Studies of Structural Gene Transcripts in Sea Urchin Embryos and Adult Tissues

Thesis by

Barbara J. Wold

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1978

(Submitted May 11, 1978)

Acknowledgements

My years in Pasadena have been most rewarding, and thanks are due to many people for making them so:

To all the members of the E. Davidson group '73-'78 for aid, inspiration and example. From them I learned most of what I know of the business of molecular biology (also tennis, cooking, cats, running, etc.). My particular thanks to my thesis advisor, Eric, and to Bob, Barbara and Bill.

To the National Science Foundation, the Danforth Foundation, the McCallum Fund, and Caltech for money (almost enough); and to Geraldine for figuring it all out.

To Connie for heroics above and beyond the call of duty in transforming my very rough drafts into final form in record time.

To Jane for gargoyles, flowers and figures.

And to Larry for everything.

Abstract

The RNA stored in the mature sea urchin oocyte includes sequences transcribed from about 6% of the single copy DNA or approximately 3.7×10^7 nucleotide pairs. It is estimated that there are about 1500 transcripts of each sequence per oocyte. A ^{3}H-labeled single copy DNA fraction highly enriched for sequences complementary to mature oocyte RNA was prepared. This DNA, referred to as oDNA, was reacted with excess polysomal mRNA from sea urchin embryos at 16-cell, blastula, and gastrula stages. Sixteen-cell embryo mRNA reacted with oDNA to a level which is 73% of that observed for the reaction of oDNA with oocyte RNA. This represents about 2.7×10^7 nucleotides of maternal sequence, or 12-15,000 different mRNAs of average size. Blastula and gastrula stage mRNA reacted with oDNA to about 56% and 53%, respectively, representing 2.1×10^7 and 1.9×10^7 nucleotides of oocyte RNA sequence. From the kinetics of hybridization, we calculate that the concentration of mRNAs belonging to the maternal sequence set is about 500 copies per embryo. This is typical of the "complex class" of embryo mRNA which contains the vast majority of diverse messenger RNA sequences but comprises only a small fraction (about 10%) of the total mRNA mass.

Total cytoplasmic RNA was extracted from 16-cell, blastula, prism and pluteus stages and reacted in excess with ^{3}H-oDNA. The amount of hybridization of oDNA with 16-cell cytoplasmic RNA was 100% of the value obtained when oDNA was reacted with oocyte RNA. Hybridization levels observed with cytoplasmic RNAs from later stages were progressively less, but were higher than the corresponding mRNA hybridization levels through the blastula stage.

To detect and quantitate embryo messenger RNA sequences which are not homologous with maternal RNAs, a labeled single copy DNA fraction entirely devoid of oocyte RNA complementary sequences was prepared. This selected DNA, termed null oDNA, was reacted with excess polysomal mRNA from 16-cell, blastula and gastrula embryos. Non-maternal sequences could not be detected in the mRNAs from 16-cell and gastrula stage embryos. Mesenchyme blastula mRNA, however, hybridized about 3.6×10^6 nucleotides of null-oDNA sequence which is sufficient to code for approximately 2,000 mRNAs of average size. These non-maternal mRNAs are complex class sequences present on blastula polysomes at about the same concentration (500 copies per embryo) as are most maternal sequence mRNAs. Thus, a significant number of new, non-maternal structural genes are expressed in blastula stage embryos.

Because the sea urchin displays large differences between embryo and adult tissue messenger RNA sequence sets, it is possible to test the proposition that structural gene sequences are transcribed only in cells where the transcripts are translated. A ^{3}H-labeled single copy DNA highly enriched for sequences complementary to blastula polysomal messenger RNA was prepared. This selected DNA fraction, referred to as mDNA, represents about 2.6×10^7 nucleotides of embryo messenger RNA sequence. A maximum of 16% of the blastula sequences are present in coelomocyte and intestine messenger RNAs while 40% of the blastula sequences are represented in gastrula mRNA. ^{3}H-mDNA was hybridized with excess nuclear RNA from coelomocytes, intestine, and gastrula embryos. Within our limits of detection, all mDNA sequences were hybridized by each of the nuclear RNAs. We conclude from this result that virtually all of the blastula mRNA sequences are transcribed in three heterologous tissues in which the transcripts are

not utilized as messenger RNAs. The kinetics of hybridization show that the blastula structural gene sequences are present in each nuclear RNA at about the same concentration as are the majority of the complex single copy nuclear transcripts.

Table of Contents

CHAPTER 1

Introduction

The experiments presented in the following chapters concern the expression of sea urchin structural genes. A structural gene is operationally defined here as a DNA sequence which codes for a polysomal messenger RNA. In the first two studies we analyze patterns of structural gene expression in sea urchin embryogenesis. The third study concerns the transcription of structural gene sequences in tissues where those sequences are not represented in the polysomal messenger RNA population.

The sea urchin Strongylocentrotus purpuratus is particularly well-suited for these studies for several reasons. First, a great deal of background information is available regarding both biological and molecular aspects of early embryogenesis. Secondly, the sea urchin is amenable to studies requiring large quantities of nucleic acids from embryos at various stages of development and differentiation. Finally, embryos efficiently take up and incorporate radioactively labeled nucleic acid precursors, making possible experiments which require in vivo labeling of RNA or DNA. The purpose of this introduction is to briefly summarize some aspects of sea urchin embryogenesis relevant to the studies presented in chapters II - IV. Included in this summary will be patterns of protein synthesis, maternal messenger RNA and its utilization, and characteristics of nuclear and messenger RNA populations.

Prelarval embryogenesis in the sea urchin takes place rapidly. In the species Strongylocentrotus purpuratus, the time from fertilization to pluteus larva is less than ninety hours. During this period cell division, differentiation and morphogenesis occur, though there is essentially no increase in embryo mass. The mature sea urchin oocyte is haploid, having completed both meiotic divisions in the ovary. Following fertilization, the first three cleavage divisions give rise to eight blastomeres of equal size. At about five hours of development, the fourth cleavage

produces a sixteen-cell embryo. This cleavage is "unequal", generating four macromeres, eight mesomeres, and four micromeres. The four micromeres include only 8% of the embryo volume. These cells later give rise to the primary mesenchyme cells of the blastula, and eventually synthesize the embryo skeleton at gastrulation. Cell division is exponential during the cleavage and early blastula stages (Hinegardner, 1967). By 10 hours of development, the embryo is composed of about 100 cells and a hollow cavity, the blastocoel, forms in the center of the embryo. This marks the beginning of the blastula stage. By about 18 hours of development, the blastula consists of approximately 350 cells in the form of a hollow sphere one cell layer thick. The rate of cell division slows markedly during the mesenchyme blastula stage which extends from about 18 to 36 hours. Gastrulation begins at about 36 hours with the invagination of the archenteron (the future digestive tract) and formation of spicules (the first skeletal structures). By gastrulation, the first major events of morphogenesis and tissue differentiation have occurred. Further development to the eighty-hour pluteus stage includes elaboration of a brachiated skeleton and a fully differentiated digestive tract as well as other specialized cell types and tissues. The pluteus larva is fully formed by about eighty hours and is ready to begin feeding. This marks the end of the prelarval period. Feeding initiates growth which is accompanied by further differentiation over a period of one to two months. Larval development terminates at metamorphosis, when a small but fully differentiated sea urchin appears. Almost nothing is known about the larval stages at the molecular level, since it is very difficult to culture the embryos beyond the unfed pluteus stage. In the studies described here, we are concerned exclusively with prelarval embryogenesis. The prelarval developmental time scale, outlined above, is summarized in Table 1.

Table 1. Time scale for S. purpuratus prelarval development at 15°C

Stage	Developmental time	Cell number[a]
First cleavage	2 hr	2
16-cell	5 hr	16
Early blastula	10 hr	∿100
Mesenchyme blastula	20 hr	∿350
Gastrula	36 hr	∿600
Pluteus (unfed)	80 hr	∿1200

[a]From Hinegardner (1967).

Compared to the embryo, the mature oocyte is translationally quiescent. It contains a very small fraction of its ribosomes in polysomes (Monroy and Tyler, 1963; MacKintosh and Bell, 1969; Humphreys, 1971; reviewed in Davidson, 1976). One to two percent of the oocyte RNA has been identified as mRNA on the basis of its activity in cell-free translation systems (Slater and Spiegelman, 1966; Gross et al., 1973; Jenkins et al., 1973; Ruderman and Pardue, 1977), its poly A content (Slater et al., 1973; Wilt, 1977), and its size distribution (Wu and Wilt, 1974; Fromson and Duchastel, 1975).

The single copy sequence complexity (the total length of diverse sequence represented in the RNA) of total RNA extracted from mature sea urchin oocytes is about 3.7×10^7 nucleotides (Anderson et al., 1976; Galau et al., 1976; Hough-Evans et al., 1977). These single copy transcripts make up about 1-2% of the total oocyte RNA mass. This is very similar to the fraction of oocyte RNA previously identified

as maternal message on the basis of in vitro translation assays and other criteria cited above. It is probable therefore that these single copy oocyte transcripts are maternal messages which are loaded into polysomes and translated during cleavage.

The maternal messenger RNA is stored in ribonucleoprotein particles in the oocyte (Gross et al., 1973; Skoultchi and Gross, 1973), and is thought to be in a "masked" form because it is not translated in the mature oocyte, even though the oocyte apparently contains a functional translational apparatus (Clegg and Denny, 1974; reviewed in Davidson, 1976).

At fertilization, maternal mRNAs are loaded into polysomes. The result is a 15-fold increase in protein synthesis in the first two hours after fertilization and before the first cleavage division (Epel, 1967; Humphreys, 1969, 1971; Fry and Gross, 1970a). At least 90% of that protein synthesis is attributable to the translation of maternal rather than newly synthesized embryo messages (Humphreys, 1971). Following the initial dramatic increase in protein synthesis at fertilization, the rate of protein synthesis reaches a level of about 0.4 to 0.8 ng per hour per embryo, and that level is maintained through the gastrula stage (Fry and Gross, 1970b; Seale and Aronson, 1973; Reiger and Kafatos, 1976; reviewed in Davidson, 1976). The amount of protein synthesized in an hour is about one percent of the total protein content of the embryo, and about the same amount of newly synthesized protein is turned over per hour (Berg and Mertes, 1970). Thus, the total protein content of the embryo remains relatively constant through early embryogenesis.

Qualitatively, the spectrum of proteins synthesized does not appear to change much until the early gastrula stage. The most sensitive measurements of protein synthesis patterns in the sea urchin have been made using two-dimensional

gel electrophoresis (Brandhorst, 1976). In this experiment, proteins were labeled in vivo with ^{35}S-methionine. About 400 distinct proteins were resolved on the two-dimensional gels. Mature oocytes, fertilized eggs, and blastula stage embryos displayed very similar electrophoretic patterns. By gastrula, however, numerous changes are observed, implying that a shift in the pattern of protein synthesis has taken place. These observations suggest that the major change in protein synthesis at fertilization is quantitative, while qualitative change is not detected until gastrulation. The proteins detected by Brandhorst and other workers using similar methods are the products of relatively prevalent embryo messenger RNAs. In sea urchin embryos, there are two classes of messenger RNA with respect to their abundance in the embryo (Galau et al., 1974, 1976; Nemer et al., 1975; Wold et al., 1978). Prevalent class messages are present at about 3-5 x 10^4 copies per embryo at the blastula stage, and there are approximately 500 to 1000 different sequences represented (Nemer et al., 1974). Earlier in development during cleavage and early blastula stages, a substantial fraction of the polysomal messenger RNA codes for histones (Moav and Nemer, 1971). As the rate of cell division slows during blastula the fraction of message coding for histone is reduced, and by gastrula histone comprises only a small fraction of total polysomal mRNA (reviewed in Davidson, 1976). In general, however, very little is known about stage specific differences in prevalent mRNA sequence sets, and therefore shifts in protein synthesis patterns cannot be directly compared with changes in the corresponding mRNA populations. It would, however, be expected that stage specific differences in major protein species reflect differences in prevalent mRNA sequences. Proteins synthesized from the rare, low abundance messenger RNAs discussed below cannot be detected on the two-dimensional gels because these proteins are minor species, each one

representing a very small fraction of the total protein synthesized.

The second group of messenger RNAs are referred to as rare or "complex class" sequences (Galau et al., 1974, 1977a). In sea urchin embryos at the blastula-pluteus stages, complex messages comprise less than 10% of the total messenger RNA mass, but include over 90% of the mRNA sequences. Thus, there are about 10,000 to 20,000 different complex class messages, each present at about 500 copies per embryo (Galau et al., 1976; Hough-Evans et al., 1977). In the blastula-pluteus stages, complex mRNAs are present at an average concentration of about one copy of each sequence per cell. This is, however, an average value, and it is not known whether specific mRNA sequences are evenly distributed among all embryo cells or are localized in a few cells.

Galau et al. (1974) measured the number of different messenger RNA sequences in gastrula polysomes by reacting excess unlabeled messenger RNA with small amounts of labeled nonrepetitive DNA. They found that about 2.7% of the single copy DNA is represented in the RNA. This is equivalent to about 1.7×10^7 nucleotide pairs of DNA sequence, enough to account for about 10^4 different mRNAs of average size. From the kinetics and extent of hybridization, they calculated that less than 10% of the messenger RNA was driving the reaction. The remaining 90% is the prevalent class mRNA discussed above.

The extent to which complex mRNA sequence sets differ at various stages of development was studied by Galau et al. (1976) and Hough-Evans et al. (1977). They found that embryo structural gene sets differ by thousands of sequences from one stage to another. A somewhat unexpected observation was that the mRNA sequence complexity decreases as embryogenesis proceeds. Thus, the complexity of cleavage stage embryo mRNA is about 2.7×10^7 nucleotides, but by gastrula the

mRNA complexity is 1.7 x 10^7 nucleotides, and at pluteus the complexity is approximately 1.3 x 10^7 nucleotides. Furthermore, the vast majority of these sequences are maternal; that is, they are homologous with RNAs found in the mature sea urchin oocyte. Galau et al. (1976) observed that at gastrula most or all of the species of message present on polysomes are also represented in oocyte RNA. Studies of other developmental stages (chapters II and III) have shown that over 80% of the diverse message sequences expressed at each prelarval stage are also present in the total RNA of the oocyte.

Even though most embryo mRNA sequences are maternal up to the pluteus stage, the messages themselves are newly transcribed by the blastula stage (Galau et al., 1977b). This is the conclusion from studies of messenger RNA synthesis and turnover which show that the steady state content of newly synthesized mRNA is about the same as the total mRNA content of the polysomes. Both prevalent and complex class mRNAs are newly synthesized and have a half-life of about five hours (Nemer et al., 1975; Galau et al., 1977b). Thus, many of the structural genes transcribed in oogenesis and represented in oocyte RNA are also transcribed during embryogenesis. The developmental significance of this observation is discussed further in chapter II.

A related question was whether there are any new, non-maternal structural genes expressed during embryogenesis. The experiments described in chapter III show that there are a substantial number of embryo specific, complex class mRNAs expressed at the mesenchyme blastula stage. Complex non-maternal sequences were not, however, detected at 16-cell or gastrula stages. Expression of a prevalent class non-maternal sequence has also been detected during the blastula-gastrula period. This non-maternal message codes for an embryo form of histone I

which replaces the oocyte form during embryogenesis (Ruderman et al., 1974; Arceci et al., 1976).

Sea urchin embryo messenger RNA is different in many respects from the nuclear RNA of the same cells. Nuclear RNA is at least two to four times longer, on average, than polysomal messenger RNA when both are measured under denaturing conditions (Kung, 1974; Fromson and Duchastel, 1975). The half-life of heterogeneous nuclear RNA at gastrula is about 20 minutes (Grainger and Wilt, 1976; Roeder and Rutter, 1970; reviewed in Davidson, 1976), compared with a half-life of about 5.5 hours for messenger RNA. The rate of synthesis of total heterogeneous RNA is about 0.5 pg per minute per embryo at cleavage and about 2.5 pg per minute per embryo at blastula and gastrula (Roeder and Rutter, 1970; Wilt, 1970; Grainger and Wilt, 1976; reviewed in Davidson, 1976). For polysomal messenger RNA, the rate of synthesis is about 0.13 pg per minute per embryo at blastula and gastrula (Galau et al., 1977b). Thus, only about five percent of the nucleotides incorporated into total heterogeneous RNA at the blastula-gastrula stage appear in messenger RNA. The remainder are incorporated in RNAs confined to the nucleus.

Nuclear RNA and messenger RNA also differ in sequence content. Heterogeneous nuclear RNA contains both repetitive and single copy sequences interspersed on the same transcript (Smith et al., 1974), but messenger RNA from gastrula stage embryos contains very little repetitive sequence transcript (Goldberg et al., 1973; McColl and Aronson, 1974; Nemer et al., 1975). The single copy sequence complexity of heterogeneous nuclear RNA is much higher than that of messenger RNA from the same tissue or embryo stage. At gastrula, for instance, the complexity of nuclear RNA is about 1.7×10^8 nucleotides (Hough et al., 1975)

compared with 1.7×10^7 nucleotides for messenger RNA. For adult sea urchin intestine tissue, the difference in complexity between mRNA and nuclear RNA is even greater. The complexity of intestine message is about 6×10^6 nucleotides (Galau et al., 1976) while the nuclear RNA complexity is almost forty times higher, about 2.3×10^8 nucleotides (Wold et al., 1978). Thus, nuclear RNA is transcribed from about one-third of the total single copy sequence in the genome, compared with values of 6% or less for messenger RNAs.

A final distinction between messenger RNA and heterogeneous nuclear RNA concerns the degree to which sequences in nuclear RNA differ from one embryo stage to another. Kleene and Humphreys (1977) reported that nuclear RNAs from blastula and pluteus stage embryos have very similar single copy complexities, each representing about one-third of the total nonrepetitive sequence in the genome. They also found that these nuclear RNAs contain no detectable stage specific single copy sequence. This is in contrast to the substantial differences in mRNA sequence sets from blastula and pluteus stage embryos. The experiments of Humphreys and Kleene do not, however, exclude the possibility that differences as large as 3 or 4×10^7 nucleotides of complexity may exist between blastula and pluteus hnRNAs. Quantitatively, this potential difference is as large as the total sequence complexity of blastula or gastrula messenger RNA, and might be important for the function of hnRNA, though that function is not presently known.

References

Anderson, D. M., Galau, G. A., Britten, R. J., and Davidson, E. H. (1976). Sequence complexity of the RNA accumulated in oocytes of Arbacia punctulata. Develop. Biol. 51, 138.

Arceci, R. J., Senger, D. R., and Gross, P. R. (1976). The programmed switch in lysine-rich histone synthesis at gastrulation. Cell 9, 171.

Berg, W. E., and Mertes, D. H. (1970). Rates of synthesis and degradation of protein in the sea urchin embryo. Exp. Cell. Res. 60, 218.

Brandhorst, B. P. (1976). Two-dimensional gel patterns of protein synthesis before and after fertilization of sea urchin eggs. Develop. Biol. 52, 310.

Clegg, K. B., and Denny, P. C. (1974). Synthesis of rabbit globin in a cell-free protein synthesis system utilizing sea urchin egg and zygote ribosomes. Develop. Biol. 37, 263.

Davidson, E. H. (1976). Gene Activity in Early Development. (New York: Academic Press).

Epel, D. (1967). Protein synthesis in sea urchin eggs. A "late" response to fertilization. Proc. Natl. Acad. Sci. U.S.A. 57, 899.

Fromson,D., and Duchastel, A. (1975). Poly(A)-containing polyribosomal RNA in sea urchin embryos: Changes in proportion during development. Biochim. Biophys. Acta 378, 394.

Fry, B. J., and Gross, P. R. (1970a). Patterns and rates of protein synthesis in sea urchin embryos. I. Uptake and incorporation of amino acids during the first cleavage cycle. Develop. Biol. 21, 105.

Fry, B. J., and Gross, P. R. (1970b). Patterns and rates of protein synthesis in sea urchin embryos. II. The calculation of absolute rates. Develop. Biol. 21, 125.

Galau, G. A., Britten, R. J., and Davidson, E. H. (1974). A measurement of the sequence complexity of polysomal messenger RNA in sea urchin embryos. Cell 2, 9.

Galau, G. A., Klein, W. H., Davis, M. M., Wold, B. J., Britten, R. J., and Davidson, E. H. (1976). Structural gene sets active in embryos and adult tissues of the sea urchin. Cell 7, 487.

Galau, G. A., Klein, W. H., Britten, R. J., and Davidson, E. H. (1977a). Functional significance of mRNA sequences in the least abundant concentration class. Arch. Biochem. Biophys. 179, 584.

Galau, G. A., Lipson, E. D., Britten, R. J., and Davidson, E. H. (1977b). Synthesis and turnover of polysomal mRNAs in sea urchin embryos. Cell 10, 415.

Goldberg, R. B., Galau, G. A., Britten, R. J., and Davidson, E. H. (1973). Non-repetitive DNA sequence representation in sea urchin embryo messenger RNA. Proc. Natl. Acad. Sci. U.S.A. 70, 3516.

Grainger, R. M., and Wilt, F. H. (1976). Incorporation of ^{13}C, ^{15}N-labeled nucleosides and measurement of RNA synthesis and turnover in sea urchin embryos. J. Mol. Biol. 104, 589.

Gross, K. W., Jacobs-Lorena, M., Baglioni, C., and Gross, P. R. (1973). Cell-free translation of maternal messenger RNA from sea urchin eggs. Proc. Natl. Acad. Sci. U.S.A. 70, 2614.

Hinegardner, R. T. (1967). Echinoderms. In: Methods in Developmental Biology. F. H. Wilt and N. K. Wessels, eds. (New York: Thomas Y. Crowell), pp. 139-155.

Hough, B. R., Smith, M. J., Britten, R. J., and Davidson, E. H. (1975). Sequence complexity of heterogeneous nuclear RNA in sea urchin embryos. Cell 5, 291.

Hough-Evans, B. R., Wold, B. J., Ernst, S. G., Britten, R. J., and Davidson, E. H. (1977). Appearance and persistence of maternal RNA sequences in sea urchin development. Develop. Biol. 60, 258.

Humphreys, T. (1969). Efficiency of translation of messenger RNA before and after fertilization in sea urchins. Develop. Biol. 20, 435.

Humphreys, T. (1971). Measurements of messenger RNA entering polysomes upon fertilization of sea urchin eggs. Develop. Biol. 26, 201.

Jenkins, N., Taylor, N. W., and Raff,F. A. (1973). In vitro translation of oogenetic messenger RNA of sea urchin eggs and picornavirus RNA with a cell-free system from sarcoma 180. Proc. Natl. Acad. Sci. U.S.A. 70, 3287.

Kleene, K. C., and Humphreys, T. (1977). Similarity of hnRNA sequences in blastula and pluteus stage sea urchin embryos. Cell 12, 143.

Kung, C. S. (1974). On the size relationship between nuclear and cytoplasmic RNA in sea urchin embryos. Develop. Biol. 36, 343.

McColl, R. S., and Aronson, A. I. (1974). Transcription from unique and redundant DNA sequences in sea urchin embryos. Biochem. Biophys. Res. Commun. 56, 47.

MacKintosh, F. R., and Bell, E. (1969). Regulation of protein synthesis in sea urchin eggs. J. Mol. Biol. 40, 365.

Moav, B., and Nemer, M. (1971). Histone synthesis. Assignment to a special class of polyribosomes in sea urchin embryos. Biochemistry 10, 881.

Monroy, A., and Tyler, A. (1963). Formation of active ribosomal aggregates (polysomes) upon fertilization and development of sea urchin eggs. Arch. Biochem. Biophys. 103, 431.

Nemer, M., Graham, M., and Dubroff, L. M. (1974). Co-existence of non-histone messenger RNA species lacking and containing polyadenylic acid in sea urchin embryos. J. Mol. Biol. 89, 435.

Nemer, M., Dubroff, L. M., and Graham, M. (1975). Properties of sea urchin embryo messenger RNA containing and lacking poly(A). Cell 6, 171.

Reiger, J.C., and Kafatos, F. C. (1976). Absolute rates of protein synthesis in sea urchins, with specific activity measurements of radioactive leucine and leucyl-7RNA. Develop. Biol. 57, 270.

Roeder, R. G., and Rutter, W. J. (1970). Multiple ribonucleic acid polymerases and ribonucleic acid synthesis during sea urchin development. Biochemistry 9, 2543.

Ruderman, J. V., Baglioni, C., and Gross, P. R. (1974). Histone mRNA and histone synthesis during embryogenesis. Nature (London) 247, 36.

Ruderman, J. V., and Pardue, M. L. (1977). Cell-free translation analysis of messenger RNA in echinoderm and amphibian early development. Devel. Biol. 60, 48.

Seale, R. L., and Aronson, A. I. (1973). Chromatin-associated proteins of the developing sea urchin embryos. I. Kinetics of synthesis and characterization of non-histone proteins. J. Mol. Biol. 75, 633.

Skoultchi, A., and Gross, P. R. (1973). Maternal histone messenger RNA: Detection by molecular hybridization. Proc. Natl. Acad. Sci. U.S.A. 70, 2840.

Slater, D. W., and Spiegelman, S. (1966). An estimation of genetic messages in the unfertilized echinoid egg. Proc. Natl. Acad. Sci. U.S.A. 56, 164.

