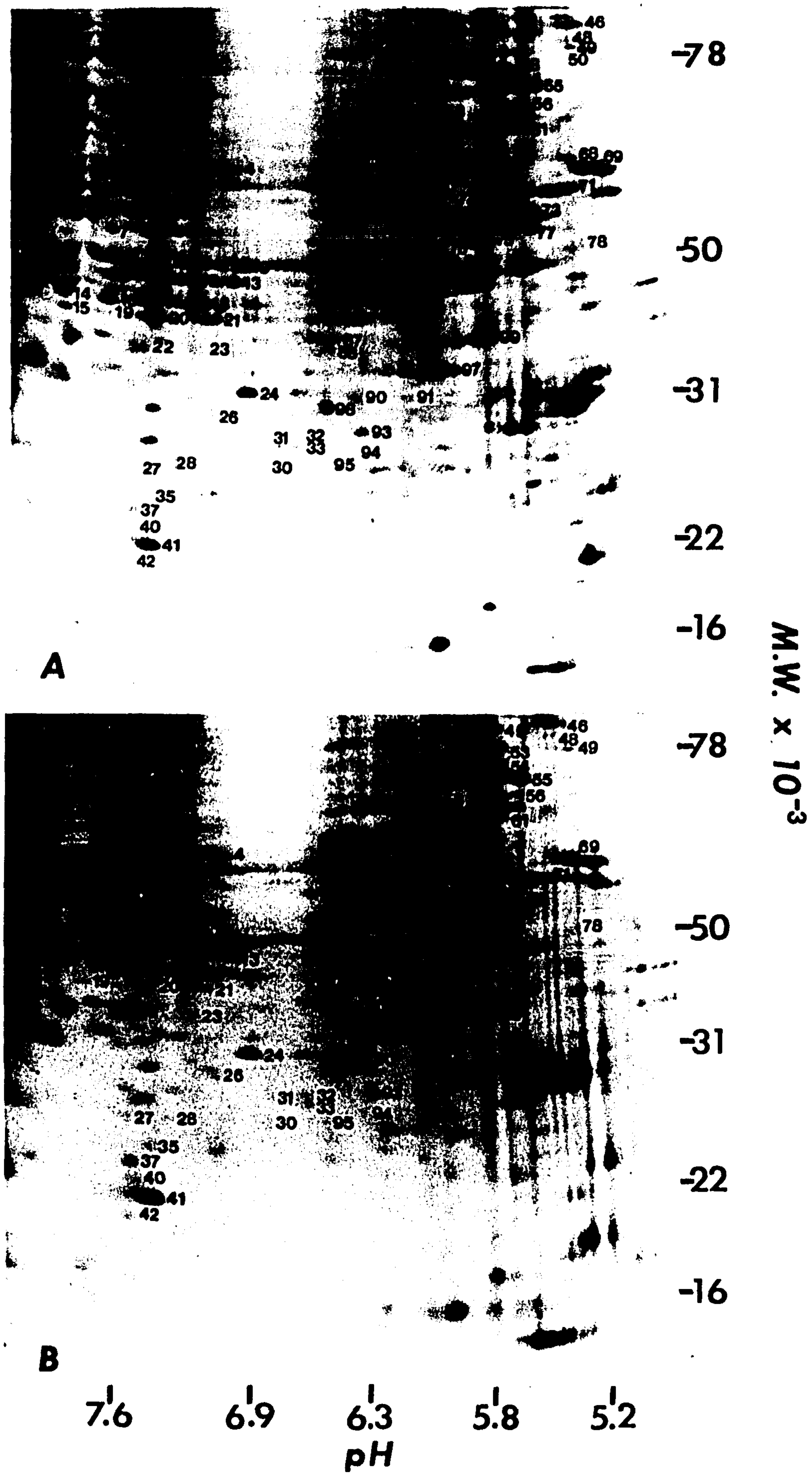
## Fewer Changes in Polypeptide Synthesis Occur at the Gastrula Stage

The polypeptides synthesized by normal gastrula stage embryos are illustrated by the fluorgram in figure 7A and the results of two replicate comparisons of polypeptides synthesized by normal mesentoblast stage embryos (Fig. 4A) with those synthesized by gastrula stage embryos are summarized in column 2 of table 6.

No new polypeptides were made by gastrula stage embryos. In contrast, twenty-four new polypeptides (8%) appeared at the mesentoblast stage. None of the polypeptides that were unique to cleavage stage embryos reappeared at the gastrula stage. Eight polypeptides disappeared between the mesentoblast and gastrula stages-a number not substantially different from the twelve polypeptides that disappeared between cleavage and mesentoblast stages. Two of the polypeptides that disappeared at the gastrula stage, 38 and 39 were among those that first appeared at the mesentoblast stage and thus were synthesized only at this period in early development.

Fig. 7A. Fluorogram of polypeptides made by normal gastrula stage embryos. Embryos were incubated for 4 hours in $^{35}$S-methionine, 5.9 x $10^5$ acid precipitable CPM were applied to the first dimension gel and second dimension gel was exposed for 4 days.

Fig. 7B. Fluorogram of polypeptides made by lobeless gastrula stage embryos. Embryos were incubated in $^{35}$S-methionine for 4 hours, 4.9 x $10^5$ acid precipitable CPM were applied to the first dimension gel and the second dimension gel was exposed for 5 days.



A
B
46
48
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56
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73
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78
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22
23
24
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27
28
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32
33
35
37
40
41
42
87
88
90
91
93
94
95
96
97
-78
-50
-31
-22
-16
M.W. x 10^-3
7.6
6.9
6.3
5.8
5.2
pH

Twelve polypeptides (4%) showed increased relative rates of accumulation at the gastrula stage--nearly twice as many polypeptides (20) increased between cleavage and the mesentoblast stage. Twenty-nine polypeptides (9.7%) decreased in relative accumulation between the mesentoblast and gastrula stages; this was the only type of stage-specific change that was more prominent at gastrulation.

The transition from the mesentoblast to the gastrula stage involves changes in a total of forty-nine polypeptides (16%) as compared to changes in seventy-four polypeptides (25%) in the transition from cleavage to the mesentoblast stage. Thus, the development of the Ilyanassa egg from early cleavage to gastrulation involves a total of forty-four (15%) qualitative changes and seventy-nine (26%) rate changes in polypeptide synthesis; none of these changes occur prior to the sixteen cell stage but significant changes occur as early as the twenty-five cell stage. The changes in polypeptide synthesis that are associated with gastrulation in the echinoderm embryo (Brandhorst, 1976) also occur in this spiralian embryo, but alterations in the pattern of polypeptide synthesis begin earlier in the Ilyanassa embryo and are correlated with the determinative events that occur at the mesentoblast stage.

Early embryogenesis depends on a store of oogenic mRNAs but at some point in development the embryo replaces and augments these with newly synthesized embryogenic mRNAs. The observation that, in most hybrid embryos, the expression of paternal characteristics begins at gastrulation (for example, Johnson, 1971; Barrett and Angelo, 1964) and that most chemically enucleated eggs arrest at or during gastrulation (Gross and Cousineau, 1964; Brachet and Denis, 1963; Morrill et al., 1976) has led to the generalization that the switch in the source of transcripts used for protein synthesis

