

RNA Complexity in Developing Sea Urchin Oocytes

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Nuclear and cytoplasmic RNAs extracted from previtellogenic and vitellogenic oocytes of the sea urchin *Strongylocentrotus purpuratus* were characterized by hybridization reactions with radioactively labeled single-copy sea urchin DNA. The complexity of nuclear RNA from previtellogenic oocytes was 1.6×10^8 nucleotides. The previtellogenic nuclear RNA sequence set is included in the hnRNA of gastrula stage embryos. The nuclear RNA of vitellogenic oocytes may also contain a class of more prevalent transcripts. A single-copy [³H]DNA tracer enriched for the sequences of mature egg RNA was reacted with cytoplasmic RNA of previtellogenic oocytes. This experiment showed that less than half of the mature egg RNA sequence set is accumulated before the onset of vitellogenesis. Therefore, a large fraction of the maternal message sequences appears in the egg during the last several weeks of oocyte development.

INTRODUCTION

The mature sea urchin egg contains RNA molecules stored for use during early development (reviewed by Davidson, 1976). In *Strongylocentrotus purpuratus* eggs, the complexity of the maternal messages is about 37×10^6 nucleotides, and there are about 1600 copies of each mRNA sequence (Hough-Evans *et al.*, 1977). Very little is known about the accumulation of the maternal mRNA sequence set during oogenesis. Galau *et al.* (1976) reported the complexity of polysomal RNA from whole immature ovaries to be 20×10^6 nucleotides, or about half the complexity of the mature egg RNA. The total cytoplasmic RNA of such ovaries contains about three-quarters of the maternal RNA sequence set (Hough-Evans *et al.*, 1977). Leahy *et al.* (1978; unpublished observations) found from oocyte counts after ovarian dissociation that ovaries of gravid *S. purpuratus* which contain $1-2 \times 10^7$ mature oocytes also contain about $0.5-2 \times 10^6$ vitellogenic oocytes and about 10^8 previtellogenic oocytes and oogonia. Vitellogenic oocytes are present in the ovaries

from a few weeks before ripe eggs first appear until just prior to the end of the gravid period. A gravid female may produce a full clutch of eggs at least four times in one season, and on each occasion she may shed over 10-fold more eggs than the steady-state content of vitellogenic oocytes in her ovaries. It follows that the vitellogenic stage is relatively short, and may last only 2-4 weeks. During the rest of the year, the ovary contains a population of around 10^8 oogonia, primary oocytes, and previtellogenic oocytes. Holland and Giese (1965) reported that premeiotic DNA synthesis was observed late in the gravid season of intertidal females. However, they were unable to demonstrate the appearance in mature oocytes of DNA labeled during premeiotic replication. Although this can probably be attributed to difficulties in maintaining animals for a sufficiently long period of time, or to other technical problems, it cannot be excluded that primary oocytes may remain in the ovary for more than a year before advancing into vitellogenesis and maturity.

Cytological examination has shown that the ovaries are covered by a peritoneal ep-

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ithelium and a wall of connective tissue and smooth muscle. The inner epithelium includes accessory cells (or "nutritive phagocytes") as well as germinal cells (Tennent and Ito, 1941; Gross *et al.*, 1965; Holland and Giese, 1965; Anderson, 1968; Chatlynne, 1969). Although some nongerminal cells are thus present in disaggregated preparations of ovaries, the majority of cells is small previtellogenic oocytes. In the first section of this paper, we report the complexity of the nuclear RNA of these small oocytes. We then compare their nuclear RNA sequences with the sequences present in gastrula-stage embryo nuclear RNA. Measurements are also described which show that the cytoplasmic RNA sequence set of mature eggs accumulates largely during the final period of oogenesis, and is mostly lacking in the cytoplasm of previtellogenic oocytes.

MATERIALS AND METHODS

Previtellogenic and vitellogenic oocytes. Mature female sea urchins (*Strongylocentrotus purpuratus*) were maintained in our laboratory holding system (as described by Leahy *et al.*, 1978). Their oocytes are classified mainly by size. Transparent previtellogenic oocytes are less than 50 μm in diameter; vitellogenic oocytes are 50–80 μm in diameter, and their cytoplasm is opaque due to the increasing amounts of yolk. Mature oocytes are 80–100 μm in diameter (see Giudice *et al.*, 1972; Giudice, 1973; Leahy *et al.*, 1978). Ovaries from nonreproductive (i.e., "out of season") animals contained only primary and small previtellogenic oocytes. The ovaries of animals about to enter the reproductive state contain, in addition, vitellogenic oocytes. Ovaries were sheared briefly in a Waring Blendor to disrupt the tissue and the cells, and to release nuclei. To some small extent, the nuclear RNA preparations were probably contaminated with cytoplasmic RNA from previtellogenic oocytes. Diphenylamine and phloroglucinol determinations of DNA and RNA content were carried out on sample nuclear pellets

(Burton, 1956; Dishe and Borenfreund, 1957).

