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Variations in Gene Transcription during Oogenesis and Early Embryogenesis of a Tubiculous Polychaete

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棲管 갯지렁이의 卵子形成과 初期發生期間에서의 遺傳子 發現의 變異

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적 요

棲管 갯지렁이(*Schizobranchia insignis*)의 卵子形成 및 初期發生 期間에 일어나는 단일유전자 발현의 변이에 대하여 DNA-RNA 分子結合法을 이용하여 연구하였다. 卵子形成 初期에 活性化되었던 유전자의 일부는, 卵子形成 과정이 진행됨에 따라 점차 억제되는 경향을 보였으며, 初期에 생성된 전사물질의 일부는 생성된 후, 卵子形成 과정에서 점차 소모되어, 末期에 잔존한 전사물질의 양은 사실상 初期에서 보다 적았다. 즉, 初期의 卵子는 末期에서는 존재하지 않는 단일유전자의 전사물질을 함유하고 있음이 확실하였다. 수정 후 발생단계를 통하여, 卵子形成 期間 중 발현된 유전자와는 다른 새로운 유전자의 발현이, Trochophore stage까지 계속 증가됨을 보여 주었다.

INTRODUCTION

It has been an established concept that gene transcripts expressed during oogenesis are stored and used during embryogenesis. This theory was supported by experiments employing physical enucleation (Brachet et al., 1963 a and b; Tyler, 1963; Denny and Tyler, 1964; Tyler, 1966) and chemical inactivation of gene transcription (Gross and Cousineau, 1964; Gross et al., 1964; Stavy and Gross, 1969). For instance, actinomycin-D-treated sea urchin eggs were able to synthesize protein as well as the normal eggs (Gross et al., 1964). RNA species present in the unfertilized eggs persist after fertilization and some RNAs are augmented by new transcription continuing throughout early development

(Whiteley et al., 1966; Glisin et al., 1966; Mizuno et al., 1973).

Conservation of various RNA species was studied in amphibians (Brown and Littna, 1964; Crippa et al., 1967; Crippa and Gross, 1969; Davidson et al., 1964; Davidson et al., 1966; Hough et al., 1973) and in sea urchin (Piatigorsky and Tyler, 1967). In amphibians ribosomal RNA and RNAs transcribed from the reiterated fractions of the genome were found to be synthesized at the lampbrush stage and retained throughout the rest of oogenesis and upto blastula stage (Davidson et al., 1966; Crippa et al., 1967). Such conserved RNAs are definitely required for the subsequent development after fertilization. However, development may not be achieved without introducing new transcripts in addition to the stored and augmented messages. In order to study whether new genes, which might be needed for the later stages of the development, are transcribed in the early stages and whether the extent of gene expression is varied during oogenesis, sequential hybridization experiments were undertaken with highly radioactive ^{125}I-labeled unique DNA.

MATERIALS AND METHODS

Collection of oocytes and embryos

Oocytes and embryos were collected from the females of *Schizobranchia insignis*, which are abundant in Friday Harbor, Washington, U.S.A. Oocytes were separated into size classes as described in details in elsewhere (Lee and Whiteley, 1979). Oocyte suspension was layered on to 280 ml of a sucrose step gradient (0.3 M, 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.825 M and 0.875 M sucrose) made up in Millipore-filtered sea water (MFSW) at 0°C. The oocytes were allowed to settle through the gradient for 60 minutes, and collected from the top by displacement with 1.5 M sucrose solution. Each size class was collected by centrifugation and frozen in RNA-homogenizing medium (0.1 M sodium acetate, 0.01 M NaCl, 10^{-3} M $MgCl_2$, pH 5.0) containing 2 mg/ml bentonite. The average size of each size class separated in this way was 25 μ, 37 μ, 50 μ, 80 μ, 100 μ, 130 μ and 180 μ in diameter, respectively.