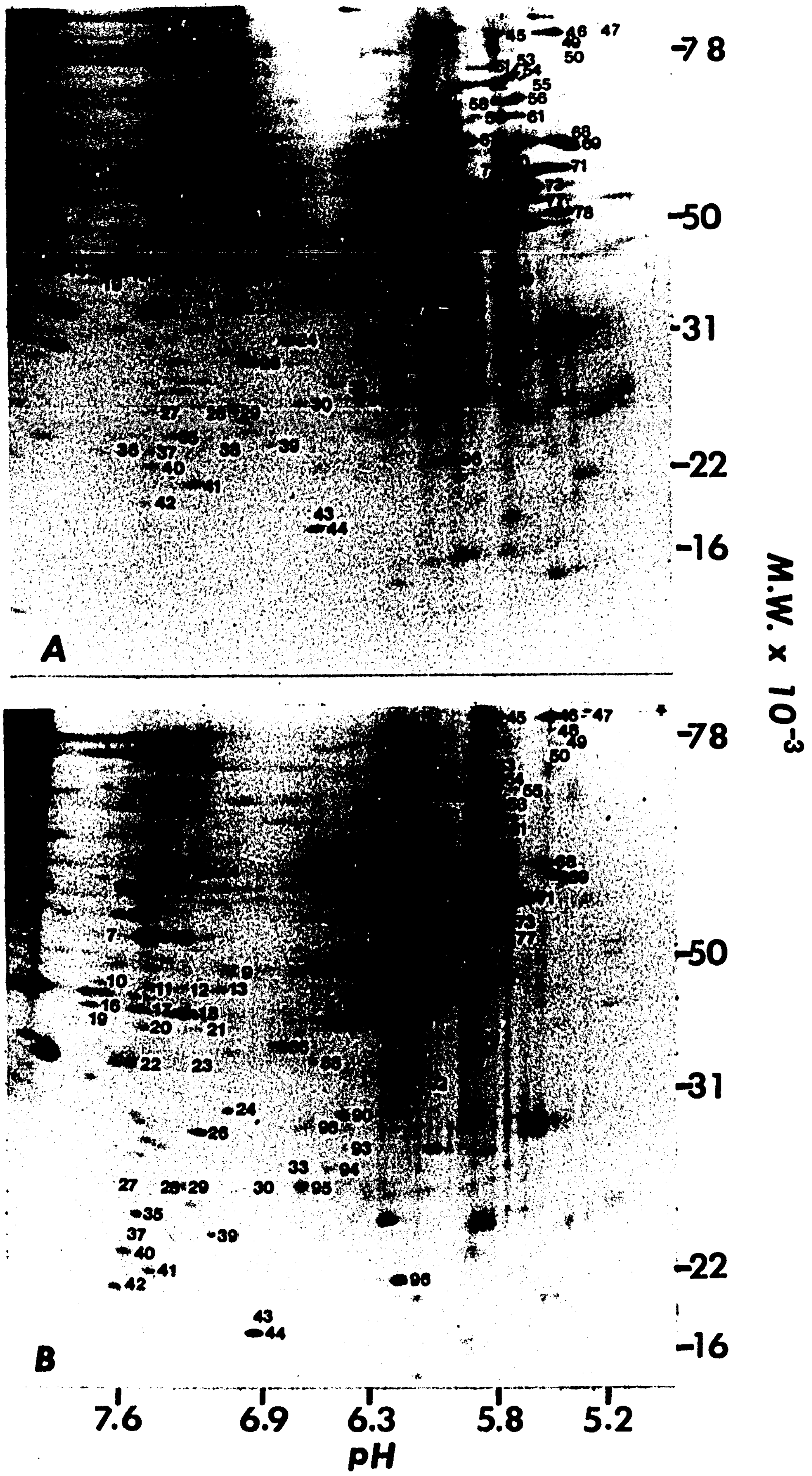
## Table 6

### POLYPEPTIDE SYNTHESIS BY NORMAL AND ACTINOMYCIN D-TREATED GASTRULA STAGE EMBRYOS

| CLEAVAGE AND MESENTOBLAST POLYPEPTIDE SYNTHESIS | NORMAL GASTRULA STAGE COMPARED WITH NORMAL MESENTOBLAST STAGE | ACTINOMYCIN D-TREATED GASTRULA COMPARED WITH NORMAL GASTRULA |
|---|---|---|
| POLYPEPTIDES UNIQUE TO CLEAVAGE STAGE<br>25 34 60 62 63 64<br>65 66 83 84 85 89<br>ABSENT IN NORMAL MESENTOBLAST STAGE | ABSENT IN NORMAL GASTRULA | ABSENT IN ACTINOMYCIN D-TREATED GASTRULA |
| POLYPEPTIDES CONSTANT BETWEEN CLEAVAGE AND MESENTOBLAST STAGES<br>1 7 22 27 30 36<br>43 44 47 49 50 53<br>59 67 75 79 81 82<br>86 88 90 92 96 98 | SAME 7 49 59 67 75 82 90 98 | SAME 7 82 90 98<br>INCREASE 67<br>DECREASE 49 59 75 |
| | INCREASE 1 53 | DECREASE 1 53 |
| | DECREASE 22 27 30 36 50 79 81 86 88 | SAME 22 27 50 79 86 88<br>INCREASE 30 36 81 |
| | ABSENT 43 44 47 96 92 | PRESENT 47 43 44 96 92 |
| POLYPEPTIDES UNIQUE TO MESENTOBLAST STAGE<br>10 12 15 21 24 28<br>31 32 33 37 38 39<br>41 42 48 51 52 54<br>55 56 74 77 93 97 | SAME 12 15 28 31 32 55 56 74 77 93 | SAME 28 56 74 93<br>DECREASE 12 55 77<br>ABSENT 15 31 32 |
| | INCREASE 10 21 24 41 51 54 97 | DECREASE 10 21 24 41 51 54 97 |
| | DECREASE 33 37 42 48 52 | SAME 33 37 42 48 52 |
| | ABSENT 38 39 | PRESENT 38 39 |
| POLYPEPTIDES WHICH CHANGE BETWEEN CLEAVAGE AND MESENTOBLAST STAGES<br>INCREASE<br>5 6 8 9 11 13<br>14 16 17 18 20 26<br>35 40 61 69 72 73<br>76 80 | SAME 5 6 8 9 13 18 61 72 73 76 80 | SAME 5 6 8 18 61 72 73 76<br>DECREASE 9 13 80 |
| | INCREASE 11 20 69 | DECREASE 11 20 69 |
| | DECREASE 14 16 17 26 35 40 | INCREASE 14 16 17 26 35 40 |
| DECREASE<br>2 3 4 19 23 29<br>45 46 57 58 68 70<br>71 78 87 91 94 95 | SAME 2 3 4 19 57 70 71 87 | SAME 2 4 19 57 70 71 87<br>DECREASE 3 |
| | DECREASE 23 45 46 58 68 78 91 94 95 | SAME 23 46 58 94 45<br>INCREASE 68 78 91 95 |
| | ABSENT 29 | PRESENT 29 |

Fig. 8A. Fluorogram of polypeptides made by actinomycin D-treated normal gastrula stage embryos. Embryos were reared from first cleavage in 30 µg/ml actinomycin D, incubated at the gastrula stage in $^{35}S$-methionine for 4 hours, 0.7 x $10^5$ acid precipitable CPM were applied to the first dimension gel and the second dimension gel was exposed for 34 days.

Fig. 8B. Fluorogram of polypeptides made by actinomycin D-treated lobeless gastrula stage embryos. Embryo culture, radioactive labeling, number of acid precipitable CPM and exposure time were as described in Fig. 8A.


A
B
78
50
31
22
16
M.W. x 10-3
7.6
6.9
6.3
5.8
5.2
pH
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9
10
11
12
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23
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28
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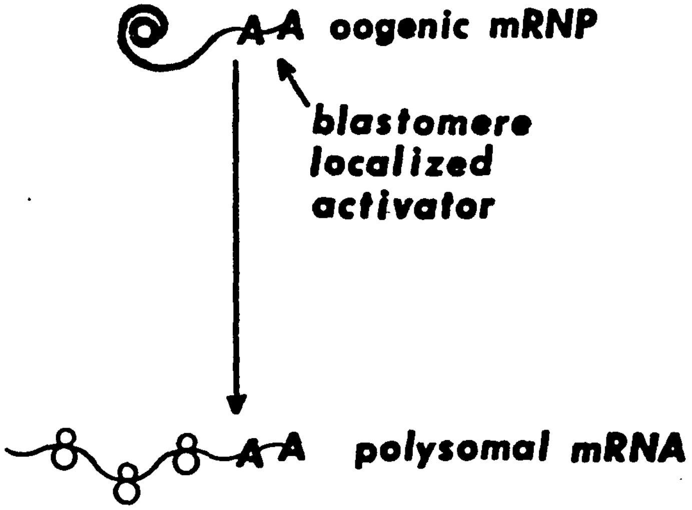
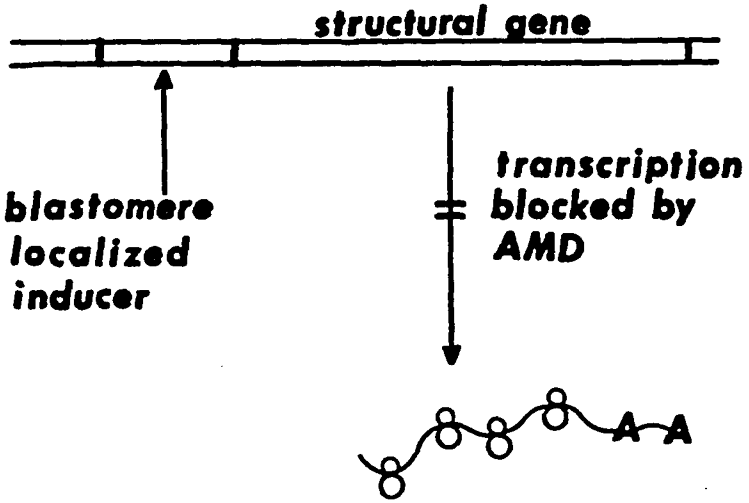
96
98

Fig. 9. Models illustrating mechanisms that may mediate some of the changes in polypeptide synthesis between cleavage and mesentoblast stages.

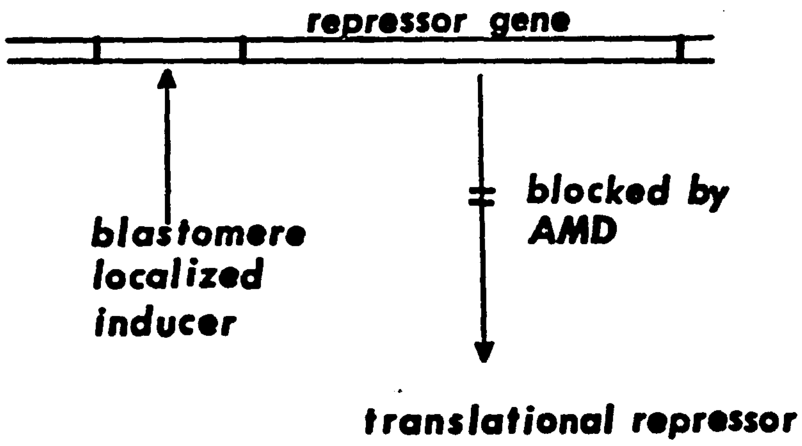
**1. ACTIVATION OF OOGENIC mRNP**
**Polypeptides 33 38 42 48 52 55 56 74 93**



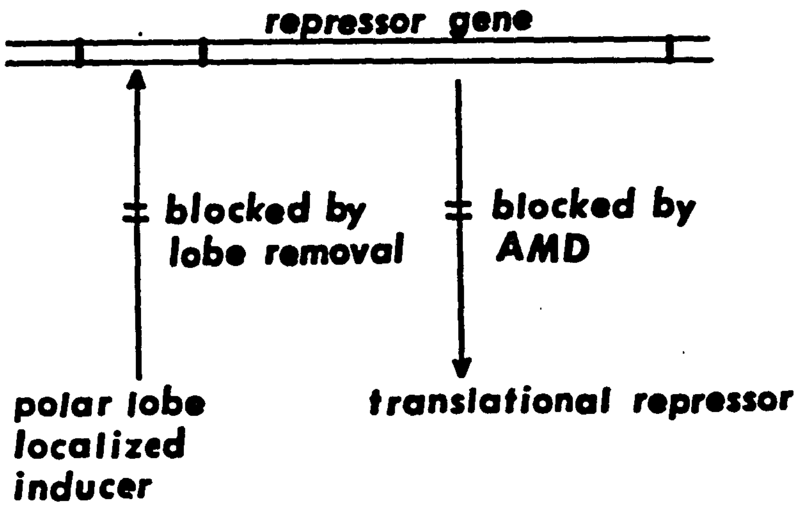
**2. ACTIVATION OF EMBRYOGENIC TRANSCRIPTS**
**Polypeptides 10 15 28 31 32 37**



**3. TRANSCRIPTION-DEPENDENT REPRESSORS**
**A) Blastomere Induction**
**Polypeptides 44 90 96 39 26 35 29 68 91 95**



**B) Polar Lobe Induction**
**Polypeptides 81 82 71 94**



occurs just prior to or during gastrulation. We have tested this assumption by a comparison of polypeptide synthesis in normal gastrulae and gastrula stage embryos reared from first cleavage in actinomycin D.

### Oogenic mRNA Remains a Primary Source of Transcripts for Gastrula Polypeptide Synthesis

A fluorogram of polypeptides synthesized by gastrula stage embryos reared from first cleavage in actinomycin D is illustrated in figure 8A, and the results of two replicate comparisons of control and actinomycin D-treated gastrula stage polypeptides are shown in table 6. At this stage, continuous incubation in actinomycin D resulted in a decrease in incorporation of $^{35}$S-methionine into acid precipitable material of 93.1% and therefore, fluorographic exposures were increased to compensate for the lowered number of acid precipitable counts per minute applied to the first dimension gel.

Of the polypeptides that changed during early development, thirty-nine showed the same relative rates of accumulation in both actinomycin D-treated and control gastrula stage embryos. Thirty-five of these were also actinomycin D-insensitive at the mesentoblast stage. Four polypeptides that increased at the mesentoblast stage in actinomycin D (71, 82, 90 and 94) showed the same level of accumulation in both control and actinomycin D-treated gastrulae. All of these thirty-nine polypeptides, as well as the unnumbered polypeptides, that continue to be synthesized at normal relative rates in actinomycin D are probably translated from oogenic mRNAs that persist for at least 48 hours after fertilization and that are regulated by transcriptionally independent mechanism(s).

Twenty-two polypeptides (7.3%) made at the gastrula stage decreased in actinomycin D-treated embryos relative to controls. As discussed above, a decrease in relative rate of accumulation in actinomycin D indicates that these polypeptides are partially dependent on new transcription to either supplement pre-existing pools of oogenic mRNAs or to activate, by transcriptionally dependent mechanism(s), the translation of oogenic mRNAs. Only three of the polypeptides that decrease in actinomycin D-treated gastrulae (49, 55 and 59) failed to show a similar decrease in actinomycin D-treated mesentoblast stage embryos. In addition, only three polypeptides (51, 31 and 32) made at the gastrula stage were absent from actinomycin D-treated gastrulae. These same polypeptides were also absent from actinomycin D-treated mesentoblast stage embryos. Their absence in actinomycin D-treated embryos indicates that they are entirely dependent on new transcription and are probably translated from embryogenic mRNAs.

While these data suggest that there is no large increase in the number of polypeptides translated from embryogenic mRNAs at gastrulation, the substantial decrease in $^{35}$S-methionine incorporation that result from actinomycin D treatment at this stage could reflect a <u>uniform</u> supplementation of <u>all</u> of the mRNAs translated at this stage. Thus, the most important finding is, not that supplementation of oogenic mRNAs by embryogenic mRNAs is not predominant at gastrulation, but rather, that oogenic mRNAs persist, in actively translated form, at least through the early stages of gastrulation and that the "switch" to embryogenic mRNA, if it occurs at this stage, must be largely non-specific.

Three polypeptides (10, 28 and 37) that were absent in actinomycin D-treated mesentoblast stage embryos, reappeared in actinomycin D-treated gastrula stage embryos. This suggests that there exists a group of oogenic messages that are stored in translationally inactive form and are activated for the first time, not at fertilization or at the mesentoblast stage, but at gastrulation.

An increase in the relative rate of accumulation of fourteen polypeptides (4.7%) was observed in actinomycin D-treated gastrula stage embryos as compared with control embryos. In addition, every one of the eight polypeptides (2.7%) that normally disappeared between the mesentoblast and gastrula stages, was present in actinomycin D-treated gastrula stage embryos. These observations indicate that the translation of the oogenic mRNAs coding for these polypeptides is normally repressed by a transcriptionally dependent mechanism and suggests that regulatory gene(s) exist whose transcripts, directly or indirectly, act as translational repressors, thus controlling the gastrula stage-specific decrease or disappearance of twenty-two polypeptides (7.4%).