Slater, I., Gillespie, D., and Slater, D. W. (1973). Cytoplasmic adenylation and processing of maternal RNA. Proc. Natl. Acad. Sci. U.S.A. 70, 406.

Smith, M. J., Hough, B. R., Chamberlin, M. E., and Davidson, E. H. (1974). Repetitive and non-repetitive sequence in sea urchin heterogeneous nuclear RNA. J. Mol. Biol. 85, 103.

Wilt, F. H.(1970). The acceleration of ribonucleic acid synthesis in cleaving sea urchin embryos. Develop. Biol. 23, 444.

Wilt, F. H.(1977). The dynamics of maternal poly(A)-containing mRNA in fertilized sea urchin eggs. Cell 11, 673.

Wold, B. J., Klein, W. H., Hough-Evans, B. R., Britten, R. J., and Davidson, E. H. (1978). Sea urchin embryo mRNA sequences expressed in the nuclear RNA of adult tissues. Submitted to Cell.

Wu, R. S., and Wilt, F. H. (1974). The synthesis and degradation of RNA containing polyriboadenylate during sea urchin embryogeny. Develop. Biol. 41, 352.

CHAPTER 2

DEVELOPMENTAL BIOLOGY 60, 258–277 (1977)

Appearance and Persistence of Maternal RNA Sequences in Sea Urchin Development

BARBARA R. HOUGH-EVANS, BARBARA J. WOLD, SUSAN G. ERNST, ROY J. BRITTEN,[1] AND ERIC H. DAVIDSON

Division of Biology, California Institute of Technology, Pasadena, California 91125, and Kerckhoff Marine Lab of the Division of Biology, California Institute of Technology, Corona del Mar, California 92625

Received April 21, 1977; accepted in revised form June 9, 1977

This paper deals with the relationship between the single copy transcripts represented in mature oocytes of the sea urchin and the RNA sequences present in immature oocytes and embryos. We term the oocyte transcripts from single copy DNA the maternal single copy sequence set. A single copy [^{3}H]DNA fraction ([^{3}H]oDNA) enriched for sequences complementary to the maternal single copy sequence set was prepared and reacted with the different RNA preparations. The complexity of the mature oocyte RNA is estimated to be 37×10^6 nucleotides. At kinetic termination, [^{3}H]oDNA reacted with the polysomal mRNA of 16-cell embryos to 73% of the reaction with mature oocyte RNA, indicating that 27×10^6 nucleotides of the maternal sequence set are present. With blastula mRNA the reaction equals about 56%, a complexity of 21×10^6 nucleotides; with gastrula mRNA, 53%, a complexity of 19×10^6 nucleotides. The relative amount of hybridization of [^{3}H]oDNA was 100% with cytoplasmic RNA of the 16-cell stage and became progressively less with the cytoplasmic RNAs of later stages. The total RNA of immature oocytes was found to include about 26×10^6 nucleotides of the maternal sequence set. Results of these experiments are discussed, and an interpretation of the pattern of utilization of structural genes during oocyte and embryo development is suggested.

INTRODUCTION

The RNA stored in mature sea urchin oocytes includes sequences transcribed from 6% of the single copy DNA, or about 37×10^6 nucleotide pairs (Galau *et al.*, 1976; Anderson *et al.*, 1976). This value was obtained by RNA excess hybridization with single copy DNA. In this paper we refer to the set of single copy DNA sequences represented in mature oocyte RNA as the maternal single copy sequence set. These RNA sequences comprise about 1% of the total oocyte RNA mass, as determined from the kinetics of the hybridization reactions. The fraction of the total oocyte RNA which can be identified as mRNA on the basis of its activity in cell-free protein synthesis systems and its poly(A) content is also 1–2% (reviewed by Davidson, 1976). It is known from the work of Galau *et al.* (1976) that about half of the maternal sequences are represented in the polysomal mRNA of gastrula stage embryos and thus can be considered to consist of structural gene transcripts. Therefore it has been argued that the oocyte RNA whose complexity is 37×10^6 nucleotides consists mainly of maternal messenger RNA (Davidson, 1976).

In this report we describe further studies regarding the developmental fate of the maternal single copy sequence set and the appearance of these sequences during oogenesis. [^{3}H]-Labeled single copy DNA enriched for sequences represented in RNA of mature oocytes ([^{3}H]oDNA) was prepared and was hybridized with RNAs extracted from oocytes and embryos of various stages. Both polysomal RNA and total cytoplasmic RNA preparations were investigated. Though we find that a large fraction of the maternal single copy sequence set is represented in the embryo polysomes

[1] Also staff member, Carnegie Institution of Washington.

throughout early development, this does not imply the persistence of the original mRNAs. Galau *et al.* (1977) showed that by the blastula-gastrula stage essentially all the polysomal mRNAs are newly transcribed.

MATERIALS AND METHODS

Sea Urchin Oocytes and Embryos

Mature oocytes of *Strongylocentrotus purpuratus* were collected, fertilized, and cultured by standard methods (Hinegardner, 1967; Smith *et al.*, 1974). The embryos were grown at 1–4 × 10^4/ml of Millipore-filtered sea water in 30 IU/ml of penicillin G and 50 μg/ml of streptomycin, with constant stirring and aeration, at 15°C. When embryos were to be harvested at the 16-cell stage, the fertilization membranes were removed by papain digestion immediately following fertilization (Hynes and Gross, 1972). The developmental stages relevant to the experiments described in this paper are as follows: At 5 hr after fertilization the embryos have reached the 16-cell stage and consist of four large and eight intermediate cells and four much smaller micromeres. Hatching occurs at 17–19 hr. Mesenchyme blastulae, from which blastula mRNA and cytoplasmic RNA were extracted, were harvested at 23–26 hr. At this stage, the embryos contain about 450 cells, including well-defined primary mesenchyme cells. At 36 hr, the embryos contain about 600 cells, have initiated gastrulation, and display small tripartite skeletal spicules. Prism stage embryos, from which 46-hr cytoplasmic RNA was prepared, have completed gastrulation and contain about 700 cells. Plutei were harvested at 72 hr. These are well differentiated larvae capable of feeding and contain complete digestive tracts and brachiated skeletons.

Mature Oocyte RNA

For each preparation, 10^7–10^8 mature oocytes were obtained by injection of female sea urchins with 0.5 *M* KCl. The oocytes were washed in sea water and resuspended at about 2.5 × 10^5 oocytes/ml in low-salt buffer containing 7 *M* urea, 50 m*M* sodium acetate (pH 5.1), 10 m*M* EDTA, 0.5% SDS (sodium dodecyl sulfate), 10 μg/ml of PVS (polyvinyl sulfate), and about 200 μg/ml of bentonite. The oocytes were homogenized with one to two strokes in a 40-ml size "B" Dounce homogenizer at 4°C.

Following lysis, additional buffer was added to about 500 ml, and the RNA was deproteinized at room temperature with an equal volume of a 1:1 mixture of [phenol:*m*-cresol:8-hydroxyquinoline (Kirby, 1965)] : [chloroform:isoamyl alcohol (24:1)]. After removal of the aqueous phase, the interface was suspended in 1 *M* sodium perchlorate, 0.1 *M* Tris (pH 8), 1% SDS, and reextracted with the phenol–chloroform mixture at 50°C. The aqueous phases were combined, extracted once at 50°C with the phenol–chloroform mixture, two times at room temperature with chloroform:isoamyl alcohol (24:1), and then precipitated at −20°C with 2 vol of 100% ethanol. The precipitate was dissolved in 10 m*M* PIPES (piperazine-*N*-*N*′-bis[2-ethanesulfonic acid]) (pH 6.5) and 5 m*M* $MgCl_2$, and DNase I (Worthington) was added to 100 μg/ml. After incubation for 2 hr at room temperature, the solution was brought to 0.1 *M* Tris (pH 8.0), 0.2% SDS and 50 m*M* EDTA, and incubated with 50 μg/ml of proteinase K (E. Merck) for 1 hr at 37°C. The solution was deproteinized with the phenol–chloroform mixture and with chloroform:isoamyl alcohol (24:1) and precipitated with ethanol. The RNA precipitate was dissolved in 0.3 *M* sodium or potassium acetate (pH 6.5) and chromatographed on Sephadex G-100 in the same buffer. The RNA in the excluded volume of the column was precipitated with ethanol and stored at −20°C in 1–10 m*M* sodium acetate.

Total Ovary RNA

Total RNA was extracted from four ovaries dissected from a single female sea urchin. One ovary was first dissected out,

minced, and examined under the phase microscope. A female was chosen whose ovaries were entirely free of mature (≤80 μm in diameter) oocytes and contained less than 1% medium-sized (50–80 μm) oocytes. We estimate that the tissue consisted principally (≤90% of the mass) of immature oocytes 30–50 μm in diameter (previtellogenic and early vitellogenic oocytes). The four intact ovaries were homogenized in low-salt buffer containing 7 *M* urea, and the total RNA was prepared as described for mature oocyte RNA.

Cytoplasmic RNA

Sixteen-cell embryos were allowed to settle in ice-cold Ca- and Mg-free sea water containing 2 m*M* EDTA, and embryos of other stages were harvested by centrifugation and washed in ice-cold 1.5 *M* glucose. Pellets were resuspended in a lysing buffer which consisted of 50 m*M* PIPES (pH 6.5), 200–500 m*M* KCl, 12 or 15 m*M* $MgCl_2$, and contained 500 μg/ml of PVS and 5 mg/ml of bentonite. The nonionic detergent NP40 was added to 0.5%. The cells were lysed by homogenization in a 40-ml size "B" Dounce homogenizer. Nuclei and cell debris were removed from the homogenate by centrifugation at 2000*g* for 10 min. RNA was extracted from the supernatant essentially as described above for mature oocyte RNA.

Messenger RNA

The mRNA preparations used in these experiments included polysomal rRNA as well as mRNA but had been extensively purified of nonpolysomal RNAs, particularly nuclear RNAs. The procedures used were described by Galau *et al.* (1976), except that the initial dextrose washes were eliminated, and the lysis buffer contained 0.5% NP40 and 5 m*M* EGTA ([ethylenebis(oxyethylenenitrilo)]tetraacetic acid).

Single Copy [^{3}H]DNA

Unlabeled single copy DNA was prepared as described by Galau *et al.* (1976). It was labeled *in vitro* by the gap translation method, also as described by Galau *et al.* (1976), except that for preparation 3 the reaction was stopped by adding EDTA to 25 m*M* and treating the mixture with Pronase (Calbiochem). Each reaction mixture was extracted with chloroform:isoamyl alcohol (24:1), and the aqueous phase was brought to 0.12 *M* phosphate buffer (equimolar Na_2HPO_4 and NaH_2PO_4). The sample was passed over an hydroxyapatite column in 0.12 *M* phosphate buffer at 60°C. Unincorporated precursor as well as any single-stranded DNA was eluted at 60°C. The double-stranded single copy [^{3}H]DNA which bound to the hydroxyapatite column was then denatured and eluted at 95°C. The 95°C fraction was cooled and passed over a second hydroxyapatite column at 60°C in 0.12 *M* phosphate buffer to remove the zero-time binding fraction, including the self-complementary "foldback" sequences generated by the enzyme during the labeling procedure. Molecules containing such sequences were bound to the column, while single-stranded DNA was eluted in 0.12 *M* phosphate buffer. This labeled DNA was further purified by passage over Sephadex G-100 in 0.3 *M* potassium acetate (pH 6.3). Measured specific activities of 4–8 × 10^6 cpm/μg were obtained under our counting conditions (40% counting efficiency). The labeled DNA tended to bind to laboratory glassware, and it was necessary to use plastic or siliconized containers for all experiments involving this tracer.

The reactivity of the labeled single copy DNA and the extent of contamination with repetitive sequences were determined from the kinetics of its reassociation with excess total sea urchin DNA. The weight average size of the labeled fragments was about 200–250 nucleotides measured in alkaline sucrose gradients.

Preparation of [^{3}H]oDNA

The single copy [^{3}H]DNA enriched for sequences represented in mature oocyte RNA ([^{3}H]oDNA) was prepared by a

method which relies on two cycles of hybridization of single copy [^{3}H]DNA with the RNA of interest. The extent of reaction was assayed after each incubation, using small aliquots of the reaction mixture. Three oocyte RNA preparations and three single copy [^{3}H]DNA preparations were used for the experiments described below. Single copy [^{3}H]DNA was incubated with excess total oocyte RNA (RNA mass/DNA mass ≥7000) in 0.42 *M* or 0.5 *M* phosphate buffer at 60°C, to an oocyte RNA equivalent C_0t >30,000 in order to assure maximum hybridization. The [^{3}H]DNA–RNA mixture was diluted to 1 mg/ml or less of RNA, and brought to 0.24 *M* phosphate buffer. RNase A (Worthington) was added to 10 μg/ml, and the unhybridized RNA was digested by 1 hr of incubation at room temperature. This treatment prevents the bulk RNA from interfering with the binding of DNA–DNA and DNA–RNA duplexes to hydroxyapatite. Following RNase digestion the preparation was adjusted to 0.12 *M* phosphate buffer, 0.06% SDS, and extracted with chloroform:isoamyl alcohol (24:1). The aqueous phase was passed over a 1-ml hydroxyapatite column in 0.12 *M* phosphate buffer, 0.06% SDS, at 60°C. The double-stranded DNA and DNA–RNA hybrids binding to the column were eluted at 95°C. About 8% of the single copy [^{3}H]DNA reacted during the first incubation, 1/3 of it (2.7% of input) with egg RNA. The eluate was then adjusted to 0.06 *M* phosphate buffer, 0.03% SDS, 0.1 *M* Tris (pH 8.0), 0.05 *M* EDTA, 50 μg/ml of proteinase K. It was incubated at 37°C for 1 hr and extracted with chloroform:isoamyl alcohol. No detectable RNase activity remained in the solution after this treatment. The aqueous phase was dialyzed against 0.3 *M* potassium acetate and coprecipitated with a second aliquot of mature oocyte RNA. After incubation to an RNA C_0t >30,000, the mixture was digested with RNase, extracted, and passed over hydroxyapatite as above. In different preparations 15–30% of the [^{3}H]DNA recovered in duplex form from the first hydroxyapatite column bound to the second hydroxyapatite column. The double-stranded material was eluted at 95°C and heated for 2 min at 100°C, quenched in ice, and again passed over a hydroxyapatite column in 0.12 *M* phosphate buffer. The purpose of this fractionation was to remove any remaining zero-time binding sequences. The DNA which did not bind at 60°C (or in the case of preparation 3, 50°C) was dialyzed and concentrated by precipitation with carrier oocyte RNA. The precipitate was treated for 45 min in 0.3 *M* KOH at 37°C to hydrolyze the residual oocyte RNA, neutralized with acetic acid, and stored in phosphate buffer (pH 6.8). The three preparations of [^{3}H]oDNA used in these experiments were characterized by measuring their reassociation kinetics with excess whole sea urchin DNA, and the final zero-time binding was measured by boiling and quenching a sample of each tracer before passing it over hydroxyapatite.

Hybridization of [^{3}H]DNAs with RNA and Analysis of Hybrid Content

Single copy [^{3}H]DNA and [^{3}H]oDNA were incubated with excess unlabeled RNA in 0.4–0.5 *M* phosphate buffer, 0.1–0.2% SDS, 5–10 m*M* EDTA at 60°C (Tables 2–4), after denaturation for 1–2 min at 98°C. RNA mass excess was ≥10^4 for single copy [^{3}H]DNA reactions, and about 10^4 cpm were included in each reaction mixture. In [^{3}H]oDNA reactions the mass excess was at least 10^5. C_0t values (*M*-sec) were calculated for the ovary, oocyte, and cytoplasmic RNAs in terms of the total RNA mass. mRNA C_0ts were calculated on the basis that 4% of the total polysomal RNA mass is mRNA (Galau *et al.*, 1977). All RNA and DNA C_0t values referred to in this paper are *equivalent* C_0ts; that is, they have been corrected for acceleration in reaction rate relative to the rate in 0.12 *M* phosphate buffer at 60°C due to higher Na^+ concentration (Britten *et al.*, 1974).

The majority of the reactions were incubated 24–48 hr, and only rarely did the incubation time exceed 90 hr.

Reaction mixtures containing RNA and total single copy [^{3}H]DNA were analyzed by the procedures described earlier, with minor modifications (Hough *et al.*, 1975; Galau *et al.*, 1974, 1976). The hybridization reactions were divided into two aliquots. Aliquot I was assayed for total duplex content on a 1-ml hydroxyapatite column in 0.12 *M* phosphate buffer, 0.06% SDS, at 60°C. Single-strand [^{3}H]DNA was eluted, and the column was washed extensively at 60°C with 0.12 *M* phosphate buffer containing 0.06% SDS. The bound DNA–DNA and DNA–RNA duplexes were eluted at 95–100°C in the same buffer. Fractions from the column were assayed for radioactivity by counting in scintillation fluid. Aliquot II was used to measure the DNA–DNA duplex content. RNA–DNA hybrids were destroyed by incubation at 37°C for 12–20 hr with 10–20 μg/ml of RNase A in 0.05 *M* phosphate buffer. The sample was then adjusted to 0.12 *M* phosphate buffer, 0.06% SDS, deproteinized with chloroform:isoamyl alcohol (24:1), and the aqueous phase was passed over hydroxyapatite as described above. The fraction of [^{3}H]DNA bound in Aliquot II is subtracted from that bound in Aliquot I to obtain the fraction hybridized with RNA.

Reaction mixtures containing RNA and [^{3}H]oDNA were analyzed in most cases by diluting the sample to 0.12 *M* phosphate buffer, 0.06% SDS, and placing it directly over a hydroxyapatite column in the same buffer. No measurement of the DNA–DNA duplex was required because the tracer self-reaction was in these cases negligible. Occasionally one or more reaction mixtures were also assayed for DNA duplex content by the "two-column" assay just described.

RESULTS

Characterization of [^{3}H]oDNA

The three [^{3}H]oDNA tracers used in this work were prepared as described in Materials and Methods from single copy DNA which had been labeled *in vitro*. To select a tracer fraction representing the maternal single copy sequence set, the single copy [^{3}H]DNA was reacted with excess mature oocyte RNA. The duplex fraction, containing both DNA–RNA hybrids and renatured DNA, was separated from single-stranded tracer by binding to hydroxyapatite and was then denatured and reacted again with oocyte RNA. Using two cycles of reaction decreases the amount of renatured DNA in the tracer fraction binding to hydroxyapatite. The bound [^{3}H]DNA was stripped of sequences binding to hydroxyapatite at $C_0t < 10^{-5}$. We refer to this material as zero-time binding DNA. The preparation was then hydrolyzed with alkali to remove residual oocyte RNA. During the hybridization reactions the single copy oocyte RNA transcripts were present in greater than 1000-fold sequence excess with respect to the complementary single copy DNA. Therefore the [^{3}H]oDNA represents the various sequences of the maternal single copy set equally, regardless of relatively minor differences in their prevalence.

The [^{3}H]oDNA tracers were tested for reactivity with DNA and oocyte RNA, and for any remaining contamination with foldback or repetitive DNA sequences, as shown in Table 1. Reactivity with DNA is defined as the extent of reaction of the [^{3}H]DNA tracer with excess sheared sea urchin DNA incubated to C_0t 20,000, measured by hydroxyapatite binding. At C_0t 20,000, 96% of the 450-nucleotide-long DNA driver had reacted. The [^{3}H]oDNA preparations contained from 15 to 42% nonreactive labeled material. The nonreactive fraction probably consists mainly of [^{3}H]DNA fragments too short to form duplexes of sufficient length to bind to hydroxyapatite (see Table 1, footnote *b*). Galau *et al.* (1976) also observed that the DNA reactivity of very high specific activity single copy DNA tends to be somewhat reduced, as a result either of radiolysis, or of degradation during preparation and se-

TABLE 1

CHARACTERIZATION OF [^{3}H]oDNA

	Preparation		
	1	2	3
Reactivity with DNA[a]	77%	85%	58%[b]
Zero-time binding[c]	1.5%	6.3%	3%
Reaction with excess whole DNA at C_0ts of 10–35 M-sec[d]	1.2%	0.7%	1%
Reaction with oocyte RNA at high RNA C_0ts[e]	68.5%	53.0%	77%
Enrichment for oocyte RNA sequences	23×	18×	26×

[a] Of the 450-nucleotide-long sea urchin DNA, ≤96% was in duplex-containing structures at C_0t 20,000. The labeled DNA reacts less completely because it contains fragments of DNA too small to form stable duplexes, and possibly other nonreactive labeled components produced during the *in vitro* labeling process (average of two or more determinations).

[b] Of [^{3}H]oDNA preparation 3, 25% consisted of [^{3}H]counts per minute which were not excluded from Sephadex G-100. The reactivity of an aliquot from the exclusion peak was 80%. This shows that much of the nonreactive labeled DNA in this tracer preparation consisted simply of fragments too short to form stable duplexes at the reaction criterion applied.

[c] The procedure by which the [^{3}H]oDNA was prepared tends to concentrate any zero-time binding and foldback sequences, and these are removed more or less effectively as described in Materials and Methods. The values given represent the average of several determinations at $C_0t < 10^{-3}$. These determinations included samples from hybridizing mixtures made up with each of the RNAs studied. The amount of [^{3}H]oDNA binding to the hydroxyapatite column was measured by the low-salt RNase procedure (see Materials and Methods).

[d] Average of two or more determinations. Zero-time binding has been subtracted.

[e] Terminal values are from Fig. 1. The extent of reaction has been corrected for reactivity with DNA (row 1 of this Table), and zero-time binding (row 2 of this Table) was subtracted.

lective purification, or both. Table 1 also shows that the procedures used to strip zero-time binding or foldback sequences from the tracer were not completely effective. A small zero-time binding fraction remains in each of the [^{3}H]oDNA preparations. The amounts of zero-time binding to hydroxyapatite (Table 1) were very reproducible for each preparation. Thus they could be routinely subtracted to obtain the quantity of duplex formed as the result of RNA–DNA hybridization.

No detectable repetitive sequence contamination existed in the [^{3}H]oDNA preparations. This was established by the reaction of each [^{3}H]oDNA preparation with excess whole DNA at C_0ts of 10 to 35. At these driver DNA C_0ts, 1–3% of the single copy sequences in whole sea urchin DNA will have renatured. Table 1 shows that the amount of reaction of [^{3}H]oDNA at low driver DNA C_0t is within expectation for tracers containing detectable quantities of only single copy sequence.

The most significant parameter shown in Table 1 is the concentration of oocyte RNA sequences in the [^{3}H]oDNA preparations. Considering only the reactive fraction of the [^{3}H]oDNA tracers, the enrichment for oocyte RNA sequences is calculated by dividing the terminal extent of reaction of [^{3}H]oDNA with oocyte RNA by the terminal extent of reaction of total single copy [^{3}H]DNA with the same RNA. The fraction of single copy [^{3}H]DNA which can be hybridized with mature oocyte RNA is about 3%. This value was reported previously by Galau *et al.* (1976) and is confirmed by data presented below. Therefore, if the [^{3}H]oDNA tracer contained only sequences complementary to oocyte RNA, then the maximum possible enrichment would be 33-fold. Table 1 shows that the three preparations were enriched for oocyte sequences 23-, 18-, and 26-fold, respectively, or from about 55% to about 79% of the maximum possible purification. We have some experimental indications that the remaining reactive [^{3}H]DNA in the [^{3}H]oDNA preparations consists at least in part of random single copy sequence. These random sequences probably derive from renatured tracer remaining from the preparation of the [^{3}H]oDNA and possibly from small quantities of single-stranded DNA contamination in the hydroxyapatite-bound fractions.

The kinetics of the reactions of the

[^{3}H]oDNA tracers with mature oocyte RNA are shown in Fig. 1. There was no detectable [^{3}H]oDNA self-reaction, and the only DNA–DNA duplex in the reaction mixture was the zero-time binding sequence. The absence of DNA self-reaction is expected from the very low [^{3}H]oDNA C_0t generated during the incubations with RNA, as well as from the asymmetric nature of the oocyte RNA transcripts. Preparation 1 was tested only at RNA C_0ts $\leq 3 \times 10^4$, when the reactions have already terminated (cf. Fig. 1). The terminal value for preparation 1 is merely an average of four determinations. Kinetic data for the reactions of [^{3}H]oDNA preparations 2 and 3 were fit by least squares methods to the pseudo-first-order function specified in the caption to Fig. 1. The terminal values obtained from the least squares solutions are listed in the figure caption.

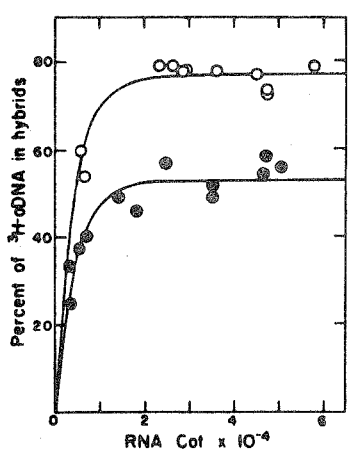


FIG. 1. Hybridization of [^{3}H]oDNA with mature oocyte RNA. The curves were fit to the data for oDNA preparations 2 (●) and 3 (○). The function used to fit the data is $D/D_0 = \exp[-C_0t\,k]$, where D/D_0 is the fraction of [^{3}H]oDNA remaining single-stranded at time t, C_0 is the RNA concentration, and k is the pseudo-first-order rate constant [see Galau *et al.* (1974) for calculations using this equation and further discussion]. The restrictions imposed on the solution were that there is a single kinetic component and that no measurable amount of hybrid forms at very low RNA C_0t; that is, that the ordinate intercept is 0. The pseudo-first-order rate constant obtained for both sets of data was $2.3 \pm 0.3 \times 10^{-4}$ M^{-1} sec^{-1}. Terminal values are 53.0 ± 1.4% for [^{3}H]oDNA preparation 2 and 77.0 ± 1.1% for [^{3}H]oDNA preparation 3. The corresponding value for [^{3}H]oDNA preparation 1 is 68.5 ± 4.8% (see text).