Previtellogenic oocyte nuclear RNA. RNA was extracted essentially as described by Scheller *et al.* (1978). Ovaries from one or two sea urchins were examined by phase microscopy; only ovaries in which all of the oocytes were less than 50 μm in diameter were used. The ovaries were minced and washed in Ca^{2+} - Mg^{2+} -free seawater brought to pH 3 with citric acid. The minced ovaries were then stirred at room temperature in Ca^{2+} - Mg^{2+} -free seawater containing 1 mg Pronase/ml for 1 hr, and the suspension of oocytes was filtered through gauze to remove ovarian membranes. Fifty to one hundred milliliters of homogenization buffer (2 mM MgCl_2 , 10 mM Pipes [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], pH 6.5, 10 $\mu\text{g}/\text{ml}$ PVS (polyvinyl sulfate), 1 M glucose) was added. The ovaries were sheared in an ice-cold Waring Blendor for about 1 min, or until few whole cells could be seen under the phase microscope. Nuclei were pelleted at 5000 rpm, and resuspended and lysed in 7 M urea, 50 mM sodium acetate (pH 5.1), 10 mM EDTA, 15 mM EGTA, 2% SDS (sodium dodecyl sulfate), 10 $\mu\text{g}/\text{ml}$ PVS. The solution 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, to which about 2 mg/ml Bentonite was added, the interface was suspended in the same 7 M urea buffer, containing, in addition, 1 M sodium perchlorate, and reextracted with the phenol-chloroform mixture. The aqueous phases were combined and extracted once with the phenol-chloroform mixture and 2 \times with chloroform:isoamyl alcohol (24:1), and then precipitated at -20°C with 2 vol 100% ethanol. The precipitate was dissolved in 10 mM Pipes (pH 6.5), 5 mM MgCl_2 . This opalescent solution was centrifuged at 20°C for 30–45 min at 50,000 rpm and the pellet was discarded. DNase I (Worthington) was pu-

rified by passage over a uridine-substituted agarose affinity column to remove ribonuclease activity (Maxwell *et al.*, 1977). The DNase was added to the supernatant to 100 $\mu\text{g}/\text{ml}$. After incubation for 2 hr at room temperature, the solution was brought to 0.1 *M* Tris (pH 8.0), 0.2% SDS, 50 *mM* EDTA, and incubated with 50 μg proteinase K (E. Merck)/ml for 1 hr at 37°C. The solution was extracted with the phenol-chloroform mixture and with chloroform: isoamyl alcohol, and precipitated with ethanol. The RNA precipitate was dissolved in 0.3 *M* sodium 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 3 *mM* sodium acetate.

Previtellogenic oocyte cytoplasmic RNA. Ovaries containing only previtellogenic oocytes were removed from four sea urchins as described above for previtellogenic oocyte nuclear RNA. The same procedures were used to wash and shear the ovaries; then the nuclei were pelleted at 10,000 rpm for 10 min, and discarded. The supernatant was added to 5 vol 7 *M* urea buffer. Extraction and purification of supernatant RNA were carried out as just described.

Vitellogenic oocyte nuclear RNA. Female sea urchins were injected with 1-2 ml 0.5 *M* KCl, and allowed to shed mature eggs for about 30 min. The ovaries were then dissected out and monitored for the presence of vitellogenic (50- to 80- μm -diameter) oocytes. These ovaries were minced and washed in Ca^{2+} - Mg^{2+} -free seawater, pH 3. The ovarian tissue was stirred with a magnetic stirrer for about 1 hr, at room temperature, in Ca^{2+} - Mg^{2+} -free seawater, pH 3, causing the oocytes to be released from ovarian tissue. The oocyte suspension was poured through gauze into an ice-cold beaker and examined microscopically. Aliquots of 5-10 ml were placed in 50-ml Nalgene tubes and underlayered with about 10 ml 1 *M* sucrose. The tubes were centrifuged at 3000 rpm in a DuPont (Sorvall) HB-4

rotor for 2 min, and the solutions separated into approximately equal supernatant, intermediate, and sucrose layers. The vitellogenic oocytes were concentrated in the intermediate layer, while previtellogenic oocytes generally pelleted. However, the separation from previtellogenic oocytes was not complete. The oocytes in the pooled intermediate layers were sheared in the Waring Blendor, and nuclear RNA was extracted as described above for previtellogenic oocytes.

Gastrula nuclear RNA. Sea urchin embryos were cultured for 36 hr to gastrula stage (Smith *et al.*, 1974). Nuclei were prepared and RNA was extracted as described in detail by Scheller *et al.* (1978).

Single-copy [³H]DNA. Unlabeled single-copy sea urchin DNA was prepared by harvesting single-stranded material after two successive incubations of total DNA to *C*₀t 200 (Graham *et al.*, 1974; Galau *et al.*, 1976). It was reassociated at a very high *C*₀t to promote hyperpolymer formation, and labeled *in vitro* by the gap translation method using *Escherichia coli* polymerase I. Labeled DNA was purified of unincorporated precursor, and of self-complementary "foldback" sequences generated during the labeling, by appropriate hydroxyapatite chromatography (Galau *et al.*, 1976; Hough-Evans *et al.*, 1977). The single-copy tracers produced had a fragment size of about 200-250 nucleotides and a specific activity of 10⁷ cpm/ μg under our (40% efficient) counting conditions.

[³H]DNA recovered from nuclear RNA hybrids. Single-copy [³H]DNA was incubated with excess nuclear RNA of previtellogenic oocytes to an RNA *C*₀t greater than 70,000. The hybridization mixture was treated with 10 μg ribonuclease A/ml in 0.24 *M* phosphate buffer for 1 hr at 4°C, and extracted with chloroform: isoamyl alcohol (24:1), before chromatography on hydroxyapatite under standard conditions. The double-stranded material was eluted from the column with 0.5 *M* phosphate buffer, diluted to 0.05 *M* phosphate buffer,

and digested with 10 μg ribonuclease A/ml at 37°C for 16 hr. Under these conditions, hybridized RNA is hydrolyzed, and the [^3H]DNA previously hybridized is left as single-stranded DNA fragments in solution. In a second passage over hydroxyapatite, this [^3H]DNA is not bound. The solution containing single-stranded [^3H]DNA was treated with proteinase K to remove ribonuclease activity, extracted with chloroform:isoamyl alcohol (24:1), and concentrated by precipitation with calf thymus DNA carrier.

oDNA. Preparation of oDNA, a single-copy DNA tracer enriched for oocyte RNA sequences, is described in detail by Hough-Evans *et al.* (1977). Single-copy [^3H]DNA was incubated with excess total egg RNA (extracted from unfertilized eggs by the same procedures as previtellogenic oocyte nuclear RNA) to an equivalent C_{ot} greater than 50,000. The hybridized sequences were further enriched by a second reaction with egg RNA, and harvested.