The unfertilized eggs, which are available only during breeding season (January—March) were obtained from the females mechanically stimulated to spawn by rubbing. The eggs were fertilized by the sperm obtained directly from the males. The eggs at 5% suspesion were cultured in MFSW at 10-12°C with gentle stirring. At this temperature the embryos reach 4-8 cell stage at about 4 hours and the trochophore larval stage at about 50 hours.

Extraction of RNA

RNA was extracted from the oocytes and embryos as described by Whiteley et al. (1966).

Extraction of DNA

DNA was extracted from ccelomic sperm of the males as described by Whiteley et al. (1966 and 1970) and subjected to deproteinization by repeated shaking with chloroform-octanol (10 : 1) and ethanol precipitation.

Labeling DNA with Iodine-125 and preparation of unique ^{125}I-DNA

The method of labeling DNA with ^{125}I-DNA was adapted from Mizuno(1973), who modified the original Cummerford's method (1971), except that sheared native DNA was used rather than denatured DNA. Fifteen μl of ^{125}I (Amersham /Searle, 5 mCi/50 μl) was treated with 35 μl of 6×10^{-5} M Na_2SO_3—0.257 N H_2SO_4 before the first incubation. This incubation was carried out at 70°C for 50 minutes in 0.3 ml of reaction mixture (0.1 M sodium acetate, pH 5.0, 7.5×10^{-4} M $TlCl_3$, 7.5×10^{-5} M KI). The iodination was terminated by introducing 0.3 ml of 0.4 N NaOH, 10^{-3} M Na_2SO_3 and the mixture was again incubated at 60°C for 20 minutes. The whole mixture was sephadexed through Sephadex G-50 column with 0.08 M phosphate buffer, 10^{-4} M Na_2SO_3. ^{125}I-DNA fractions were loaded on hydroxylapatite column and the excessive free ^{125}I was washed out with 0.08 M phosphate buffer. Finally ^{125}I-DNA was eluted with 0.4 M phosphate buffer.

Double-stranded ^{125}I-DNA was denatured by boiling and renatured in 0.4 M phosphate buffer, 40% formamide at 37°C to Cot 100, since the renaturation kinetics has shown that the unique DNA sequences comprise for the DNAs renatured at Cot value greater than 100 (Lee and Whiteley, 1979). This renatured ^{125}I-DNA was absorbed onto hydroxylapatite column (Biorad, HTP gel) at 60°C and the unabsorbed single-stranded DNA was collected.

Preparation of S1-nuclease

S1-nuclease, which specifically hydrolyze single-stranded DNA, was prepared from α-amylase (Sigma, crude, type IV-A) by the method developed by Sutton (1971) and modified on the basis of Vogt's method (1973). The enzyme prepared by Sutton's method was finally eluted from DEAE-cellulose column (Whatman DE-52) with 0.3 M NaCl, 0.04 M KH_2PO_4. The eluate was recylced through another column of DEAE-cellulose with Vogt's Tris buffer (1973), washing the column extensively with Tris buffer and the enzyme being eluted with Tris buffer supplemented with 0.25 M NaCl. The S1-nuclease was stored at −20°C with an equal volume of 50% glycerol.

Sequential molecular hybridization

Sequential molecular hybridization reactions were carried out by hybridizing ^{125}I-DNA with RNA from one stage and additionally with the RNA from the other stage still in the presence of the first RNA. The extents of the ^{125}I-DNA-