The rates of the reactions illustrated in Fig. 1 agree well with those reported in previous studies from this laboratory. Figure 1 shows that the rate constant for the oocyte RNA-[^{3}H]oDNA reaction is about 2.3×10^{-4} M^{-1} sec^{-1}. Similarly Galau *et al.* (1976) reported rate constants for oocyte RNA reactions with various single copy DNA tracers of 1.2×10^{-4} M^{-1} sec^{-1} and 2.2×10^{-4} M^{-1} sec^{-1}. Analysis of the relatively extensive measurements presented here shows that about 1% of the total mature oocyte RNA constitutes the complex sequence class which drives the hybridization reaction. This conclusion is consistent with earlier work (Anderson *et al.*, 1976; Galau *et al.*, 1976). The complexity of *S. purpuratus* oocyte RNA has been reported previously as 37×10^6 nucleotides (Galau *et al.*, 1976). Data which confirm this value are shown in Table 2. The terminal value for the reaction of each of the [^{3}H]oDNA preparations with oocyte RNA thus is taken to represent 37×10^6 nucleotides of complexity. If the [^{3}H]oDNA reacts with RNA from another stage of development to an extent which is a fraction α of the terminal reaction of that tracer preparation with oocyte RNA, the complexity of the maternal single copy sequence set in this RNA is therefore calculated as (α) (37×10^6) nucleotides.

The yields of [^{3}H]oDNA obtained in each preparation were estimated by dividing the quantity of [^{3}H]oDNA recovered by the amount of single copy [^{3}H]DNA complementary to oocyte RNA which was present in the starting total single copy DNA (i.e., 3%, or 37×10^6 nucleotides per haploid genome, from Galau *et al.*, 1976, and Table 2). This ratio must then be adjusted to exclude the fraction of the [^{3}H]oDNA which fails to react with oocyte RNA. The yields ranged from about 1% for preparation 1 to about 10% for preparation 3. These yields are approximately what is expected considering that the yield of each

of the 20 individual steps in the [^{3}H]oDNA preparation is 80–90%. Evidence that the losses of oocyte sequences occurring during the tracer preparation are random with respect to sequence can be obtained from the extent of reaction of [^{3}H]oDNA with gastrula mRNA since all gastrula mRNA sequences are also represented in oocyte RNA (Galau *et al.*, 1976) (these experiments are described in detail below). Thus the complexity of the gastrula mRNA as calculated from reactions with [^{3}H]oDNA agrees well with previous determinations of the complexity of gastrula polysomal RNA calculated from reactions with total single copy DNA tracer (Galau *et al.*, 1974).

RNA Complexity Measurements with Single Copy [^{3}H]DNA

In following sections of this paper we describe reactions of [^{3}H]oDNA with cytoplasmic RNA preparations from sea urchin ovaries and embryos. A serious concern is the possibility that the RNAs reacting with the [^{3}H]oDNA are contaminating nuclear RNAs rather than bona fide cytoplasmic RNAs. This could occur easily if nuclei are damaged and release their contents during cell lysis. The hybridization of single copy [^{3}H]DNA with the cytoplasmic RNA preparations, summarized in Table 2, provides a sensitive test for contamination with hnRNA. Hough *et al.* (1975) showed that the complexity of sea urchin gastrula hnRNA is close to 1.7×10^9 nucleotides, or about four times greater than that of oocyte RNA, and 10 times greater than that of the polysomal mRNA populations found in sea urchin gastrulae (Galau *et al.*, 1974). According to Kleene and Humphreys (1977), the complexity of the hnRNA of blastula and pluteus stage embryos is about the same as that of the gastrula stage. Were the cytoplasmic RNA preparations contaminated with hnRNA, therefore, the fraction of single copy [^{3}H]DNA hybridized would rise continuously at high RNA C_0t toward a level several times that expected for an hnRNA-free preparation. On the other hand, we expect that bona fide cytoplasmic RNAs will be found in roughly the same numbers of copies per embryo as in the mature oocyte (Galau *et al.*, 1974; 1976), so that a *terminal* RNA C_0t will be reached at around 20,000 or 30,000. The quantitative estimation of hnRNA contamination in message preparations by this method was discussed by Hough *et al.* (1975). We showed earlier that our mRNA preparations are free of hnRNA contamination detectable by the single copy hybridization procedure as well as by other criteria (Goldberg *et al.*, 1973; Galau *et al.*, 1974; 1976; Hough *et al.*, 1975). However the cytoplasmic RNA preparations are more susceptible to hnRNA contamination than are mRNA preparations, since the mRNA is further purified following removal of the nuclei. Hybridization with single copy [^{3}H]DNA can reveal only contamination with a major fraction of the hnRNA sequences, such as might occur if nuclei were broken during embryo lysis or not removed in the subsequent pelleting step. Selective leakage of a subset of the hnRNA sequences into the cytoplasm during lysis would not be detectable in most cases, and in fact would be very difficult to distinguish from a physiological process.

Table 2 provides terminal reaction data for the hybridization of single copy [^{3}H]DNA with the cytoplasmic RNA preparations of interest. In the case of the pluteus cytoplasmic RNA, it is clear that there is no large change in the extent of single copy DNA hybridization with increasing RNA C_0t. Thus there is no evidence for total hnRNA contamination at a level which could affect the hybridization of the [^{3}H]oDNA. Though it is by no means certain, due to scatter in the data, the blastula and 16-cell cytoplasmic RNA preparations could have included small amounts of hnRNA. A least squares solution to the data shown in Table 2 for the blastula RNA suggests that if total hnRNA sequences are present, their con-

TABLE 2

HYBRIDIZATION OF SINGLE COPY [^{3}H]DNA WITH MATURE OOCYTE, OVARY, AND EMBRYO CYTOPLASMIC RNAs

RNA	C_0t		[^{3}H]DNA in duplex (%)			Terminal value (%)[b]	Complexity (nucleotides)[c]
	RNA	[^{3}H]DNA	Total	DNA-DNA	DNA-RNA[a]		
Mature oocyte RNA (preparation)	(1) 25,700	1.4	3.14	0.97	2.65	3.01 ± 0.16	37 ± 2.2 × 10^6
	(3) 30,400	2.5	7.37	4.66	2.85		
	(2) 32,900	2.5	3.46	0.84	3.09		
	(2) 40,000	0.07	2.82	0.29	2.98		
	(2) 44,800	4.0	3.71	0.93	3.27		
	(1) 78,000	40	6.86	6.20	2.85		
Ovary total RNA	18,000	2.9	6.09	4.14	2.07	2.10 ± 0.04	26 ± 0.4 × 10^6
	28,000	4.6	12.2	10.2	2.13		
16-Cell cytoplasmic RNA	14,400	0.09	3.02	0.45	2.82	4.56 ± 0.43	37 ± 3.4 × 10^6[d]
	18,300	0.11	3.28	0.49	3.07		
	30,500	1.0	4.58	0.97	3.97		
	33,400	0.20	4.56	0.52	4.44		
	46,100	0.28	4.81	0.65	4.57		
	68,100	2.3	5.58	0.88	5.16		
	74,100	2.5	5.11	0.85	4.68		
Blastula cytoplasmic RNA	17,200	3.0	7.68	3.73	4.10	3.84 ± 0.64	47 ± 7.8 × 10^6[e]
	22,300	3.8	6.98	4.00	3.37		
	26,800	4.6	6.44	3.54	3.09		
	44,200	7.6	8.22	4.54	3.91		
	44,200	7.6	8.21	3.77	4.72		
Prism cytoplasmic RNA	33,400	1.3	3.11	0.68	2.64	2.38 ± 0.37	29 ± 4.5 × 10^6
	37,100	1.5	2.42	0.48	2.11		
Pluteus cytoplasmic RNA	20,600	2.0	1.14	0.25	1.04	0.98 ± 0.06	12 ± 0.4 × 10^6
	30,200	3.0	1.22	0.41	0.95		
	36,200	3.6	1.18	0.38	0.94		

[a] Calculated by subtracting DNA-DNA duplex from total duplex and correcting for the reactivity of the single copy [^{3}H]DNA. This was measured by reaction with total DNA and was >80% in every case.

[b] The terminal value was determined for each RNA by averaging all the data shown, except for the case of mature oocyte RNA and 16-cell embryo RNA. In these cases data obtained at RNA $C_0t > 3 \times 10^4$ were used to obtain the terminal value. Error estimates shown represent one standard deviation around the best parameter values. The error estimates do not include any nonrandomly occurring systematic errors which could have affected the data.

[c] Complexity was calculated (assuming single-strand transcription) as follows: (terminal fraction of reactive single copy [^{3}H]DNA hybridized) × 2 × (single copy complexity of *S. purpuratus* genome). The complexity of the single copy sequence fraction of the genome is 6.1 × 10^8 nucleotide pairs (Graham *et al.*, 1974).

[d] From data obtained at RNA C_0t's greater than 30,000. This series of observations had a high background, and simultaneous measurements with oocyte RNA also terminated when 4.6% of the single copy DNA had hybridized. The complexity of the 16-cell cytoplasmic RNA was therefore also about 37 × 10^6 nucleotides: (4.6/4.56) × 37 × 10^6 = 37 × 10^6.

[e] These data were fit by least squares methods assuming two pseudo-first-order kinetic components. The purpose of this exercise is to distinguish the kinetic component due to trace contamination with hnRNA from the kinetic component due to reaction of RNAs present at about the same prevalence as oocyte RNAs. The rate constant of the first component, representing the cytoplasmic RNA, was fixed at 1.73 × 10^{-4} M^{-1} sec^{-1}, similar to that of oocyte RNA and the other cytoplasmic RNAs studied. The second component was fixed at a complexity of 1.7 × 10^6 nucleotides to represent hnRNA (Hough *et al.*, 1975; Kleene and Humphreys, 1977). The rate constant for this component provided by the least squares solution was 2.09 × 10^{-6} M^{-1} sec^{-1}, or about 1.2 × 10^{-2} times that for the cytoplasmic sequences. Since the hnRNA complexity is about four times greater than oocyte RNA complexity, the sequence concentrations of the putative hnRNA would be around (1.2 × 10^{-2}) 0.25 or approximately 3 × 10^{-3} that of the complex cytoplasmic RNA sequences.

centration is 20-fold lower than that of the cytoplasmic RNA sequences reactable with [^{3}H]oDNA (see footnotes to Table 2). Even if real, this level of contamination is too low to affect the reaction of [^{3}H]oDNA significantly. Cytoplasmic RNA preparations which displayed evidence of significantly greater amounts of hnRNA contamination were discarded.

Table 2 shows that the complexity of pluteus cytoplasmic RNA is about 12 × 10^6 nucleotides. This value is similar to the complexity of pluteus mRNA reported by Galau *et al.* (1976), 13–15 × 10^6 nucleo-

tides. Thus most of the diverse species of RNA in the pluteus cytoplasm can be accounted for as mRNA. For the earlier embryonic stages, however, the cytoplasmic RNA complexities listed in Table 2 exceed the polysomal mRNA complexities measured by Galau *et al.* (1974, 1976), as discussed below.

Reaction of [³H]oDNA with RNA from Ovary Containing Only Immature Oocytes

It is not known when during oogenesis the maternal mRNA stored in the mature oocyte is transcribed. To determine whether or not the full set of maternal RNA sequences is already present in previtellogenic oocytes we carried out reactions of [³H]oDNA with the total RNA extracted from the ovaries of out-of-season females. Sea urchins produce mature gametes only at certain times of the year, and ovaries were selected which contained no mature oocytes. Cytological examination revealed a small amount of connective tissue and clusters of immature oocytes, easily identifiable by their large nuclei. Almost all of the ovarian tissue appeared to consist of oocytes. At least 99% of these measured less than 50 μm in diameter and are classified as previtellogenic or very early vitellogenic oocytes.

RNA extracted from immature ovaries was hybridized with single copy [³H]DNA and with [³H]oDNA, preparation 2. At an RNA C_0t which would be terminal for the mature oocyte RNA-driven reaction, 2.1% of the reactable single copy [³H]DNA was found in RNA-DNA duplexes. Thus the complexity of the RNA in the immature oocytes which reacts by C_0t 18,000 (Table 2) is about $^2/_3$ that of mature oocyte RNA. The reactions of [³H]oDNA with the ovary RNA are shown in Fig. 2. In Fig. 2 the immature ovary RNA–[³H]oDNA reaction is compared to the [³H]oDNA reaction with mature oocyte RNA, reproduced from Fig. 1. When fit by linear regression analysis these data display a slight positive slope, indicative of a low prevalence RNA of higher complexity. This can probably be accounted for as immature oocyte nuclear RNA. However, it is not clear that this small kinetic component is really present, and by far the major portion of the reaction is evidently due to RNA sequences present at the same (or greater) concentration as in mature oocyte RNA. The horizontal dashed line shown in Fig. 2 represents the best estimate of the fraction of the maternal single copy sequence set which is already present in the RNA of previtellogenic oocytes, i.e., at a concentration similar to that of most single copy transcripts in mature oocyte RNA. Calculated from the reaction with [³H]oDNA, the complexity of this sequence set is about 26×10^6 nucleotides, which agrees very closely with the total RNA complexity measured for the same RNA by reaction with single copy [³H]DNA (26×10^6 nucleotides; Table 2). It follows that in the immature ovary all or almost all of the sequences, whose prevalence is similar to that of the single copy transcripts in mature oocytes, are homologous to these mature oocyte RNAs. Since other cell types are present in the ovary, these measurements provide a

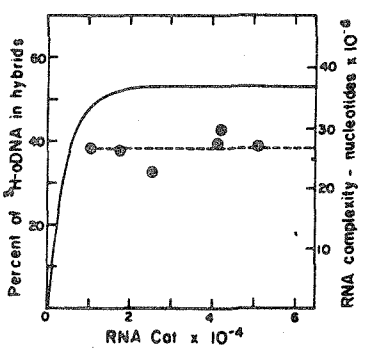


FIG. 2. Hybridization of [³H]oDNA with total immature ovary RNA. The solid line is the pseudo-first-order curve fitted to the hybridization data for [³H]oDNA preparation 2 with oocyte RNA, from Fig. 1. The dashed line represents the average of the data for the immature ovary RNA (38 ± 3.2%). Complexity of the reacting RNA ($26 \pm 2.2 \times 10^6$ nucleotides) is indicated on the right hand ordinate. Complexity was calculated as (terminal value/53%) × 37 × 10^6 nucleotides.

maximum estimate of the homology between the RNA of immature oocytes and mature oocyte RNA.

Reactions of [³H]oDNA with Embryo Cytoplasmic RNA

The [³H]oDNA tracer was reacted with cytoplasmic RNAs of 16-cell cleavage stage embryos, 26-hr mesenchyme blastulae, 46-hr prism stage embryos, and 72-hr plutei. The RNA preparations used were those whose reactions with single copy [³H]DNA were discussed above (Table 2). The reaction of the [³H]oDNA with 16-cell cytoplasmic RNA is indistinguishable from its reaction with mature oocyte RNA (data not shown). At 5 hr of development all of the complex maternal RNA sequences are apparently still present in the cytoplasm. However, after this stage the maternal single copy sequence set begins to disappear from the cytoplasmic fraction of the embryo RNA. This trend is shown in Fig. 3. Only terminal reaction data were obtained, except for the pluteus cytoplasmic RNA where measurements were also carried out at low RNA C_0t. The data scatter was greater in the reactions shown in Fig. 3 than we customarily observe for mRNA-driven reactions, for reasons we do not understand. The standard deviations for the terminal values shown in Fig. 3 are of the order of 10% of the terminal value, rather than the 2 to 5% obtained in the excess polysomal RNA reactions described below.

The main conclusion from this series of experiments is clear, despite the variations among the individual measurements. Figure 3a indicates that a significant decrease in the size of the cytoplasmic maternal single copy sequence set has occurred by the mesenchyme blastula stage. Progressively smaller fractions of the initial maternal single copy sequence set persist to the prism (Fig. 3b) and pluteus stages (Fig. 3c). Expressed as percentages of the reaction of the [³H]oDNA tracer with oocyte RNA, the terminal values obtained for the embryonic RNA reactions

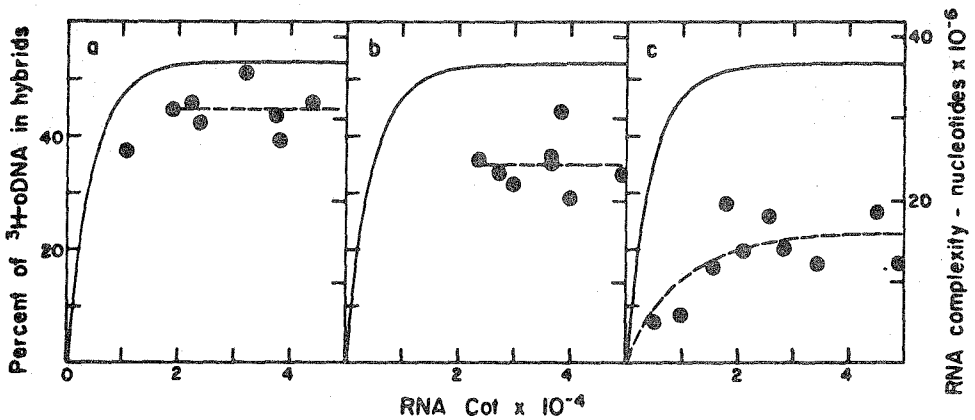


FIG. 3. Hybridization of [³H]oDNA with blastula, prism, and pluteus cytoplasmic RNAs. The left ordinate gives the percentage of [³H]oDNA in duplex, and the right ordinate is calibrated in terms of complexity. (a) 26-hr mesenchyme blastula cytoplasmic RNA; (b) 46-hr prism cytoplasmic RNA; (c) 72-hr pluteus cytoplasmic RNA. In (a) and (b) the dashed lines show the terminal extent of reaction, 44.8 ± 4.0% (31 ± 2.8 × 10^6 nucleotides) and 35 ± 4.6% (24 ± 3.2 × 10^6 nucleotides), respectively. The dashed curve in (c) describes a pseudo-first-order function fitted to the pluteus cytoplasmic RNA data. The terminal value of this curve is 23.3 ± 3% (16 ± 2.6 × 10^6 nucleotides), and the rate constant of the reaction is 1 ± 0.5 × 10^{-4} M^{-1} sec^{-1} This is only a factor of 2 smaller than the rate constant for the reaction of the [³H]oDNA tracer with mature oocyte RNA. For comparison the reaction of [³H]oDNA preparation 2 with mature oocyte RNA is reproduced from Fig. 1 in each panel (solid curves).

were 100% at the 16-cell stage, about 85% at the blastula stage, ~66% at the prism stage, and ~44% at the pluteus stage.

Reactions of [³H]oDNA with Embryo Polysomal RNAs

The extent of reaction of the [³H]oDNA tracer with polysomal mRNA extracted from cleavage stage embryos provides a minimum estimate of the fraction of the oocyte RNA single copy sequence set which can be attributed to a structural gene sequence. The earliest stage studied here was a 5-hr 16-cell cleavage stage embryo. Polysomal RNA was prepared from demembranated 16-cell embryos after two cycles of sucrose gradient centrifugation as described in Materials and Methods. This mRNA preparation and mature oocyte RNA were hybridized with [³H]oDNA (preparation 2) as paired samples. The data are listed in Table 3. In this series of reactions about 58% of the [³H]oDNA tracer hybridized with the mature oocyte RNA at termination, rather than the 53% seen in other experiments. Table 3 indicates that the fraction of the [³H]oDNA reacting with the 16-cell mRNA preparation is about 73% of that reacting with the mature oocyte RNA. Thus at least 73% of the maternal single copy sequence set can be accounted for as an embryonic mRNA sequence set. This value is to be regarded as an underestimate, since additional oocyte sequences might have been included in the polysomal message at periods earlier than the 5-hr stage studied here, and since some maternal mRNA species not used by 5 hr could conceivably be loaded onto the polysomes later in cleavage.

The three [³H]oDNA tracers were reacted with polysomal RNA preparations from blastula and gastrula stage embryos. The data are shown in Table 4. For the calculation of terminal values and analysis of the reaction kinetics, the data obtained with different DNA tracers for each mRNA preparation were pooled. This was done by normalizing the results from [³H]oDNA tracers 1 and 2 to the reactivity with oocyte RNA of [³H]oDNA preparation 3 (this computation is described in Note *c* of Table 4). The reaction kinetics obtained with the pooled data are shown in Fig. 4. In terms of the total polysomal RNA concentrations, the rate constants describing the mRNA–[³H]oDNA reactions lie within a factor of 2 of the rate constant for the reaction of total oocyte RNA with [³H]oDNA. Therefore the reacting RNA species in the polysomal RNA are about as prevalent as the same sequences in total

TABLE 3

HYBRIDIZATION OF [³H]oDNA WITH MATURE OOCYTE RNA AND 16-CELL EMBRYO POLYSOMAL RNA[a]

RNA	RNA C_0t	[³H]oDNA in hybrid (%)[b]	Terminal value in hybird (%)[c]	Percentage of oocyte RNA reaction
Mature oocyte RNA	28,300	56.9	58 ± 2.9	100
	49,500	55.4		
	54,500	62.1		
	76,300	58.4		
16-Cell polysomal RNA	20,000	40.7	42 ± 2.0	73
	35,700	43.5		
	39,600	44.6		
	55,700	40.8		

[a] A different aliquot of [³H]oDNA preparation 2 was used for these reactions, and the samples were analyzed by a slightly different hydroxyapatite procedure. This may account for the slightly higher oocyte RNA reaction than that shown in Table 1.

[b] Data have been corrected for 6.3% zero-time binding and 85% tracer reactivity.

[c] Determined by averaging the data, since RNA C_0t is terminal. Standard deviations are indicated.

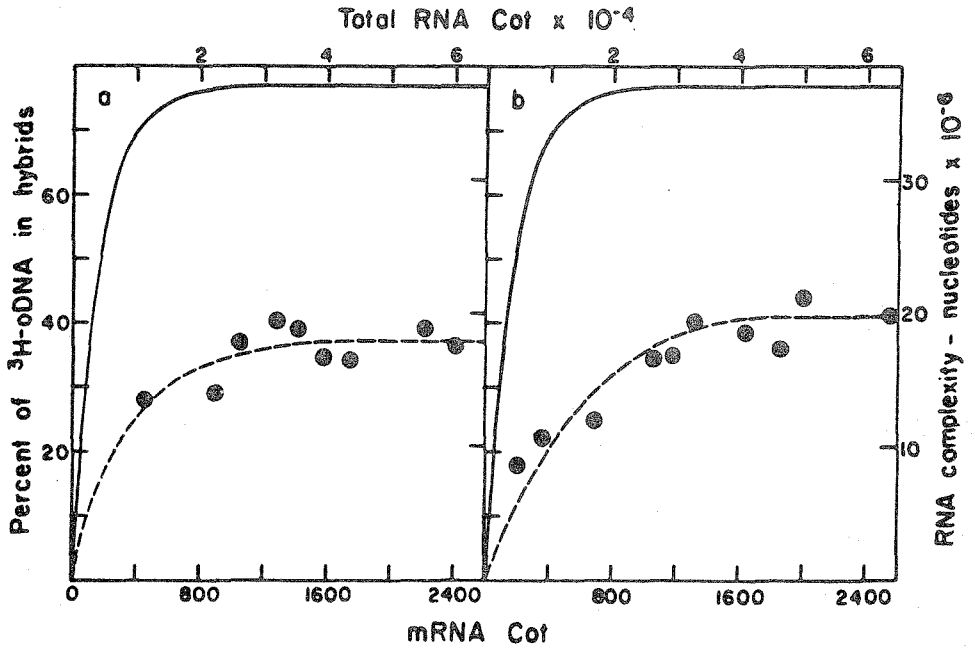


FIG. 4. Hybridization of [³H]oDNA with blastula and gastrula polysomal RNAs. The lower abscissa shows the mRNA C_0t calculated on the basis that mRNA comprises 4% of the mass of polysomal RNA (Galau *et al.*, 1977). The left ordinate gives the percentage of [³H]oDNA in hybrid. The right ordinate is calibrated in terms of RNA complexity. Solid curves are reproduced from the reaction of [³H]oDNA preparation 3 with mature oocyte RNA (Fig. 1). Data from reactions with [³H]oDNA preparations 1, 2, and 3 (Table 4) were pooled by normalization to the reactivity of preparation 3 (see note c of Table 4). The dashed curves represent least squares solutions assuming one component pseudo-first-order hybridization kinetics, with the additional assumption that the ordinate intercept is zero. (a) Blastula mRNA. At termination 37.4 ± 1.3% of the [³H]oDNA has reacted. The rate constant is $2.7 \pm 0.6 \times 10^{-3}$ M^{-1} sec^{-1} with respect to mRNA, or 1.08×10^{-4} M^{-1} sec^{-1} with respect to total polysomal RNA. (b) Gastrula mRNA. At termination 41 ± 2% of the [³H]oDNA has reacted. The rate constant is $1.8 \pm 0.3 \times 10^{-3}$ M^{-1} sec^{-1} with respect to mRNA, or 0.72×10^{-4} M^{-1} sec^{-1} with respect to total polysomal RNA. Errors shown indicate one standard deviation.

iments shown in Table 4 the reaction of the gastrula mRNA with [³H]oDNA was 53% of that with oocyte RNA. This result is satisfactorily close to prediction and shows that losses during preparation of oDNA were not sequence-specific.

