Metabolism of Low Molecular Weight Ribonucleic Acids in Early Sea Urchin Embryos†

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ABSTRACT: There are three major low molecular weight RNAs (150–300 nucleotides) larger than 5S rRNA present in sea urchin embryos. Two of these are localized in the nucleus and one is localized in the cytoplasm. The nuclear species contain "capped" 5′ termini, with a cap I structure. These RNAs are synthesized starting in late cleavage and continuing through pluteus. Relative to 5S RNA there is a 10-fold change in the rate of synthesis of these RNAs, due primarily to a decrease in their rate of transcription after blastula. The RNAs are metabolically stable and the nuclear RNA genes are reiterated 50–100 times in the genome. Significant amounts of these RNAs are present in sea urchin eggs, enough to supply the embryo during early cleavage, prior to initiation of their synthesis.

Animal cell nuclei contain a number of small homogenous RNA molecules (Weinberg & Penman, 1968; Moriyama et al., 1969; Marzluff et al., 1975; Frederiksen et al., 1974). On a molar basis these RNAs are the most abundant in the cell except for the rRNAs. These molecules are found exclusively in nuclei (Brown & Marzluff, 1978), possibly associated with the nuclear matrix (Miller et al., 1978). In mammalian cells they are highly modified and at least four of them contain a "cap" structure similar to that found in mRNA except that the 7-methylguanosine found in mRNA is replaced by 2,-2,7-trimethylguanosine (Ro-Choi et al., 1975; Fernandez-Munoz et al., 1977). The role of these small nuclear RNA molecules remains unknown, but their relative constancy from one tissue to another, their homogeneity, and their limited number combine to imply a structural role rather than a regulatory role, or possibly a general role in rRNA and/or in mRNA formation.

Early sea urchin embryos provide a system in which gene expression is continually changing as is the rate of cell division. In particular, structural RNAs, rRNA and tRNAs, are not prominent products early in development (Emerson & Humphreys, 1970; Sconzo & Giudice, 1971), while there is substantial mRNA accumulation during this period (Galau et al., 1977; Brandhorst & Humphreys, 1971). Thus a role for these RNAs in either mRNA production or in rRNA synthesis might be apparent from study of changes in metabolism of these RNAs during development.

In addition, a mechanism must exist to supply the large number of RNA molecules which are needed during the rapid cell divisions in the first few hours of embryogenesis. These could be supplied either by storing a large number of molecules in the egg or by a rapid rate of synthesis in early cleavage. We describe here the metabolism of low molecular weight nuclear RNAs in early sea urchin embryos. The synthesis of these RNAs is initiated in late cleavage (morula) and continues at a high rate until blastula at which time the rate declines sharply. A large amount of these RNAs is found in unfertilized eggs, possibly serving to supply the nuclei in early cleavage.

Materials and Methods

Materials

[^{3}H]Uridine (25 Ci/mM) and $^{32}PO_4$ were obtained from Schwarz/Mann. Nuclease P1 was obtained from Yamasa-Shoyu. Nucleotide pyrophosphatase, alkaline phosphatase, RNase A, and T2 RNase were from Sigma. Phenol, triethylamine, and pyridine were redistilled before use.

Methods

Growth of Urchins. Stronglyocentrolus purpuratus was obtained from Pacific Bio-Marine and maintained at 15 °C. The embryos were developed at 15 °C at a concentration of 0.5% or less in sea water from the Florida State University Marine Laboratory containing 50 μg/mL of streptomycin. For $^{32}PO_4$ labeling, the embryos were cultured in artificial sea water containing no phosphate.

Lytechinus variegatus were collected at the Florida State University Marine Laboratory and grown at room temperature, 22–25 °C. At least 95% of the eggs fertilized and developed normally in all cultures.

The gametes were collected after injection of 0.5 M KCl into the coelomic cavity, and the eggs were washed three times in filtered sea water. The sperm was stored dry at 4 °C until needed. To a suspension of 5 mL of eggs in 100 mL of sea water was added 100 mL of sea water containing 1 mL of a 0.5% suspension of sperm. After 5 min the fertilized eggs were collected, washed two times in sea water, and then cultured, while being stirred at 60 rpm with gentle aeration.