The oDNA used in these experiments reacted 93% at a high C_{ot} with excess whole sea urchin DNA, and reassociated less than 7% at C_{ot} values of 10–35 $M\text{-sec}$. The residual zero-time binding measured 4%. The extent of reaction with egg RNA was 63%. A pseudo-first-order function with a rate constant of $10^{-4} M^{-1} \text{sec}^{-1}$ fit the data from this reaction with a root mean square error of 3.6%. This rate is close to that measured for the reaction of other single-copy [^3H]DNA tracers with egg RNA (Galau *et al.*, 1976; Hough-Evans *et al.*, 1977). The yield of oDNA in this preparation was 10% (see Hough-Evans *et al.*, 1977, for a detailed discussion of oDNA preparation and yield).

Hybridization of [^3H]DNAs with RNA and analysis of hybrid content. Single-copy [^3H]DNA and oDNA were incubated with excess unlabeled RNA in 0.4–0.5 M phosphate buffer, 0.1% SDS, at 60°C, after denaturation for 1–2 min at 98°C. RNA mass excess was $\geq 10^4$ for single-copy [^3H]DNA reactions. In oDNA reactions, the mass excess was at least 10^5 . RNA C_{ot} values ($M\text{-sec}$)

were calculated in terms of the total RNA mass. All RNA and DNA C_{ot} values referred to in this paper are *equivalent* C_{ot} values; 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^+ concentrations (Britten *et al.*, 1974).

Reaction mixtures containing RNA and total single-copy [^3H]DNA were analyzed by procedures described earlier, with minor modifications (Hough *et al.*, 1975; Hough-Evans *et al.*, 1977; Galau *et al.*, 1974, 1976). Reaction mixtures containing RNA and 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. Zero-time binding of 4% was subtracted from the total for each determination. Data were reduced by nonlinear least-squares analysis assuming pseudo-first-order kinetics, using the computer program described by Pearson *et al.* (1977).

RESULTS

Complexity of Previtellogenic Oocyte Nuclear RNA

RNA was extracted from nuclei of ovaries which contained no oocytes $>50 \mu\text{m}$ in diameter. This RNA was reacted in excess with a single-copy [^3H]DNA tracer, and tracer hybridization was analyzed by hydroxyapatite chromatography. The kinetics of these reactions are shown in Fig. 1. The kinetics are severalfold slower than usually observed with sea urchin nuclear RNAs, due apparently to the low fraction of the total RNA in the preparation which is heterogeneous in sequence. The reaction has not quite terminated even at the highest RNA C_{ot} attainable (1.2×10^5). If a single pseudo-first-order kinetic component is arbitrarily assumed, the data of Fig. 1 show that about 13.5% of the single-copy tracer is represented in the nuclear RNA. However, it is clear that this could be an underestimate, since the kinetics could be more complex.

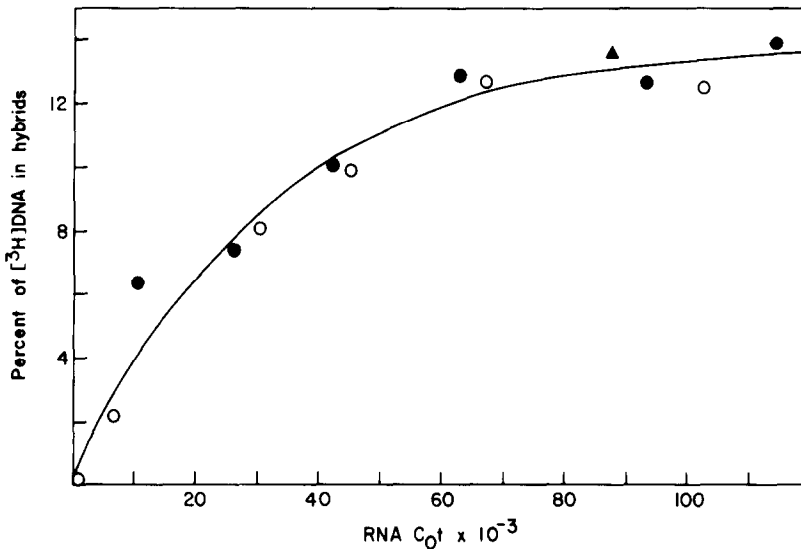


FIG. 1. Hybridization of single-copy $[^3\text{H}]$ DNA with excess nuclear RNA of previtellogenic oocytes. The pseudo-first-order function used to fit the data is $D/D_0 = \exp[-k C_0 t]$, where D/D_0 is the fraction of $[^3\text{H}]$ DNA remaining single stranded at time t , C_0 is the RNA concentration, and k is the pseudo-first-order rate constant. The terminal value obtained from this analysis is $13.5 \pm 0.9\%$ of the $[^3\text{H}]$ DNA hybridized, with a rate $k = 3.2 \pm 0.6 \times 10^{-5} M^{-1} \text{sec}^{-1}$. The root mean square error (RMS) was 0.9%. The value for $[^3\text{H}]$ DNA bound was corrected in each case for reactivity with whole sea urchin DNA of the particular single-copy $[^3\text{H}]$ DNA preparation hybridized. Reactivity of these tracers varied from 80 to 95%. Three preparations of previtellogenic oocyte nuclear RNA were hybridized in separate experiments: 1 (●), 2 (▲), and 3 (○). The RNA of preparation 2 was extracted from nuclei of isolated previtellogenic oocytes, and RNAs 1 and 3 from nuclei of whole ovaries containing only previtellogenic oocytes.