## <u>The Mode of Regulation Used at the Gastrula Stage is Established at the Mesentoblast Stage</u>

In an attempt to detect coupling of regulatory mechanisms between the mesentoblast and gastrula stage, the relative rates of accumulation of all numbered polypeptides in control and actinomycin D-treated mesentoblast and gastrula stage embryos were plotted diagramatically, as shown in figure 10. The level of accumulation of each polypeptide (determined from the density of spots on the fluorogram) at the mesentoblast stage was used as a baseline. While there are twenty-four possible permutations of actinomycin D and stage-specific changes in relative rates of accumulation, it was found that all of the numbered polypeptides exhibited a total of only eight patterns.

In figure 10, the eight observed patterns are given a numerical designation in column 1. Column 2 compares the relative rates of accumulation of the polypeptides listed in column 3 in control mesentoblast, actinomycin D-treated mesentoblast, gastrula and actinomycin D-treated gastrula stage embryos.

Fig. 10. Patterns of polypeptide synthesis in mesentoblast and gastrula stage embryos. Eight patterns of polypeptide synthesis observed in normal embryos are shown with a numerical designation for each in column 1. Column 2 compares diagramatically the relative rates of accumulation of the polypeptides listed in column 3 in control mesentoblast, actinomycin D-treated mesentoblast, gastrula and actinomycin D-treated gastrula stage embryos. The polypeptides that are absent in actinomycin D are designated in column 3 by the superscript "1" and, in column 2, by an "x". In column 2, increases or decreases in accumulation are symbolized by the position of each square--if one square is higher than another, an increase is indicated, if lower, a relative decrease is indicated. The effect of actinomycin D on polypeptide synthesis at the mesentoblast and gastrula stages is summarized in column 4.

| Pattern | Relative Synthesis<br>mesentoblast | <br>mesentoblast AMD | <br>gastrula | <br>gastrula AMD | Polypeptides | Response to Actinomycin D<br>Mesentoblast | <br>Gastrula | Source of mRNA and Mode of Regulation |
|---|---|---|---|---|---|---|---|---|
| 1 | ■ | ■ | ■ | ■ | 2 4 5 6 7 8 18 19 33 56 57 61 70 72 73 76 87 93 98 74 | $AMD^{0}$ | $AMD^{0}$ | consistent translation of oogenic mRNA at both stages |
| 2 | ■ | ■ | ■ | ■ | 22 23 27 42 45 46 48 50 52 58 79 86 88 | $AMD^{0}$ | $AMD^{0}$ | translation of oogenic mRNA at both stages, transcriptionally independent repression at gastrula stage |
| 3 | ■ | ■ | ■<br>x | ■ | 26, 29[1] 35 44[1] 68 81 91 95 96[1] | $AMD^{+}$ | $AMD^{+}$ | consistent control of oogenic mRNA by transcriptionally dependent repressor(s) |
| 4 | ■ | ■<br>x | ■ | ■ | 1 10[1] 11 20 21 24 28[1] 41 51 53 54 97 69 | $AMD^{-}$ | $AMD^{-}$ | supplementation by embryogenic mRNA or transcription-dependent activation of mRNP plus translational activation at gastrula stage |
| 5 | ■ | ■<br>x | ■ | ■<br>x | 3 12 15[1] 31[1] 32[1] 37[1] 75 77 80 | $AMD^{-}$ | $AMD^{-}$ | same as in (4) above without translational activation |
| 6 | ■ | ■ | ■<br>x | ■ | 14 16 17 36 38[1] 40 43[1] 47[1] 67 78 92[1] 30 39 | $AMD^{0}$ | $AMD^{+}$ | translation of oogenic mRNA controlled by transcriptionally dependent repressor(s) at gastrula stage only |
| 7 | ■<br>X | ■ | ■<br>X | ■<br>X | 71 82 90 94 84[1] 89[1] | $AMD^{+}$ | $AMD^{0}$ | translation of oogenic mRNA controlled by transcriptionally dependent repressor(s) at mesentoblast stage only |
| 8 | ■ | ■ | ■ | ■ | 49 55 59 | $AMD^{0}$ | $AMD^{-}$ | supplementation of oogenic mRNA by embryogenic mRNA or transcriptionally dependent activation of mRNP at gastrula stage only |

[1] Polypeptides absent where indicated with "x"

The polypeptides that are absent in actinomycin D are designated in column 3 by the superscript "1" and, in column 2, by an "x". The effect of actinomycin D on polypeptide synthesis at the mesentoblast and gastrula stages is summarized in column 4.

Two types of actinomycin D-insensitive polypeptide synthesis are illustrated by patterns 1 and 2. In pattern 1, no change in the relative rates of accumulation of twenty polypeptides (6.7%) was observed between the mesentoblast and gastrula stages and actinomycin D had no effect at either stage. Pattern 2 showed a similar transcriptional independence, but, unlike pattern 1, was associated with an actinomycin D-insensitive decrease in relative accumulation between the mesentoblast and gastrula stages; thirteen polypeptides (4.3%) exhibited this mode of regulation. A total of thirty-two (10.7%) of the numbered polypeptides are thus translated from oogenic mRNA and regulated by transcriptionally independent mechanisms at both the mesentoblast and gastrula stages.

Three groups of polypeptides (Patterns 6, 7 and 3) showed at one or both stages, increased relative rates of accumulation in the presence of actinomycin D. In pattern 6, which was characteristic of thirteen polypeptides (4.3%), the transcriptionally dependent repression of polypeptide synthesis affected only gastrula stage embryos; synthesis at the mesentoblast stage was actinomycin D-insensitive. This pattern was invariably associated with a stage-specific decrease or disappearance of polypeptides between the mesentoblast and gastrula stages that failed to occur in the presence of actinomycin D. Actinomycin D caused an increase in the relative rates of accumulation of six polypeptides at the mesentoblast stage, but not at the gastrula stage (pattern 7). No stage-specific change in relative accumulation was seen in the polypeptides that exhibited this pattern. As indicated by an increase in relative accumulation of a polypeptide in the presence of actinomycin D, transcriptionally dependent repression of oogenic mRNA translation occurs at both the mesentoblast and gastrula stages in pattern 3. All of the nine polypeptides that exhibited this pattern of actinomycin D sensitivity showed a decrease in relative accumulation between mesentoblast and gastrula stages. Only part of this stage-specific decrease was actinomycin D sensitive; the remainder resulted from a transcriptionally independent translational repression. Throughout the mesentoblast and gastrula stages, the synthesis of 26 polypeptides (8.6%) was controlled by one of these three modes of regulation.

Actinomycin D treatment resulted in a decrease in the relative rates of accumulation (or disappearance), at one or both stages, of twenty-seven polypeptides (9%). As discussed above, a decrease in the accumulation of a polypeptide in the presence of actinomycin D indicates that either the polypeptide is translated from both embryogenic and oogenic mRNA or that transcription is required for the translational activation of oogenic mRNA. The interpretation of absences occurring as a result of transcriptional inhibition is similarly ambiguous. Nevertheless, a significant percentage of the total number of polypeptides made at the mesentoblast and gastrula stages depend wholly or partially on transcription during embryogenesis to achieve normal levels of accumulation.

A decrease in relative accumulation resulting from actinomycin D treatment and affecting only gastrula stage embryos was observed for only five polypeptides (pattern 8). More commonly, actinomycin D treatment resulted in a decrease in relative accumulation (or absence) at both the mesentoblast and gastrula stages. Pattern 5 was characteristic of nine polypeptides; these polypeptides did not show a change in relative accumulation between stages, but did show a decrease of the same magnitude at both stages in the presence of actinomycin D.

Pattern 4 was characteristic of thirteen polypeptides. These polypeptides showed a decreased relative accumulation in actinomycin D at both stages and all of these polypeptides also showed an increased relative accumulation between the mesentoblast and gastrula stages. This increase occurred whether or not transcription was inhibited with actinomycin D, however, the final levels of accumulation of these polypeptides reached at the gastrula stage and at the mesentoblast stage were significantly lower in the presence of actinomycin D. This finding suggests that, while transcription is required to obtain normal levels of accumulation of these polypeptides at both the mesentoblast and gastrula stages, the stage-specific increase is regulated by a transcriptionally independent mechanism(s). Thus, in patterns 3 and 4, a transcriptionally independent mechanism that regulates a stage-specific change is coupled with a transcriptionally dependent mechanism that controls the relative rate of accumulation at both stages.

Sixty-two of the polypeptides surveyed showed a uniform response to actinomycin D at both the mesentoblast and gastrula stages, which suggests that a mechanism exists to determine that the same source of mRNA and mode of regulation are used at both stages.

A similar higher-level control may operate to switch, between the mesentoblast and gastrula stages, the source of mRNA or regulatory mechanisms controlling the synthesis of the twenty-two polypeptides that exhibit patterns 6, 7 and 8. Why, for example, a particular oogenic mRNA should be under the control of transcriptionally dependent repressors at one stage, but not at another (patterns 6 and 7), is an intriguing question whose answer may have developmental significance. Similar shifts in the form, translatability and half-life of myosin mRNA have been reported to accompany myoblast fusion and differentiation (Whalen <u>et al</u>., 1976) and such

# Table 7

POLYPEPTIDE SYNTHESIS BY LOBELESS GASTRULA STAGE EMBRYOS

| Cleavage and Mesentoblast Polypeptide Synthesis | Normal Gastrula Compared with Normal Mesentoblast Stage | Lobeless Gastrula Compared with Normal Gastrula | Actinomycin D-Treated Lobeless Gastrula Compared with Lobeless Gastrula Control |
|---|---|---|---|
| Polypeptides Unique to Cleavage Stage 25 34 60 62 63 64 65 66 83 84 85 89 absent in normal mesentoblast stage | absent in normal gastrula | absent in lobeless gastrula stage | absent in actinomycin D-treated lobeless gastrula |
| Polypeptides Constant between Cleavage and Mesentoblast Stages 1 7 22 27 30 36 43 44 47 49 50 53 59 67 75 79 81 82 86 88 90 92 96 98 99 | SAME 7 49 59 67 75 82 90 98 | SAME 7 49 59 67 75 82 90 98 | SAME 7 90 98 |
| | | | INCREASE 67 82 |
| | | | DECREASE 49 59 75 |
| | INCREASE 1 53 99 | SAME 1 53 | DECREASE 1 53 |
| | | INCREASE 99 | DECREASE 99 |
| | DECREASE 22 27 30 36 50 79 81 86 88 | SAME 22 27 30 36 50 81 86 88 | SAME 22 27 50 86 88 |
| | | | INCREASE 30 36 81 |
| | | DECREASE 79 | INCREASE 79 |
| | ABSENT 43 44 47 92 96 | ABSENT 43 44 47 92 96 | PRESENT 43 44 47 92 96 |
| Polypeptides Unique to Mesentoblast Stage 10 12 15 21 24 28 31 32 33 37 38 39 41 42 48 51 52 54 55 56 74 77 93 97 | SAME 12 15 28 31 32 55 56 74 77 93 | SAME 12 28 31 32 55 56 74 77 93 | SAME 28 56 74 93 |
| | | | DECREASE 12 55 77 |
| | | | ABSENT 31 32 |
| | | ABSENT 15 | ABSENT 15 |
| | INCREASE 10 21 24 41 51 54 97 | SAME 10 24 41 51 54 97 | DECREASE 10 24 41 51 54 97 |
| | | DECREASE 21 | SAME 21 |
| | DECREASE 33 37 42 48 52 | SAME 33 37 42 48 52 | SAME 33 37 42 48 52 |
| | ABSENT 38 39 | ABSENT 38 39 | PRESENT 38 39 |
| Polypeptides which Change between Cleavage and Mesentoblast Stages<br>INCREASE 5 6 8 9 11 13 14 16 17 18 20 26 35 40 61 69 72 73 76 80 | SAME 5 6 8 9 13 18 61 72 73 76 80 | SAME 5 6 8 9 13 18 61 72 73 76 80 | SAME 8 61 72 73 76 |
| | | | DECREASE 5 6 9 13 18 80 |
| | INCREASE 11 20 69 | SAME 11 69 | DECREASE 11 69 |
| | | DECREASE 20 | SAME 20 |
| | DECREASE 14 16 17 26 35 40 | SAME 26 35 40 | INCREASE 26 35 40 |
| | | DECREASE 16 17 | SAME 16 17 |
| | | ABSENT 14 | ABSENT 14 |
| DECREASE 2 3 4 19 23 29 45 46 57 58 68 70 71 78 87 91 94 95 | SAME 2 3 4 19 57 70 71 87 | SAME 2 3 4 19 57 70 71 87 | SAME 2 4 19 57 70 |
| | | | INCREASE 71 |
| | | | DECREASE 3 |
| | | | ABSENT 87 |
| | DECREASE 23 45 46 58 68 78 91 94 95 | SAME 23 45 46 58 91 94 95 | SAME 23 45 46 58 94 |
| | | | INCREASE 91 95 |
| | | DECREASE 78 | SAME 78 |
| | | ABSENT 68 | PRESENT 68 |
| | ABSENT 29 | ABSENT 29 | PRESENT 29 |