For labeling the RNA, the embryos were settled (preblastula) or collected by centrifugation, washed with fresh sea water, and resuspended at a concentration of 0.5–1.0% for 1 h before addition of the label. Routinely, 5–10 μCi/mL of [^{3}H]uridine was used. Twenty-five microcuries/milliliter was used in the 30-min pulses and in labeling the early cleavage stages. For labeling with $^{32}PO_4$ the embryos were grown at 0.5% concentration, 10 μCi/mL of $^{32}PO_4$ was added at the mesenchyme blastula stage, and another 10 μCi/mL was added 8 h later. The embryos were harvested at the prism stage. In all cases the presence of the label did not affect the growth of the embryos.

Chase experiments were performed by washing the embryos twice in sea water after labeling and then growing them for an additional 16 h in fresh sea water.

Subcellular Fractionation. The embryos were collected by centrifugation and washed two times in 0.55 M KCl. They were washed once with 10 volumes of 0.25 M sucrose, 0.001 M EDTA,[1] 0.01 M Tris, pH 8.0, and then suspended in 5–10 volumes of 0.25 M sucrose, 0.002 M $Mg(CH_3COO)_2$, 0.003 M $CaCl_2$, 0.01 M Tris, pH 8, and homogenized in a Dounce

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; LMW, low molecular weight; hnRNA, heterogeneous nuclear RNA.

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homogenizer 20–40 strokes with the B pestle. Cell breakage was monitored by microscopy. The homogenate was centrifuged at 1000*g* for 10 min, the cytoplasm (supernatant) was removed, and RNA was prepared. The crude nuclear pellet was resuspended by homogenization (10–15 mL/g of original embryos) in 1.6 M sucrose, 0.005 M $Mg(CH_3COO)_2$, 0.01 M Tris, pH 8, layered over an 8-mL pad of 2 M sucrose in the same buffer, and centrifuged 45 min at 20 000 rpm in the A627 rotor of the Sorvall OTD-2 centrifuge. The purified nuclei (uncontaminated by cytoplasm by phase microscopy) were suspended in 0.25 M sucrose, 0.005 M $Mg(CH_3COO)_2$, 0.01 M Tris, pH 8, for RNA preparation.

Preparation of RNA. The embryos were washed as described above and suspended in 5–10 volumes of 0.25 M sucrose, 0.001 M EDTA, 0.01 M Tris, pH 8. An equal volume of 1% $NaDodSO_4$, 0.01 M EDTA was added, and the cells were lysed by homogenization. The solution was adjusted to 0.1 M $NaCH_3COO$, pH 5.0, and extracted with an equal volume of water-saturated phenol–chloroform (2:1) at room temperature. The phases were separated, and the phenol and interphase layer were reextracted with an equal volume of the aqueous solution. The combined aqueous layers were reextracted two additional times with phenol, adjusted to 0.3 M NaCl, and precipitated with 2.5 volumes of ethanol. For preparation of RNA after a 30-min label, the same procedure was followed except that the extraction was done at 55 °C to extract nRNA efficiently. No difference was found in recovery of low molecular weight RNA whether extraction was done at 25 or 55 °C, except that the 5.8S rRNA (Sy & McCarty, 1970) was recovered in the low molecular weight RNA fraction when extraction was done at 55 °C. nRNA and cytoplasmic RNA were prepared in a similar way by extraction at 25 °C.

Analysis of RNA. RNA was first fractionated on 38-mL 5–20% sucrose gradients in 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris, pH 7.5, for 16 h at 24 000 rpm in the A627 rotor of the Sorvall OTD-2 centrifuge. The low molecular weight region (4–8 S) was recovered by EtOH precipitation and analyzed on polyacrylamide gels. Routinely all RNA samples were analyzed on both 10% polyacrylamide gel under aqueous conditions and 12% gels in 70% formamide as previously described (Marzluff et al., 1975). In some cases RNA was also analyzed on 12.5% acrylamide–0.33% bis(acrylamide) gels prepared according to Laemmli (1970).

Gels were processed for fluorography as described by Laskey & Mills (1975) and exposed to prefogged X-ray film (A_{550} = 0.15) at −70 °C under conditions where the density of the band was proportional to the amount of radioactivity. The films were scanned in a densitometer and the areas under the peaks determined by planimetry.