We next prepared a $[^3\text{H}]$ DNA tracer consisting primarily of the single-copy sequences represented in previtellogenic oocyte nuclear RNA. This was accomplished by annealing single-copy $[^3\text{H}]$ DNA with the nuclear RNA and isolating the hybridizing sequences, as described in Materials and Methods. Figure 2 shows reactions of this $[^3\text{H}]$ DNA fraction with excess total sea urchin DNA. The kinetics of this reaction are as expected for a single-copy tracer, except that a few percent of the hybridized DNA could consist of repetitive sequences. However, at least 85% of the reactable $[^3\text{H}]$ DNA obtained from the nuclear RNA hybrids consists of nonrepetitive sequences. Therefore, the extent of reaction in the experiment of Fig. 1 is a measure of the complexity of the previtellogenic oocyte nuclear RNA. Assuming asymmetric transcription of nuclear RNA (Hough *et al.*, 1975), and a single-copy complexity of the *Strongylo-*

centrotus purpuratus genome equal to 6.1×10^8 nucleotide pairs (Graham *et al.*, 1974), the complexity of the nuclear RNA is at least 1.6×10^8 nucleotides. From the rate of the reaction in Fig. 1, the concentration of most of the reacting sequences can be calculated (see, e.g., Galau *et al.*, 1974) to be about 0.5% of the total RNA in the preparation. To estimate the number of transcripts of each such sequence present per nucleus, the DNA and RNA contents of previtellogenic oocyte nuclear pellets were measured in two different preparations by the diphenylamine and phloroglucinol procedures. These data provide an estimate of 2.2 and 7.7 pg RNA/4C nuclear amount DNA [i.e., per 3.56 pg DNA; the haploid genome contains 0.89 pg DNA (Hinegardner, 1968)]. If only 0.5% of the RNA consists of the complex reacting sequences, there would be about 0.2–0.8 copy of each complex hnRNA sequence per av-

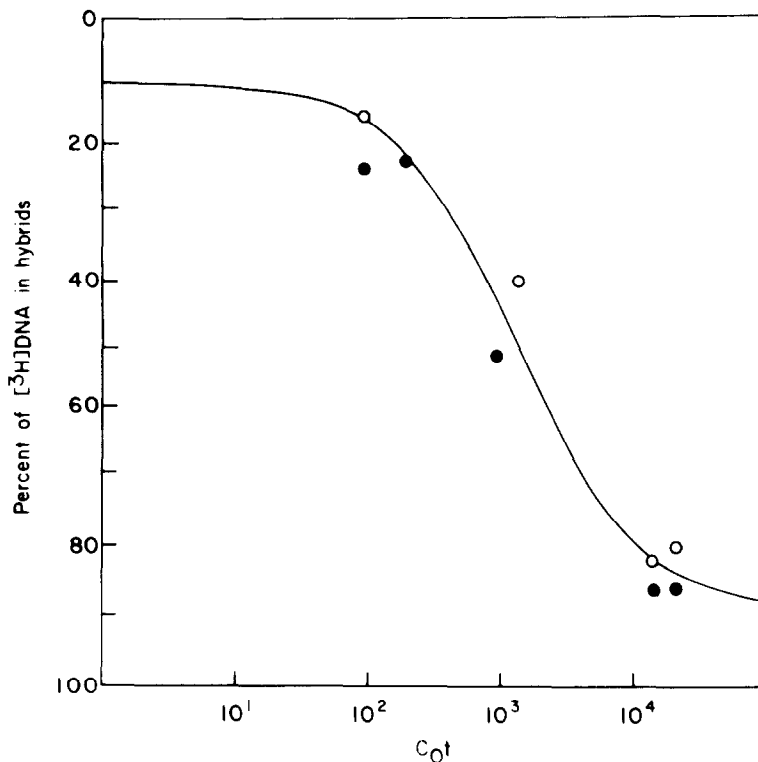


FIG. 2. Hybridization with excess total sea urchin DNA of $[^3H]DNA$ recovered from RNA-DNA hybrids with previtellogenic oocyte nuclear RNA. Second-order reaction kinetics were assumed (solid line). The rate constant is $0.7 \pm .3 \times 10^{-3} M^{-1} sec^{-1}$, which is close to the standard reaction rate of single-copy $[^3H]DNA$ with excess total sea urchin DNA (Galau *et al.*, 1974; Hough *et al.*, 1975). The RMS for the least-squares analysis was 6%. Two separate preparations of previously hybridized $[^3H]DNA$ were made: one (●) using nuclear RNA 1 of Fig. 1, and the other (O) using nuclear RNA 3 of Fig. 1. At very low C_0t values, less than 1% of the tracer bound to hydroxyapatite.

erage previtellogenic oocyte nucleus. However, this is to be regarded as a very rough calculation, since we assume that there is insignificant amplified rDNA in the sea urchin oocyte nuclei, as in those of other marine invertebrates (Brown and Dawid, 1968). Some of the nuclei in the preparation may not be 4C, having been derived from oogonia or accessory cells, and these might not contain all of the nuclear RNA sequences. Furthermore, a small contamination of the nuclear pellet with whole cells would increase the RNA/DNA ratio. The point we wish to make is that the complex nuclear RNA sequences of the immature oocyte are probably present in very few copies per nucleus, possibly less than one. In this respect, the nuclear RNA of those

oocytes is similar to that of embryos and adult somatic tissues (Hough *et al.*, 1975; Kleene and Humphreys, 1977).