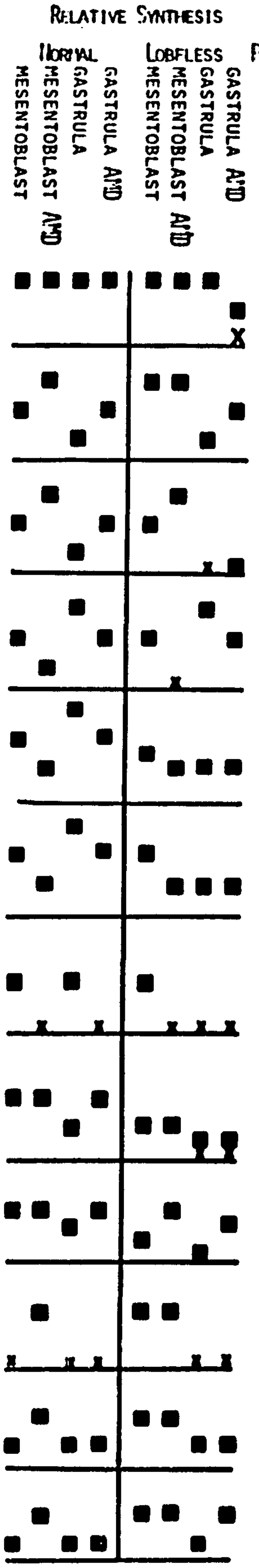
translational-level switches may emerge as a common feature of differentiating cells.

## Removing the Polar Lobe Affects Both Oogenic and Embryogenic mRNA at the Gastrula Stage

Conklin (1897) has described the lineage of the mesentoblast or 4d cell in Crepidula fornicata, whose development is very similar to that of Ilyanassa (Clement, 1952). Shortly after its formation from the 3D macromere, the 4d cell divides into right and left halves ($ME^1$ and $ME^2$). Six subsequent divisions bring about the complete segregation of the mesoblast and entoblast lines and, by the time the embryo consists of 88 cells (an early gastrula stage), eight progeny of the original mesentoblast cell have formed. These cells are absent from the lobeless embryo and defects in polypeptide synthesis in lobeless gastrulae may be correlated with this developmental abnormality.

Fluorograms of the polypeptides synthesized by lobeless gastrulae and actinomycin D-treated lobeless gastrula stage embryos are shown in figures 7B and 8B, respectively. The results of two replicate comparisons of normal and lobeless gastrulae are shown in column 3, table 7 and the results of two comparisons of untreated lobeless gastrula stage embryos with lobeless embryos raised in actinomycin D are summarized in column 4 of table 7. The defects in polypeptide synthesis seen in the lobeless embryo at both the mesentoblast and gastrula stages are described diagrammatically in figure 11. Eight polypeptides that displayed abnormal rates of accumulation in lobeless mesentoblast stage embryos appeared to "recover" in gastrula stage lobeless embryos, seven polypeptides showed abnormal patterns of accumulation as a result of polar lobe removal at both the mesentoblast and gastrula stages and seven polypeptides exhibited abnormal

Fig. 11. Summary of defects in polypeptide synthesis in lobeless mesentoblast and gastrula stage embryos. Each pattern of polypeptide synthesis in lobeless embryos is designated by a number, which refers to the normal pattern of polypeptide synthesis (see Fig. 10) and a letter, which refers to the specific defect seen in the lobeless embryo. The polypeptides that exhibit each pattern are listed in column 3 and the relative accumulations of these polypeptides under 8 different conditions are illustrated diagramatically in column 2. A brief summary of possible interpretations of these patterns of polypeptide synthesis is provided in column 4.



| PATTERN | POLYPEPTIDES | INTERPRETATION OF LOBELESS DEFECT |
|---|---|---|
| 1A | 5 6 18<br>87[1] | OOGENIC POLYSOMAL mRNA REDUCED AT GASTRULA STAGE. |
| 3A | 81 | ABSENCE OF POLAR LOBE-LOCALIZED INDUCER. |
| 3B | 68 | OOGENIC POLYSOMAL mRNA REDUCED AT GASTRULA STAGE. |
| 4A | 51 53 59<br>97 | LOBELESS EMBRYO FAILS TO ACTIVATE OOGENIC mRNP AT MESENTOBLAST STAGE. |
| 4B | 20 | OOGENIC AND/OR EMBRYOGENIC POLYSOMAL mRNA REDUCED AT BOTH STAGES. |
| 4C | 21 | OOGENIC AND/OR EMBRYOGENIC POLYSOMAL mRNA REDUCED AT GASTRULA STAGE. |
| 5A | 15 | ABSENCE OF EMBRYOGENIC POLYSOMAL mRNA AT GASTRULA STAGE. |
| 6A | 14[1] 78 | OOGENIC POLYSOMAL mRNA REDUCED AT BOTH STAGES. |
| 6B | 16 17 | LOBELESS EMBRYO USES INCORRECT MODE OF REGULATION (PATTERN 3 RATHER THAN PATTERN 6). |
| 7A | 84 89 | ABSENCE OF POLAR LOBE-LOCALIZED INDUCER. |
| 7B | 94 | ABSENCE OF POLAR LOBE-LOCALIZED INDUCER. |
| 7C | 71 82 | ABSENCE OF POLAR LOBE-LOCALIZED INDUCER. LOBELESS USES INCORRECT MODE OF REGULATION (PATTERN 6 RATHER THAN PATTERN 7). |

[1] ABSENT WHERE INDICATED BY "X"

patterns of synthesis in gastrula stage lobeless embryos, but not at the mesentoblast stage.

Eight polypeptides (81, 51, 53, 54, 97, 84, 89, 94) that showed aberrant relative accumulation rates in lobeless mesentoblast stage embryos and/or in actinomycin D-treated lobeless mesentoblast stage embryos appeared to be regulated normally at the gastrula stage. Polypeptides 81, 84, 89, and 94 showed increased relative rates of accumulation at the mesentoblast stage in normal and lobeless actinomycin D-treated embryos and in untreated lobeless embryos. As discussed above, this pattern suggests that the lobeless embryo fails to synthesize or activate transcriptionally dependent translational repressor(s) and, because these four polypeptides show, at the gastrula stage, no abnormal patterns of synthesis, this polar lobe dependent regulatory mechanism would appear to operate only at the time when the mesentoblast cell is formed. Polypeptides 51, 53, 54 and 97 were, at the mesentoblast stage, absent in actinomycin D-treated lobeless embryos, but were only reduced in actinomycin D-treated normal embryos. At the gastrula stage, these polypeptides undergo a stage-specific increase in both normal and lobeless embryos and, in both classes, are reduced but not absent in actinomycin D. That both normal and lobeless embryos are capable of synthesizing these polypeptides at the gastrula stage despite the inhibition of RNA synthesis from first cleavage indicates that they both contain stable oogenic mRNAs coding for these polypeptides; however, the absence of these polypeptides in lobeless actinomycin D-treated mesentoblast stage embryos suggests that at this stage the oogenic mRNAs coding for these polypeptides are translationally inactive. Two alternative (and presently indistinguishable) explanations of these observations are:
(1) two classes of oogenic mRNAs coding for these polypeptides exist, both stored in translationally inactive form. One class, destined to be

activated at the mesentoblast stage, is localized in the polar lobe, the other class, activated at the gastrula stage, is localized in the lobeless blastomeres, or (2) the lobeless blastomeres contain factors which activate stored oogenic mRNA at the gastrula stage, but those factors that activate stored oogenic mRNA at the mesentoblast stage are localized in the polar lobe and are, therefore, absent from the lobeless embryo. There is no reason to exclude either of these hypotheses and, indeed, both may be correct.

Seven polypeptides (20, 14, 78, 16, 17, 71 and 82) are affected by polar lobe removal at both the mesentoblast and gastrula stages. At the mesentoblast stage, polypeptide 20 showed a decreased relative accumulation in lobeless control embryos in comparison to normal embryos, but showed the same level of relative accumulation in the lobeless embryo in the presence of actinomycin D as did the normal embryo, suggesting that, at the mesentoblast stage, the lobeless embryo fails to supplement or activate oogenic mRNA by new transcription. At the gastrula stage, the relative rate of accumulation of polypeptide 20 is reduced in comparison to the normal embryo in both actinomycin D-treated and untreated lobeless embryos. That the levels of accumulation of polypeptide 20 are reduced in lobeless actinomycin D-treated gastrulae when compared to normal actinomycin D-treated gastrulae, suggests that the transcriptionally independent activation of oogenic mRNA, which normally mediates the stage-specific increase of polypeptide 20 at gastrulation, fails to occur in the lobeless embryo. In addition, the level of accumulation of this polypeptide in control lobeless embryos is the same as in actinomycin D-treated lobeless embryos, which represents a substantial reduction in comparison to the level of accumulation in control normal embryos. This suggests that, at the gastrula stage, as well as at the mesentoblast stage, the lobeless embryo fails to either (1) activate some oogenic mRNA by a transcriptionally dependent mechanism, (2) transcribe supplementary embryogenic mRNA coding for polypeptide 20, or (3) translate newly transcribed mRNA coding for polypeptide 20.

At both the mesentoblast and gastrula stages, polypeptides 14 and 78 were decreased in comparison with normal embryos, but not in comparison with lobeless actinomycin D-treated embryos. At both stages, and in both normal and lobeless embryos, the synthesis of these polypeptides is unaffected by actinomycin D and therefore, it is likely that these polypeptides are translated from oogenic mRNAs and regulated by transcriptionally independent mechanism(s). The decrease in accumulation of these polypeptides in the lobeless embryo at both stages, suggests that the oogenic mRNAs coding for these polypeptides are translated less efficiently or are decreased in concentration. The primary difference in the synthesis of these polypeptides in the lobeless embryo at the mesentoblast and at the gastrula stage is that the defect is much more severe at the gastrula stage.

Similarly, for polypeptides 16 and 17, the type of defect shown by the lobeless embryo is the same at both the mesentoblast and the gastrula stage, but the magnitude of the defect is much greater at the gastrula stage. At both stages, polypeptides 16 and 17 showed increased relative rates of accumulation in the lobeless embryo in the presence of actinomycin D, indicating that they are translated from oogenic mRNAs and that the translation of these mRNAs is repressed by a transcriptionally dependent mechanism. In the normal mesentoblast stage embryo, the synthesis of these polypeptides is independent of transcription and it is not until the gastrula stage that the normal embryo utilizes new transcription to repress the translation of the oogenic mRNAs coding for these polypeptides and the normal embryo uses only this mechanism to control the stage-specific decrease in these polypeptides at the gastrula stage. In contrast, the decrease in the relative accumulation of these polypeptides between the mesentoblast and gastrula stages in the lobeless embryo, occurs in both actinomycin D-treated and in untreated embryos, indicating that, while the final levels of accumulation of polypeptides 16 and 17 are controlled by transcription, the decrease between stages in the lobeless embryo is due to a transcriptionally <u>independent</u> translational repression. This pattern of regulation, which uses both transcriptionally dependent repressor at both stages coupled to a transcriptionally independent repressor that operates between stages, is not an abnormal one; nine polypeptides in the normal embryo exhibit this mode of regulation (pattern 3, figure 10). But the incorrect use of this mode of regulation to control the translation of the mRNAs coding for polypeptides 16 and 17 results in an abnormal decrease in accumulation in the lobeless embryo.