Purification and Analysis of [$^{32}PO_4$]*RNA.* Gels containing [$^{32}PO_4$]RNA were covered with saran wrap and exposed directly to X-ray film. The bands were excised and the RNA was eluted as previously described (Marzluff et al., 1975). The pure RNAs were precipitated in the presence of 40 μg of mouse 28S rRNA and digested in 50 μL with 1 μg of RNase A and 5 units of T2 RNase in 0.01 M sodium acetate, pH 5.5. The digest was diluted to 1 mL with 7 M urea, 0.01 M Tris, pH 7.5, and 500 μg of a pancreatic RNase digest of mouse rRNA was added, and the mixture was applied to a 30-cm (8-mL bed volume) column of DEAE-cellulose equilibrated in the same buffer and eluted with a linear gradient (100 mL) of 0–0.35 M NaCl in the same buffer (Tomlison & Tener, 1963). Fractions (2 mL) were collected and counted directly by Cerenkov radiation in a Packard 3255 scintillation counter. The material eluting with charge −5.5 was diluted with four volumes of water and absorbed to a 1-cm column of DEAE-cellulose in a pasteur pipette. The column was washed with 4 mL of water, and the nucleotides were eluted with 2 M triethylammonium bicarbonate, pH 8.0. The nucleotides were lyophilized, digested with nuclease P1 and alkaline phosphatase, and analyzed by electrophoresis at pH 3.5 on cellulose thin-layer plates, and the nucleotides were detected by autoradiography, as previously described (Brown & Marzluff, 1978). The radioactive material was eluted with water, the amounts of radioactivity in each spot were determined by Cerenkov radiation, and the caps were recovered by lyophilization. The caps were digested with nucleotide pyrophosphatase, and the digestion products were resolved by pH 3.5 electrophoresis.

RNA–DNA Hybridization. The method of Birnstiel et al. (1972) was used exactly as previously described (Marzluff et al., 1975). RNA concentration was 0.04 μg/mL, and 40 μg/mL of mouse 28S rRNA was included in all hybridizations. Blank filters were taken at each time point and bound less than 5 cpm over machine background.

The specific activity was determined by direct measurement of the specific activity of 5S RNA and RNAs N1 and N2. In addition, the relative amounts of each RNA were determined by scanning the gel at 260 nm in a Gilford gel scanner and the X-ray film in a densitometer. In most preparations the specific activity of RNAs N1 and N2 was twice that of 5S rRNA.

The amount of DNA on each filter and the melting points of the hybrids were determined as previously described (Marzluff et al., 1975).

Competition hybridization experiments were performed for 9 h in exactly the same conditions as the saturation hybridization.

Rates of RNA Synthesis. Embryos were labeled for 30 min, and the RNA was prepared and fractionated on sucrose gradients. The counts in each fraction were determined and the low molecular weight fractions pooled. A portion of the sample was run on a 10 cm × 7 mm 10% polyacrylamide cylindrical gel, and radioactivity in each 1-mm fraction was determined as previously described (Marzluff et al., 1974). The same sample was run on a slab gel and analyzed by fluorography as described above. The percentage of counts in a major band, 5S and/or 4S RNA, was determined from the cylindrical gel, and the percentage of counts in each of the other bands was determined by comparison of the areas under the peak of the fluorogram. This procedure was necessary since it was not possible to determine the amount of material at the top of the gel by fluorography and the amount of radioactivity in the minor components could not be accurately determined by cutting up the gel.

For calculation of the rates of RNA synthesis, the measured percentage of RNA was divided by 2 to take into account the turnover of RNA with a half-life of about 5–20 min (Brandhorst & Humphreys, 1971; Grainger & Wilt, 1976) and the linear increase in specific activity of the triphosphate pool (Galau et al., 1977) during the first 30 min of labeling. Following Galau et al. (1977), the accumulation of total RNA in any time t, with a linear increase in specific activity of the precursor with time, will be

$$k_s = \int_0^t t' e^{-k_d(t-t')}\, dt'$$

In 30 min, if k_d is chosen to give a 10-min half-life for the hnRNA (90% of the synthesis) (Brandhorst & Humphreys, 1971), then the percent of the newly made RNA which is the