Comparison of Oocyte Nuclear RNA Sequences with *Gastrula* Nuclear RNA Sequences

The $[^3H]DNA$ fraction recovered from hybrids with previtellogenic oocyte nuclear RNA was reacted again with this RNA, as shown in Fig. 3. Nuclear RNA prepared from sea urchin gastrulas was then reacted with the same selected $[^3H]DNA$ tracer. These data are also shown in Fig. 3. Note that the extent of reaction is, within error, the same in the two experiments of Fig. 3. It follows that the single-copy sequence set transcribed in the nuclei of embryos at the

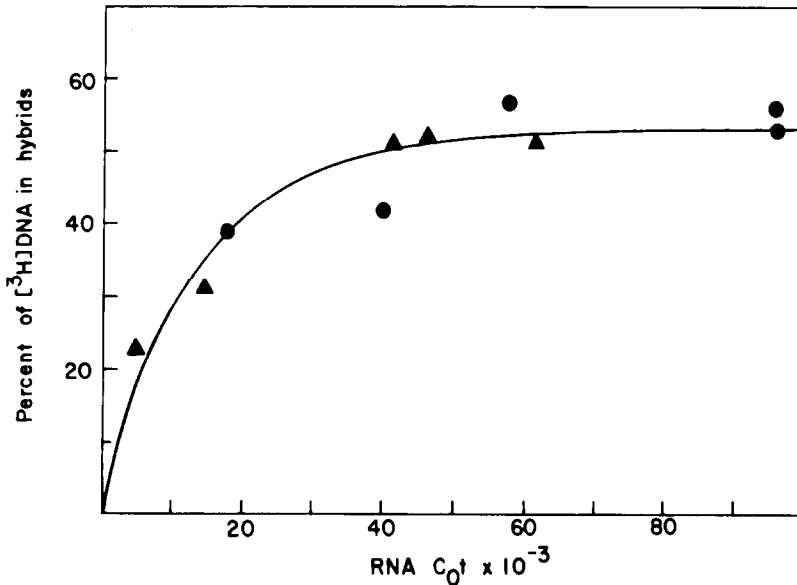


FIG. 3. Hybridization with gastrula and previtellogenic oocyte nuclear RNAs of single-copy $[^3H]DNA$ recovered from duplexes with previtellogenic oocyte nuclear RNA. A pseudo-first-order function was used to fit data from the reaction of this $[^3H]DNA$ with previtellogenic oocyte nuclear RNA (●), and with gastrula nuclear RNA (▲), with the assumptions that there is a single kinetic component and that the ordinate intercept is zero. The termination value of $53 \pm 2\%$ and rate constant of $7 \pm 1.4 \times 10^{-5} M^{-1} sec^{-1}$ were obtained by least-squares analysis.

gastrula stage includes essentially all of the single-copy sequences transcribed in the nuclei of previtellogenic oocytes. This conclusion, of course, does not refer to oocyte nuclear RNA sequences which are so rare that their hybridization would be detected only at an RNA C_0t beyond $10^5 M$ -sec.

Nuclear RNA of Vitellogenic Oocytes

One to two months before the appearance of mature eggs, vitellogenic oocytes are found in the ovaries, both in wild populations and in animals maintained over long periods in our laboratory culture system (Chatlynne, 1969; Gonor, 1973; Leahy *et al.*, 1978). Ovaries containing vitellogenic oocytes were dissociated, and the suspended oocytes were centrifuged over a cushion of 1 M sucrose, as described in Materials and Methods. Most of the previtellogenic oocytes pelleted through the sucrose, while the larger vitellogenic oocytes remained in the middle layer between the sucrose and the seawater. However, it was

not possible to exclude all previtellogenic oocytes. The ratio of vitellogenic oocytes to previtellogenic oocytes was increased about 10- to 20-fold from their starting ratio in the ovary, which is about $0.5-1 \times 10^{-2}$.

Hybridization of single-copy $[^3H]DNA$ with the nuclear RNA of the enriched vitellogenic oocyte preparation is shown in Fig. 4. The hybridization curve rises toward about 13% hybridization. However, the complex nuclear RNA sequence class responsible for this portion of the reaction could have derived from the previtellogenic oocytes also present (Fig. 1). Thus, the total complexity of the *vitellogenic* oocyte nuclear RNA per se is not established. An interesting feature of this RNA preparation, however, is that it may contain a set of prevalent transcripts. This is suggested by the form of the low RNA C_0t data (solid symbols) and by experiments (not shown) in which the tracer reacting at a low RNA C_0t was isolated and reacted again with the enriched vitellogenic oocyte nuclear RNA preparation.

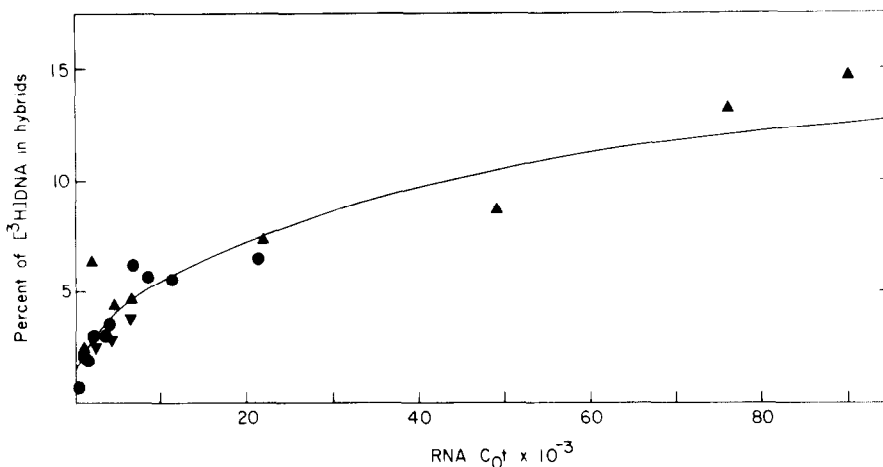


FIG. 4. Hybridization of total single-copy $[^3H]DNA$ with nuclear RNA from preparations enriched for vitellogenic oocytes. RNA of preparations A (●) and C (▼) was extracted from nuclei of oocytes selected and enriched for vitellogenic oocytes as described in the text. Preparation B (▲) RNA was extracted from nuclei of a total ovary homogenate, made from ovaries with a high vitellogenic oocyte content. Data from hybridizations of single-copy $[^3H]DNA$ with the three RNA preparations were analyzed assuming two pseudo-first-order kinetic components. Terminal extent of reaction was fixed at 14% (from Fig. 1). The kinetic components were 2.0% of the $[^3H]DNA$, reacting with a rate constant set at $3.5 \times 10^{-4} M^{-1} sec^{-1}$; and 10.6% of the $[^3H]DNA$, with a rate constant set at $2.3 \times 10^{-5} M^{-1} sec^{-1}$.