The complexity of blastula mRNA belonging to the maternal single copy sequence set is about 18×10^6 nucleotides. This is well below the 31×10^6 nucleotides of maternal sequence observed in total cytoplasmic RNA from blastulae. It follows that maternal RNA sequences are present in the cytoplasm at the blastula stage which are not loaded on the polysomes. Furthermore, while the complexities of the maternal single copy sequence set represented in the mRNAs of blastula and gastrula stage embryos are very similar, the overall complexity of blastula mRNA is significantly higher (Galau *et al.*, 1976). We conclude that there are at least three classes of complex blastula cytoplasmic RNA: mRNAs belonging to the maternal single copy sequence set; mRNAs absent from the maternal single copy sequence set; and cytoplasmic RNAs belonging to the maternal single copy sequence set but not represented in the polysomes.

DISCUSSION

Our findings are summarized in Fig. 5. Here the extents of [³H]oDNA reaction with oocyte RNA, embryo cytoplasmic

TABLE 4

HYBRIDIZATION OF [^{3}H]oDNA WITH BLASTULA AND GASTRULA mRNAs

RNA	[^{3}H]oDNA preparation	mRNA C_0t[a]	[^{3}H]DNA in hybrid (%)[b]	Terminal value in hybrid (%)[c]	Complexity of mature oocyte single copy sequence set represented in embyro mRNA as	
					Nucleotides[d]	Percentage of oocyte RNA reaction[e]
Blastula mRNA	1	460	25.1	37.4 ± 1.3	18 ± 0.6 × 10^6	49
		1070	33.1			
		1420	34.8			
		1750	30.4			
	2	900	20.1			
		1290	27.9			
		2220	26.9			
	3	1590	34.8			
		2410	36.7			
Gastrula mRNA	1	1060	30.0	41.0 ± 2.0	19 ± 1.0 × 10^6	53
		1650	34.2			
	3	200	18.2			
		350	22.2			
		700	25.0			
		1180	35.3			
		1320	40.5			
		1860	36.0			
		2070	44.3			
		2570	40.9			

[a] mRNA C_0t was calculated as 4% of total polysomal RNA C_0t.

[b] Zero-time binding for each [^{3}H]oDNA preparation (Table 1) was subtracted from total duplex, except for a few cases where a direct DNA-DNA determination was made. These were always in agreement with the values shown in Table 1. The values shown were corrected for the differing reactivities of the tracer DNA preparations (Table 1).

[c] Data were pooled for the calculation of terminal values by normalizing to the reactivity with oocyte RNA of [^{3}H]oDNA tracer preparation 3 (77%). The normalization factors (Table 1) were thus 0.77/0.685 for preparation 1 and 0.77/0.53 for preparation 2. The hybridization rate and terminal value were extracted from the pooled data by least squares procedures assuming pseudo-first-order hybridization kinetics. Parameters are shown in the legend to Fig. 4.

[d] (Terminal value/77%) × 37 × 10^6 nucleotides.

[e] (Terminal value/77%) × 100.

oocyte RNA. There are an average of 1600 copies of each species of maternal RNA belonging to the single copy sequence set per oocyte. A similar number of copies of each RNA species reacting with the [^{3}H]oDNA must exist in the polysomes of an embryo. At the 600-cell gastrula stage this means there are an average of only one to a few copies of each such mRNA species per cell. The same conclusion was reached in the case of the gastrula mRNA population whose complexity was measured at 17 × 10^6 nucleotides by Galau *et al.* (1974, 1976). Furthermore these authors showed that essentially all of the gastrula mRNA species are included in the maternal sequence set. Therefore we would expect that the reaction of [^{3}H]oDNA with gastrula polysomal RNA would terminate at a level equal to 47% of the reaction of the same tracer with oocyte RNA (i.e., 17 × 10^6/37 × 10^6). In the exper-

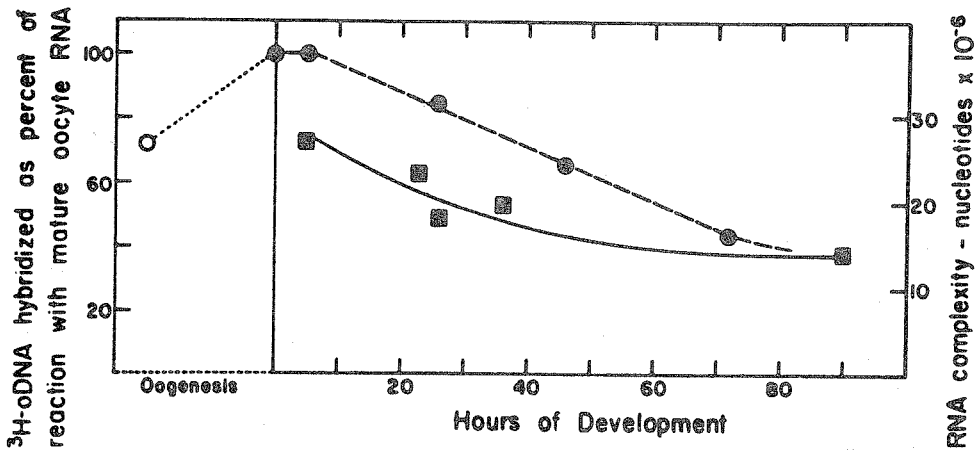


FIG. 5. The maternal single copy sequence set during cogenesis and early development. Terminal values for [^{3}H]oDNA hybridization with each RNA preparation are plotted as percentages of [^{3}H]oDNA reaction with mature oocyte RNA (left ordinate). The right ordinate represents the RNA complexity. ○, Total ovary RNA; ●, mature oocyte and cytoplasmic RNAs; ■, mRNAs. Data and sources are as follows.

Time of development	RNA	Complexity of RNA of the maternal single copy sequence set		Source
		As a percentage of mature oocyte RNA	As nucleotides ($\times 10^6$)	
Mid-oogenesis	Total	72	2	Fig. 2
Mature oocyte	Total	100	37	Table 2 Galau *et al.*, 1976
16-Cell embryo (5 hr)	Cytoplasmic	100	37	Text
	mRNA	73	27	Table 3
Blastula (23 hr)	mRNA	63	23	Wold, Britten, and Davidson, unpublished observations.
Blastula (26 hr)	Cytoplasmic	85	31	Fig. 3
	mRNA	49	18	Table 4
Gastrula (36 hr)	mRNA	53	19	Table 4
			17	Galau *et al.*, 1974
Prism (46 hr)	Cytoplasmic	66	24	Fig. 3
Pluteus (72 hr)	Cytoplasmic	44	16	Fig. 3
(90 hr)	mRNA	38	13–15	Galau *et al.*, 1976

RNA, and polysomal mRNA are displayed as functions of time. Of course we are ignorant of the detailed shape of these functions and have simply assumed smooth curves which connect the points in development where measurements were made. The left ordinate of Fig. 5 shows the fraction of the mature oocyte single copy sequence set on a scale extending from 0 to 100%, and on the right is indicated the complexity of the maternal single copy sequence set on a scale ranging from 0 to 37 $\times$ 10^6 nucleotides. The [^{3}H]oDNA reactions with each embryo RNA illustrated in Fig. 5 indicate only the complexity of those embryo RNA sequences which are also

represented in the mature oocyte, i.e., the maternal single copy sequence set. The *total* complexity of the embryo RNA is, at least in the case of the blastula mRNA, significantly higher (Galau *et al.*, 1976; Wold, Britten and Davidson, in preparation).

Comparison with Previous Results

Before discussing the significance of Fig. 5 it is important to consider the consistency of the present data with prior measurements (Galau *et al.*, 1974, 1976). Direct comparisons can be made at several points (see caption to Fig. 5). These are (1) the complexity of mature oocyte RNA, (2) the complexity of the polysomal mRNA at the gastrula stage, and (3) the complexity of the polysomal RNA at the pluteus stage. The complexity of oocyte RNA was calculated by Galau *et al.* (1976) as 37×10^6 nucleotides, and almost exactly the same value was obtained here. This agreement provides important support for the single copy measurements presented in Table 2. As noted in Results the total complexity of the gastrula mRNA measured by Galau *et al.* (1974) is also about the same as that calculated from the amount of reaction with the [^{3}H]oDNA tracer. Galau *et al.* (1976) found no sequences in the mRNA of pluteus stage embryos which are not also represented in gastrula mRNA. Therefore the pluteus mRNA sequence set should also be included in oocyte RNA, and the complexity of pluteus mRNA measured directly should agree with that obtained by reaction with [^{3}H]oDNA. In this study we did not measure the pluteus mRNA reaction with [^{3}H]oDNA. However, we did carry out reactions between the [^{3}H]oDNA tracer and pluteus cytoplasmic RNA. Figure 3 shows that the complexity of the maternal sequence in 72-hr pluteus cytoplasm is about 16×10^6 nucleotides, while Galau *et al.* (1976) found a complexity of $13–15 \times 10^6$ nucleotides for the mRNA of slightly later embryos, again a satisfactory agreement. The consistency of the present complexity estimates with earlier ones is noteworthy, since they are based on completely independent sets of DNA tracers, with differing reactivities and differing specificities. Thus, Galau *et al.* (1976) utilized a tracer selected to represent gastrula mRNAs, while in the present work the tracer was selected to represent the complex class sequences of mature oocyte RNA. The comparisons summarized here suggest that the various sets of measurements agree within 10%, though additional systematic errors could exist. We can conceive of no way that the complexities measured could be too high. However, RNAs present at $^1/_{10}$ to $^1/_{100}$ of the prevalence of the dominant maternal single copy transcripts would be missed.

Cytoplasmic Transcripts of the Maternal Single Copy Sequence Set

The level of certainty we can attribute to the polysomal mRNA measurements does not extend to the measurements shown in Fig. 5 for total cytoplasmic RNA. It is clear from much earlier work that the polysomal mRNA preparations are free of significant hnRNA contamination (Goldberg *et al.*, 1973; Galau *et al.*, 1974, 1976; Hough *et al.*, 1975). To some extent such contamination in the cytoplasmic RNA preparations would have been detected in the single copy DNA reactions summarized in Table 2. These reactions show that there is no significant contamination with *total* hnRNA, but we cannot preclude "leakage" of specific hnRNA sequences. As an unlikely example, selective stage-specific "leakage" of a diminishing subset of hnRNA sequences homologous to oocyte RNA could explain the results obtained with cytoplasmic RNA.

If the cytoplasmic RNA measurements in Fig. 3 are physiologically meaningful, an unexpected result is the disparity at earlier embryonic stages between the set of sequences present in the cytoplasm and

that represented in polysomal mRNA. At the 16-cell cleavage stage, 100% of the oocyte sequences are present in the cytoplasm, while 73% are represented in the polysomal RNA. Similarly, at the 26-hr mesenchyme blastula stage, 83% of the oocyte sequences are present in the cytoplasm, and 49% are represented in the polysomal mRNA. As Fig. 5 indicates, this distinction disappears by the end of embryogenesis. At the late pluteus stage approximately all cytoplasmic RNA sequences can be accounted for as polysomal mRNA sequences. The difference between polysomal and cytoplasmic RNA sequence sets could also vanish at the earliest stages of development. Our earliest observations were made at 5 hr after fertilization. Conceivably the whole maternal single copy sequence set is represented in polysomes during the first few hours of development.

Transcription of Embryo Structural Genes and the Role of Maternal mRNA

A major conclusion from this study is that about half of the maternal single copy sequence set persists in the polysomal mRNA, all the way through the pluteus stage. The remaining maternal sequences disappear gradually from the polysomes. Interpretation of these results depends, in part, on the recent demonstration that both the complex and prevalent classes of polysomal mRNA are newly synthesized in blastula–gastrula stage embryos (Galau *et al.*, 1977). That is, from the blastula stage on, embryo structural genes are being transcribed which were also utilized during oogenesis for the synthesis of maternal mRNA. This relation may apply earlier than the blastula stage as well. Kedes and Gross (1969), Humphreys (1971), and Wu and Wilt (1974), among others, have shown that new mRNA enters the polysomes during early cleavage, but as yet there is no evidence for the synthesis of complex class mRNAs at this stage. In Fig. 5 we observe a gradual decline in the size of the maternal single copy sequence set in the polysomal mRNA after the blastula stage. Since the reacting polysomal mRNAs are new transcripts rather than surviving maternal mRNAs, this decline results from a narrowing pattern of embryo structural gene transcription. It is *not* the result of a gradual loss from the polysomes of different classes of stored maternal mRNA molecules.

The origin and disposition of the nonpolysomal cytoplasmic sequences which react with [^{3}H]oDNA may be quite different. Thus we cannot distinguish between the alternatives that these sequences are surviving maternal mRNAs which gradually disappear, or that these sequences are new transcripts. If they are maternal, there must be a *sequence-specific* persistence time for the nonloaded RNAs. That is, certain of the maternal mRNA sequences disappear sooner than others from the embryo cytoplasm.

Maternal RNA Sequences in Growing Oocytes

Figure 5 shows that the maternal single copy sequence set is not fully complete in previtellogenic oocytes. The amount of time required for this stage of oocyte to become mature is not specified in Fig. 5 and is probably variable. Our unpublished observations suggest that a period of at least 1 month is required for the maturation of 30- to 50-μm early vitellogenic oocytes. The complexity of the immature oocyte RNA is about 26×10^6 nucleotides as measured by reaction with [^{3}H]oDNA (Fig. 2), and is also 26×10^6 nucleotides as measured directly by single copy [^{3}H]DNA reaction (Table 2). Therefore the immature ovary RNA contains few if any detectable sequences absent from mature oocyte RNA (relatively rare hnRNA sequences could not have been detected in these reactions). However, these results imply that about a third of the mature oocyte sequences are accumulated to a detectable extent only later in oogenesis. It is interesting to compare the situation in amphib-

ian oogenesis. Rosbash and Ford (1974) found that the poly(A) RNA content of *Xenopus* oocytes, i.e., the maternal mRNA complement, reaches its final level by the beginning of vitellogenesis, early in the maximum lampbrush chromosome stage.

It is believed that lampbrush chromosomes are present in previtellogenic and early vitellogenic sea urchin oocytes (Jörgenssen, 1913; Davidson, 1968; Giudice, 1973; Hough-Evans, unpublished observations). Echinoderm lampbrush chromosomes appear to consist of looped-out transcription units maximally packed with polymerases and nascent RNA transcripts (DeLobel, 1971). Their structure is similar to other oocyte lampbrush chromosomes (reviewed by Davidson, 1976). Since at least $^2/_3$ of the maternal single copy sequence set can already be detected in the lampbrush stage sea urchin oocytes, these structural gene sequences are probably being transcribed at this time and perhaps earlier as well. With respect to this set of transcripts, the sea urchin oocyte appears to behave as does the *Xenopus* oocyte. Galau *et al.* (1976) measured the complexity of the mRNA of ovaries containing only immature oocytes to be 20×10^6 nucleotides. Therefore we may conclude that most (about 80%) of the previtellogenic oocyte RNA sequences detected by reaction with [^{3}H]oDNA are represented in the oocyte polysomal RNA. It follows that a majority of the immature oocyte sequence set is required for translation during oogenesis and is also included in the stored mRNA of the mature oocyte.

RNA elongation rates have been measured for sea urchin embryos at 6–9 nucleotides sec^{-1} per polymerase (Aronson and Chen, 1977). Assuming this rate for the oocyte and the close polymerase packing typical of lampbrush chromosomes, it would require only a matter of hours to synthesize the 1600 copies of each sequence found in the mature oocyte. However, the immature oocytes we are discussing here are still at least 1–2 months away from maturity.

Overview and Interpretation: Utilization of Structural Genes Needed for Development

Much previous work demonstrates that the polysomes of the early cleavage stage embryos are loaded mainly with maternal mRNAs (Infante and Nemer, 1967; Humphreys, 1969, 1971; see review in Davidson, 1976). Combining this fact with the findings of Galau *et al.* (1977) and the data reported here, we may construct the following overall picture of events. During the first hours after fertilization, all or most of the stored species of maternal mRNA destined for use in the embryo are loaded onto polysomes and begin to be translated. Once located on the polysomes the mRNAs probably decay stochastically. At least from the blastula stage on, their average half-life is 5.7 hr (Galau *et al.*, 1977). The role of maternal mRNA is primarily to initiate embryogenesis (including perhaps the synthesis of regulatory elements) and to maintain protein synthesis at the very beginning of development. This role may be important only during cleavage, and soon the embryo begins to replace the *polysomal* maternal mRNAs with its own transcripts.

By the blastula stage essentially all of the polysomal mRNAs are the product of embryo structural genes. Most of these are also expressed during oogenesis, since the mRNAs they produce are homologous to oocyte RNAs. This is true of the complex class mRNAs, according to the results presented here and by Galau *et al.* (1976), and it is also true of prevalent class mRNAs. Thus, Brandhorst (1976) reported that of about 400 species of newly synthesized proteins resolvable on two-dimensional gels, almost all are synthesized from prior to fertilization until at least the gastrula stage. These proteins are the product of prevalent maternal and embryo mRNAs.

Some qualitatively different, nonmater-

nal transcripts must also be present in the polysomes during the blastula stage. However, such transcripts have disappeared from the polysomes by the gastrula stage. By this point all of the newly transcribed mRNA sequences are also included in the maternal single copy sequence set. The size of the maternal and total single copy sequence set present at each stage is smaller than at the previous stage. Thus, change in the mRNA sequence sets during development depends on the continuous turnover of the newly synthesized mRNA and on change in the patterns of structural gene transcription.

We now discuss the origin and role of the maternal mRNAs. Once we know that the early embryo synthesizes, and turns over, mRNAs which belong to the same sequence set as do the mature oocyte RNAs, a similar explanation suggests itself for the oocyte. Thus we might suppose that many of the same mRNAs are constantly being synthesized, translated, and degraded in growing oocytes as in early embryos. It is striking that on a per-embryo basis the prevalence of individual complex class mRNA sequences is about the same as the prevalence of individual oocyte sequences. The whole period, from early oogenesis until well into embryological development, can thus be considered as one in which a certain set of structural genes presumably required for early morphogenesis is being transcribed, while their protein products slowly accumulate. The small number of copies of each such mRNA (and possibly the rate of oogenesis itself) could thus be a consequence of the very large number of different mRNA species required for early development. We now know that the mRNA complexity at the very beginning of development is greater than at any later time. Thereafter it declines, but even in previtellogenic oocytes or in late plutei the structural gene sets in use exceed by several-fold those present in several adult tissues (Galau *et al.*, 1976).

The basic unexplained aspect of these findings is the requirement for an enormous diversity of relatively rare proteins in early embryos. The complexity of the maternal single copy sequence set is sufficient to provide at least 2×10^4 different protein species. The processes of embryonic morphogenesis are apparently very expensive in genomic information. Perhaps this is not surprising when one considers that the morphogenesis of a structure as simple as the T4 phage requires over 50 different structural gene products (reviewed by Wood and Revel, 1976). Our present state of knowledge indicates that the large class of what we surmise to be "morphogenesis proteins" required to build a sea urchin embryo begin to be accumulated during early oogenesis, long before their morphogenetic utilization. Following fertilization, the progressive utilization of the hypothetical "morphogenesis proteins" could be a sequential process, requiring a relatively minor number of new stage-specific proteins which appear only during embryogenesis.

We are pleased to acknowledge the competent and intelligent technical assistance of Michael R. Kozlowski. This research was supported by grants from the NIH (Child Health and Human Development and Research Resources) and the NSF. B.J.W. has been supported by an NSF Fellowship and by the McCallum and Danforth Funds. S.G.E. was supported by the National Foundation-March of Dimes and by an NIH Fellowship.

REFERENCES

ANDERSON, D. M., GALAU, G. A., BRITTEN, R. J., and DAVIDSON, E. H. (1976). Sequence complexity of the RNA accumulated in oocytes of *Arbacia punctulata*. *Develop. Biol.* 51, 138–145.

ARONSON, A. I., and CHEN, K. (1977). Rates of RNA chain growth in developing sea urchin embryos. *Develop. Biol.* 59, 39–48.

BRANDHORST, B. P. (1976). Two-dimensional gel patterns of protein synthesis before and after fertilization of sea urchin eggs. *Develop. Biol.* 52, 310–317.

BRITTEN, R. J., GRAHAM, D. E., and NEUFELD, B. R. (1974). Analysis of repeating DNA sequences by reassociation. *In* "Methods of Enzymology" (L. Grossman and K. Moldave, eds.) Vol. 29E, pp.

363–418. Academic Press, New York.

DAVIDSON, E. H. (1968). "Gene Activity in Early Development," 1st Ed. Academic Press, New York.

DAVIDSON, E. H. (1976). "Gene Activity in Early Development," 2nd Ed. Academic Press, New York.

DELOBEL, N. (1971). Étude descriptive des chromosomes en écouvillon chez *Echinaster sepositus* (Echinoderme, Astéride). *Ann. Embryol. Morphol.* 4, 383–396.

GALAU, G. A., BRITTEN, R. J., and DAVIDSON, E. H. (1974). A measurement of the sequence complexity of polysomal messenger RNA in sea urchin embryos. *Cell* 2, 9–21.

GALAU, G. A., KLEIN, W. H., DAVIS, M. M., WOLD, B. J., BRITTEN, R. J., and DAVIDSON, E. H. (1976). Structural gene sets active in embryos and adult tissues of the sea urchin. *Cell* 7, 487–505.

GALAU, G. A., LIPSON, E. D., BRITTEN, R. J., and DAVIDSON, E. H. (1977). Synthesis and turnover of polysomal mRNAs in sea urchin embryos. *Cell* 10, 415–432.

GIUDICE, G. (1973). "Developmental Biology of the Sea Urchin Embryo." Academic Press, New York.

GOLDBERG, R. B., GALAU, G. A., BRITTEN, R. J., and DAVIDSON, E. H. (1973). Nonrepetitive DNA sequence representation in sea urchin embryo messenger RNA. *Proc. Nat. Acad. Sci. USA* 70, 3516–3520.

GRAHAM, D. E., NEUFELD, B. R., DAVIDSON, E. H., and BRITTEN, R. J. (1974). Interspersion of repetitive and nonrepetitive DNA sequences in the sea urchin genome. *Cell* 1, 127–137.

HINEGARDNER, R. T. (1967). Echinoderms. *In* "Methods in Developmental Biology" (F. H. Wilt and N. K. Wessells, eds.), pp. 139–155. T. Y. Crowell, New York.

HOUGH, B. R., SMITH, M. J., BRITTEN, R. J., and DAVIDSON, E. H. (1975). Sequence complexity of heterogeneous nuclear RNA in sea urchin embryos. *Cell* 5, 291–299.

HUMPHREYS, T. (1969). Efficiency of translation of messenger RNA before and after fertilization in sea urchins. *Develop. Biol.* 20, 435–458.

HUMPHREYS, T. (1971). Measurements of messenger RNA entering polysomes upon fertilization of sea urchin eggs. *Develop. Biol.* 26, 201–208.

HYNES, R. O., and GROSS, P. R. (1972). Informational RNA sequences in early sea urchin embryos. *Biochim. Biophys. Acta* 259, 104–111.

INFANTE, A. A., and NEMER, M. (1967). Accumulation of newly synthesized RNA templates in a unique class of polyribosomes during embryogenesis. *Proc. Nat. Acad. Sci. USA* 58, 681–688.

JÖRGENSSEN, M. (1913). Die Ei-und Nährzellen von *Pisciola*. *Arch. Zellforsch.* 10, 1–161.

KEDES, L. H., and GROSS, P. R. (1969). Synthesis and function of messenger RNA during early embryonic development. *J. Mol. Biol.* 42, 559–575.

KIRBY, K. S. (1965). Isolation and characterization of ribosomal ribonucleic acid. *Biochem. J.* 96, 266–269.

KLEENE, K. C., and HUMPHREYS, T. (1977). Similarity of hnRNA sequences in blastula and pluteus stage sea urchin embryos. *Cell*, in press.

ROSBASH, M., and FORD, P. J. (1974). Polyadenylic acid-containing RNA in *Xenopus laevis* oocytes. *J. Mol. Biol.* 85, 87–101.

SMITH, M. J., HOUGH, B. R. CHAMBERLIN, M. E., and DAVIDSON, E. H. (1974). Repetitive and nonrepetitive sequences in sea urchin hnRNA. *J. Mol. Biol.* 85, 103–126.

WOOD, W. B., and REVEL, H. R. (1976). The genome of bacteriophage T4. *Bacteriol. Rev.* 40, 847–868.

WU, R. S., and WILT, F. H. (1974). The synthesis and degradation of RNA containing polyriboadenylate during sea urchin embryogeny. *Develop. Biol.* 41, 352–370.