RNA hybrids formed during the sequential hybridization reactions were determined by counting the radioactivity of the hybrids. For the first hybridization 0.36 μg of ^{125}I-DNA and 2.88 mg of RNA from one stage were mixed in 720 μl of 0.3 M Nacl, 0.05 M phosphate buffer in a sealed glass tube (0.8×5 cm) on ice. The reaction mixture was boiled for 6 minutes, transferred to 66°C water bath and incubated for 30 hours. This hybridization condition was found to lead the reactions to the saturation level from the preliminary experiment. After the first hybridization the glass tube containing the reaction mixture was subdivided into 6 hybridization tubes, each receiving 100 μl. To each hybridization tube was added 600 μg RNA in 50 μl from homologous or heterologous stages. The hybridization tube containing 4 mg/ml of the second RNA in a total voulme of 150 μl was sealed and incubated for 30 hours at 66°C. At the end of the second hybridization reaction each tube was chilled in ice water, opened by scribing with a diamond pencil, and the contents tested for DNA—RNA duplexes. The contents were washed out of the tube directly into a test tube (1×10cm) containing 2 ml of S1-nuclease medium (0.03 M sodium acetate, pH 4.4, 0.15 M NaCl, 10^{-4} M $ZnSO_4$), and treated with 100 μl of S1-nuclease at 50°C for one hour in the presence of 50 μg of denatured *S. insingnis* DNA. ^{125}I-DNA—RNA hybrids were precipitated on ice with 200 μl of cold 100% trichloroacetic acid in the presence of 280 μg of yeast RNA as a carrier. Fifteen minutes later the precipitate was collected on Gelman glass fiber filter (type A, 25 mm diameter), washed with 3 ml of cold 10% TCA and 10 ml of cold 95% ethanol. Glass filters carrying ^{125}I-DNA-RNA were dried at 85°C for 1 hour. Radioactivity was counted in toluene-based scintillant with a Nuclear Chicago Scintillation counter.

The blank tubes were made by incubating 0.05 μg of ^{125}I-DNA in the hybridization buffer for zero time, 18 hours and 33 hours. S1-nucelase resistance was measured by counting TCA-precipitable radioactivity at the end of incubation, as done for the assay of the hybrids. The blank values were constant from zero to 33 hours at about 300±20 cpm and have been subtracted from the data presented below.

RESULTS

Kinetics and purity of S1-nuclease

Kinetic studies on S1-nuclease were carried out by incubating single-or double-stranded ^{125}I-DNA with the enzyme for various durations in order to establish experimental conditions for the optimal enzyme activity. The conditions examined were time of incubation and purity of the enzyme with respect to single-strand specificity. The S1-nuclease activity was assayed as described in the Methods. These experiments were undertaken in the presence of 500 μg of

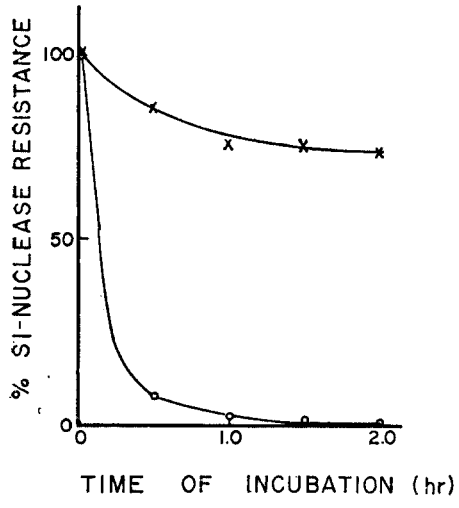

Fig. 1. Kenetic studies on S1-nuclease activity on single-and double-stranded DNAs. Repeated and unique ^{125}I-DNAs fractionated at Cot 100 were used as double-(x-x) and single-DNAs(o-o), respectively. The DNAs were digested with 40 μl of of S1-nuclease at 50°C in the presence of 500 μg of yeast RNA.

yeast RNA, since RNA introduced into the hybridization reactions might affect digestability of S1-nuclease. The results showed that the digestability of the single-stranded ^{125}I-DNA by the enzyme rapidly increases in 30 minutes and then asymptotically approaches to complete digestion with 98% digestion in one hour. (Fig. 1) Identical tests, in which sheared double-stranded ^{125}I-DNA was digested with the enzyme, resulted in about 25% digestion in one hour, and then reaching a plateau. Those ^{125}I-DNA fragments digested by S1-nuclease may not be double-stranded sequences but single-stranded DNA "tails" retained at the end of double-strands, when the native DNAs were sheared.