Accumulation of Single-Copy DNA Sequence Transcripts in Oocytes

Hough-Evans *et al.* (1977) showed that most of the single-copy transcripts stored in the cytoplasm of mature sea urchin eggs are maternal messages, since the same sequences appear on embryo polysomes at the beginning of development. In that study, embryo RNAs were hybridized with an "oDNA" (oocyte DNA) tracer highly enriched for the maternal RNA single-copy sequences set. A similar tracer was prepared and reacted with cytoplasmic RNA extracted from ovaries which had been examined carefully and found to contain no oocytes larger than $50 \mu m$ in diameter. These ovaries were washed, minced, and sheared in a Waring Blender. The nuclei were centrifuged out of solution at 1.5 times the force and for twice the time as in the preparation of nuclear RNA, to minimize contamination of the cytoplasmic supernatant with nuclei or whole cells. The cytoplasmic RNA was extracted from the post-nuclear supernatant. The oDNA tracer used in these experiments was enriched

about 25-fold for the 3% of total single-copy DNA which hybridizes with egg RNA. The tracer fraction was selected as those single-copy sequences bound to hydroxyapatite through two cycles of hybridization with egg RNA, as described in Hough-Evans *et al.* (1977). Reactions of the oDNA tracer with cytoplasmic RNA prepared from previtellogenic oocytes are shown in Fig. 5. Less than half of the mature egg RNA sequence set can be found in the previtellogenic oocyte cytoplasm. The oDNA does not hybridize with possible contaminating nuclear RNAs, since the reaction terminates at a level far lower than when oDNA is reacted with nuclear RNA (Fig. 6). Nor is there evidence of a gradual increase in hybridization at high RNA C_0t values. The upper curve in Fig. 5 shows the reaction of oDNA with mature oocyte RNA (normalized from 59% terminal reaction to 100%). The rate of reaction of oDNA with previtellogenic oocyte cytoplasmic RNA appears to be similar to that of the mature oocyte RNA. The implication is that maternal mRNA sequences are present at about the

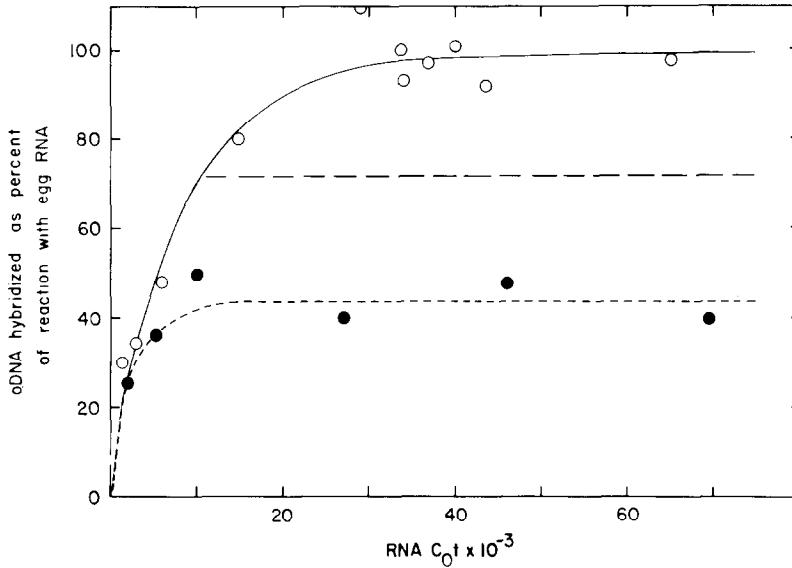


FIG. 5. Reactions of oDNA with egg and oocyte RNAs. Upper curve: oDNA hybridization with total mature egg RNA. The rate constant is $10^{-4} M^{-1} \text{sec}^{-1}$. Middle line: Reaction of oDNA with total RNA of immature ovaries (from Hough-Evans *et al.*, 1977). Lower curve: Hybridization of oDNA with cytoplasmic RNA of previtellogenic oocytes, assuming an ordinate intercept of zero. The fraction hybridized is $44 \pm 5\%$ of that reacting with egg RNA; the reaction rate constant was $4 \times 10^{-4} M^{-1} \text{sec}^{-1}$. This oDNA preparation reacted 59% with egg RNA and 26% with cytoplasmic RNA of previtellogenic oocytes, after the 4% zero-time binding was subtracted. The normalized data are shown as percentages of the total reaction of oDNA with egg RNA.