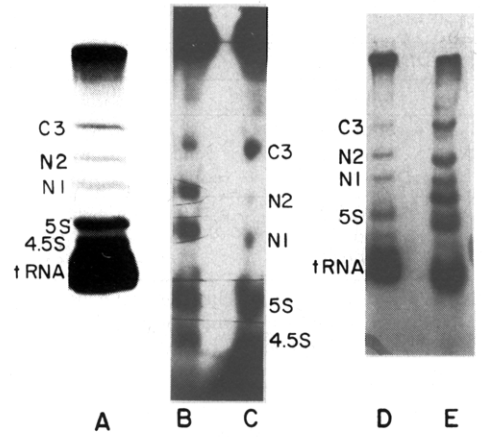

FIGURE 1: Subcellular localization of RNAs. *L. variegatus* embryos were grown in $^{32}PO_4$ from blastula to pluteus. From one portion total RNA was prepared. The remaining embryos were fractionated into nuclei and cytoplasm, and RNA was prepared from each fraction. The RNA was analyzed on 10% polyacrylamide gels. (A) Total RNA; (B) RNA; (C) cytoplasmic RNA; (D) total RNA; (E) mouse myeloma low molecular weight RNA. Samples B and C were run until tRNA ran off the gel.

small amount of stable RNA will be overestimated by a factor of 2.

Results

Subcellular Localization of LMW RNA. Analysis of total RNA prepared from embryos (*L. variegatus*) grown in $^{32}PO_4$ revealed three major low molecular weight RNA species larger than 5S rRNA. In addition there is at least one RNA migrating between 4S and 5S rRNA (Figure 1). These same RNAs were the only prominent LMW RNAs (other than 4S and 5S rRNA) observed when total embryo RNA was analyzed when we scanned the polyacrylamide gels at 260 nm (not shown).

Embryos were grown in $^{32}PO_4$ and fractionated into purified nuclei and cytoplasm. The RNA was analyzed, and RNAs N1 and N2 were found predominantly in the nucleus while RNA C3 was present in the cytoplasm (Figure 1). The 4.5S RNA was also predominantly nuclear. Comparison of the electrophoretic mobility of these RNAs with the corresponding RNAs from mouse myeloma cells showed that these nuclear RNAs N1 and N2 were distinct from the mouse RNAs (of which there are three major species), while the 4.5S RNA and the cytoplasmic RNA had identical electrophoretic mobility to the corresponding species from the mouse. A relatively large proportion of the labeled 5S rRNA was associated with the nucleus. A similar situation has been found in mammalian cells (Knight & Darnell, 1967; Marzluff et al., 1975; Brown & Marzluff, 1978). The purified sea urchin nuclei (which have not been treated with detergent) contain rRNA (but less than 5% of the total in the cell). A much higher percentage of the labeled 5S rRNA than expected, if it was all associated with ribosomes, was found in the nuclear fractions.

The RNAs from three sea urchin species were compared, and the RNAs of *Arbacia punctualata* and *L. variegatus* were identical. However, *S. purpuratus* had a third nuclear RNA (N1a) resolved on the aqueous polyacrylamide gels (Figure 2). Analysis of the RNAs on a gel system in high ionic strength or in 70% formamide resulted in the N1 and N1a RNA in *S. purpuratus* migrating as a single component with an overall electrophoretic pattern identical with that of *L. variegatus*. No new major RNAs were detected in the other gel systems; however, a minor species intermediate in mobility between N1 and N2 was apparent. Another LMW RNA in these cells is the 5.8S rRNA (Sy & McCarty, 1970) which is hydrogen-bonded to the 26S rRNA. It is released when the solution is heated and normally is not present in significant amounts in our preparations. It had an identical mobility with RNA N1 in the standard aqueous gel system, but migrated slightly more slowly in formamide and slightly more rapidly in the high ionic strength system (Figure 2).

We feel the two N1 components present in *S. purpuratus* most likely represented conformers of the same RNA. The chain lengths were determined from the mobility in formamide acrylamide gels and were approximately 190 for N1, 230 for N2, and 280 for C3. The mouse low molecular weight nuclear RNAs were used as markers.