It was not, however, confirmed in the kinetic studies whether the double-stranded DNAs may be digested, even though their S1-nuclease resistance remains constant beyond 25% digestion. Since S1-nuclease preparation might have been contaminated with a trace amount of double-strand-specific DNAse, the extent of such enzyme activity was determined. For this purpose pure double-stranded ^{3}H-DNA with no single stranded DNA fragments was prepared. DNA of trochophore of *S. insignis* was labeled with ^{3}H-thymidine, extracted, sheared and then treated with S1-nuclease. This DNA was then absorbed onto hydroxyl-apatite previously equilibrated with 0.12 M phosphate buffer, and washed with 20 volumes of the same buffer at 60°C to remove single-stranded DNAs which must have been digested. Finally double-stranded DNAs were eluted with 0.4 M phosphate buffer. These ^{3}H-DNAs, which presumably have no tails at either end, were used to assay the presence of double-strand-specific DNAse activity in S1-nuclease preparation. Table I shows the results of the tests, in which pure double-stranded ^{3}H-DNA is 98.5% resistant to the enzyme and single-stranded DNA only 2% resistant. Single-stranded unique ^{125}I-DNA digested under the same conditions was 0.63% resistant. The enzyme as used is therefore largely specific for single-strands.

Table 1. Digestion of single and "tailless" double-stranded DNA by S1-nuclease

DNA	Strandedness of the DNA	Amount of S1-nuclease (μl)	TCA-precipitable counts (cpm)	S1-nuclease resistence (%)
^{3}H-DNA*	double	none	48,331	100 (input)
^{3}H-DNA*	double	40	47,608	98.5
^{3}H-DNA*	single	40	965	2.0
^{125}I-DNA**	single	none	41,778	100 (input)
^{125}I-DNA**	single	40	262	0.63

*H^{3}-DNA, which had been extracted from ^{3}H-thymidine-labeled trochophore larvae, was sheared, digested by S1-nuclease and recycled through hydroxylapatite, as described in the text.
**^{125}I-DNA is single-stranded unique DNA isolated at Cot 100.

Vairations in gene transcription during oogenesis and embryogenesis

Sequential hybridization experiments were undertaken to ask whether gene transcription varies during oogenesis and embryogenesis, and whether transcripts which are conserved throughout oogenesis are solely responsible for embryogenesis or new genes are transcribed in the early embryonic stages.

In the first series of experiments unique ^{125}I-DNA was first hybridized with RNA from 180 μ oocytes and additionally with RNA from each of 37 μ and 80 μ oocytes, 4~8 cell stage embryos and trochophore larvae. The results of the hybridization experiments are given in Fig. 2 and Table II. The extent of ^{125}I-DNA—RNA hybridization in the 180 μ oocytes corresponds to 2.4% of the unique DNA sequences. Since these transcripts present in 180 μ oocytes can not be entirely made at the same stage but retained from the young stage oocytes, the extent of the hybridization reflects the complexity of the genes transcribed in the early stages, and a fraction of early gene products may be conserved in 180 μ oocytes. In 37 μ previtellogenic oocytes and in 80 μ oocytes, just beginning to form yolk platelets, the extent of the hybridization was measured to be 4.12% and 3.02%, respectively, indicating that more species of RNAs transcribed in the previtellogenic stages than in the later stages. All of the genetic messages which have not been expressed in the early stages are not retained throughout the rest of the oogenesis, suggesting that some RNAs are utilized probably for driving oogenesis and degraded. Although the transcripts of the fully-grown oocytes may be the remnants of the previtellogenic oocytes, it was clearly shown that some genes which have been activated in the early stages are gradually suppressed as oogenesis proceeds.