Polypeptides 71 and 82, like polypeptides 81, 84, 89, and 94, showed, at the mesentoblast stage, the same increase in relative accumulation in three experimental classes (normal and lobeless actinomycin D and lobeless control embryos) in comparison with normal mesentoblast stage embryos. As discussed above, these observations suggest that a factor, localized in the polar lobe, induces the synthesis of, or activates, at the mesentoblast stage, a transcriptionally dependent translational repressor(s). While polypeptides 81, 84, 89 and 94 are subsequently regulated in an apparently normal fashion, polypeptides 71 and 82, show, at the gastrula stage, a new type of defect in the lobeless embryo. As can be seen in figure 11, the relative rates of accumulation of these two polypeptides in the normal gastrula, are unaffected by actinomycin D; in the lobeless embryo, in contrast, the application of actinomycin D results in an increase in the relative accumulation of these polypeptides. This increase suggests that the translation of the maternal mRNAs coding for these two polypeptides is repressed in the lobeless gastrula but not in the normal gastrula by a transcriptionally dependent mechanism. This case and that of polypeptides 16 and 17 suggest that factor(s) in the polar lobe may block, for a specific set of mRNAs, the synthesis or activity of a transcriptionally dependent translational repressor(s).

It is likely that the differences in polypeptide synthesis in normal and lobeless mesentoblast stage embryos are primary defects that result directly from the removal of the polar lobe cytoplasm and, with it, the removal of factors controlling polypeptide synthesis. This is a reasonable assumption because (1) the isolated polar lobe has been shown to contain stable mRNAs and at least some factors that regulate translation in 24-hour old lobes and (2) the determinative effect of the polar lobe cytoplasm is largely delayed until the formation of the 4d cell (Clement, 1967). Does this reasoning apply to those defects in polypeptide synthesis that appear for the first time in gastrula stage lobeless embryo? Probably not.

Seven polypeptides are affected by polar lobe removal for the first time at the gastrula stage. The defects in the synthesis of these polypeptides in the lobeless embryo are extremely varied; this and the late stage at which these abnormalities appear suggest that they may be secondary rather than primary effects of polar lobe removal. That is, they may be the end result of a chain of events initiated by the lack of polar lobe factors which might, at the mesentoblast stage, alter the synthesis of a factor needed to regulate protein synthesis at the gastrula stage.

Four of the polypeptides (5, 6, 18 and 87) that show abnormal patterns of accumulation in lobeless gastrulae show similar defects. Polypeptides 5, 6 and 18 accumulate at normal relative rates in the lobeless gastrula but in the presence of actinomycin D, are substantially reduced. Polypeptide 87 is also apparently normal in lobeless control gastrulae but is absent from actinomycin D-treated lobeless gastrulae. In the normal embryo, the synthesis of these polypeptides is actinomycin D-insensitive, indicating that they are translated from oogenic mRNAs and that their translational regulation does not require new transcription. The decrease or disappearance of these polypeptides in actinomycin D-treated lobeless embryos suggests that the oogenic mRNAs coding for these polypeptides are reduced in concentration or are translated less efficiently in the lobeless embryo. That the level of accumulation of these polypeptides is normal in control gastrulae suggests that the lobeless embryo either (1) supplements with embryogenic mRNA the oogenic mRNAs for these polypeptides or (2) is able to activate the translation of existing oogenic mRNAs by a transcriptionally dependent mechanism--neither of these devices appear to be necessary in the normal embryo.

Two of the polypeptides (21 and 68) that show aberrent patterns of accumulation in lobeless gastrulae show a decrease in relative accumulation in both actinomycin D-treated and untreated lobeless embryos compared with actinomycin D-treated and untreated normal embryos. There are, however, subtle differences in the defects exhibited by each of these polypeptides in the lobeless embryo. The synthesis of polypeptide 68 may be regulated in the normal embryo by a transcriptionally dependent repressor system, which, in conjunction with a transcriptionally independent repressor, controls the translation of the oogenic mRNA coding for this polypeptide--as indicated by an increase in accumulation at both the mesentoblast and gastrula stages in the presence of actinomycin D and an actinomycin D-insensitive decrease in accumulation at the gastrula stage (see fig. 10, pattern 3). In the lobeless embryo, this pattern of accumulation was adhered to at the mesentoblast stage but at the gastrula stage, polypeptide 68 was absent from the lobeless control embryo and in the lobeless actinomycin D-treated embryo, was present but reduced to below the level of accumulation seen in untreated normal gastrulae. The presence of polypeptide 68 in the actinomycin D-treated lobeless gastrula suggests that some of the oogenic mRNA coding for this polypeptide must be present in translatable form but in such reduced amounts that only when the transcriptionally dependent repressor system is inactivated by actinomycin D is this mRNA translated in detectable quantities.

The pattern of accumulation of polypeptide 21 in both normal and lobeless embryos is very similar to that of polypeptide 20, except that polypeptide 20 showed a decreased relative accumulation in mesentoblast stage lobeless embryos that did not occur for polypeptide 21. In the lobeless gastrula, the relative accumulation of polypeptide 21 was reduced to the same extent in comparison to that of the normal embryo in both actinomycin D-treated and untreated embryos. For a discussion of the interpretation of these observations, the reader is referred to the section above, which describes the synthesis of polypeptide 20 in the lobeless gastrula.

Polypeptide 15 may be an example of an effect on the transcription of a structural gene resulting from polar lobe removal. In the normal embryo, polypeptide 15 was, at both the mesentoblast and gastrula stages, absent from actinomycin D-treated embryos. This complete dependence on new transcription suggests that this polypeptide is translated entirely from embryogenic mRNA. In the lobeless mesentoblast stage embryo, polypeptide 15 was synthesized at normal relative rates and was, as expected, absent from actinomycin D-treated lobeless embryos. By the gastrula stage, however, polypeptide 15 was absent from both actinomycin D-treated and <u>untreated</u> lobeless embryos. The two most reasonable explanations of these observations are that (1) the lobeless embryo, at the gastrula stage, ceases to transcribe the mRNA coding for polypeptide 15 or (2) the newly transcribed mRNA coding for polypeptide 15 is present in the lobeless embryo but cannot be translated. It should be noted that defects at any one of several points in the processing, transport or translation of embryogenic mRNA could be consistent with the latter interpretation.

At the gastrula stage, the lobeless embryo fails to (1) translationally activate or stabilize oogenic mRNAs (polypeptides 5, 6, 18, 87, 20, 21, 14, 78, 68) and (2) transcribe or translate embryogenic mRNAs (polypeptides 20, 21, 15). In addition, the lobeless embryo represses the translation of oogenic mRNA by both transcriptionally independent (polypeptides 16, 17) and transcriptionally dependent (polypeptides 16, 17, 71, 82) mechanisms.

Fifteen polypeptides show abnormal patterns of accumulation at the mesentoblast stage and fourteen at the gastrula stage. While these polypeptides are a small fraction of the total number of polypeptides made, they represent a significant 20% and 29% of the polypeptides that change at the mesentoblast and gastrula stages, respectively. It is not possible to establish from this study whether the differences in polypeptide synthesis in normal and lobeless embryos are causal, but they are strongly correlated with the failure of the lobeless embryo to form the mesentoblast cell and its progeny and suggests that the polar lobe cytoplasm plays a key role in regulating the changes in polypeptide synthesis that occur between cleavage and gastrulation.

# DISCUSSION

The results described above provide a portrait of the regulation of gene expression during early embryogenesis in a spiralian embryo. Through a comparison of polypeptides synthesized by early cleavage, mesentoblast and gastrula stage Ilyanassa embryos, it has been demonstrated that significant changes in the pattern of polypeptide synthesis occur as early as the twenty-five cell stage, which are correlated with the determinative events of this period in development, and that further changes in polypeptide synthesis are correlated with the morphogenetic movements of gastrulation.

The analysis of polypeptide synthesis in embryos reared in actinomycin D has demonstrated that (1) all of the polypeptides made during early cleavage and detected by two-dimensional electrophoresis are coded for by oogenic mRNAs, (2) stored oogenic mRNA is activated at the mesentoblast and at the gastrula stage, (3) the continued translation of most species of maternal mRNA is regulated by transcriptionally independent mechanisms throughout early development, (4) oogenic mRNA remains a primary source of transcripts for protein synthesis through gastrulation, and (5) transcription of embryogenic mRNA is activated at the mesentoblast stage. It is suggested that the transcription of regulatory genes is activated at the mesentoblast stage; the products of these genes repress the translation of oogenic mRNA and play a critical role in mediating stage-specific decreases and disappearances of polypeptides made throughout early development.

An analysis of polypeptide synthesis in the isolated polar lobe and in the lobeless blastomeres throughout early development has shown that (1) the polar lobe contains at least 300 species of maternally transcribed mRNAs, many with half-lifes of at least 12 hours, (2) the polar lobe translates all of the oogenic mRNAs translated in cleavage stage lobeless blastomeres, (3) the polar lobe is capable of activating stored oogenic mRNA and of regulating the translation of some maternal mRNAs, (4) the effect of the polar lobe on polypeptide synthesis is delayed until the mesentoblast stage and (5) at the mesentoblast and gastrula stages, the polar lobe cytoplasm acts to (a) regulate the activation of oogenic mRNA, (b) activate the synthesis or translation of embryogenic mRNA and (c) control the transcription of regulatory genes whose products repress the translation of maternal mRNAs.

## Early Cleavage Stage

Early embryogenesis is a maternal _tour de force_. _Ilyanassa_ embryos reared from the time of first polar body formation in concentrations of actinomycin D that inhibit 85% of uridine incorporation into acid precipitable material (table 1) continue to synthesize, without selective change in relative rates of accumulation, all of the 300 polypeptides characteristic of untreated early cleavage stage embryos. These results demonstrate that the polypeptides made during early cleavage and detected by two-dimensional electrophoresis are translated from oogenic mRNAs and that the regulation of their translation is not dependent on new transcription.

This is hardly surprising. That cleavage is independent of nuclear contribution was first demonstrated by E.B. Harvey in 1940 with the observation that enucleate merogones of _Arbacia_ eggs would cleave and form irregular blastulae when activated parthenogenetically. Similar capacities were shown by ennucleated amphibian eggs fertilized with irradiated sperm (Briggs _et al_., 1951). The independence of cleavage from nuclear activity, coupled with the observation that protein synthesis is required for cleavage in sea urchin eggs (Hultin, 1961) suggested that the egg might contain a store of preformed templates that would permit protein synthesis to continue in the absence of concurrent transcription. Consistent with this hypothesis was the finding, by Gross and Cousineau (1964) that the post-fertilization rise in protein synthesis in *Arbacia* eggs is unaffected by actinomycin D and the observation that protein synthesis occurs in activated enucleate merogones of sea urchin eggs (Denny and Tyler, 1964). Subsequent studies have extended these observations to a variety of organisms, including the pulmonate snail, *Limnaea palustris* (Morrill, 1965) and *Ilyanassa* (Feigenbaum and Goldberg, 1965; Collier, 1966; Newrock and Raff, 1976).

That the patterns of proteins synthesized by early cleavage stage embryos are largely unaffected by physical or chemical enucleation has been shown, using one-dimensional electrophoresis, for sea urchins (Spiegel *et al.*, 1965; Terman and Gross, 1965), *Rana pipiens* (Ecker and Smith, 1971) and *Limnaea palustris* (Morrill *et al.*, 1976). In addition, it has been demonstrated that the synthesis of some specific proteins, for example histones in sea urchins (Johnson and Hnilica, 1970; Ruderman and Gross, 1974) and tubulins in *Ilyanassa* and sea urchin embryos (Raff *et al.*, 1976, 1971) occurs in the absence of transcriptional activity.

More recently, the presence and persistence of RNA transcribed during oogenesis has been directly demonstrated in the eggs and early embryos of sea urchins (Humphreys, 1969, 1971), amphibians (Hough *et al.*, 1973) and mice (Bachvarova and De Leon, 1980).