5′ Termini of the RNA. In experiments where RNA was labeled with [*methyl*-^{3}H]methionine only RNA species N1 and N2 were labeled other than tRNA (not shown). In *S. purpuratus* both N1 and N1a were labeled. To determine if unusual 5′ termini were present the $^{32}PO_4$-labeled RNAs were digested with RNase T2 and chromatographed on DEAE-cellulose. RNAs N1 and N2 gave similar profiles with no detectable di- or trinucleotides but with a single, highly charged component eluting with an apparent charge of −5.5 (Figure 3). In contrast, 5S rRNA gave a single component with a charge of −4, consistent with the known pGp terminus (not shown). The highly charged material from RNA N1 and N2, representing 2–2.5% of the total $^{32}PO_4$, was digested with nuclease P1 and alkaline phosphatase and subjected to electrophoresis at pH 3.5. Forty percent of the radioactivity was present as inorganic phosphate while the remaining 60% ran as a single component, identical with m^{7}GpppA (Figure 3, lower). These data indicate a cap I structure, mGpppXmpYp, for the T2 resistant material. The isolated "cap" was completely resistant to redigestion with nuclease

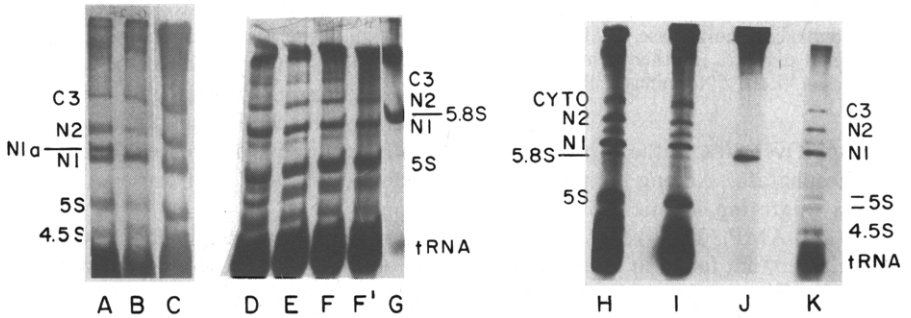

FIGURE 2: Species specificity of low molecular weight RNA. Total low molecular weight RNA from embryos labeled in blastula stage with [^{3}H]uridine for 4 h was analyzed in different gel systems. Left, 10% polyacrylamide gels as in Figure 1: (A) *S. purpuratus*; (B) *L. variegatus*; (C) *A. punctualata*. Middle, 12.5% polyacrylamide gels in 70% formamide: (D) *S. purpuratus*; (E) *L. variegatus*; (F and F′) *A. punctualata*; (G) 5.8S rRNA from *L. variegatus*. Right, 12.5% polyacrylamide gel prepared according to Leammli (1970): (H) *S. purpuratus*; (I) *L. variegatus*; (K) *A. punctualata*; (J) 5.8S rRNA from *L. variegatus*.

Table I: Gene Copies of the Low Molecular Weight RNAs[a]

RNA	% genome (μg of RNA/μg of DNA)						
	expt 1			expt 2			
	sp act. (cpm/μg of RNA)	% genome (μg of RNA/μg of DNA × 10^{-2})	gene copies	sp act. (cpm/μg of RNA)	% genome (μg of RNA/μg of DNA × 10^{-2})	gene copies	av gene copies
5S	145000	0.0064	190	100000	0.0052	160	175
N1	262000	0.0035	68	200000	0.0028	56	62
N2	262000	0.0039	64	200000	0.0018	31	48

[a] Purified $^{32}PO_4$-labeled RNAs were hybridized to immobilized sea urchin sperm DNA. The saturation hybridization value was determined from the plateau value constant between 6 and 18 h of hybridization, as expected for the RNA concentrations and conditions used (Birnstiel et al., 1972; Marzluff et al., 1975). The following molecular weights were used: 5S, 40 000; N1, 62 000; and N2, 74 000. These values shown are for *L. variegatus,* but a single experiment with *S. purpuratus* gave similar results.