The results of the experiments in which unique ^{125}I-DNA was saturated with the RNA of 180 μ oocytes and hybridized with the RNAs of the embryonic stages are also given in Fig. 2 and Table II. The extent of hybridization is 4.85% in the cell stage embryos and 10.37% in the trochophore larvae. These

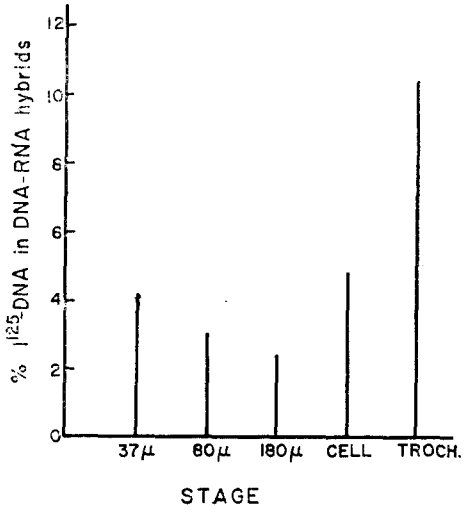

Fig. 2. Extents of hybrid formation of unique ^{125}I—DNA with RNAs obtained from various stages. ^{125}I-DNA was first hybridized with 4 mg/ml of homologous RNA from 180 μ oocytes for 30 hours and additionally hybridized with 4 mg/ml of heterologous RNA from 37 μ and 80 μ oocytes, 4—8 cell stage embryos (cell), and trochophore larvae (troch).

results indicate that new genes different from those producing the transcripts in 180 μ oocytes are activated even in the 4~8 cell stage and throughout the early developmental stages at least upto trochophore stage. The results obtained from the second series of hybridization experiments in which ^{125}I-DNA was first hybridized with the RNA from 37 μ oocytes and then rehybridized with the RNA from each of the various stages are given in Fig. 3 and Table III. A similar conclusion could be derived from these experiments; that is, previtllogenesis-specific genes are transcribed in the young oocytes and those transcripts are not entirely retained throughout the rest of oogenesis while the genes activated in the early stages are suppressed toward the end of oogenesis, and new gene informations are expressed during developmental stages in addition to the stored messages. It was, however, difficult to answer whether the vitellogenic oocytes transcribe the same genes that have been expressed during previtellogenic stages

Table 2. Sequential hybridization of ^{125}I—DNA with the RNA from various stages.

RNA for first hybridization	RNA for second hybridization	Actual counts in hybrids (cpm)	Extent of* hybridization (%)
180 μ oocytes	37 μ oocytes	1,543	4.12
180 μ oocytes	80 μ oocytes	1,130	3.02
180 μ oocytes	180 μ oocytes	899	2.40
180 μ oocytes	4—8 cell stage embryos	1,813	4.85
180 μ oocytes	Trochophore larvae	3,890	10.37

*The input count was 37,502 cpm.

(augmentation) or new gene transcription is introduced into the later oogenetic stages while those genes which have been activated are gradually suppressed. Such difficulty arose from an unexpected nature of the hybridization reaction, which is an equilibrium shift between complementary ^{125}I—DNA sequences and RNAs by the dilution of the first RNA with the addition of the second RNA. Such dilution effect was demonstrated in the second series of experiments (Fig. 3 and Table III). The extent of hybridization with RNAs of 80 μ and 180 μ oocytes is 4.70% and 4.28 %, respectively, which are lower than that of the homologous hybridizaiton (5.45%). In these hybridization reactions the concentration of RNA of 37 μ oocytes was first 4 mg/ml and shifted to 2.67 mg/ml in the second hybridization. Such dilution effect must have been included in every hybridization reaction, but the lowering effect was not always shown in every reaction. When there exist new transcripts in the RNA species for the second hybridization, the extent of hybridization was higher than that of the homologous hybridiza-

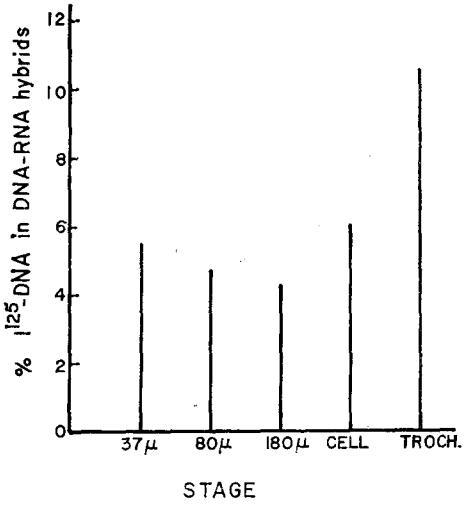

Fig. 3. Extents of hybrid formation of unique ^{125}I-DNA with RNAs obtained from various stages. The description is the same as in Fig. 2 except that the homologous RNA for the first hybridization was obtained from 37 μ oocytes.