CHAPTER 3

Non-maternal Messenger RNA Sequences in Sea Urchin Embryos

Barbara J. Wold, Roy J. Britten[1], and Eric H. Davidson

Division of Biology

California Institute of Technology

Pasadena, California 91125

Running title: Non-maternal mRNA Sequences

Send proofs to: Eric H. Davidson, Division of Biology, California Institute of Technology, Pasadena, California 91125.

[1]Also staff member, Carnegie Institution of Washington

ABSTRACT

A set of messenger RNA sequences not represented in the RNA of the mature oocyte was detected in blastula stage sea urchin embryos. This observation was made by reacting embryo polysomal RNAs with a single copy tracer lacking mature oocyte RNA sequences. No significant reaction was obtained with cleavage stage or gastrula mRNA. The non-maternal blastula message set could code for as many as 2000 different messages, though it represents no more than 15% of the total complexity of the mRNA.

INTRODUCTION

The following experiments were designed to test the proposition that all structural genes utilized in sea urchin embryos are represented in the RNA of the mature oocyte. Galau et al. (1977) found that by the mesenchyme blastula stage the polysomal mRNA molecules of Strongylocentrotus purpuratus embryos are newly synthesized rather than maternal in origin. However, the investigations of Galau et al. (1976) and Hough-Evans et al. (1977) showed that the complexity of the polysomal mRNA present in embryos of any given stage is less than that of mature oocyte RNA, and that a major fraction, if not all, of the embryo mRNA sequence set is also included in the oocyte maternal message. It appeared possible from these results that the structural genes active during embryonic development are entirely a subset of those utilized in the production of maternal message. We describe briefly hybridization experiments with a single copy DNA tracer lacking oocyte RNA sequences which disprove the latter hypothesis. Blastula stage embryos are found to contain a set of non-maternal polysomal mRNAs which includes up to 15% of the total complexity of the message. This would be sufficient to code for as many as 2000 diverse proteins.

MATERIALS AND METHODS

Sea urchin embryos and oocytes

Mature oocytes of Strongylocentrotus purpuratus were collected, fertilized and cultured by standard methods (Hinegardner, 1967; Smith et al., 1974) in Millipore-filtered sea water at 15°C. Embryos harvested at the 16-cell stage were demembranated with papain at fertilization (Hynes and Gross, 1972).

Mature oocyte RNA and embryo messenger RNAs

Total RNA was extracted from mature oocytes as described by Hough-Evans et al. (1977).

Polysomal messenger RNA was extracted from 16-cell embryos (about 5 hr of development), mesenchyme blastulae (23 hr of development), early gastrula stage embryos (36 hr of development). The RNAs were prepared by puromycin or EDTA release from purified polysomes (Hough-Evans et al., 1977; Galau et al., 1976; Galau et al., 1974).

Single copy [^{3}H]DNA

The single copy [^{3}H]DNA tracer was labeled in vitro to a specific activity of 8×10^6 cpm/μg (Galau et al., 1976; Hough-Evans et al., 1977).

Preparation of Null [^{3}H] oDNA

Single copy [^{3}H]DNA (8×10^6 cpm/μg) was reacted with excess total oocyte RNA to C_0t 3.5×10^4. The reaction was terminated by freezing, the mixture treated with 10 μg/ml RNase A in 0.24 M phosphate buffer (an equimolar solution of monobasic and dibasic sodium phosphate) at room temperature for 1 hr to digest unhybridized RNA but leave RNA-DNA duplex intact. The digest was adjusted to 0.12 M phosphate buffer, 0.2% SDS, extracted with chloroform: isoamyl alochol (24:1) and fractionated over a 1 ml bed of hydroxyapatite at 60°C. Unreacted DNA was eluted at 60°C and the bound material, consisting of RNA-DNA hybrids and DNA-DNA duplexes, was eluted at 95°C. Residual RNase A was removed from the unbound fraction by proteinase K digestion followed by extraction with organic solvents (Hough-Evans et al., 1977). The unreacted DNA fraction was concentrated, oocyte RNA was added, and the mixture was reacted to an RNA C_0t of 4.8×10^4. The hydroxyapatite fractionation described above was

repeated. The unreacted DNA from the second round of hybridization and fractionation was again reacted with oocyte RNA. The final hybridization was taken to RNA C_0t 4 x 10^4. On termination, the reaction mixture was diluted to 0.12 M phosphate buffer, 0.2% SDS, and fractionated over a 30 ml bed of hydroxyapatite at 53°C in 0.12 M phosphate buffer, 0.2% SDS. Under these conditions, most of the ribosomal RNA binds along with DNA:DNA duplexes and RNA:DNA hybrids. The unbound fraction was immediately passed over a second hydroxyapatite column (20 ml bed, 53°C, 0.12 M phosphate buffer). The unbound material was concentrated, and residual RNA hydrolyzed with potassium hydroxide. The tracer was passed over a Sephadex G-100 column equilibrated with 0.12 M phosphate buffer, 0.05% SDS and the excluded fractions pooled. The tracer was stored at 4°C in 0.5 M phosphate buffer, 10 mM EDTA, 0.2% SDS.

Hybridization of Null [^{3}H] oDNA with oocyte RNA and embryo messenger RNAs

All hybridizations were performed in 0.5 M phosphate buffer, 5 mM EDTA and 0.2% SDS at 60°C. All RNA and DNA C_0ts referred to in this paper are equivalent C_0ts; that is, they have been corrected for accelerations in reaction rate due to Na^+ concentration (Britten et al., 1974) relative to the rate in 0.12 M phosphate buffer at 60°C. All analytical hybridizations contained 4-8 x 10^4 cpm of tracer and were adjusted so as to contain 20 to 80 cpm of DNA-DNA duplex. The amount of RNA-DNA hybrid was assayed by the "split column" protocol described in Galau et al. (1976) for reactions with null mDNA, except that the reaction mixtures were not chromatographed over Sephadex before RNase treatment.

Preparation of null oDNA enriched for blastula complementary sequences

Null [^{3}H] oDNA tracer (about 1.2 x 10^6 cpm) was reacted with excess mesenchyme blastula mRNA to mRNA C_0t of 1,830. On termination, the reaction

treated with RNase A as described above (Galau et al., 1974). The mixture was fractionated by hydroxyapatite chromatography. The bound material was eluted at 60°C with 0.5 M phosphate buffer, diluted to 0.05 M phosphate buffer, and digested with 10 µg/ml RNase A at 37°C for 10 hr to destroy RNA in RNA-DNA hybrids. The digest was deproteinized and fractionated over hydroxyapatite at 55°C, in 0.12 M phosphate buffer, 0.2% SDS. The unbound material was passed immediately over a second hydroxyapatite column (1 ml bed volume) at 55°C. The unbound material from this column was treated with proteinase K, extracted with organic solvents and concentrated as described previously for "mDNA" (Wold et al., 1978).

RESULTS

To detect non-maternal species of messenger RNA, a single copy [^{3}H]DNA tracer lacking sequences complementary to total oocyte RNA was prepared. The selected DNA fraction is referred to as a "null oocyte DNA" or "null oDNA" tracer. The preparation of this tracer is described in Materials and Methods. The starting material was a total single copy [^{3}H]DNA which had been labeled with E. coli DNA polymerase I to a specific activity of about 8 x 10^6 cpm/µg (Galau et al., 1976; Hough-Evans et al., 1977). To remove sequences complementary to oocyte RNA, three successive hybridizations of the tracer with total oocyte RNA were performed. After each reaction the mixture was fractionated by hydroxyapatite chromatography, and the nonreacted, single-stranded [^{3}H]DNA was saved for the next step. The hybridizations were performed at RNA to DNA sequence excess $\geq$ 500-fold. To ensure nearly complete hybridization of sequences represented in the RNA, the reactions were taken to an RNA C_0t of 3.5-5 x 10^4 M sec. These values are over 10 times the half C_0t for the

pseudo-first order reaction of oocyte RNA with single copy DNA (Hough-Evans et al., 1977). The weight mean single-strand fragment length of the final null oDNA preparation was about 200 nucleotides as measured in alkaline sucrose gradients. The null oDNA was 80% reactive. This was determined by hydroxyapatite binding of samples reacted to C_0t 30,000 with excess 450 nucleotide sea urchin DNA. The second order rate constant for the reaction with sea urchin DNA was 1.3×10^{-3} M^{-1} sec^{-1}, as expected for single copy sequence according to earlier studies (Graham et al., 1974; Galau et al., 1974). There was no detectable repetitive sequence contamination.

To establish that the null oDNA preparation was effectively stripped of maternal sequence, the tracer was hybridized with excess total oocyte RNA. The reactions were assayed by hydroxyapatite chromatography as described previously. Each hybridization mixture was divided into two aliquots, one of which was used to determine DNA-DNA duplex content while the total duplex content, i.e., DNA-RNA hybrid plus DNA-DNA duplex, was measured in the other. The difference gives the fraction of the tracer included in RNA-DNA hybrid. Data for the oocyte RNA reactions with null oDNA are presented in the upper portion of Table 1 and in Fig. 1 (open circles). These measurements show that there is very little, if any, oocyte sequence remaining in the null oDNA tracer. About 3% of unfractionated single copy DNA can be hybridized by mature S. purpuratus oocyte RNA (Hough-Evans et al., 1977). Table 1 indicates that an average of 0.02% of the null oDNA tracer hybridizes with the oocyte RNA, and this minute amount of reaction may not be real. The concentration of oocyte sequences in the null oDNA preparation has thus been reduced at least 100-fold with respect to the starting single copy tracer.

The null oDNA was reacted with excess polysomal RNAs from 16-cell cleavage stage embryos, mesenchyme blastula and gastrulae. Evidence that the polysomal mRNA preparations are essentially free of nuclear RNA contamination has been presented earlier (Goldberg et al., 1973; Galau et al., 1974; 1976; Hough et al., 1975; Hough-Evans et al., 1977). Results of the null oDNA reactions with the embryo polysomal mRNAs are listed in Table 1. Here and in Fig. 1 (closed circles) it can be seen that the 16-cell cleavage stage message appears to consist entirely of maternal sequences. A few hundred new message sequences would of course not be detected, considering that the total complexity of 16-cell polysomal RNA is 2.7 x 10^7 nucleotides (Hough-Evans et al., 1977). The null oDNA hybridization values for the gastrula mRNA preparation are only marginally higher than those for oocyte RNA or 16-cell mRNA (Fig. 1, squares). Though there may be a very small amount of non-maternal sequence in the gastrula polysomes, the signal obtained could easily be due to experimental fluctuation. Even if real, it would amount to no more than 3% of the total polysomal mRNA complexity (0.5 x 10^6 nucleotides of non-maternal sequence complexity compared to a total complexity of 17 x 10^6 nucleotides [Galau et al., 1977]). Galau et al. (1976) and Hough-Evans et al. (1977) showed that all or almost all gastrula mRNA sequences are represented in mature oocyte RNA. Therefore, the gastrula results in Table 1 are perfectly consistent with previous knowledge.

A significant reaction with the null oDNA is obtained only with mesenchyme blastula mRNA. Approximately 0.3% of the reactable tracer is hybridized at kinetic termination. The pseudo-first order rate constant for the reaction is 1.4 x $10^{-3} M^{-1} sec^{-1}$. Though not well determined, this rate constant is consistent with that observed by Wold et al. (1978) for the reaction of blastula mRNA with

unfractionated single copy tracer (1.6×10^{-3} M^{-1} sec^{-1}). It follows that the non-maternal sequences are represented in blastula polysomal mRNA at essentially the same concentration as are most other species of mRNA. Therefore, the non-maternal messages belong to the complex class of the mRNA population, which as a whole contains over 90% of the sequence diversity, but includes only 10% of the mRNA mass (Galau et al., 1974; 1977). We know from the studies cited above that each complex class message species is present in mesenchyme blastula at about 500 copies per embryo, or on average once per cell.

Though only 0.3% of the null oDNA hybridizes with blastula mRNA, this could represent a significant amount of sequence complexity. If the hybridizing non-maternal sequences are single copy, their complexity (assuming asymmetric transcription) would be 3.4×10^6 nucleotides (see Table 1 for calculation). This is sufficient for as many as 2000 diverse mRNAs. While there is no detectable repetitive sequence in the null oDNA tracer, the measurements cited earlier are too insensitive to exclude the possibility that the 0.3% of the tracer reacting with non-maternal blastula mRNA sequences is repetitive. In order to determine more accurately the repetitive sequence content, we attempted to prepare a null oDNA subfraction enriched for the non-maternal blastula sequence. A small amount of enriched tracer, referred to as "Bl-null oDNA", was obtained by reacting excess blastula mRNA with null oDNA and isolating the hybrid fraction by hydroxyapatite binding. A 45 to 60-fold concentration of the non-maternal blastula mRNA sequences was obtained. Thus, 11-16% of the blastula null oDNA hybridized with blastula mRNA compared to 0.3% of the starting null oDNA. Data from these reactions are listed in Table 2, where it can also be seen that the blastula null oDNA does not hybridize appreciably with oocyte RNA. The reaction of Bl-null

oDNA with blastula mRNA is therefore due to non-maternal sequence. To determine whether the non-maternal mRNA sequence is repetitive, the blastula null oDNA was reacted to C_0t 20 with 450 nucleotide driver DNA. Only 2.2% of the tracer reacted (Table 2), as predicted from the rate constant for single copy sequence reaction. The sensitivity of this determination is decreased by the fraction of the Bl-null oDNA tracer which is complementary to blastula mRNA. Nonetheless, significantly more reaction of the tracer at C_0t 20 would have been obtained were the blastula mRNA sequences moderately repetitive. The small amount of reaction observed at C_0t 20 was not due to tracer degradation, since after incubation to driver DNA C_0t 30,000 the tracer reacted 88%. The non-maternal blastula mRNA sequences are probably all single copy transcripts, though the possibility that they are transcripts of low frequency repeats cannot be excluded. In either case, the complexity of the non-maternal sequence set is clearly large enough to code for a large number of new proteins not available prior to blastula stage.

DISCUSSION

The maximum possible size of the non-maternal sequence set is shown comparatively in Fig. 2. Here the open portion of each bar represents the fraction which is maternal sequence, and the solid portion represents non-maternal sequence. The hatched areas show the complexity of non-maternal sequences from 16-cell and gastrula stage embryos, assuming that the small signals reported in Table 2 for these stages are real. As shown earlier (Galau et al., 1976; Hough-Evans et al., 1977), the vast majority of the diverse mRNA sequences utilized in the course of embryogenesis are also present in oocyte RNA. Even at the mesenchyme

blastula stage, the non-maternal sequences constitute only about 15% of the total blastula mRNA sequence set. We know from the study of Galau et al. (1977) that after blastula stage all the embryo messages are newly synthesized. Therefore, the maternal sequences of the embryo message population are transcribed during embryogenesis from structural genes which were also utilized in oogenesis, while the non-maternal blastula mRNA sequences are transcribed from structural genes not utilized in the production of oocyte maternal message.

We were not able to carry out a kinetic study of the blastula null oDNA reaction with driver DNA due to the difficulty of obtaining sufficient amounts of this tracer. Thus, it is not proved that the non-maternal sequence set is single copy. However, the data of Table 2 show that these mRNAs cannot derive from repetitive sequences unless they are of relatively low repetition frequency. In fact, there is small likelihood that the non-maternal messages are repetitive to any extent. The complex class sea urchin embryo messages investigated in detail in earlier studies show virtually no repetitive sequence representation (Galau et al., 1974; 1976; 1977; Hough-Evans et al., 1977; Wold et al., 1978); nor could the non-maternal blastula sequences consist of nuclear RNA contaminants, since the complexity of nuclear RNA is almost 50 times greater than that of the reactive null oDNA fraction. We conclude that the most probable interpretation of these data is that blastula stage embryos possess a significant number of single copy messenger RNA sequences which were not utilized earlier in development or in oogenesis.

These experiments eliminate the possibility that in sea urchins all the genetic information required for development is represented in mature oocyte RNA. In interpreting their observation that hemoglobin mRNAs are present in

Xenopus oocytes, Perlman et al. (1977) suggested that oogenesis is a period of "promiscuous" structural gene transcription. The stored messages of an oocyte may not represent all the structural gene sequences transcribed earlier in oogenesis. However, we doubt that the maternal message pool of sea urchin embryos is anything but functional. The maternal messages are utilized extensively after fertilization, and a particular subset of the maternal sequence set is represented in embryo polysomes at each stage of development (Hough-Evans et al., 1977; see Davidson, 1976). Neither these observations nor the finding that a non-maternal set of messages is utilized specifically at the blastula stage are consistent with the idea that transcription of maternal messages is random or "promiscuous."

Our observations focus attention on the blastula stage of development. The morphogenetic events which culminate in gastrulation are already beginning at the mesenchyme blastula stage Brandriff et al. (1975) showed that blastulation is the only pre-larval period when lethal mutations which arrest development are frequently detected in homozygous diploid sea urchin embryos formed by experimental induction of parthenogenesis. Brandhorst (1976) demonstrated that the pattern of protein synthesis has changed noticeably several hours after the mesenchyme blastula stage, i.e., in early gastrulae. This observation cannot be directly related to the non-maternal mRNA sequences studied here since the latter are a complex class message set. The individual proteins whose synthesis was detected on two dimensional gels by Brandhorst all derive from more prevalent messages (see review in Davidson, 1976). The appearance of a non-maternal polysomal message set, and the phenomena observed by Brandhorst and by Brandriff et al. may be manifestations of a special process occurring at the blastula stage. It is reasonable to conclude that this is the first and only period in the embryonic

development of the sea urchin when a large new set of structural genes is expressed.

ACKNOWLEDGEMENTS

This research was supported by a grant from the NIH (Child Health and Human Development). B.J.W. was supported by an NSF predoctoral fellowship and by a Danforth Foundation fellowship.

REFERENCES

Brandhorst, B. P. (1976). Two-dimensional gel patterns of protein synthesis before and after fertilization of sea urchin eggs. Develop. Biol. 52, 310-317.

Brandriff, B., Hinegardner, R. T., and Steinhardt, R. (1975). Development and life cycle of the parthenogenetically activated sea urchin embryo. J. Exp. Zool. 192, 13-24.

Britten, R. J., Graham, D. E., and Neufeld, B. R. (1974). Analysis of repeating DNA sequences by reassociation. In "Methods of Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 29E, pp. 363-418. Academic Press, New York.

Davidson, E. H. (1976). "Gene Activity in Early Development," 2nd Ed. Academic Press, New York.

Galau, G. A., Britten, R. J., and Davidson, E. H. (1974). A measurement of the sequence complexity of polysomal messenger RNA in sea urchin embryos. Cell, 2, 9-21.

Galau, G. A., Klein, W. H., Davis, M. M., Wold, B. J., Britten, R. J., and Davidson, E. H. (1976). Structural gene sets active in embryos and adult tissues of the sea urchin. Cell 2, 487-505.

Galau, G. A., Lipson, E. D., Britten, R. J., and Davidson, E. H. (1977). Synthesis and turnover of polysomal mRNAs in sea urchin embryos. Cell 10, 415-432.

Goldberg, R. B., Galau, G. A., Britten, R. J., and Davidson, E. H. (1973). Non-repetitive DNA sequence representation in sea urchin embryo messenger RNA. Proc. Nat. Acad. Sci. USA 70, 3516-3520.

Graham, D. E., Neufeld, B. R., Davidson, E. H., and Britten, R. J. (1974). Interspersion of repetitive and nonrepetitive DNA sequences in the sea urchin genome. Cell 1, 127-137.

Hinegardner, R. T. (1967). Echinoderms. In "Methods in Developmental Biology" (F. H. Wilt and N. K. Wessells, eds.), pp. 139-155. Y. Crowell, New York.

Hough, B. R., Smith, M. J., Britten, R. J., and Davidson, E. H. (1975). Sequence complexity of heterogeneous nuclear RNA in sea urchin embryos. Cell 5, 291-299.

Hough-Evans, B. R., Wold, B. J., Ernst, S. G., Britten, R. J., and Davidson, E. H. (1977). Appearance and persistence of maternal RNA sequences in sea urchin development. Develop. Biol. 60, 258-277.

Hynes, R. O., and Gross, P. R. (1972). Informational RNA sequences in early sea urchin embryos. Biochim. Biophys. Acta 259, 104-111.

Perlman, S. M., Ford, P. J., and Rosbash, M. M. (1977). Presence of tadpole and adult globin RNA sequences in oocytes of Xenopus laevis. Proc. Nat. Acad. Sci. USA 74, 3835-3839.

Smith, M. J., Hough, B. R., Chamberlin, M. E., and Davidson, E. H. (1974). Repetitive and nonrepetitive sequences in sea urchin hnRNA. J. Mol. Biol. 85, 103-126.

Wold, B. J., Klein, W. H., Hough-Evans, B. R., Britten, R. J., and Davidson, E. H. (1978). Sea urchin embryo mRNA sequences expressed in the nuclear RNA of adult tissues. Cell, submitted for publication.

Table 1. Hybridization of ^{3}H -null oDNA with Total Oocyte RNA and Embryo mRNAs

RNA	Total RNA Cot	mRNA Cot	Percent of ^{3}H-DNA in Duplex			Complexity (nucleotides)
			Total	DNA-DNA	DNA-RNA	
Total oocyte RNA	7,800		0.09	0.07	0.02	
	16,700		0.19	0.18	0.01	
	27,500		0.14	0.15	-0.01	
	31,200		0.52	0.46	0.06	
	45,700		0.21	0.21	0.00	
16-cell mRNA	9,300	370	0.25	0.23	0.02	———
	26,300	1,050	0.32	0.33	-0.01	
	43,000	1,800	0.25	0.24	0.01	Undetectable
Mesenchyme blastula mRNA	4,000	160	0.24	0.20	0.04	
	25,000	1,000	0.39	0.22	0.17	
	45,300	1,810	0.59	0.36	0.23	
	59,000	2,360	0.64	0.41	0.23	[a] 3.4×10^{6}
Gastrula mRNA	18,700	750	0.32	0.28	0.04	
	30,200	1,210	0.40	0.36	0.04	$\_ 0.5 \times 10^{6}$

Table 1 (continued)

[a]The complexity of 3.4×10^6 nucleotides is calculated as:

$$\frac{2.3 \times 10^{-3}}{0.8} \times 2 \times 5.7 \times 10^8$$

where 2.3×10^{-3} is the fraction of null oDNA tracer hybridized at kinetic termination: 0.8 is the reactivity of the [^{3}H]-null oDNA (see Results); the factor of 2 accounts for asymmetric transcription of messenger RNA; and 5.7×10^8 nucleotide pairs is the complexity of null oDNA. That is, 6% of the DNA sequence complexity has been removed from the total single copy tracer, the complexity of which is 6.1×10^8 nucleotide pairs (Graham et al., 1974).

Table 2. Reaction of ^{3}H -Null oDNA Enriched for Blastula Sequences with Oocyte RNA, Blastula mRNA and Total DNA

RNA	Total RNA Cot	mRNA Cot	% ^{3}H-DNA in Duplex		
			Total	DNA-DNA	RNA-DNA
Blastula mRNA	40,100	1,600	16.8	1.2	15.6
Blastula mRNA	40,100	1,600	11.7	1.2	10.5
Oocyte RNA	41,400	——	2.4	1.3	1.1

Excess Sea Urchin DNA	DNA Cot	% ^{3}H-DNA in Duplex
	21	2.2
	30,000	88

Figure 1. The Hybridization of Total Oocyte RNA and Embryo mRNAs with [^{3}H] null oDNA

The left ordinate is percent of [^{3}H] null-oDNA in hybrid. The lower abscissa is total RNA Cot and the upper abscissa mRNA Cot. Messenger RNA Cot is calculated on the basis that 4% of the polysomal RNA mass is mRNA (Galau et al., 1977). (O) Total oocyte RNA; (●) 16-cell embryo polysomal mRNA; (■) Mesenchyme blastula polysomal mRNA: (▲) Gastrula polysomal mRNA. The curve is a least squares fit to the blastula data, assuming a single pseudo-first order component with an ordinate intercept of zero (Pearson et al., 1977). The function used is D/Do = exp [-Cot k] where D/Do is the fraction of ^{3}H-DNA remaining single stranded at time t, Co is the RNA concentration and k is the rate constant. The pseudo-first order rate constant is 1.4×10^{-3} M^{-1} sec^{-1} with respect to mRNA and 6×10^{-5} M^{-1} sec^{-1} with respect to total polysomal RNA. At kinetic termination 0.23% of [^{3}H] null-oDNA was hybridized. The reactivity of null oDNA with excess total sea urchin DNA at Cots $\geq 2.5 \times 10^4$ was 80%. Normalizing for tracer reactivity, 0.3% of null-oDNA sequence reacts with blastula mRNA.