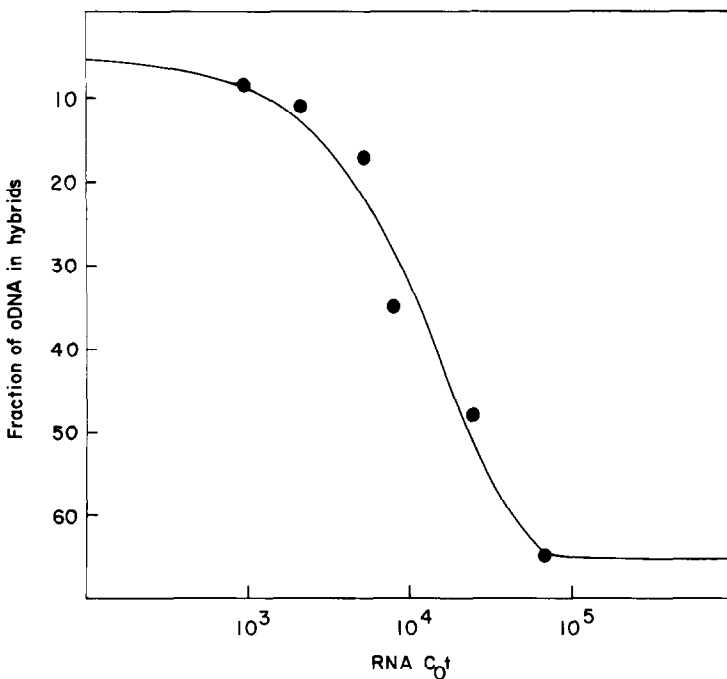


FIG. 6. Hybridization of oDNA with nuclear RNA of vitellogenic oocytes. A pseudo-first-order function was used to fit the data. The analysis showed $60 \pm 6\%$ of the oDNA reacting with a rate constant of $6 \times 10^{-5} M^{-1} \text{sec}^{-1}$. The oDNA reacted 59% with egg RNA.

same concentrations with respect to the total stored RNA at early stages as in the mature egg. The experiment shows that most of the single-copy sequences characteristic of the mature egg RNA are accumulated later in oogenesis, during the vitellogenic stages of oocyte development. The level of oDNA hybridization with total RNA of immature ovaries is also reproduced in Fig. 5, from Hough-Evans *et al.* (1977). This reaction amounted to 72% of the oDNA hybridization with mature egg RNA. The ovaries used in the latter experiment contained oocytes which were more mature than those of the present study. Apparently the storage of complex sequences of maternal RNA in oocyte cytoplasm is *sequential*, with most of the transcripts and most of the sequence diversity being added during vitellogenesis.

Since new maternal mRNA sequences are accumulated during vitellogenesis, it is conceivable that they are a major product of transcription during this period and are present at higher concentrations in vitellogenic oocyte nuclear RNA than are other single-copy sequence transcripts. To test this, the oDNA was reacted with the same vitellogenic oocyte-enriched nuclear RNA as used for the experiments in the preceding section. Figure 6 shows these reactions (●). The oDNA sequence set is completely represented in the nuclear RNA preparation, that is, at least 60% of it reacts, which is comparable to its reaction with mature oocyte RNA. This reaction could be due in part to a low concentration of contaminating cytoplasmic RNAs. Whatever the source of the RNAs reacting with the oDNA tracer, however, the kinetics of this reaction show that the concentration of transcripts complementary to oDNA (maternal message sequences) is similar to that of single-copy transcripts, in general, in this nuclear RNA.

DISCUSSION

Little information has been available regarding the transcriptional activities of sea

urchin oocytes during the long previtellogenic stages, other than autoradiographic evidence that some RNA synthesis occurs. Sconzo *et al.* (1972) showed that ribosomal RNA synthesis can be detected in vitellogenic oocytes of *Paracentrotus lividus*, and Gross *et al.* (1965) obtained evidence that heterogeneous repetitive sequence transcripts as well as ribosomal RNAs are labeled in near-terminal (i.e., vitellogenic) oocytes. On the other hand, the characteristics of the RNA stored in the mature sea urchin egg are relatively well known (see review in Davidson, 1976). This egg contains a complex set of maternal messenger RNAs which has been shown to include an enormous variety of single-copy structural gene transcripts, as well as large quantities of histone mRNA. Recently, Costantini *et al.* (1978) found that sea urchin egg RNA also contains a specific and heterogeneous population of transcripts complementary to interspersed repetitive sequences, which is different from that of the other sea urchin tissues studied. Almost certainly, the RNAs stored in the mature egg are of major significance in the processes of early development.

As a first step in examining the transcriptional activities of immature oocyte nuclei, we measured the complexity of their nuclear RNA. Figure 1 shows that the complexity of the previtellogenic oocyte nuclear RNA is at least 1.6×10^8 nucleotides, close to that of gastrula-stage embryo nuclear RNA (Hough *et al.*, 1975). The experiment of Fig. 3 demonstrates that the previtellogenic oocyte nuclear RNA sequence set is totally included in the gastrula nuclear RNA sequence set. While it is clearly possible that we have underestimated the complexity of the previtellogenic oocyte nuclear RNA (see Results), our calculations suggest that any transcripts not included in these measurements must be present at far less than one copy per immature oocyte nucleus. The complete inclusion of the measured previtellogenic oocyte germinal vesicle RNA sequence set in the gastrula nuclear

RNA sequence set must be considered in light of the fact that the cytoplasmic RNA (presumably message) sequence set of the previtellogenic oocyte and that of gastrula message are of similar complexity and overlap significantly (Galau *et al.*, 1976). On the other hand, it may be that nuclear RNAs of sea urchin cells in general contain largely overlapping single-copy sequence sets, regardless of differences in their mRNA populations (Kleene and Humphreys, 1977; Wold *et al.*, 1978).