Table 3. Sequential hybridization of ^{125}I—DNA with the RNA from various stages.

RNA for first hybridization	RNA for second hybridization	Actual counts in hybrids (cpm)	Extent of hybridization (%)*
37 μ oocytes	37 μ oocytes	1,910	5.45
37 μ oocytes	80 μ oocytes	1,624	4.70
37 μ oocytes	180 μ oocytes	1,494	4.28
37 μ oocytes	4—8 cell stage embryos	2,111	6.03
37 μ oocytes	Trochophore larvae	3,712	10.60

*The input count was 35,062 cpm.

tion (Fig. 3 and Table III). Although this fact may not be always true, the lowering effect tends to demonstrate that some of the gene transcripts present in the 180 μ oocytes are the same as those of 37 μ oocyes, whether these gene products are derived from the preservation of the previtellogenic transcripts or from the augmentation of the genes which were activated in the early stages of oogenesis.

DISCUSSION

Evidence that stored messages is preserved in the mature oocytes and becomes functional during postfertilization period has been supported by the studies on sea urchin eggs (Brachet et al., 1963 a and b; Denny and Tyler, 1964; Gross et al., 1964; Gross and Cousineau, 1964; Tyler, 1963) and on *Spisula* eggs (Gabrielli and Baglioni, 1975). In sea urchin eggs these egg RNAs have been shown to encode histones (Farquhar and McCarthy, 1973; Skoultchi and Gross, 1973), microtubule protein (Raff et al., 1972) and ribonucleotide reductase (Noronha et al., 1972). In addition a large number of uridentified peptides have been translated *in vivo* from this RNA (Gross et al., 1973). Gabrielli and Baglioni (1975) showed that stored RNA isolated from the polysomes of the *Spisula* eggs can be translated *in vitro* into many peptides. The amount of information encoded in the RNA of *Schizobranchia* eggs potentially sufficient for many peptides than have so far been revealed for the urchin and surf clam, but the actual number of different messages and the identity of proteins encoded are unknown.

However. the sequential hybridization experiments showed that the early stage embryos transcribe new unique genes, which have not been transcribed in the early and/or late stages of oogenesis. The stored maternal messages may be sufficient to direct the early development upto certain stage, but new genetic information seems to be reqired for the late development. A few studies on the polysomal RNA in the early embryos of sea urchin (Rinaldi and Monroy, 1969, Kedes and Gross, 1969), of *Spisula* (Firtel and Monroy, 1970), and of the coot clam (Kidder, 1972 a and b) strongly suggested that the newly synthesized RNAs are translatable in the early stages. Direct evidence for the synthesis of new RNA was derived from the molecular hybridization studies of micromere RNA of 16-cell stage in the sand dollar (Mizuno et al., 1974). The micromeres synthesize specific RNA different from the egg RNA. This consideration and the present results suggest that the early stage embryos need transcription of new genes more than those inherited from the oocytes.

SUMMARY

Variations in expressions of unique genes during oogenesis and early embryogenesis of a tubiculous polychaete were studied by determining the extents of

gene transcriptions by sequential DNA—RNA molecular hybridizations. The genes which had been activated in the early stages of oogenesis (previtellogenesis) were gradualy suppressed during the subsequent stages of oogenesis. The transcripts that had been synthesized upto the stages examined were utilized and degraded throughout the vitellogenic stages, and thus, the amount of the transcripts remaining in the fully-grown oocytes was much smaller than that of the previtellogenic oocytes. During the post-fertilization period new genes were transcribed even in the 4—8 cell stage embryos, and the extent of transcription of new genes continues to increase at least upto the trochophore stage.

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