Because the third polar lobe in the *Ilyanassa* egg forms at a time when the genome is probably transcriptionally inactive, the observation that protein synthesis occurs in this isolated enucleate egg fragment (Clement and Tyler, 1967; Geuskins, 1968; Teitelman, 1973) has been used to substantiate the conclusion that egg cytoplasm contains actively translated mRNAs of oogenic origin. The results presented here (and by Collier and McCarthy, 1979, 1980) confirm and extend these earlier observations with the demonstration that the isolated polar lobe synthesizes an average of 300 distinct polypeptide species--the same polypeptides that are made by intact cleavage stage embryos whether or not transcription is inhibited with actinomycin D. While it has been reported that mitochondria in Ilyanassa polar lobes incorporate $^{3}$H-uridine into acid-insoluble, ribonuclease-sensitive material (Geuskins and Jonghe d'Ardoye, 1971), a significant mitochondrial contribution to protein synthesis in early cleavage stage embryos is unlikely, since cyclohexamide inhibits 96% of the incorporation of $^{35}$S-methionine into acid precipitable material in early cleavage stage embryos (Collier, unpublished data). Thus the results presented here strongly suggest that the early cleavage stage embryo and the isolated polar lobe contain a minimum of 300 distinct species of mRNAs transcribed during oogenesis and the observation that isolated 24-hour old polar lobes continue to synthesize the majority of polypeptides made by freshly isolated lobes attests to the stability of these maternal mRNAs.

### Mesentoblast and Gastrula Stages

Between the twelve and twenty-five cell stage, approximately sixteen hours elapse, during which time, the segregation of the polar lobe cytoplasm determines the formation of the mesentoblast or 4d cell and the fates of the first three quartets are determined by induction and ooplasmic segregation (Clement, 1952, 1967, 1976). During the next twenty-four hours, the 4d cell divides, forming the mesoblast and entoblast cells and gastrulation occurs by epiboly of the ectoblasts (Clement, 1952, 1971).

Thus, two events that occur simultaneously in deuterostomes--the segregation of germ layers and the morphogenetic movements that establish their ultimate positions--are termporally separate in the spiralian embryo. As summarized in tables 8 and 9, changes in polypeptide synthesis and in the mechanisms that regulate polypeptide synthesis accompany the determination of the ectoblasts and mesentoblast at the twenty-five cell stage.

## TABLE 8

## SUMMARY OF STAGE SPECIFIC CHANGES IN POLYPEPTIDE SYNTHESIS AT THE MESENTOBLAST AND GASTRULA STAGES

| TYPE OF CHANGE | POLYPEPTIDES THAT CHANGE[1] | | | |
|---|---|---|---|---|
| | MESENTOBLAST STAGE | | GASTRULA STAGE | |
| Disappearance | 25 34 60 62 63 64 65 66 83 84 85 89 | (12) | 29 38 39 43 44 47 92 96 | (8) |
| Appearance | 10 12 15 21 24 28 31 32 33 37 38 39 41 42 48 51 52 54 55 56 74 77 93 97 | (24) | none | |
| Increase | 5 6 8 9 11 13 14 16 17 18 20 26 35 40 61 69 72 73 76 80 | (20) | 1 10 11 20 21 24 41 51 53 54 69 97 | (12) |
| Decrease | 2 3 4 19 23 29 45 46 57 58 68 70 71 78 87 91 94 95 | (18) | 14 16 17 22 23 26 27 30 33 35 36 37 40 42 45 46 48 50 52 58 68 78 79 81 86 88 91 94 95 | (29) |
| Total number of polypeptides that change | | 74 | | 49 |

[1] numbers refer to individual polypeptides listed in tables 4 and 6 and shown in Figs. 3, 4 and 7

These events are homologous to the segregation of the germ layers at gastrulation in deuterostomes, although, as emphasized by Conklin (1897), these cells are not "layers", nor do they occupy their ultimate positions at the time of their determination. The proliferation of these stem cells and their morphogenetic movements begin later, at gastrulation; changes in polypeptide synthesis also occur at this stage and are regulated by transcriptional dependent and independent mechanisms (tables 8 and 9).

The transition from early cleavage to the mesentoblast stage involves changes in twenty-five percent of the polypeptides made; twelve polypeptides disappear, twenty-five appear for the first time, twenty show increases in relative accumulation and eighteen decrease.

Between the mesentoblast and gastrula stages, forty-nine polypeptides undergo stage-specific changes: eight disappear, twelve increase in relative accumulation and twenty-nine decrease. There are no new polypeptides made by gastrula stage embryos.

The events of determination and morphogenetic cell movements at the mesentoblast and gastrula stages are, therefore, correlated with significant changes in polypeptide synthesis but the function of these polypeptides is unknown. Some, for example the polypeptides that decrease or disappear at the mesentoblast stage, may function to maintain the mature oocyte in a dormant state. An interesting possibility is that some of these polypeptides may act to maintain the totipotency of the egg--to hold open an array of developmental options. The elimination of these polypeptides could be an important mechanism by which the prospective potencies of embryonic cells become restricted. Some polypeptides, for example those that increase or appear at the mesentoblast stage and decrease or disappear at gastrulation, might be important in maintaining specialized patterns of cell adhesion or in restricting cell motility prior to gastrulation. Similar speculations can be made regarding the increases and new appearances of polypeptides during early development.

Changes in polypeptide synthesis have been reported during early development in the embryos of mice (Cullen et al., 1980; Handyside and Johnson, 1978; Levinson et al., 1978; Dewey et al., 1978; Braude 1979), rabbits (Van Blerkom, 1979; Van Blerkom and McGaughey, 1978) and *Xenopus* (Bravo and Knowland, 1979; Brock and Reeves, 1978) and some of the functions proposed for the polypeptides that change during early development in *Ilyanassa* embryos may be shared by these embryos. The sea urchin may be an exception since Branhorst (1976) has reported that very few changes in the pattern of polypeptide synthesis occur in this embryo prior to gastrulation, however, a more detailed analysis may reveal that in this embryo, as well, early developmental changes in polypeptide synthesis do occur. The function of the polypeptides that change during early development in the *Ilyanassa* embryo, and in other embryos, may be secondary to the fact that they provide probes of the regulatory mechanisms that control gene expression during early embryogenesis--the unseen events by which the egg transforms itself into an orderly assemblage of somatic cells.

What are the mechanisms that control the stage-specific changes in polypeptide synthesis in the *Ilyanassa* embryo? The analysis of the ability of embryos reared in actinomycin D to accomplish these changes has shown that combinations of four distinct regulatory modes are used to effect changes in polypeptide synthesis between cleavage and gastrulation: (1) translational control of oogenic mRNA, (2) activation of stored oogenic mRNP, (3) activation of embryogenic mRNA transcription and (4) the transcriptionally dependent repression of oogenic mRNA translation.

The polypeptides that remain constant between cleavage and gastrulation represent seventy percent of the polypeptides made during early development

# TABLE 9
# MODES OF REGULATION OF POLYPEPTIDE SYNTHESIS

| PROBABLE MODE OF REGULATION | CRITERIA[1] | SPECIFIC POLYPEPTIDES[2] | | | |
|---|---|---|---|---|---|
| | | Mesentoblast Stage | | Gastrula Stage | |
| A. Transcriptionally Independent | | | | | |
| 1. Translational repression | AMD insensitive disappearance | 25 34 60 62 63 64 65 66 83 85 | (10) | none | |
| 2. Same | AMD insensitive decrease | 2 4 19 23 45 46 57 58 70 78 87 | (11) | 22 23 27 33 37 42 45 46 48 50 52 58 79 86 88 94 | (16) |
| 3. mRNA or mRNP activation | AMD insensitive increase | 5 6 8 14 16 17 18 40 61 72 73 76 | (16) | none | |
| 4. mRNP activation | AMD insensitive new appearance | 33 38 42 48 52 55 74 93 | (9) | 10 28 37 | (3) |
| B. Transcriptionally Dependent | | | | | |
| 1. Embryogenic mRNA | absent in AMD | 10 15 28 31 32 37 | (6) | 15 31 32 | (3) |
| 2. Supplementation by embryogenic mRNA or Transcriptionally dependent activation of oogenic mRNA/mRNP | decrease in AMD | 1 3 9 11 12 13 20 21 24 41 51 53 54 69 75 77 80 97 | (18) | 1 3 9 10 11 12 13 20 21 41 49 51 53 54 55 59 69 75 77 80 97 | (22) |
| 3. transcriptionally dependent repression of translation | increase in AMD | 26 29 35 39 44 68 71 81 82 84 89 90 91 94 95 96 | (16) | 14 16 17 26 29 30 35 36 38 40 43 44 47 67 68 78 81 91 92 95 96. | (22) |

[1] Criteria for descriptions of modes of action are based on the type of stage-specific changes that occur in the synthesis of specific polypeptides between stages and/or in response to actinomycin D treatment.

[2] Numbers refer to individual polypeptides illustrates in Figs. 2-8.

(they include all of the polypeptides that did not receive number designations and eight of the numbered polypeptides--table 6, column 2, line 2). With four exceptions at the gastrula stage and two exceptions at the mesentoblast stage, (table 6, column 3, lines 2 and 3; table 4, column 3 lines 2 and 3) all of these polypeptides are insensitive to actinomycin D, indicating that they are probably translated from oogenic mRNAs activated at fertilization, and that these oogenic mRNAs have half-lifes of at least twenty-four hours. The imperturbable synthesis of these polypeptides and the fact that they do not change during early development suggest that they are "housekeeping" proteins whose function is required by both the egg and the cells of the early embryo. It is not clear that the translation of this group of oogenic mRNAs is "regulated" in the strict sense, but the very long half-life of these transcripts suggests that active measures must be taken to ensure their stability.

Evidence does exist for the operation of transcriptionally independent regulation of oogenic mRNA translation in the Ilyanassa embryo--i.e. for transcriptionally independent translational control, a term used here in its broadest sense to describe a variety of mechanisms operating at the level of the mRNA and the ribosome to affect the stability of mRNAs and the rate of initiation of polypeptide synthesis and the rate of ribosome migration (Lodish, 1976; Revel and Groner, 1978). It is suggested that the actinomycin D-insensitive changes in polypeptide synthesis between cleavage and mesentoblast stage which include the disappearance of ten polypeptides (table 9, line A1), the decrease in accumulation of eleven (table 9, line A2 and the increase in accumulation of twelve (table 9, line A3) reflect the action of transcriptionally independent repressors and activators of oogenic mRNA translation.

Transcriptionally independent mechanisms play a continuing, but diminished role in the regulation of polypeptide synthesis at the gastrula stage. Sixteen polypeptides undergo a gastrula stage-specific decrease in accumulation that is transcriptionally independent (Table 9, line A2), however, none of the increases or disappearances of polypeptides at this stage are regulated entirely by transcriptionally independent mechanisms.

That the translation of the oogenic mRNAs coding for these polypeptides are under the selective control of the egg cytoplasm, at least at the mesentoblast stage, is supported by comparisons of polypeptide synthesis in freshly isolated and 24-hour old polar lobes, which show that some of these stage-specific changes occur normally in 24-hour old polar lobes (table 3, lines A, C and E).

The synthesis of eighty-four percent of the polypeptides synthesized throughout the entire period from cleavage to the early gastrula stage is transcriptionally independent and, as noted above, the oogenic mRNAs coding for these polypeptides have half-lifes of at least twenty-four hours.

It is not improbable, however, that mRNAs could persist for forty-eight hours. Whalen et al. (1976) report that in postfusion myoblasts, myosin mRNA is actively translated and is stable for ten to twenty-four hours and Palmiter (1975) reports that ovalbumin mRNA has an average lifetime of thirty-five hours. In the mouse embryo, Bachvarova and De Leon (1980) have shown that sixty percent of bulk maternal RNA (including a constant proportion of poly(A) and RNA) is stable through the first two days of development.

The activation of stored oogenic mRNA is a special case of translational control. The appearance of nine new polypeptides, on schedule in actinomycin D-treated mesentoblast stage embryos (table 9, line A5) suggests that they are coded for by oogenic mRNAs stored in translationally inactive form and activated, twenty-four hours after first cleavage, at the mesentoblast stage. Similarly, three polypeptides, absent from actinomycin D-treated mesentoblast stage embryos, were present in actinomycin D-treated gastrulae, suggesting that some stored mRNAs are activated not at fertilization, nor at the mesentoblast stage, but at gastrulation, forty-eight hours after first cleavage. It is likely that these oogenic mRNAs are stored in the form of ribonucleoprotein particles, (mRNPs) the "masked messenger RNAs" (Tyler, 1967) or "informosomes" (Spirin, 1966) that have recently been directly demonstrated in unfertilized sea urchin eggs (Kaumeyer _et_ _al_., 1978; Young and Raff, 1979).