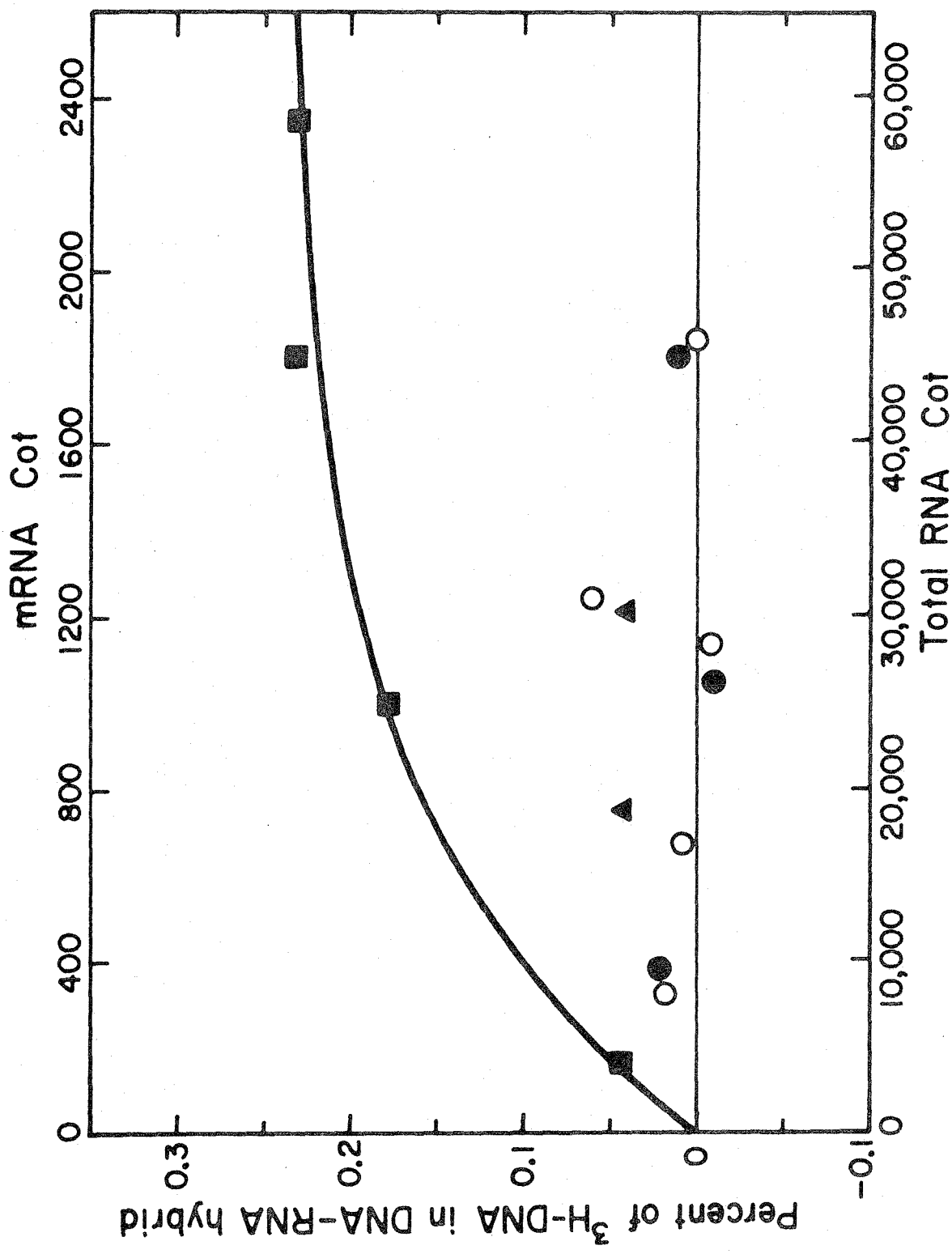
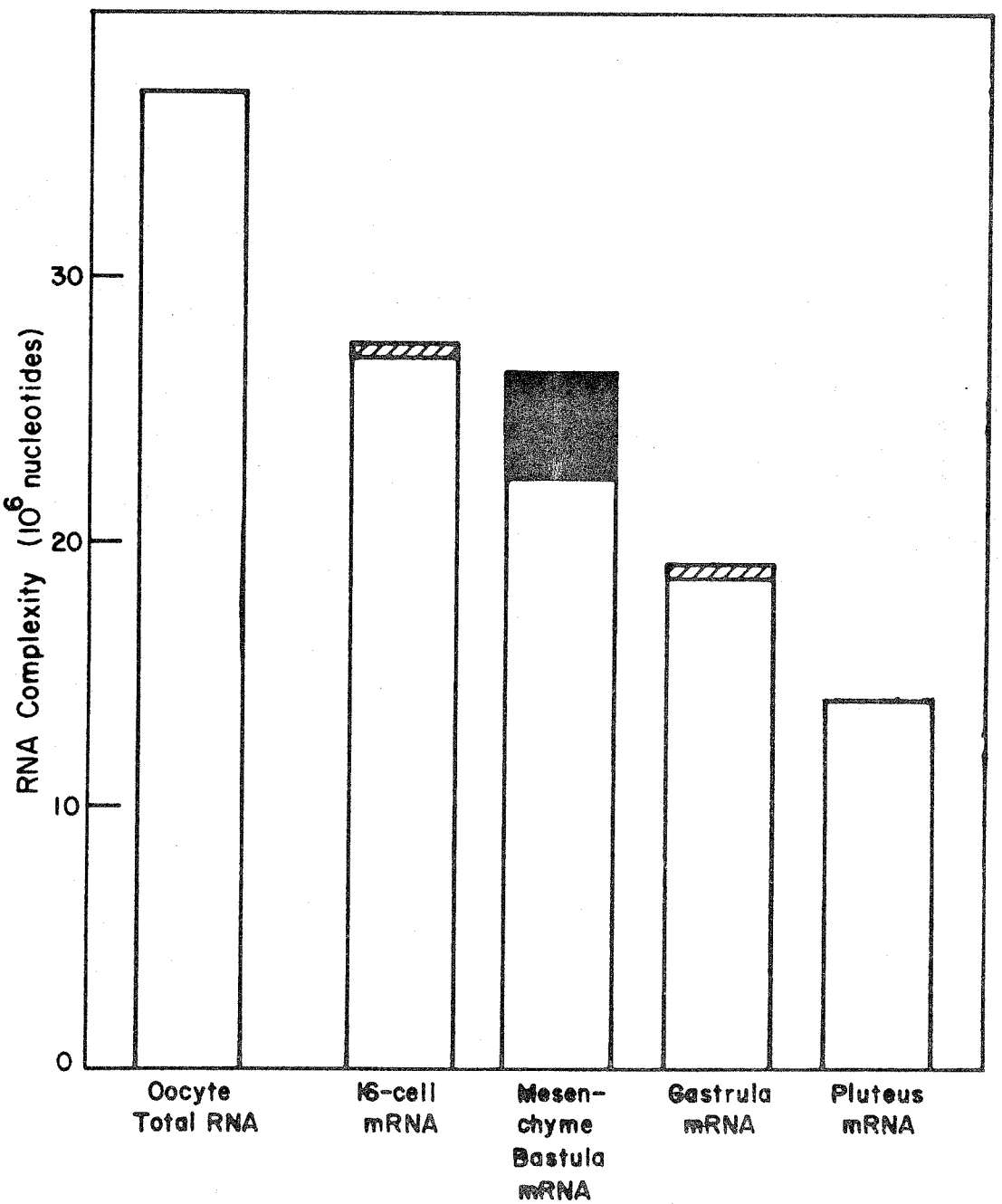

Percent of ^{3}H-DNA in DNA-RNA hybrid
0.3
0.2
0.1
0
-0.1
mRNA Cot
0
400
800
1200
1600
2000
2400
Total RNA Cot
0
10,000
20,000
30,000
40,000
50,000
60,000

Figure 2. Maternal and Non-maternal Sequence Complexity in Embryo mRNAs

The overall height of each bar represents the total RNA sequence complexity. The open portion of each bar represents maternal sequence and the solid portion represents non-maternal sequence. The hatched portion indicates the maximum amount of non-maternal sequence which could have been present according to the data of Table 1 and Figure 1. The complexity values and the source of the data for each RNA are as follows: Oocyte RNA has a total complexity of 3.7×10^7 nucleotides (Hough-Evans et al., 1977; Galau et al., 1976). Sixteen-cell mRNA has a maternal sequence complexity of 2.7×10^7 nucleotides (Hough-Evans et al., 1977), and a maximum possible non-maternal complexity of 3×10^5 nucleotides based on an average of the data from Table 1. Mesenchyme blastula has a maternal sequence complexity of approximately 2.3×10^7 nucleotides (Hough-Evans et al., 1977; and unpublished observations) and a non-maternal complexity of about 3.4×10^6 nucleotides (Table 1). The total blastula mRNA compelxity was measured to be 2.6 to 2.7×10^7 nucleotides (Wold et al., 1978; Galau et al., 1976). Gastrula stage mRNA has a maternal sequence complexity of 1.7 to 1.9×10^7 nucleotides (Hough-Evans et al., 1977; Galau et al., 1974; 1976), and a maximum possible non-maternal complexity of $\leq 5 \times 10^5$ nucleotides. Unfed pluteus mRNA has a total sequence complexity of 1.3×10^7 nucleotides (Galau et al., 1976). Based on the data of Galau et al. (1976) and Hough-Evans et al. (1977), there is no evidence for non-maternal sequence in unfed pluteus stage embryos.


RNA Complexity (10^6 nucleotides)
30
20
10
0
Oocyte Total RNA
16-cell mRNA
Mesen-chyme Bastula mRNA
Gastrula mRNA
Pluteus mRNA

CHAPTER 4

Sea Urchin Embryo mRNA Sequences Expressed in the Nuclear RNA of Adult Tissues

Barbara J. Wold, William H. Klein, Barbara R. Hough-Evans,
Roy J. Britten*, and Eric H. Davidson

Division of Biology
California Institute of Technology
Pasadena, California 91125

Running title: mRNA sequences in nuclear RNA

*Also Staff Member, Carnegie Institution of Washington

Summary

The representation of message sequences in nuclear RNA was studied in sea urchin tissues which utilize these messages in their polysomes and in tissues which do not. A ^{3}H-labeled single copy DNA tracer highly enriched for sequences complementary to blastula embryo mRNA was prepared. This tracer (mDNA) reacted to 78% with excess blastula mRNA, compared to 2.1% for the starting single copy DNA. As expected from previous data, the mDNA reacted to only 12% with cytoplasmic RNA of adult intestine, since most embryo mRNA sequences are not detectable in this tissue. However, the mDNA reacted to the same extent with nuclear RNA from either adult intestine, adult coelomocytes or gastrula embryos as with polysomal mRNA from blastula embryos. Therefore, virtually all of the blastula mRNA sequences are present in adult tissue nuclear RNAs, though most of these mRNA sequences are absent from cytoplasmic or polysomal RNA in the adult cells. The blastula mRNA sequences are present in heterogeneous nuclear RNAs at the same concentrations as are nuclear transcripts of most single copy DNA sequences. Calculations based on the steady state concentration of structural gene transcripts in the embryo nuclei suggest that a majority of these molecules do not serve as message precursors.

Introduction

Large differences exist between the sets of single copy sequence represented in the polysomal mRNA of sea urchin embryos and adult tissues. This was shown by Galau et al. (1976), and further evidence was obtained by Hough-Evans et al. (1977). According to these studies, a specific population of mRNAs is loaded on polysomes at each embryonic stage. Only a minor fraction of the embryo mRNA sequence set is expressed in the mRNA of three adult tissues. It is reasonable to assume that specific mRNA populations are required for the ontogeny and maintenance of various states of cell differentiation. The large differences between embryo and adult mRNA sequence sets provide an opportunity to test the hypothesis that differences in the mRNA sequence sets are a simple and direct reflection of the regions of the genome transcribed into nuclear RNA in each cell type. According to this hypothesis, structural gene sequences would be transcribed only in cells in which their transcripts are being translated. A single copy ^{3}H-DNA tracer reacting specifically with blastula stage embryo mRNA was prepared. As expected, it reacts to only a small extent with mRNAs of adult intestine cells. We find, however, that the reaction of this tracer can be driven to completion by nuclear RNAs from intestine and other adult cell types, as well as by embryo nuclear RNA. It follows that the nuclear RNA includes transcripts of the embryo structural gene set, irrespective of whether the corresponding mRNAs are present in the polysomes, as in the embryo, or are mainly absent, as in the adult cell types.

Results

Characterization of Blastula mRNA

The single copy sequence complexity of polysomal mRNA from mesenchyme blastula stage embryos was measured previously to be about 2.7×10^7 nucleotides (Galau et al., 1976). In Figure 1, we present data from two separate determinations which confirm this value. The mRNA sequence complexity was measured by reacting excess blastula polysomal mRNA with trace quantities of highly labeled single copy DNA. These reactions were performed under standard conditions at RNA to DNA sequence excesses of 50 to 1000 fold, and the amount of hybrid in each reaction mixture was assayed by binding to hydroxyapatite (see Experimental Procedures). Data obtained with two different single copy ^{3}H-DNA tracers, labeled in vivo and in vitro, were pooled and fit by least squares methods assuming a single pseudo-first order kinetic component. At termination, 2.1% of the reactable ^{3}H-DNA is hybridized. This represents about 2.6×10^7 nucleotide pairs of single copy sequence, calculated as described in the legend to Figure 1. The rate of the reaction is consistent with those reported previously by Galau et al. (1976) and Hough-Evans et al. (1977). From this rate and the single copy sequence complexity, we calculate the fraction of the RNA driving the hybridization reaction to be about 10%. This fraction is the "complex class" of the message population (Galau et al., 1974), which includes over 90% of the diverse mRNA sequences, but only a minor fraction of the mRNA mass. In the mesenchyme blastula, complex class mRNAs are present at about 500 copies per embryo or, on the average, 1 copy per cell. The calculation of the driver RNA fraction and of the average concentration of transcripts per cell is described by Galau et al. (1974, 1976). A small fraction of the mRNA mass has been demonstrated to contain most

of the sequence complexity in gastrula, cleavage, and pluteus stage embryo message populations, and in several adult tissues including the coelomocytes and intestine cells used in the present investigation (Galau et al., 1974, 1976; Hough-Evans et al., 1977).

The data presented in Figure 1 also indicate that there is no detectable nuclear RNA in the blastula polysomal mRNA preparations. Hybridization of messenger RNA with total single copy DNA provides a highly sensitive test for hnRNA contamination. The sequence complexity of blastula nuclear RNA is almost 10 fold higher than that of blastula mRNA (Kleene and Humphreys, 1977; and unpublished data). If the mRNAs were contaminated by nuclear RNA, the fraction of single copy ^{3}H-DNA hybridized would rise continuously with increasing RNA Cot toward the 10 fold higher nuclear RNA termination value. We estimate that a maximum of 5% of the hybridization observed at total RNA Cot less than 10^{5} M sec could be attributed to nuclear RNA. Therefore, $\geq$ 95% of the single copy DNA fraction which hybridizes with blastula mRNA represents polysomal mRNA sequence rather than hnRNA.

Preparation and Characterization of Blastula mDNA

The ^{3}H-DNA fraction complementary to blastula mRNA (mDNA) was isolated from total single copy ^{3}H-DNA by two successive hybridization reactions with blastula polysomal mRNA. A single copy tracer labeled in vitro to 8 x 10^{6} cpm/µg was the starting material for the mDNA preparation. The first reaction was taken to mRNA Cot 1900 and the second to mRNA Cot 1600 (see Figure 1). After each hybridization, the reaction mixture was fractionated by binding to hydroxyapatite to separate DNA in RNA-DNA hybrids from unreacted DNA. The

method used was based on Galau et al. (1976) and Hough-Evans et al. (1977), as described in detail in Experimental Procedures.

Characteristics of the blastula mDNA tracer are presented in Table 1. Less than 1% of the mDNA was bound to hydroxyapatite at Cots $\leq 1 \times 10^{-3}$. To show that the mDNA is not enriched for repetitive DNA sequence, the tracer was reacted with excess unfractionated 500 nucleotide sea urchin "driver" DNA. Less than 4% of the mDNA tracer had reacted by Cot 55, when essentially all of the repetitive sequence of the driver DNA is in duplex form (Graham et al., 1974; Eden et al., 1977). The reactivity of the mDNA preparation was 92%, as measured by reassociation with excess sea urchin DNA to Cot 30,000.

Figure 2 shows the reaction of mDNA with the same blastula mRNA used for the preparation of the tracer. At kinetic termination, 78% of the reactable ^{3}H-DNA is hybridized by blastula mRNA. This represents a 37 fold purification of the blastula mRNA sequences with respect to the total single copy DNA. A pure blastula mDNA tracer would represent an enrichment of 47 fold. The 22% of the ^{3}H-mDNA preparation which reacts with excess DNA but does not react with blastula mRNA probably consists of random sequence contamination which was not successfully removed in the course of mDNA preparation, and perhaps some DNA fragments containing very short regions of blastula sequence which do not always form stable hybrid structures. All of the observed mDNA reaction with mRNA is due to RNA-DNA hybridization, since the duplexes are labile to RNAase treatment under conditions which digest RNA in DNA-RNA duplex structures (Figure 2, open circles).

Overlap of Blastula with Adult Tissue mRNA Sequence Sets

Galau et al. (1976) showed that the complexity of intestine polysomal

mRNA is 6×10^6 nucleotide pairs. Based on the single copy sequence complexities of blastula mRNA and intestine mRNA, the fraction of mDNA expected to be hybridized if all intestine mRNA sequences are also in blastula mRNA is:

$$\frac{6 \times 10^6}{2.6 \times 10^7} \times 0.78 \text{ (the fraction of the mDNA preparation which is blastula sequence)} = 18\%$$

This is the maximum reaction expected if intestine mRNA is a sequence subset of blastula mRNA.

In Figure 2, the reaction of excess adult intestine cytoplasmic RNA with mDNA is shown. At kinetic termination, only 12% of the mDNA is hybridized by intestine cytoplasmic RNA. The amount of sequence overlap between the blastula and intestine structural gene sets is thus about 4×10^6 nucleotide pairs ($12/78 \times 2.6 \times 10^7$). By measuring the reaction of intestine cytoplasmic RNA with blastula mDNA, we also test the possibility that a signficant number of blastula structural genes are represented as non-polysomal cytoplasmic RNAs. The data presented in Figure 2 demonstrate that most blastula RNA sequences (greater than 85%) are not detectable in intestine cytoplasmic RNA. The measurement is in good agreement with that predicted from earlier studies with polysomal RNAs which showed that approximately 2×10^6 nucleotides of sequence are common to the gastrula and adult intestine mRNA populations (Galau et al., 1976).

RNAs extracted from adult sea urchin coelomocytes were also used in the following experiments. About five different cell types are present in the coelomic fluid (Boolootian and Giese, 1958). Our earlier measurements showed that the sequence complexity of coelomocyte mRNA is $\leq 4.3 \times 10^6$ nucleotides. It follows that the maximum reaction of coelomocyte mRNA with mDNA would be about 16%.

Characterization of Gastrula and Intestine Nuclear RNAs

The complexity of the nuclear RNA of gastrula stage embryos is at least 1.7×10^8 nucleotides, almost one-third of the genomic single copy sequence (Hough et al., 1975). Gastrula nuclear RNA reacts with single copy DNA with a rate constant of approximately $10^{-4}M^{-1}sec^{-1}$. From its complexity and rate of reaction, it may be calculated that the single copy transcripts are present at an average concentration of about 0.5 to 1 copy per cell in gastrula stage embryos (Hough et al., 1975; Scheller et al., 1978).

The complexity of sea urchin adult tissue nuclear RNAs has not previously been reported. RNA was extracted from intestine cell nuclei as described in Experimental Procedures and reacted with single copy ^{3}H-DNA. Data are shown in Figure 3. The reaction kinetics are best fit by a single pseudo-first order component, implying that within a factor of two or three, most of the hybridizing sequences are represented in nuclear RNA at similar concentrations. The rate constant for the hybridization is $2.5 \times 10^{-4}M^{-1}sec^{-1}$ and at termination 17.9% of the reactable ^{3}H-DNA is in hybrid. From these data, we calculate that the fraction of the RNA preparation driving the hybridization is about 10%, compared to 2-3% for our gastrula nuclear RNA preparations. Hough et al. (1975) showed that the single copy sequence transcripts of gastrula nuclear RNA are asymmetric. Assuming this holds for intestine nuclear RNA as well, its sequence complexity is approximately 2.3×10^8 nucleotides.

The average steady-state concentration of the nuclear transcripts can be estimated by dividing the mass of complex single copy RNA per nucleus by its sequence complexity. The total RNA content of isolated intestine nuclei was measured by the phloroglucinol procedure (Dishe and Borenfreund, 1957) and the

DNA content by the diphenylamine procedure (Burton, 1956). These measurements yielded a DNA to RNA mass ratio of 10:1. The diploid DNA content for S. purpuratus is 1.78 pg per nucleus (Hinegardner, 1967). Assuming that all DNA in the nuclear preparation is chromosomal and that intestine cells are diploid, the total RNA per nucleus in our preparations is approximately 0.18 pg. Since about 10% of the RNA drives the hybridization reaction, the mass of complex class RNA per nucleus is approximately 1.8×10^{-2}pg or 3×10^{7} nucleotides. For intestine tissue, then, the steady-state concentration of complex nuclear transcripts is about 0.1 copy per cell (3×10^{7} nucleotides of complex RNA per cell/2.3×10^{8} nucleotides complexity).

To summarize, we find that both gastrula and intestine nuclear RNAs represent about one-third of the genomic single copy sequence, though the concentration of these complex transcripts differs somewhat in the two types of nuclei. Gastrula embryos have 0.5 to 1 copies per cell and intestine cells about 0.1 copies. We do not know from the complexity measurements what fraction of the sequences represented are common to both nuclear RNAs. Kleene and Humphreys (1977) have shown that the nuclear RNAs of blastula and pluteus stage embryos display a high degree of sequence overlap, and this may be generally true of sea urchin nuclear RNAs.

Reaction of Gastrula, Intestine and Coelomocyte Nuclear RNAs with Blastula mDNA

As indicated above, the maximum overlap of the coelomocyte mRNA sequence set with the blastula mRNA sequence set is about 16%, while for gastrula mRNA there is about 40% overlap with the blastula mRNA sequence set (Galau et al., 1976). Since 78% of the mDNA tracer reacts with blastula mRNA, the maximum levels of hybridization which could be expected for reactions of this

tracer with coelomocyte and gastrula mRNAs are 13% for coelomocytes and 32% for gastrula. The data of Figure 2 show a 12% reaction with intestine cytoplasmic RNA. Twenty-two percent of the reactable ^{3}H-DNA in the mDNA tracer may be random single copy sequence, as noted earlier. Its contribution in reactions with nuclear RNAs will be relatively insignificant, as it cannot exceed 4% (the single copy reaction with the nuclear RNA, 18%, x the fraction of the tracer not reacting with blastula mRNA, 22%). If structural gene sequences are transcribed only when the transcripts are utilized as messengers, then the heterologous tissue nuclear RNA will hybridize the same fraction of mDNA as does the mRNA. Should the nuclear RNA hybridize any additional mDNA, then that additional hybridization must be attributed to transcripts containing mRNA sequences which do not become functional messengers.

The reaction of blastula mDNA with excess nuclear RNA from heterologous tissues is shown in Figure 4. Virtually all of the blastula mDNA which can react with blastula mRNA is driven into hybrid by each of the nuclear RNAs. The mDNA reactions are not due to DNA contaminating the nuclear RNA preparations, since these contain no reactable DNA. This was demonstrated for each of the nuclear RNAs by means of separate hybridizations with a single copy tracer (not shown). The DNA-DNA duplex content of these control reaction mixtures was measured after low salt ribonuclease treatment to destroy RNA-DNA hybrids. Incubation was to RNA Cots equivalent to those shown in Figure 4. DNA-DNA duplex content never exceeded 1%, thus demonstrating the absence of detectable quantities of reactable DNA in the nuclear RNA. The hybridization of each nuclear RNA with mDNA is best fit by a single pseudo-first order reaction. At kinetic termination, 76%, 79% and 82% of the blastula mDNA was hybridized by intestine,

coelomocyte and gastrula nuclear RNA, as compared with 78% for the homologous reaction of blastula mRNA with mDNA. Therefore, at least 90% of the blastula structural gene set is transcribed in the nuclei of three very different cell populations, representing both embryo and adult tissues.

In Table 2, the rate constants for the hybridization of intestine and gastrula nuclear RNAs with mDNA are given. These rates, reflecting the concentration of mDNA sequences in each nuclear RNA, can be compared with the rate constants derived for the reactions of nuclear RNAs with total single copy ^{3}H-DNA tracer (Table 2). For gastrula nuclear RNA, the mDNA reaction rate constant is 1.3×10^{-4} $M^{-1}sec^{-1}$ while the rate constant for total single copy tracer is 0.8×10^{-4} $M^{-1}sec^{-1}$. This agreement shows that the sequences reacting with the mDNA are present in gastrula nuclear RNA at about the same concentration as are the majority of the single copy sequence transcripts. Similar agreement is observed for the hybridization of intestine nuclear RNA with mDNA and single copy DNA. This appears to be the case whether or not the sequences reacting with the blastula mDNA appear on polysomes. Thus, a substantial fraction (about 40%) of the blastula mRNA sequences are also present in the mRNA of gastrula stage embryos. Were these polysomal sequences represented in gastrula nuclear RNA at concentrations significantly different from the non-polysomal sequences (60% of the mDNA), the reaction of gastrula hnRNA with mDNA would be kinetically inhomogeneous. We would expect to see 40% of the mDNA driven into hybrid at a rate different from the remaining 60%. Within our limits of detection, which we estimate to be a factor of perhaps 3 in concentration, all of the blastula sequences are present in gastrula nuclear RNA at about the same concentration, i.e., that of most single copy transcripts.