The mature sea urchin oocyte contains about 50–100 pg maternal messenger RNA (reviewed in Davidson, 1976). About 1600 copies of each mRNA sequence transcribed from single-copy genes are present per egg (Hough-Evans *et al.*, 1977). The maternal mRNAs of the mature oocyte could derive from synthesis earlier in oogenesis, as in amphibians (Rosbash and Ford, 1974), although Davidson (1976) calculated that most of the maternal mRNA species could be synthesized within only a few hours. Our results, together with those presented earlier, show that this complex set of mRNAs is accumulated sequentially during oogenesis. Less than half the mature egg sequence set is represented in previtellogenic oocyte cytoplasmic RNA. The complexity of the latter RNA is about 1.6×10^7 nucleotides (44% of 3.7×10^7 nucleotides, the complexity of mature egg RNA; from Fig. 5). We estimate that there are about 600 copies of these mature egg RNA sequences present in previtellogenic oocyte cytoplasm. Of course, it is not known whether these are stored while new copies are slowly added, or whether there is a steady-state population of polysomal messages which continues to turn over until the polysomes finally disaggregate at the end of oogenesis. In either case, the remaining 56% of the maternal RNA sequence set is not loaded into the egg cytoplasm until after vitellogenesis is underway. This must be true even if all of the mature egg sequences are being transcribed as hnRNA in previtellogenic oocyte germinal vesicles. This seems likely from

the hybridization of oDNA with nuclear RNA of vitellogenic and previtellogenic oocytes, shown in Fig. 6, and from the results of Wold *et al.* (1978). The latter authors showed that the complete blastula mRNA sequences set is represented in adult intestine nuclear RNA, although 85% of it is absent from intestine cell polysomes.

The small number of copies of each complex maternal mRNA sequence in the egg argues against the idea that synthesis and storage of maternal message are quantitatively a major aspect of transcription in vitellogenic oocytes. However, it appeared worthwhile to test whether the prevalent nuclear RNA in oocyte preparations enriched for vitellogenic oocytes includes the maternal mRNA sequences. The data in Fig. 6 show that this is not the case. It follows that the maternal mRNAs are probably transcribed as typical rare nuclear RNA sequences present at steady state in only a few copies per nucleus. Were this so, a rough calculation suggests that it could require as long as 20 days, the approximate minimum length of the vitellogenic period, to accumulate the 1600 copies of each single-copy sequence transcript which are present on the average in the mature egg.

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REFERENCES

- ANDERSON, E. (1968). Oocyte differentiation in the sea urchin *Arbacia punctulata*, with particular reference to the origin of cortical granules and their participation in the cortical reaction. *J. Cell Biol.* **37**, 514–539.
- BRITTEN, R. J., GRAHAM, D. E., AND NEUFELD, B. R. (1974). Analysis of repeating DNA sequences by reassociation. In "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), 29E, pp. 363–418. Academic Press, New York.
- BROWN, D. D., AND DAWID, I. B. (1968). Specific gene amplification in oocytes. *Science* **160**, 272–280.

- BURTON, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Proc. Natl. Acad. Sci. USA* **62**, 315-323.
- CHATLYNNE, L. G. (1969). A histochemical study of oogenesis in the sea urchin *Strongylocentrotus purpuratus*. *Biol. Bull* **136**, 167-184.
- COSTANTINI, F. D., SCHELLER, R. H., BRITTEN, R. J., AND DAVIDSON, E. H. (1978). Repetitive sequence transcripts in the mature sea urchin oocyte. *Cell* **15**, 173-187.
- DAVIDSON, E. H. (1976). "Gene Activity in Early Development," 2nd ed. Academic Press, New York.
- 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.
- 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.
- GIUDICE, G. (1973). "Developmental Biology of the Sea Urchin Embryo." Academic Press, New York.
- GIUDICE, G., SCONZO, G., BONO, A., AND ALBANESE, I. (1972). Studies on sea urchin oocytes. I. Purification and cell fractionation. *Exp. Cell Res.* **72**, 90-94.
- GONOR, J. J. (1973). Reproductive cycles in Oregon populations of the Echinoid, *Strongylocentrotus purpuratus* (Stimpson). II. Seasonal changes in oocyte growth and in abundance of gametogenic stages in the ovary. *J. Exp. Mar. Biol. Ecol.* **12**, 65-78.
- 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.
- GROSS, P. R., MALKIN, L. I., AND HUBBARD, M. (1965). Synthesis of RNA during oogenesis in the sea urchin. *J. Mol. Biol.* **13**, 463-481.
- HINEGARDNER, R. T. (1968). Evolution of cellular DNA content in teleost fishes. *Amer. Natur.* **102**, 517-523.
- HOLLAND, N. D., AND GIESE, A. C. (1965). An autoradiographic investigation of the gonads of the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.* **128**, 241-258.
- 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.
- 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.
- LEAHY, P. S., TUTSCHULTE, T. C., BRITTEN, R. J., AND DAVIDSON, E. H. (1978). A large-scale laboratory maintenance system for gravid purple sea urchins (*Strongylocentrotus purpuratus*). *J. Exp. Zool.* **204**, 369-380.
- MAXWELL, I. H., MAXWELL, F., AND HAHN, W. E. (1977). Removal of RNase activity from DNase by affinity chromatography on agarose-coupled aminophenylphosphoryl-uridine 2'(3') phosphate. *Nucleic Acids Res.* **4**, 241-246.
- PEARSON, W. R., DAVIDSON, E. H., AND BRITTEN, R. J. (1977). A program for least squares analysis of reassociation and hybridization data. *Nucleic Acids Res.* **4**, 1727-1737.
- ROSBASH, M., AND FORD, P. J. (1974). Polyadenylic acid-containing RNA in *Xenopus laevis* oocytes. *J. Mol. Biol.* **85**, 87-101.
- 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* **15**, 189-203.
- SCONZO, G., BONO, A., ALBANESE, I., AND GIUDICE, G. (1972). Studies on sea urchin oocytes. II. Synthesis of RNA during oogenesis. *Exp. Cell Res.* **72**, 95-100.
- 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.
- TENNENT, D. H., AND ITO, T. (1941). A study of the oogenesis of *Mespilia globulus* (Linné). *J. Morphol.* **69**, 347-404.
- 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* **14**, 941-950.