Our data demonstrates that (1) not all mRNPs are activated at fertilization and (2) that a mechanism exists to control the selective, stage-specific release of oogenic mRNPs. These observations are supported by the finding that two polypeptides unique to mesentoblast stage embryos also appear for the first time in 24-hour old polar lobes (table 3, line G). An additional nine polypeptides that were unique to mesentoblast stage embryos were also synthesized in actinomycin D-treated embryos, although their rates of accumulation differed from those observed in control embryos (table 5, column 3, lines 6 and 7); at least some of the mRNAs coding for these polypeptides are probably oogenic in origin and stored during cleavage as mRNPs.

The results presented here suggest that transcriptional level "switches" play a rather minor role in the regulation of polypeptide synthesis during early development in the _Ilyanassa_ embryo, yet, as early as the four-cell stage, this embryo is actively engaged in RNA transcription (Collier, 1976, 1975a, 1977), including the synthesis of DNA-like RNA (Collier and Yuyama, 1969; Koser and Collier, 1976) and polyadenylated RNA (Collier, 1975b; Kidder _et_ _al_., 1977; Clark and Kidder, 1977).

What is this RNA doing?

The failure of actinomycin D to alter the synthesis of any of the polypeptides detected during early cleavage suggests that either (1) polypeptide synthesis is <u>uniformly</u> affected by new transcription (see also p.58) (2) newly synthesized RNAs are stored in inactive form and, in a manner analogous to the release of oogenic mRNP, activated at a later stage or (3) the newly synthesized mRNAs belong to the complex class of messages whose sequences are present at such low frequency that they are not detected by the methods used here (Davidson and Britten, 1979).

It is not until the mesentoblast stage that the inhibition of RNA syntheses begins to affect the relative accumulation of specific polypeptides, although only forty polypeptides in all show some transcriptional dependence. While this is a small fraction of the polypeptides detected and leaves the function of a very substantial amount of RNA synthesis unexplained, it is a significant forty-four percent of the polypeptides that change during early development. By the gastrula stage, forty-seven polypeptides (fifty-two percent of the polypeptides that change during development) are wholly or partially dependent on new transcription.

Nevertheless, the number of polypeptides that are coded for by embryogenic mRNAs is exceedingly small. At the mesentoblast stage, only six polypeptides, and at the gastrula stage, only three polypeptides, are absent in actinomycin D-treated embryos and threfore translated from embryogenic mRNAs (table 9, line B1). Eighteen polypeptides showed decreased relative rates of accumulation in actinomycin D-treated mesentoblast stage embryos and twenty-two polypeptides decreased in actinomycin D-treated gastrulae. Either these polypeptides are translated from both oogenic and supplementary embryogenic mRNA or they are translated entirely from oogenic mRNA but require the presence of a transcriptionally dependent activator of oogenic mRNA of mRNP to achieve normal levels of accumulation (table 9, line B2). These alternatives cannot be distinguished by the methods used in this study.

One of the most unexpected findings in this study was that some polypeptides showed increased relative rates of accumulation or failed to disappear on schedule in actinomycin D-treated embryos. At the mesentoblast stage, two polypeptides failed to disappear and sixteen increased in the presence of actinomycin D. At gastrulation, fourteen polypeptides showed actinomycin D-induced increases and all of the eight polypeptides that would normally disappear at this stage continued to be synthesized. We suggest that these data provide evidence for a group of regulatory genes whose products, directly or indirectly, repress the translation of specific mRNAs.

Similar observations have been made by Braude (1979) who reports that several polypeptides that normally disappear in mouse embryos between the morula and blastocyst stage, fail to do so in the presence of $\alpha$-amanitin.

While Braude (1979) suggests, on the basis of the specificity of $\alpha$-amanitin for RNA polymerase II, that the inhibitor-induced increase in several polypeptides results from the failure of amanitin-treated embryos to synthesize an mRNA whose polypeptide product represses translation or degrades specific mRNAs, it is not clear that all translational repressors are proteins. Some, of course, may be; this is suggested by the observation that "superinduction" of tyrosine aminotransferase and interferon occur as a result of both inhibition of RNA synthesis by actinomycin D and of protein synthesis by cycloheximide (Tompkins et al., 1972, Vilcek and Navell, 1973). Alternatively, actinomycin D may inhibit the synthesis of one or more translational control RNAs (tcRNAs), such as those described in embryonic skeletal and heart muscle (Heywood and Kennedy, 1976; Deshpande et al., 1977), and in dormant Artemia gastrulae (Lee Huang et al., 1977).

It has also been suggested (Lodish 1976), that the phenomenon of "superinduction" of specific proteins by inhibitors of protein and RNA synthesis, may result from decreased competition among mRNAs for limiting factors such as tRNAs, initiation factors or ribosomes. The failure of actinomycin D to selectively alter the synthesis of any polypeptides detected during cleavage suggests that this alternative is the least probable. Further, two recent studies demonstrate that the concentration of mRNAs has little or no effect on the relative rates of their translation: Mermod et al. (1980) have shown that fourfold variations in the concentration of Drosophila embryo mRNAs in an in vitro translation system has no effect on the relative concentration of polypeptides translated and Berger et al. (1980) have shown that the amount of interferon made by Xenopus oocytes is linear, over a range of two orders of magnitude, to the amount of interferon mRNA injected, even when a large excess of other mRNAs was added. Thus, it seems likely that the increase observed in Ilyanassa embryo polypeptides in the presence of actinomycin D results from the failure of these embryos to transcribe either a translational control RNA or an mRNA whose product acts to repress the translation of specific mRNAs.

That two organisms as phylogenetically distant as mud snails and mice should require transcriptionally dependent translational repressors to mediate the decrease or disappearance of specific polypeptides during their development, suggests that this mechanism has a venerable evolutionary history and may be fundamental to the control of early embryogenesis.

In summary, it should be emphasized that in the Ilyanassa embryo, one important function of new RNA synthesis appears to be regulatory--that is, rather than controlling polypeptide synthesis by differential transcription of structural genes, this embryo transcribes regulatory genes whose products control the differential translation of oogenic mRNAs. As will be demonstrated below, even the expression of these regulatory genes is under the control of the egg cytoplasm, underlining the dominance of ooplasmic regulation during early embryogenesis.

Detailed comparisons of the effect of actinomycin D on the relative rates of accumulation of specific polypeptides at the mesentoblast and gastrula stages (Fig. 10) have revealed eight patterns of stage-specific and actinomycin D-induced changes in polypeptide synthesis, each of which reflect the operation of combinations of the basic control mechanisms described above. Using the same basic transcriptionally dependent and independent controls, in different combinations, twenty-four permutations of stage-specific changes and regulatory modes can be generated. Since the spiralia are among the most ancient of metazoans, it is predicted that the regulatory mechanisms used to control polypeptide synthesis in the Ilyanassa embryo will be found in the embryos of other organisms, but that they may be used at different times in development or in different combinations to generate new patterns of polypeptide synthesis.

Evidence has been presented (see p. 64) that suggests the operation of higher level control mechanisms that (1) ensure that, for some polypeptides, the same source of mRNA and the same transcriptionally dependent or independent regulatory mechanisms will be used at both the mesentoblast and gastrula stage, or (2) control a switch in the mode of regulation or in the source of mRNA between the mesentoblast and gastrula stages. Whatever these controls may be, changes in the timing or mode of their operation may be keys to the evolution of new patterns of ontogeny.

As shown in figure 10 (patterns 3 and 4), an important device used by this embryo to regulate gastrula stage polypeptide synthesis is the joint action of transcriptionally dependent and independent regulatory mechanisms. This coupling illustrates the way the embryo gradually assumes the burden of regulating its own development and of how, for this brief and critical time, maternal and embryogenic information co-exist, forming a complex web of interacting regulatory mechanisms that sometimes reinforce, and sometimes change, the program of gene expression during early embryogenesis.

## Ooplasmic Segregation and the Regulation of Polypeptide Synthesis

The experimental embryology of the spiralians has a venerable history. Conklin's (1897) studies of cell lineage in the gastropod mollusc, Crepidula demonstrated the remarkable regularity of cleavage in this egg and showed that virtually all of the mesoderm and endoderm is derived from but a single cell, the mesentoblast, formed as the fourth division product of the D macromere. In 1896, Crampton established the role of the vegetal cytoplasm in the determination of the mesoderm in the Ilyanassa egg by removing a large cytoplasmic "yolk-lobe" or "polar lobe" formed at first cleavage from the vegetal hemisphere. He demonstrated that the primary defect exhibited by these "lobeless" embryos was the absence of the mesoblast cells of the normal larva--a striking finding in view of the fact that the substance of the polar lobe is passed ultimately into the D macromere, which forms the mesentoblast or 4d cell. The effect of the polar lobe on the determination of the mesentoblast and its derivatives was confirmed by Wilson (1904) with the egg of the scaphopod mollusc, Dentalium. Subsequent studies by Clement have extended these early observations to demonstrate that the third polar lobe of the Ilyanassa egg determines the cleavage pattern of the D quandrant (Clement, 1952, 1962) has an inductive effect on eyes, foot and velum (Clement, 1976), establishes the anteroposterior axis (Clement, 1962) and has an organizing effect on the formation of the external shell (Clement, 1976).

The importance of egg cytoplasm was recognized by Wilson (1924), who wrote:

> "... the specification of the blastomeres is due to the nature of the specific cytoplasmic materials which they receive during cleavage....(p. 1065)

> ... The egg cytoplasm has a definite molecular organization directly handed down from the parent; cleavage sunders the various "physiological molecules" and isolates them in particular cells....(p. 1041)"

Over half a century later, the nature of these "physiological molecules" remains unknown.

Since the observation, by Clement and Tyler (1967), that the isolated polar lobe is capable of protein synthesis, it has been repeatedly suggested that specific mRNAs are selectively localized in the polar lobe. Does the polar lobe contain a unique set of maternal mRNAs? The observation that the freshly isolated polar lobe synthesizes the same three hundred species of polypeptides made by the lobeless blastomeres indicates that there is no selective localization of the mRNAs translated during cleavage. At the mesentoblast and gastrula stages, however, several polypeptides were synthesized in actinomycin D-treated normal embryos, but were reduced or absent in actinomycin D-treated lobeless embryos, suggesting that either (1) the oogenic mRNAs that code for these polypeptides are localized in the polar lobe and thus are absent from the lobeless embryo or (2) the lobeless embryo contains the oogenic mRNPs coding for these polypeptides, but lacks polar lobe-localized factor(s) required to activate their translation (Table 10, line A). These alternatives are equally probable, and indistinguishable, by the methods used in this study.

Whittaker (1977) has demonstrated the appearance of localized endodermal alkaline phosphatase activity in specific blastomeres of the ascidian embryo, Ciona, and has shown that the delayed appearance of this enzyme is actinomycin D-insensitive, suggesting that either the maternal mRNA for this enzyme, or a factor regulating its translation, is segregated during early cleavage into the endodermal cell line.

The question of whether specific mRNAs are localized during polar lobe formation might be resolved by a comparison of the *in vitro* translation products of mRNAs isolated from polar lobes and lobeless blastomeres. This approach was used by Cheney and Ruderman (1978) to show some differences in the polypeptides translated *in vitro* from RNA of AB and CD half-embryos of the mollusc, *Spisula*. When this work is published in detail, it may provide the first clear evidence for localization of mRNAs during cleavage.

The comparison of polypeptide synthesis in freshly isolated and twenty-four hour old polar lobes has revealed that some of the mesentoblast stage-specific changes in polypeptide synthesis that occur in intact embryos, also occur in this cytoplasmic egg fragment. The analysis of these changes indicates that the polar lobe contains factors that (1) repress the translation of oogenic mRNA (table 3, lines A and C) and (2) activate oogenic mRNP and/or enhance the translation of oogenic mRNA (table 3, lines E and G).