Discussion

Nuclear Transcription of Complex Class mRNA Sequences

The blastula mRNA sequence set appears to be completely represented in the nuclear RNA of adult cells which utilize only a minor fraction of these sequences in their polysomal message. This is the clear result of the experiments reported in Figure 4. Our results disprove the proposition that the only structural genes whose sequence is represented in nuclear RNA are those whose messages are being translated in the cytoplasm.

There are two respects in which this somewhat unexpected conclusion may be limited. First, we do not know that the blastula message set is completely absent from the polysomes of intestine cells or coelomocytes. Second, the mDNA tracer is essentially a complex class mRNA tracer, and the developmental significance of complex class mRNAs is only inferential. That is, the specific protein products of these rare messages cannot yet be demonstrated. The embryonic mRNA molecules reacting with the tracers used in this work are present on the average in only 1 or a few copies per cell (Figure 1, and Galau et al., 1974, 1976, 1977a). Calculations based on the kinetics of intestine cytoplasmic and mRNA reactions (Figure 2, and Galau et al., 1976) indicate that the complex class intestine messages are present in similarly low numbers per cell. To estimate the likely concentration of the putative messages not detected in our measurements with intestine cytoplasmic RNA, we carried out a least squares analysis of the data in Figure 2. We find that a second component of mRNA sequences which would ultimately bring the complexity of the intestine cytoplasmic RNA to that of the blastula mRNA would be present at a concentration about 1000 times less than that of typical complex class messages. In other words, this mRNA would occur in

intestine polysomes (if at all) at frequencies less than 0.001 times per cell.

The more serious issue is whether regulation of complex class mRNA sets is physiologically meaningful. Galau et al. (1976) demonstrated sharp and specific differences in the complex class mRNA sequence sets of sea urchin embryos at various stages, and of adult tissues. Differences involving thousands of distinct structural genes have also been seen in other biological systems when diverse cell types are compared (e.g., Ryffel and McCarthy, 1975; Hastie and Bishop, 1976; Axel et al., 1976). These large-scale changes would appear to be the direct manifestation of gene regulation. Galau et al. (1977a) also reviewed evidence that complex class messages in mammalian liver serve as templates for particular histospecific proteins. However, these arguments are all indirect. We have no inescapable proof that the data presented in this paper mean there is general nuclear transcription of physiologically significant message sequences.

To our knowledge, similar investigations have been carried out only for two specific structural genes which produce prevalent messages, those coding for ovalbumin and hemoglobin. So far the results are equivocal. Groudine and Weintraub (1975) concluded that hemoglobin gene transcripts are not detectable in the total RNA of primary cultured chick fibroblasts, but Humphries et al. (1976) reported that mouse globin transcripts are present at low concentrations in liver, brain, and tissue culture cells. Globin transcripts have also been detected in the total RNA and nuclear RNA of uninduced Friend cells (Gilmour et al., 1974; Gottesfeld and Partington, 1977), and adult globin mRNAs were reported in Xenopus oocyte RNA (Perlman et al., 1977). In some cases, the globin transcripts may have been due to minute levels of contamination of tissue preparations with reticulocytes, and in others to derangement of normal gene control mechanisms in

long-term tissue culture cells. Low concentrations of ovalbumin gene transcripts were found in the cytoplasmic RNA of chicken liver by Axel et al. (1976), but no ovalbumin sequences could be detected in the total RNA of untransformed chick fibroblasts by either Groudine and Weintraub (1975) or Spector et al. (1978). Thus, it is not clear from the literature whether these prevalent message sequences appear in nuclear RNA of cells in which these genes are not expressed.

Rate of Nuclear Transcription of Structural Gene Sequences Compared to Rate of mRNA Synthesis

The kinetic measurements in Table 2 and Figure 4 show that the steady-state concentration of nuclear RNAs hybridizing with the mDNA tracer is the same as the concentration of most single copy transcripts. Sea urchin embryo heterogeneous nuclear RNA consists mainly of single copy sequence transcript (Smith et al., 1974; Hough et al., 1975), and is synthesized and turned over rapidly. The rate of transcription of nuclear RNA in S. purpuratus embryos is fairly well known (reviewed in Davidson, 1976). The average rate of synthesis of complex class messages in sea urchin embryos has also been estimated (Galau et al., 1977b). By rate of message synthesis is meant the rate at which new mRNA molecules flow into the polysomes, irrespective of their nuclear rate of transcription. In Table 3, the rates of nuclear single copy sequence transcription and of complex class mRNA synthesis are compared. Given the synthesis rate of gastrula nuclear RNA and its complexity, it can be seen that on the average each structural gene sequence included in the mDNA is transcribed about 1.5 times per hour. Thus, there are about 3 transcripts of each sequence produced in each diploid nucleus per hour (see Table 3). On the other hand, an average of only about 0.2 molecules of each

complex class mRNA sequence appear to enter the polysomes of each cell per hour. The data used for the calculation in Table 3 are the product of direct measurements made in several laboratories with S. purpuratus embryos, as noted there. This calculation indicates that in S. purpuratus embryos 15 times more transcripts of the mDNA sequences are made than are actually loaded on polysomes. Since all of the values used for the calculation in Table 3 are subject to some uncertainty, this result should probably be regarded as tentative, though it seems unlikely that the 15 fold difference in calculated synthesis rates can be explained away as measurement error. We conclude that a large majority of the complex class structural gene transcripts may not be serving as mRNA precursors, even in embryo cells expressing these genes at the polysomal level. One way of considering these results is to suppose that in gastrula cells, about 10% of those mDNA transcripts represented in the polysomes are utilized as message precursors, while in intestine cells none, or only a minute fraction, of the blastula-specific transcripts present in the nucleus are utilized as message precursors.

The rates calculated for nuclear transcription of the mDNA sequences are within a factor of 3 of the rates of appearance of prevalent mRNAs in the polysomes. Galau et al. (1977b) reported that on the average, about 9 molecules of newly synthesized prevalent class message appear in the polysomes of each embryo cell per hour. The average steady-state concentration of prevalent mRNAs is about 60 copies of each sequence per cell, compared to about 1 per cell for complex class messages in gastrula stage embryos (Nemer et al., 1974; Galau et al., 1977b). It is conceivable that prevalent class messages, complex class messages, or no messages at all could be produced from a given structural gene sequence, according only to the fraction of the nuclear transcripts which are processed and

exported. This may be considered the "extreme processing model". It implies that all or most structural genes are being transcribed all the time at about the same rate, in all cell types, and that the diverse sets of mRNA sequences in the polysomes of various cells derive solely from controlled post-transcriptional events.

The Single Copy Sequences of Nuclear RNA

Hough et al. (1975) showed that at gastrula stage, the complexity of nuclear RNA is 10 times that of the polysomal mRNA in the same cells. The measurements reported here indicate an even higher ratio for adult intestine cells. The results we have obtained in this investigation suggest that in any given cell type a significant fraction of the single copy sequence transcribed into nuclear RNA could be structural genes used in other cell types. The total complexity of the structural genes used throughout the life cycle of the sea urchin is, of course, not known. However, the results of Galau et al. (1976) imply that less than 10% of the single copy sequence in the genome is included in the structural gene set needed to support most of oogenesis and all of prelarval embryonic development, as well as a few differentiated adult tissues. While 10% is a small fraction, it is an appreciable proportion of the nuclear RNA complexity. Nuclear RNAs from the adult tissues and embryo stages so far examined display similar complexities, representing 30 to 40% of total sea urchin single copy sequence (Hough et al., 1975; Kleene and Humphreys, 1977; and unpublished data). Therefore, about one-fourth of the single copy transcript derives from the structural gene sequence so far observed to be represented in polysomal mRNAs. The measurements now in hand could easily underestimate the aggregate complexity of the structural genes used throughout larval and adult life. The extreme processing model suggests that the single copy sequence transcripts of nuclear RNA can be wholly accounted for as

structural gene sequence plus leader sequence, intragenic sequences if these exist in sea urchin genes, etc; in other words, that each nuclear RNA molecule is a potential mRNA precursor.

It is erroneous to conclude that the data we present here require the extreme processing model or exclude transcription-level control of structural gene sequences. A simple alternative is that there are (at least) two forms of nuclear RNA transcript which include mRNA sequences or portions thereof. One form could consist of the true mRNA precursors, or "pre-mRNAs", and transcription of these may indeed be regulated quantitatively at the initiation level. A second form of nuclear RNA, including a major fraction of the newly transcribed molecules in any cell, could consist of a relatively complex set of transcripts which reads across structural genes as well as other single copy sequences, and performs a function other than serving as potential message precursor. The rapid synthesis and turnover of most nuclear RNA sequences, as well as various other properties, suggest an intranuclear role, perhaps regulatory in nature (see Davidson et al., 1977; Scheller et al., 1978).

Whatever the function of nuclear RNA, the results presented in this paper suggest caution in the identification of nuclear precursors of mRNAs. It is clear that many mRNA sequences exist as nuclear transcripts which have no role as "pre-mRNAs". Evidently, this may be true of a majority of transcripts containing a message sequence even in cells expressing that sequence. The physical characteristics and initiation sites, as well as the fate and function of the "non pre-mRNAs" may well be quite different from those of true message precursors.

Experimental Procedures

Growth of Sea Urchin Embryos

Eggs of Strongylocentrotus purpuratus were collected, fertilized, and cultured at 15°C by standard methods (see Smith et al., 1974). The embryos were grown at 1-4 x 10^4/ml of Millipore-filtered sea water made 30 units/ml penicillin G, 50 µg/ml streptomycin with constant stirring and aeration. Mesenchyme blastula embryos were harvested at 23 hr (about 450 cells) and early gastrula embryos at 36 hr (about 600 cells).

Isolation of Blastula mRNA

The mRNA preparations used in these experiments included polysomal rRNA as well as mRNA but had been extensively purified of nonpolysomal RNAs, particularly nuclear RNAs (see text, and references below). The isolation procedure used here is essentially that described by Goldberg et al. (1973), modified as described by Galau et al. (1976) and by Hough-Evans et al. (1977).

Isolation of Nuclear and Cytoplasmic RNAs

Gastrula nuclear RNA. Nuclear RNA from gastrula stage embryos was prepared as described previously (Smith et al., 1974) with the modifications detailed by Scheller et al. (1978).

Intestine nuclear RNA. Intestine tissue was dissected from 100 sea urchins. The test was cut on the oral surface, the Aristotle's lantern removed, coelomic fluid decanted and the body cavity washed twice with a solution containing 4 parts cold Millipore-filtered sea water (MFSW) and one part 0.5 M EGTA (ethyleneglycol-bis- [β -amino-ethyl ether]N,N'-tetra acetic acid) (pH 8.0).

Dissected intestine was rinsed twice in cold MFSW solution taking care to remove any contaminating testicular or ovarian tissue. Intestine tissue from two animals was pooled in a 100 ml beaker containing 60 ml cold intestine lysing buffer (50 mM Tris, pH 8.0, 5 mM magnesium chloride, 50 mM ammonium chloride, 5 mM EGTA, 1% Triton X-100), and stirred for one to two min at high speed on ice. The cell lysate was filtered through six layers of cotton gauze into a 250 ml Nalgene centrifuge bottle. Lysate from six animals was pooled in each bottle and underlayered with 50 ml cold 0.5 M sucrose containing 2 mM magnesium chloride. The lysate was centrifuged at 3000 g for 10 min at 4°C.

Each nuclear pellet was suspended in 5 ml of 7 M urea buffer: 7 M urea (Schwarz-Mann ultra pure grade urea), 50 mM sodium acetate (pH 5.1), 10 mM EDTA, 0.5% SDS, 10 µg/ml PVS [polyvinyl sulfate], and an equal volume of a 1:1 mixture of [phenol:m-cresol:8-hydroxyquinoline (Kirby, 1965)]:[chloroform: isoamyl alcohol (24:1)]. The mixture was deproteinized by stirring at room temperature for at least 30 min. The aqueous phase was removed, and the interface was suspended in 7 M urea buffer made 1 M in sodium perchlorate, 0.1 M Tris (pH 8), 1% SDS, and reextracted with the phenol:chloroform mixture at room temperature. The aqueous phases were combined, extracted twice at room temperature with the phenol-chloroform mixture, once with chloroform:isoamyl alcohol (24:1), and then precipitated at -20°C with 2 vol of 100% ethanol. The precipitate was dissolved in 25 mM PIPES (pH 6.5) and 10 mM magnesium chloride and incubated with 50 µg/ml of "RNAase-free" DNAase I (Worthington) which had been further purified by passage over 5'-(p-aminophenyl phosphoryl)-uridine-2'(3')-phosphate agarose according to Maxwell et al. (1977). After incubation for 1 hr at room temperature, the solution was brought to 0.1 M Tris (pH 8.0), 0.2% SDS and 50 mM EDTA, and

incubated with 50 μg/ml of proteinase K (E. Merck) for 1 hr at 37°C. The solution was deproteinized with the phenol-chloroform mixture and with chloroform:isoamyl alcohol (24:1) and precipitated with ethanol. The RNA precipitate was dissolved in 0.3 M sodium acetate, 5 mM EDTA (pH 6.5), and chromatographed on Sephadex G-100 in the same buffer at 5°C. The RNA in the excluded volume of the column was precipitated with ethanol, suspended in 25 mM PIPES, 5 mM magnesium chloride at 500 μ g/ml. The DNAase digestion was repeated using 50 μg/ml enzyme. The proteinase K digest and subsequent deproteinization, precipitation and G-100 procedures were all repeated as described above. The final RNA preparation was precipitated with 2 vol of 100% ethanol at -20°C. The RNA was suspended in 10 mM sodium acetate (pH 6.5), 2 mM EDTA and 0.001% SDS at 25 mg/ml and stored frozen at -20°C.

Intestine cytoplasmic RNA. Intestine tissue was dissected from adult sea urchins as described above. Intestine tissue pooled from 5 animals was lysed in 40 ml of lysing buffer containing 100 units/ml heparin, 0.5% diethylpyrocarbonate, and 2.5 mg/ml bentonite by rapid stirring for 5 min at 4°C. The lysate was centrifuged for 20 min at 12,000 rpm (Sorvall HB4 rotor) and the top two-thirds of the supernatant was added to 100 ml of 7 M urea buffer. The preparation was deproteinized, precipitated, treated with DNAase and proteinase K, reprecipitated and placed over Sephadex G-100, all as described above. The RNA excluded from the column was suspended in 3 mM sodium acetate (pH 6.5), at 18 mg/ml and stored at -70°C.

Coelomocyte nuclear RNA. Coelomocytes were harvested from about 150 sea urchins by opening the animals around the peristomal membrane and quickly decanting the fluid contents of each sea urchin through four layers of gauze into

about 10 ml of solution containing 8 ml of ice cold MFSW and 2 ml of 0.5 M EGTA (pH 8.0). The mixture was immediately centrifuged for 0.75-1 min in an International table top centrifuge at the highest speed setting. The cellular pellet was suspended in 5 ml of 50 mM ammonium chloride, 10 mM Tris (pH 8), 2 mM magnesium chloride, 5 mM EGTA, 1% Triton X-100, 2 mg/ml bentonite. The cells were lysed by two strokes in a Dounce homogenizer, and the nuclei pelleted by 5 min of centrifugation at 5,000 rpm, 4°C, in a Sorvall HB4 rotor. The nuclear pellet was taken up in 7 M urea buffer, extracted with organic solvents, and further purified as described for intestine nuclear RNA above.

Single copy ^{3}H-DNA. In vivo ^{3}H-labeled nonrepetitive DNA was prepared as described by Galau et al. (1974). The tracer specific activity was 5×10^{5} cpm/μg and its weight mean single strand fragment length was about 300 nucleotides.

In vitro ^{3}H-labeled single copy DNA was prepared from unlabeled single copy DNA (Galau et al., 1974) by "gap translation" using E. coli DNA polymerase I. The method was that described by Galau et al. (1976), modified according to Hough-Evans et al. (1977). The DNA was labeled to a specific activity of 8×10^{6} cpm/μg and its weight mean single strand length was 200-300 nucleotides.

RNA-DNA Hybridization

Single copy ^{3}H-DNA and ^{3}H-mDNA were hybridized with excess unlabeled RNA in 0.5 M phosphate buffer (PB), 0.05% SDS, 5 mM EDTA at 67°C after denaturation for 40 sec to 1 min at 105°C. The RNA mass excess was 10^{4} for reactions with total single copy ^{3}H-DNA. In ^{3}H-mDNA reactions, the RNA mass excess was at least 5×10^{5}. RNA Cot values (M sec) were calculated for cytoplasmic and nuclear RNAs in terms of total RNA mass. mRNA Cots were calculated on the basis that 4% of the total polysomal RNA mass is mRNA (Galau et al., 1977b). All

RNA and DNA Cot values referred to in this paper are equivalent Cots; that is, they have been adjusted to account for rate acceleration during hybridization relative to the rate in 0.12 M PB at 60°C due to higher Na^+ concentration (Britten et al., 1974). The majority of the reactions were incubated 0.5 to 60 hr, and only rarely did the incubation time exceed 90 hr.

Reaction mixtures containing RNA and total single copy ^{3}H-DNA were analyzed by the procedures described earlier, with minor modifications (Galau et al., 1976; Hough-Evans et al., 1977). The hybridization reactions were divided into two aliquots. Aliquot I was assayed for total duplex content on a 1 ml hydroxyapatite column (Bio Rad DNA-grade, Lot 15535) in 0.12 M PB, 0.05% SDS, at 60°C. Single-strand ^{3}H-DNA was eluted, and the column was washed extensively at 60°C with 0.12 M PB containing 0.05% SDS. The bound DNA-DNA and DNA-RNA duplexes were eluted at 95-100°C in the same buffer. Fractions from the column were assayed for radioactivity by counting in scintillation fluid. Aliquot II was used to measure the DNA-DNA duplex content. RNA-DNA hybrids were destroyed by incubation at 37°C for 1-2 hr with 50 µg/ml of RNAase A in 0.01 M PB. The sample was then adjusted to 0.12 M PB, 0.05% SDS, deproteinized with chloroform: isoamyl alcohol (24:1), and the aqueous phase was passed over hydroxyapatite as described above. The fraction of ^{3}H-DNA bound in Aliquot II is subtracted from that bound in Aliquot I to obtain the fraction hybridized with RNA.

Reaction mixtures containing RNA and ^{3}H-mDNA were analyzed in most cases by diluting the sample to 0.12 M PB, 0.05% SDS, and placing it directly over a hydroxyapatite column in the same buffer. No measurement of the DNA-DNA duplex was required because the tracer self-reaction was, in these cases, negligible. Occasionally one or more reaction mixtures were also assayed for DNA duplex content by the "two-column" assay just described.

Preparation of Blastula mDNA

Single copy ^{3}H-DNA enriched for sequences complementary to blastula mRNA was prepared by two cycles of hybridization of nonrepetitive ^{3}H-DNA with blastula mRNA. The procedure used here is essentially that described in detail by Hough-Evans et al. (1977) and is outlined here with minor modifications noted.

The "parent" single copy DNA tracer was labeled in vitro to a specific activity of 8×10^{6} cpm by the gap translation method (Galau et al., 1976). The DNA was hybridized with excess blastula polysomal mRNA (mass excess of 5000) to an mRNA Cot of 1900 (total RNA Cot of 48,000) in 0.5 M PB, 0.05% SDS at 67°C. The reaction mixture was diluted to 500 µg/ml RNA and digested with 10 µg/ml RNAase A in 0.24 M PB for 1 hr at room temperature. This treatment digests unhybridized RNA, but leaves RNA in DNA-RNA duplexes intact. The mixture was adjusted to 0.12 M PB, 0.05% SDS and extracted with an equal volume of chloroform:isoamyl alcohol (24:1) solution. The aqueous phase was fractionated by hydroxyapatite chromatography in 0.12 M PB, 0.05% SDS at 60°C. The bound material (6% of the input cpm) was eluted with 0.5 M PB, 0.01% SDS at 60°C, leaving RNA-DNA and DNA-DNA duplexes intact. The duplex mixture was diluted to 0.05 M PB and RNAase A added to 10 µg/ml. It was digested at 37°C for 12 hr. This treatment destroys RNA in RNA-DNA hybrids but leaves DNA-DNA duplexes intact (Galau et al., 1974). The digest was adjusted to 0.12 M PB, 0.05% SDS and extracted with chloroform:isoamyl alcohol (24:1). The aqueous phase was fractionated on hydroxyapatite in 0.12 M PB, 0.05% SDS at 60°C. The unbound fraction contained DNA released from RNA-DNA hybrids, about 20% of the input DNA cpm. This fraction was treated with proteinase K as described by Hough-Evans et al. (1977) to remove residual RNAase A before the second round of hy-

bridization with blastula mRNA. The bound fraction contained DNA from DNA-DNA duplexes formed in the first hybridization reaction and any DNA fragments containing self complementary regions [a by-product of the in vitro labeling procedure (see Hough-Evans et al., 1977)]. It amounted to 3.5% of the input single copy DNA.

The second hybridization reaction was carried to mRNA Cot 1600, treated with RNAase A in 0.24 M PB for 1 hr and again fractionated over hydroxyapatite. Fifty-five percent of the ^{3}H-DNA bound and was eluted with 0.12 M PB, 0.05% SDS at 98^{o}C. Since no appreciable DNA self reaction was generated during the course of the second hybridization (due to the low DNA Cot attained), the bound material was essentially all RNA-DNA hybrids. This material was again treated with proteinase K and finally concentrated by ethanol precipitation. The tracer was hydrolyzed with 0.3 M KOH for 1 hr to destroy residual blastula mRNA, neutralized and stored in 0.5 M PB, 0.05% SDS, 2.5 mM EDTA at 4^{o}C.

Acknowledgements

This research was supported by an NIH grant (NICHHD). B.J.W. was supported by an NSF predoctoral fellowship and a Danforth Foundation fellowship. W.H.K. was supported by a Lievre Fellowship from the American Cancer Society, California Division.

References

Axel, R., Feigelson, P. and Schutz, G. (1976). Analysis of the complexity and diversity of mRNA from chicken liver and oviduct. Cell 7, 247-254.

Boolootian, R. A. and Giese, A. C. (1958). Coelomic corpuscles of echinoderms. Biol. Bull. 115, 53-63.

Britten, R. J., Graham, D. E. and Neufeld, B. R. (1974). Analysis of repeating DNA sequences by reassociation. In Methods in Enzymology, 29E, L. Grossman and K. Moldave, eds. (New York: Academic Press), pp. 363-406.

Burton, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Proc. Nat. Acad. Sci. USA 62, 315-323.

Davidson, E. H. (1976). Gene Activity in Early Development. (New York: Academic Press).

Davidson, E. H., Klein, W. H. and Britten, R. J. (1977). Sequence organization in animal DNA and a speculation on hnRNA as a coordinate regulatory transcript. Dev. Biol. 55, 69-84.

Dishe, Z. and Borenfreund, E. (1957). A new color reaction for the determination of aldopentose in the presence of other saccharides. Biochim, Biophys. Acta 23, 639-642.

Eden, F. C., Graham, D. E., Davidson, E. H. and Britten, R. J. (1977). Exploration of long and short repetitive sequence relationships in the sea urchin genome. Nucleic Acids Res. 4, 1553-1567.

Galau, G. A., Britten, R. J. and Davidson, E. H. (1974). A measurement of the sequence complexity of polysomal messenger RNA in sea urchin embryos. Cell 2, 9-21.

Galau, G. A., Klein, W. H., Davis, M. M., Wold, B. J., Britten, R. J. and Davidson, E. H. (1976). Structural gene sets active in embryos and adult tissues of the sea urchin. Cell 7, 487-505.

Galau, G. A., Klein, W. H., Britten, R. J. and Davidson, E. H. (1977a). Functional significance of mRNA sequences in the least abundant concentration class. Arch. Biochem. Biophys. 179, 584-599.

Galau, G. A., Lipson, E. D., Britten, R. J. and Davidson, E. H. (1977b). Synthesis and turnover of polysomal mRNAs in sea urchin embryos. Cell 10, 415-432.

Gilmour, R. S., Harrison, P. R., Windass, J. D., Affara, N. A. and Paul, J. (1974). Globin messenger RNA synthesis and processing during haemoglobin induction in Friend cells. I. Evidence for transcriptional control in clone M2. Cell Differen. 3, 9-22.

Goldberg, R. B., Galau, G. A., Britten, R. J. and Davidson, E. H. (1973). Non-repetitive DNA sequence representation in sea urchin embryo messenger RNA. Proc. Nat. Acad. Sci. USA 70, 3516-3520.

Gottesfeld, J. M. and Partington, G. A. (1977). Distribution of messenger RNA-coding sequences in fractionated chromatin. Cell 12, 953-962.

Graham, D. E., Neufeld, B. R., Davidson, E. H. and Britten, R. J. (1974). Interspersion of repetitive and non-repetitive DNA sequences in the sea urchin genome. Cell 1, 127-137.

Grainger, R. M. and Wilt, F. H. (1976). Incorporation of ^{13}C, ^{15}N-labeled nucleosides and measurement of RNA synthesis and turnover in sea urchin embryos. J. Mol. Biol. 104, 589-601.

Groudine, M. and Weintraub, H. (1975). Rous sarcoma virus activates embryonic globin genes in chicken fibroblasts. Proc. Nat. Acad. Sci. USA 72, 4464-4468.