Some of the stage-specific changes in polypeptide synthesis observed in normal and lobeless embryos failed to occur in aging polar lobes (table 3, lines B, H and I) and some of the changes detected in 24-hour old polar lobes were not characteristic of normal embryos (table 3, lines D and F). These data indicate that the interaction of both animal and vegetal halfs of the egg is required for the normal regulation of stage-specific changes in polypeptide synthesis and demonstrate that different regions of the egg cytoplasm, at different times in development, selectively translate the same species of oogenic mRNAs. This differential expression of maternal information in space and in time may well be the key to the mechanism by which segregation of the ooplasm exerts its determinative effect. This is supported by the analysis of polypeptide synthesis in the lobeless embryo.

The effect of the polar lobe on polypeptide synthesis is delayed until the mesentoblast stage. By the mesentoblast stage, fifteen polypeptides show abnormal patterns of accumulation in the lobeless embryo and at the gastrula stage, the synthesis of fourteen polypeptides is affected by polar lobe removal (Fig. 11, table, 10). While these polypeptides represent a small fraction of the total number of polypeptides made, they are a significant 20% and 29% of the polypeptides that change at the mesentoblast and gastrula stages, respectively. The defects in polypeptide synthesis that occur in lobeless embryos are correlated in time with the failure of the lobeless embryo to form the mesentoblast cell and its derivatives, suggesting that these polypeptides are causally related to the determination of the mesentoblast and the maintenance of the determined state, and that the regulation of the synthesis of these polypeptides is, at least at the mesentoblast stage (see p. 73 ), directly controlled by factors localized in the polar lobe.

The data presented above (and summarized in table 3 and table 10, line A) demonstrate that the egg cytoplasm controls the synthesis of some polypeptides by the differential distribution of mRNAs or by the selective, transcriptionally-independent translation of oogenic mRNA, and that these processes may control determination. The next section will demonstrate that the ooplasm also controls the expression of <u>regulatory</u> genes whose products mediate the selective translation of oogenic mRNAs.

The polypeptides listed in table 10, lines B-D are all partially dependent, in either the normal or the lobeless embryo, on transcription, yet, with only one exception (polypeptide 15, line D ), all are translated, entirely or in part, from oogenic mRNA. The defects in their patterns of accumulation in the lobeless embryo reflect the failure of this embryo to regulate the transcriptionally dependent control of mRNA translation.

All of the polypeptides listed in line B of table 10 show, in comparison

## TABLE 10

## SUMMARY OF POLAR LOBE DEPENDENT REGULATION OF POLYPEPTIDE SYNTHESIS

| PROBABLE MODE[1] OF ACTION OF POLAR LOBE | SPECIFIC POLYPEPTIDES[2] | |
|---|---|---|
| | MESENTOBLAST STAGE | GASTRULA STAGE |
| A. Transcriptionally independent activation of oogenic mRNA or mRNP translation, or, mRNP localization | 14 51 53 54 97 78 | 5 6 14 18 20 21 68 78 87 |
| B. Induces transcription of regulatory gene that represses oogenic mRNA translation or activates transcriptionally dependent repressor | 81 82 84 89 71 94 | none |
| C. Blocks the synthesis or activity of a transcriptionally dependent repressor of oogenic mRNA translation | 16 17 | 16 17 71 82 |
| D. Controls transcription of embryogenic mRNA, or, induces transcription of a translational activator of oogenic mRNA or mRNP | 20 | 15 20 21 |

[1] Criteria for descriptions of the mode of action of the polar lobe are the differences in relative rates of accumulation of polypeptides in control and actinomycin D-treated lobeless embryos when compared to normal embryos. These data are summarized in Fig. 11.

[2] numbers refer to individual polypeptides shown in figures 2-8.

with normal untreated mesentoblast stage embryos, increased relative rates of accumulation in three experimental classes (actinomycin D-treated normal, actinomycin D-treated lobeless and untreated lobeless mesentoblast stage embryos), indicating that the expression of a transcriptionally dependent translational repressor has been eliminated. That these polypeptides increase in untreated lobeless embryos suggests that the polar lobe contains a factor that (1) induces the transcription of a repressor gene or (2) controls the post-transcriptional processing or activation of the repressor gene product. Whichever alternative is correct, these data indicate that the expression of a regulatory gene that represses translation is under the control of oogenic factors localized in a region of egg cytoplasm crucial to determination and suggests that the control of these regulatory genes may be causally related to determination.

The polypeptides listed in line C of table 10 show a defect in the lobeless embryo that is the inverse of that discussed above; all of these polypeptides are, in the lobeless embryo, repressed by a transcriptionally dependent mechanism that is blocked in the normal embryo. In the normal embryo, these polypeptides are translated from oogenic mRNAs and their synthesis is controlled by transcriptionally independent mechanisms, but in the lobeless embryo, these polypeptides show increased relative rates of accumulation in actinomycin D, indicating the operation of a transcriptionally dependent translational repressor. These data suggest that factors localized in the polar lobe act to (1) block the transcription of a regulatory gene whose product represses oogenic mRNA translation or (2) prevent the processing or activation of the product of a repressor gene. As in the previous case, it is clear that the expression of a regulatory gene whose product represses translation of oogenic mRNAs is under the control of maternal factors localized in the polar lobe.

Does the polar lobe regulate the transcription of structural genes? Collier (1977) has demonstrated that removing the polar lobe results in a significant 43.7% and 48.0% decrease in the absolute rates of RNA synthesis at the mesentoblast and gastrula stages respectively, but removing the polar lobe does not significantly alter the proportion of poly(A)-RNA synthesized during early embryogenesis (Collier, 1975b). Koser and Collier (1976) showed that the lobeless embryo accumulates proportionally less high molecular weight RNA (possibly hnRNA) and more low molecular weight RNA (mRNA) than did the normal embryo, but that these differences were statistically significant only after gastrulation.

While it is clear that the polar lobe does have an effect on transcription as early as the 25-cell stage, the data presented here do not support the notion that the polar lobe controls the differential transcription of structural genes used during early development. Throughout the entire period from first cleavage to early gastrulation, only three polypeptides (table 10, line D) illustrate a possible effect by the polar lobe on mRNA transcription.

Polypeptides 20 and 21 showed a decrease in relative accumulation in lobeless control embryos when compared with normal control embryos, but the same decrease was not apparent when actinomycinD-treated lobeless embryos were compared with normal actinomycin D-treated embryos, indicating that the difference in accumulation is due to a transcriptional effect. These observations suggest two possible interpretations, which are equally plausible: either the polar lobe (1) controls the transcription, processing or translation of embryogenic mRNAs or (2) induces the transcription or activity of a regulatory gene product that activates the translation of oogenic mRNPs.

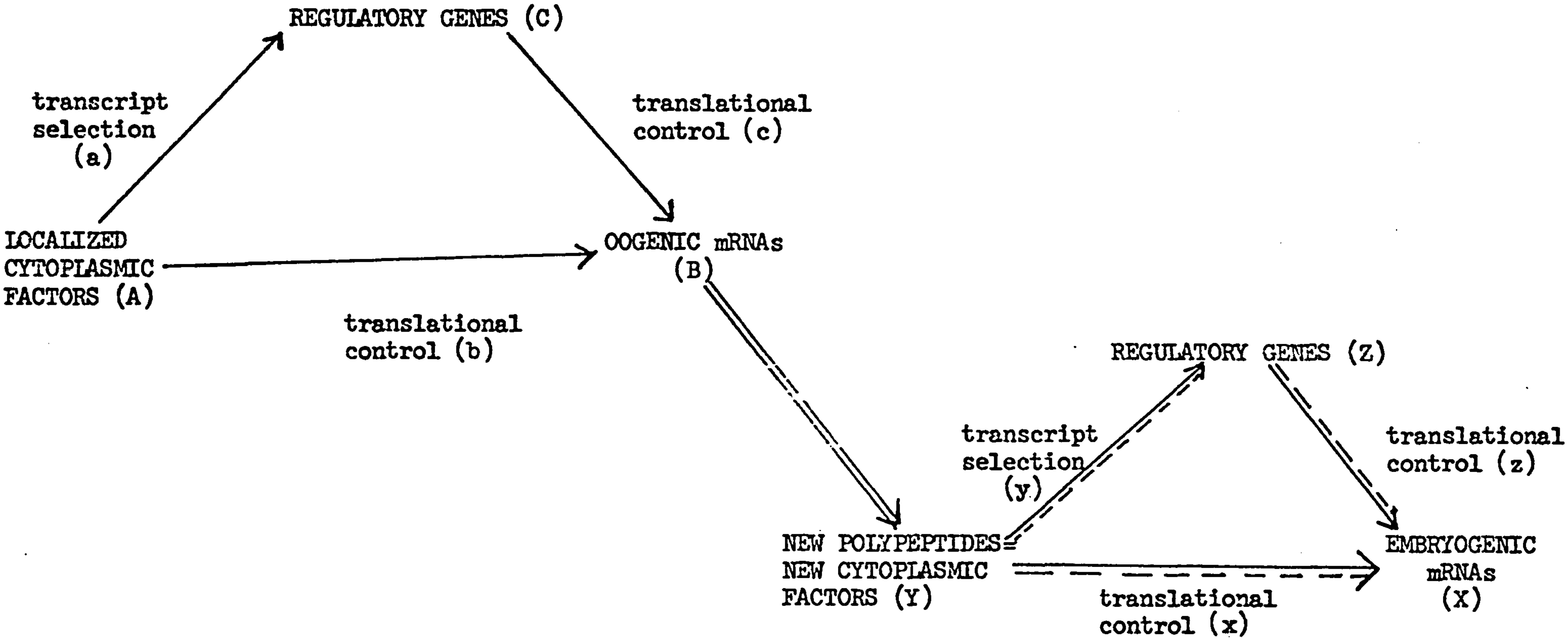
The interpretation of the effect of the polar lobe on the synthesis of polypeptide 15 is somewhat less ambiguous. This polypeptide is always absent in actinomycin D-treated embryos and is probably coded for by embryogenic mRNA. While present in lobeless mesentoblast stage embryos, polypeptide 15 is absent in untreated gastrula stage lobeless embryos. These data indicate that the gastrula stage lobeless embryo is unable to activate the transcription, processing or translation of the embryogenic mRNA coding for this polypeptide. The third alternative is particularly interesting since the increased accumulation of low molecular weight RNAs in the lobeless embryo may occur because these RNAs are not translated (Koser and Collier, 1976).

The fate of individual blastomeres is specified by the localization of ooplasmic factors that control the expression of maternal information directly, through the selective translation of oogenic mRNAs, and indirectly, by controlling the expression of regulatory genes whose products, in turn, regulate the differential translation of oogenic mRNA. These relationships are summarized in figure 12.

The critical elements in this relationship are maternal in origin and, although both oogenic mRNA and ooplasmic factors may be segregated, only one element need be selectively localized for the relationship to function. The segregation of ooplasmic factors (A) establishes an initial asymmetry that results in the differential expression of oogenic information. This occurs through "transcript selection" --- the control of gene expression by selective transport or processing of indiscriminately transcribed genes, as well as by differential transcription. The result of transcript selection by ooplasmic factors is the discriminate expression of regulatory genes (C) whose products, in turn, control the selective translation (c) of oogenic mRNAs (B).

Ooplasmic factors can also act directly to control the translation (b) of

FIGURE 12


REGULATORY GENES (C)
transcript selection (a)
translational control (c)
LOCALIZED CYTOPLASMIC FACTORS (A)
OOGENIC mRNAs (B)
translational control (b)
REGULATORY GENES (Z)
transcript selection (y)
translational control (z)
NEW POLYPEPTIDES=
NEW CYTOPLASMIC FACTORS (Y)
EMBRYOGENIC mRNAs (X)
translational control (x)

oogenic mRNAs (B).

Through these mechanisms, the differential expression of maternal information in specific blastomeres is accomplished. At this point, the system becomes self-perpetuating, i.e. determined, since the differential translation of oogenic mRNA results in the production of new polypeptides (Y) that may control subsequent gene expression (dotted lines x, y, and z) without the intervention of ooplasmic factors.

Thus, the relationships that regulate early development by selective translation, may regulate later developmental events as well. The extensive transcription that occurs during early development may be precocious and largely non-selective. Once determination has occurred, the embryo may begin to repeat the translational regulatory processes of early development, this time selecting from a store of previously transcribed and untranslated embryogenic mRNA or mRNP (X); again regulating the translational selection of specific mRNAs by cytoplasmic factors (Y), directly (x) and indirectly through the selection of regulatory gene transcripts (y) that control translation (z).

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