Hastie, N. D. and Bishop, J. O. (1976). The expression of three abundance classes of messenger RNA in mouse tissues. Cell 9, 761-774.

Hinegardner, R. T. (1967). Echinoderms. In Methods in Developmental Biology, F. H. Wilt and N. K. Wessells, eds. (New York: Thomas Y. Crowell), pp. 139-155.

Hough, B. R., Smith, M. J., Britten, R. J. and Davidson, E. H. (1975). Sequence complexity of heterogeneous nuclear RNA in sea urchin embryos. Cell 5, 291-299.

Hough-Evans, B. R., Wold, B. J., Ernst, S. G., Britten, R. J. and Davidson, E. H. (1977). Appearance and persistence of maternal RNA sequences in sea urchin development. Dev. Biol. 60, 258-277.

Humphries, S., Windass, J. and Williamson, R. (1976). Mouse globin gene expression in erythroid and non-erythroid tissues. Cell 7, 267-277.

Kirby, K. S. (1965). Isolation and characterization of ribosomal ribonucleic acid. Biochem. J. 96, 266-269.

Kleene, K. C. and Humphreys, T. (1977). Similarity of hnRNA sequences in blastula and pluteus stage sea urchin embryos. Cell 12, 143-155.

Maxwell, I. H., Maxwell, F. and Hahn, W. E. (1977). Removal of RNase activity from DNase by affinity chromatography on agarose-coupled aminophenyl-phosphoryl-uridine-2'(3')-phosphate. Nucleic Acids Res. 4, 241-246.

Nemer, M., Graham, M. and Dubroff, L. M. (1974). Co-existence of non-histone messenger RNA species lacking and containing polyadenylic acid in sea urchin embryos. J. Mol. Biol. 89, 435-454.

Perlman, S. M., Ford, P. J. and Rosbash, M. M. (1977). Presence of tadpole and adult globin RNA sequences in oocytes of Xenopus laevis. Proc. Nat. Acad. Sci. USA 74, 3835-3839.

Roeder, R. G. and Rutter, W. J. (1970). Multiple ribonucleic acid polymerases and ribonucleic acid synthesis during sea urchin development. Biochemistry 9, 2543-2553.

Ryffel, G. U. and McCarthy, B. J. (1975). Complexity of cytoplasmic RNA in different mouse tissues measured by hybridization of polyadenylated RNA to complementary DNA. Biochemistry 14, 1379-1385.

Scheller, R. H., Costantini, F. D., Kozlowski, M. R., Britten, R. J. and Davidson, E. H. (1978). Specific representation of cloned repetitive DNA sequences in sea urchin RNAs. Cell, submitted for publication.

Smith, M. J., Chamberlin, M. E., Hough, B. R. and Davidson, E. H. (1974). Repetitive and non-repetitive sequence in sea urchin heterogeneous nuclear RNA. J. Mol. Biol. 85, 103-126.

Spector, D. H., Smith, K., Padgett, T., McCombe, P., Roulland-Dussoix, D., Moscovici, C., Varmus, H. E. and Bishop, J. M. (1978). Uninfected avian cells contain RNA related to the transforming gene of avian sarcoma viruses. Cell 13, 371-379.

Table 1. Characteristics of Blastula mDNA

Specific activity	8×10^6 cpm/μg
"Zero-time" binding to hydroxyapatite[a]	$\leq$ 1%
Reaction with whole DNA at Cot 55	3.5%
Reaction with whole DNA at Cot 30,000	92%
Reaction with blastula mRNA[b]	73%
Blastula sequence purity[c]	78%
Purification[d]	37

[a]The percent of ^{3}H-mDNA which binds to hydroxyapatite when the tracer is denatured by boiling, quenched and immediately fractionated over hydroxyapatite under standard conditions (see Experimental Procedures).

[b]The value listed here is the percentage of ^{3}H-mDNA tracer hybridized by blastula mRNA at kinetic termination, and is derived from the data of Figure 2 before correction for tracer reactivity.

[c]Reaction of mDNA with blastula mRNA at kinetic termination, normalized for the 92% reactivity of the ^{3}H-mDNA preparation.

[d]Calculated by dividing the fraction of ^{3}H-mDNA which reacts with blastula mRNA by the fraction of total single copy DNA which reacts with the mRNA: 78/2.1 = 37.

Table 2. Kinetics of Nuclear RNA Hybridizations with Total Single Copy DNA and Blastula mDNA

Nuclear RNA	Single Copy ^{3}H-DNA k ($M^{-1}sec^{-1}$)	Blastula ^{3}H-mDNA k ($M^{-1}sec^{-1}$)
Gastrula	0.8×10^{-4}[a]	1.3×10^{-4}
Intestine	2.5×10^{-4}	3.1×10^{-4}

[a]This measurement was made with the same gastrula nuclear RNA preparation which was hybridized with blastula mDNA (column 2 and Figure 4).

Table 3. Transcription of Nuclear RNA and Complex Class mRNA Sequences in Sea Urchin Gastrula

Absolute synthesis rate for nuclear RNA	[a] 5×10^8 nucleotides $hr^{-1}nucleus^{-1}$
Single copy sequence complexity of nuclear RNA	[b] 1.7×10^8 nucleotides
Average number of nuclear transcripts of each sequence synthesized per hr	[c] 3 transcripts $hr^{-1}nucleus^{-1}$
Average number of transcripts of a complex class mRNA appearing in polysomes	[d] 0.2 transcripts $hr^{-1}cell^{-1}$
Ratio of transcripts of an mRNA sequence synthesized in nucleus to transcripts appearing in polysomes	[e] 15

[a]Data of Roeder and Rutter (1970) and Grainger and Wilt (1976) reviewed by Davidson (1976). This determination refers to the synthesis of total, rapidly turning over nuclear RNA.

[b]Hough et al. (1975).

[c]This value was obtained by dividing the absolute synthesis rate by the single copy sequence complexity of nuclear RNA. No correction has been made for

the 10 to 30% of nuclear RNA which is transcribed from repetitive sequences (Smith et al., 1974) or for the possible existence of a fraction of total nuclear RNA synthesis in a low complexity, prevalent class of transcripts (Hough et al., 1975). Each of these factors would lower the average number of transcripts synthesized per unit time. However, we believe their combined effect could not exceed a factor of three, and it is not at all clear that these factors are quantitatively significant for our present purposes.

[d]Galau et al. (1977b).

[e]Kleene and Humphreys (1977) calculated that in a Hawaiian sea urchin, Tripneustes gratilla, the nuclear rate of single copy sequence transcription is about equal to the rate of complex class mRNA synthesis. Their calculation depended on nuclear RNA synthesis rates which they inferred indirectly for T. gratilla embryos. These rates are much lower than those obtained by direct measurement for S. purpuratus (reviewed in Davidson, 1976). Kleene and Humphreys then compared their low T. gratilla nuclear RNA synthesis rate, 0.15 pg hr^{-1} per cell, with the complex class mRNA synthesis rate reported by Galau et al. (1977b) for S. purpuratus. Since according to Kleene and Humphreys these two species differ significantly with regard to the rate of nuclear RNA synthesis, it does not seem safe to assume that they do <u>not</u> differ with regard to mRNA synthesis rates.

Figure Legends

Figure 1. Hybridization of Single Copy ^{3}H-DNA with Blastula mRNA

The curve represents a least squares fit to the data assuming a single pseudo-first order reaction with an ordinate intercept of 0. Messenger RNA Cot is calculated on the basis that 4% of the polysomal RNA mass is mRNA (Galau et al., 1977b). The rate constant is $2.0 \times 10^{-3} M^{-1} sec^{-1}$ with respect to mRNA. The terminal hybridization value is 2.1%. Assuming asymmetric transcription, 4.2% of the total single copy complexity is represented. Since the complexity of the non-repetitive fraction of the S. purpuratus genome is 6.1×10^{8} nucleotide pairs (Graham et al., 1974), the complexity of the blastula mRNA is about 2.6×10^{7} nucleotide pairs. The right ordinate is in units of nucleotide pairs of complexity.

Three preparations of blastula mRNA were used, each obtained by puromycin release from purified polysomes. These RNAs were hybridized with two single copy ^{3}H-DNA tracers. Tracer preparation I was purified by standard methods (Galau et al., 1974) from total sea urchin ^{3}H-DNA which had been labeled in vivo to a specific activity of about 3×10^{5} cpm/μg. Single copy DNA preparation II was ^{3}H-labeled in vitro to approximately 8×10^{6} cpm/μg by "gap translation" (Galau et al., 1976; Hough-Evans et al., 1977) of purified single copy DNA with E. coli DNA polymerase I. Each single copy ^{3}H-DNA preparation was shown to be free of detectable repetitive sequence by the kinetics of its reassociation with excess total sea urchin DNA. The reactivity of the labeled DNA preparations was determined by the extent of reaction of the tracers with excess total DNA at Cot > 2.5×10^{4}. The data have been corrected for the reactivities of the ^{3}HDNA tracers.

Figure 1 - continued

(●) Polysomal mRNA preparation 1 and, (▲), polysomal mRNA preparation 2 reacted with single copy ^{3}H-DNA (specific activity = 3 x 10^{5} cpm/μg; reactivity with excess sea urchin DNA driver is 91%). (■) Polysomal mRNA preparation 3 reacted with single copy ^{3}H-DNA (specific activity = 8 x 10^{6} cpm/μg; reactivity with excess sea urchin DNA is 83%.

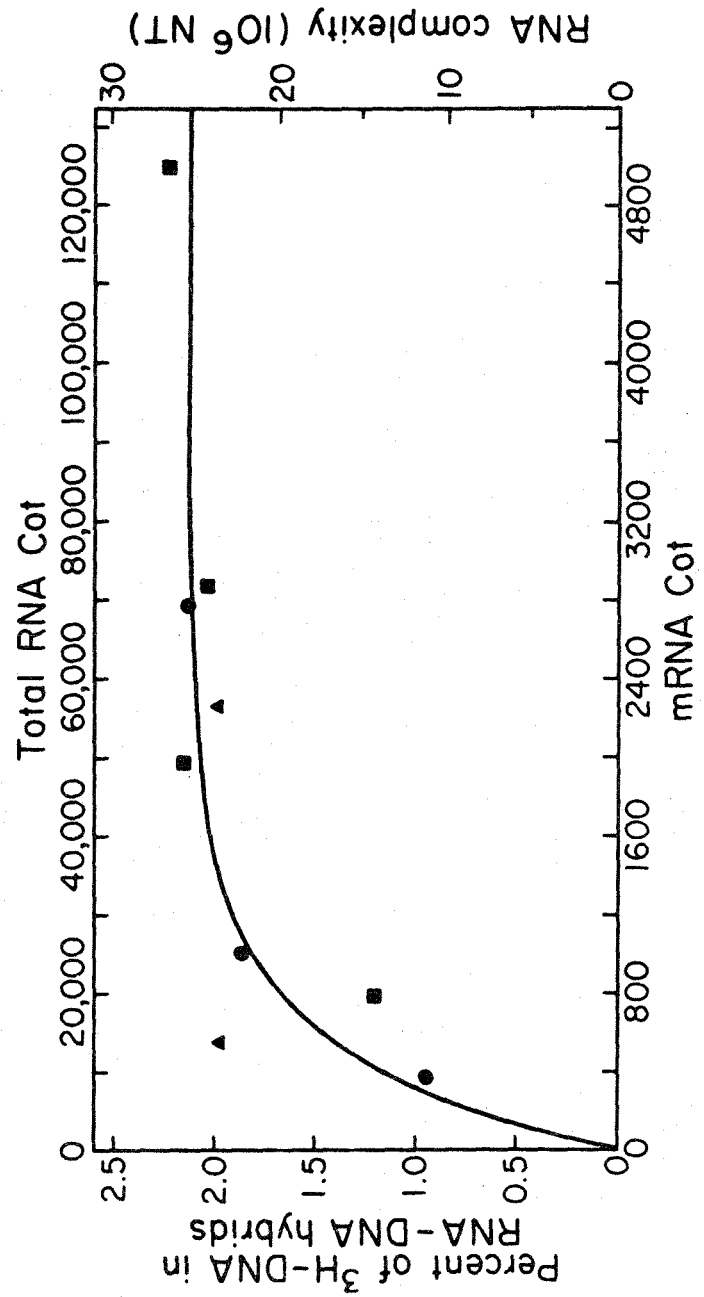

Percent of ^{3}H-DNA in RNA-DNA hybrids
2.5
2.0
1.5
1.0
0.5
0
0
800
1600
2400
3200
4000
4800
mRNA Cot
0
20,000
40,000
60,000
80,000
100,000
120,000
Total RNA Cot
30
20
10
0
RNA complexity (10^6 NT)

Figure 2. Hybridization of Blastula mDNA with Blastula Polysomal mRNA and Intestine Cytoplasmic RNA

The curves represent single pseudo-first order kinetic components fit to the data by least squares methods. (●) Blastula polysomal mRNA: at termination 78% of the ^{3}H-mDNA is in hybrid; the rate constant with respect to messenger RNA is $2.5 \times 10^{-3} M^{-1} sec^{-1}$, or $1.0 \times 10^{-4} M^{-1} sec^{-1}$ with respect to total polysomal RNA (see Experimental Procedures). (o) Blastula polysomal mRNA: before hydroxyapatite fractionation, the reaction mixture was treated with RNAase under low salt conditions which destroy the RNA in RNA-DNA hybrids (Galau et al., 1974). (■) Intestine cytoplasmic RNA: at termination, 12% of ^{3}H-mDNA is in hybrid; the rate constant, k, is $4.4 \times 10^{-5} M^{-1} sec^{-1}$. The data have been normalized for the 92% reactivity of the mDNA tracer. The right ordinate is calibrated in terms of RNA complexity, on the basis that the terminal hybridization value of 78% observed for blastula mRNA represents 2.6×10^{7} nucleotides.

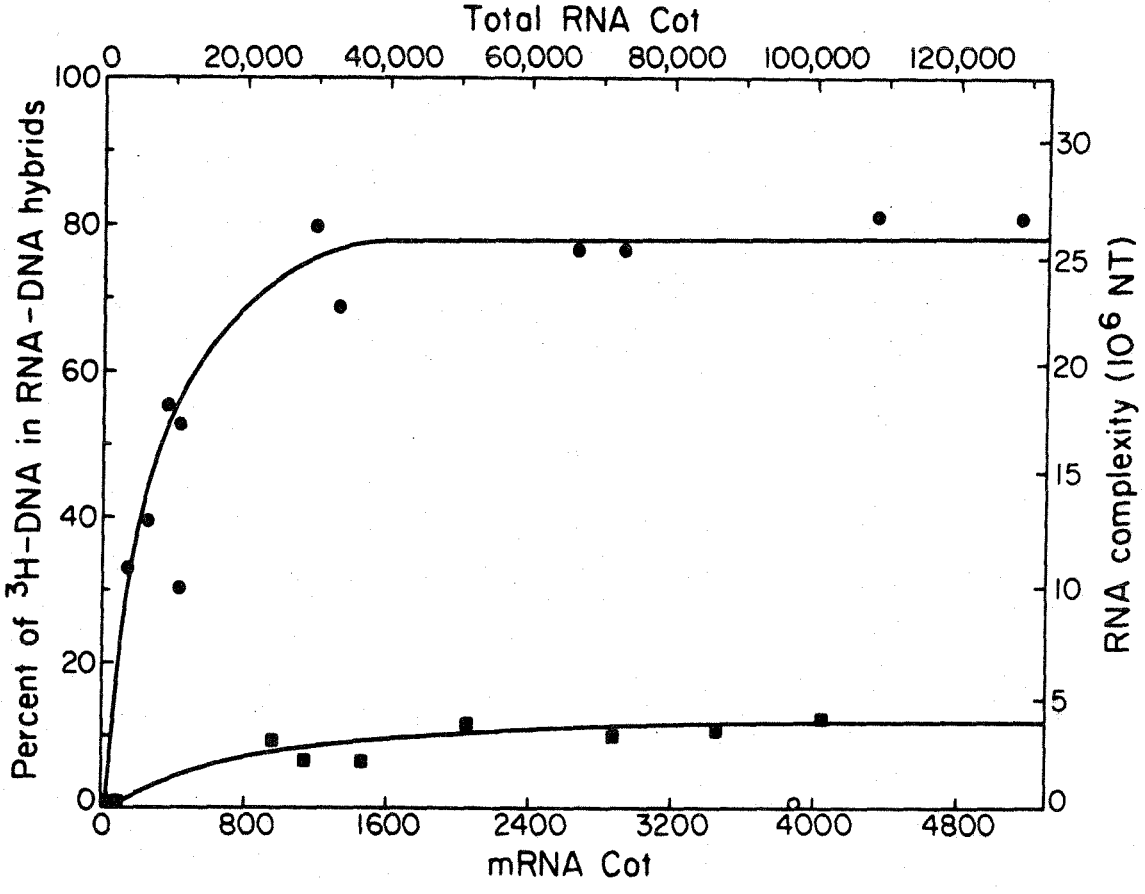

Total RNA Cot
0
20,000
40,000
60,000
80,000
100,000
120,000
Percent of ^{3}H-DNA in RNA-DNA hybrids
100
80
60
40
20
0
RNA complexity (10^6 NT)
30
25
20
15
10
5
0
0
800
1600
2400
3200
4000
4800
mRNA Cot

Figure 3. Hybridization of Intestine Nuclear RNA with Single Copy ^{3}H-DNA

The curve represents a single pseudo-first order reaction fit by least squares methods with the assumption that the ordinate intercept is 0. The solution shown yields a rate constant of $2.5 \times 10^{-4} M^{-1} sec^{-1}$ and a terminal value of 17.9%. The specific activity of the single copy tracer used was 8×10^{6} cpm/μg and its reactivity with excess sea urchin DNA at kinetic termination was 94%. The data have been normalized for the reactivity of the ^{3}H-DNA tracer.

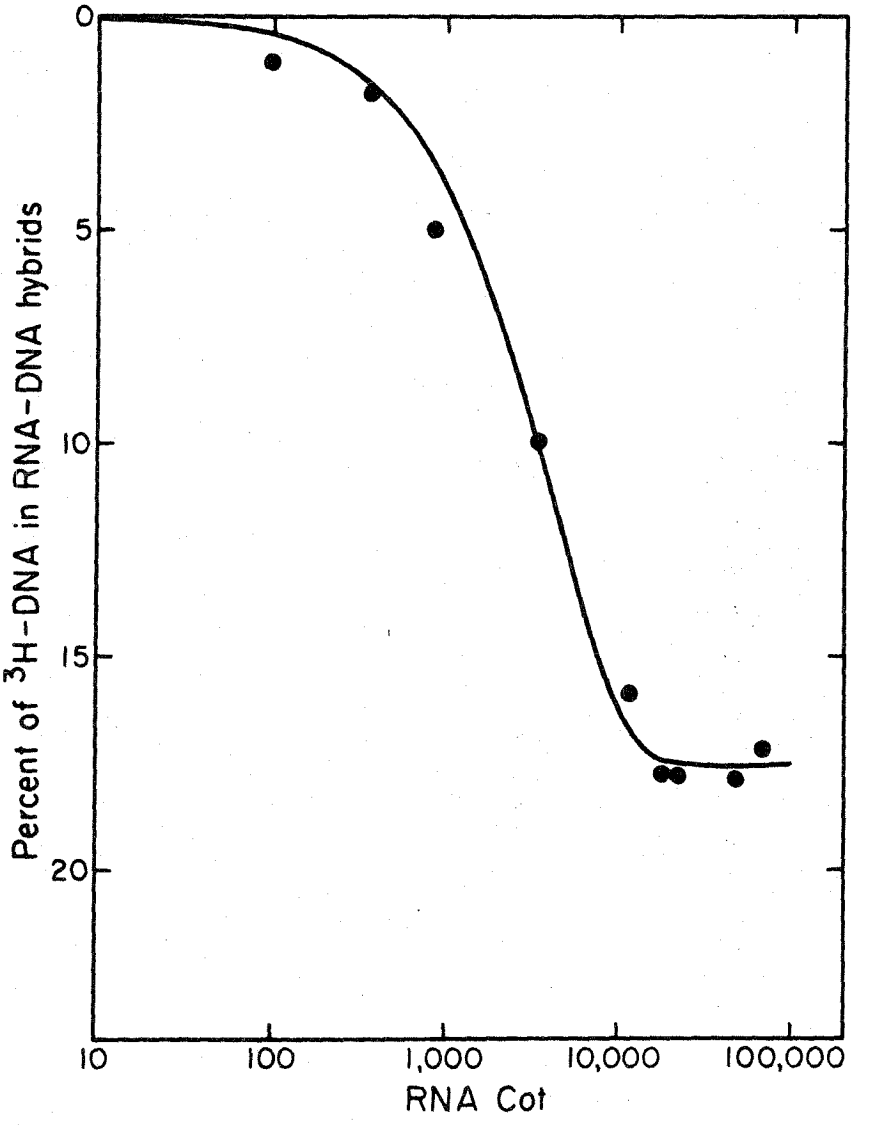
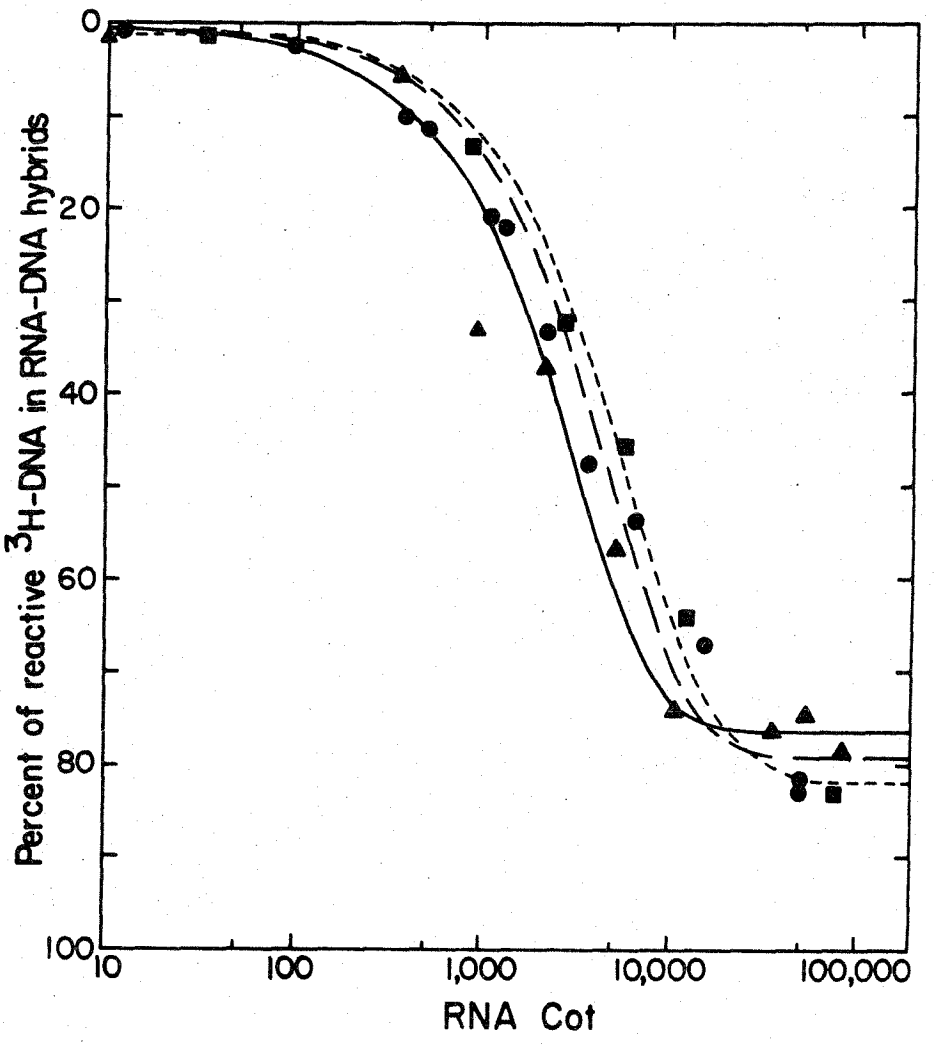

0
5
10
15
20
Percent of ^{3}H-DNA in RNA-DNA hybrids
10
100
1,000
10,000
100,000
RNA Cot

Figure 4. Hybridization of Blastula mDNA with Nuclear RNAs from Intestine, Coelomocytes and Gastrula Stage Embryos

The hybridization of each nuclear RNA with blastula mDNA is described by a single pseudo-first order reaction fit to the data by least squares methods. (— — ● — —) Coelomocyte nuclear RNA: the rate constant is $2.0 \times 10^{-4} M^{-1} sec^{-1}$; at termination 79% of the mDNA is hybridized. (- - - - ■ - - - -) Gastrula nuclear RNA: the rate constant is $1.3 \times 10^{-4} M^{-1} sec^{-1}$; at termination 82% of the mDNA is hybridized. (—— ▲ ——) Intestine nuclear RNA: the rate constant is $3.1 \times 10^{-4} M^{-1} sec^{-1}$; at termination 76% of the mDNA is hybridized. The data have been normalized for the 92% reactivity of the mDNA tracer.


Percent of reactive ^{3}H-DNA in RNA-DNA hybrids
0
20
40
60
80
100
10
100
1,000
10,000
100,000
RNA Cot