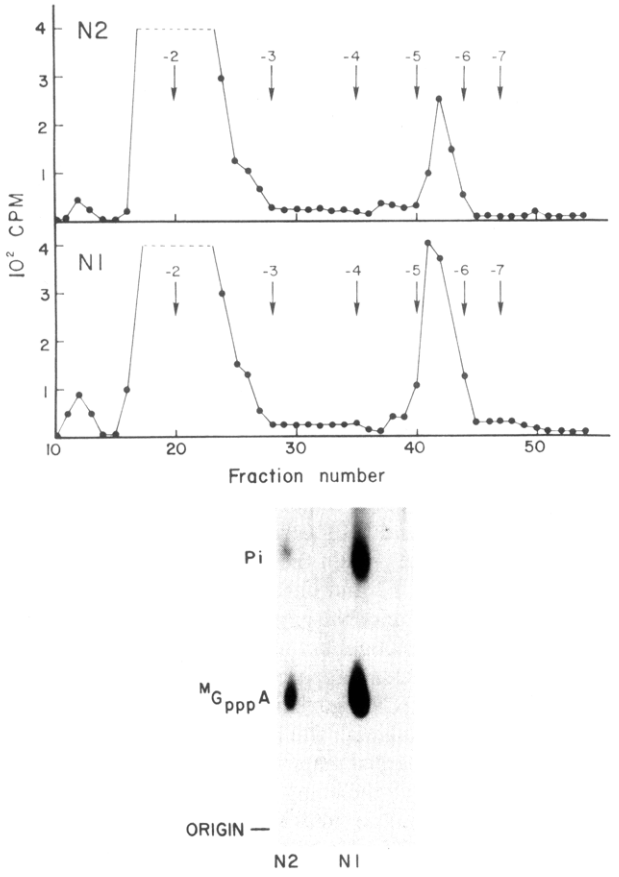

FIGURE 3: 5′ termini of RNA. (Upper) Purified N1 and N2 RNA was digested with RNase T2 and pancreatic RNase, and the digest was chromatographed on DEAE-cellulose as described under Materials and Methods. The position of charge markers (from a pancreatic RNase digest of rRNA) is shown. Radioactivity in each fraction was determined by Cerenkov radiation. Top: RNA N2. Bottom: RNA N1. (Lower) The material eluting with a charge of −5.5 (fractions were digested with RNase P1 and alkaline phosphatase, and the digest was analyzed by electrophoresis at pH 3.5). Authentic m^7GpppA was electrophoresed in a parallel slot. Left: 5′ terminus of RNA N2. Right: 5′ terminus of RNA N1.

P1 and alkaline phosphatase. Over 80% of the cap was digested by nucleotide pyrophosphatase. Among the products identified were components migrating identically with 7-methylguanosine phosphate and AMP. The likely structure of the cap is *GpppAm, similar to that found in mammalian cells (Ro-Choi et al., 1975). The methylated guanosine derivative has not yet been identified.

Gene Copies of the nRNAs. Highly purified $^{32}PO_4$-labeled RNAs (specific activity 2–4 × 10^5 cpm/μg) were hybridized to sea urchin DNA immobilized on nitrocellulose filters. Hybridization was carried out at low RNA concentrations,

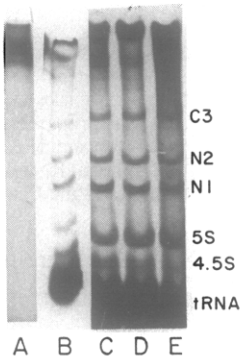

FIGURE 4: RNA synthesis during development. *L. variegatus* embryos were labeled for 4 h with [^{3}H]uridine at various stages, total RNA was isolated, and the low molecular weight RNA was analyzed by gel electrophoresis as described in Figure 1 and radioactivity was detected by fluorography. Left to Right: (A) 1–32 cells; (B) morula (200 cells); (C) blastula; (D) gastrula; (E) pluteus.

under conditions where saturation will be reached in a relatively short time if the RNA is of low complexity. 5S RNA and N1 and N2 RNA each reached 90% of saturation within 4–5 h of hybridization at 0.04 μg/mL of RNA. The hybrids all melted as single transitions between 70 and 75 °C in 0.015 M NaCl. RNAs N1 and N2 were repetitive, present 50–100 times in the genome, while 5S rRNA was present in about 200 copies (Table I). Significant hybridization was not observed with RNA C3, indicating it was present in less than 20 copies per genome.

Synthesis during Development. The pattern of RNA accumulation changes dramatically during early sea urchin development. In a 4-h labeling period, the major RNAs accumulated do not represent the structural RNAs until the pluteus stage when the rRNAs are the major stable RNAs accumulated (Emerson & Humphreys, 1970). However, some low molecular weight RNA accumulated from cleavage on, although during late cleavage (morula) it was overshadowed by the predominant histone mRNA synthesis.

To study the possible changes in low molecular weight RNA metabolism, embryos were labeled for 4-h periods from 1 to 32 cells, during morula and early blastula (just prior to hatching) and continuing to pluteus. During this period there was no qualitative difference in the RNAs which accumulated during a 4-h label, but there was a dramatic quantitative change.

Low molecular weight RNA was isolated by cold phenol extraction and sucrose gradient centrifugation. The RNAs were analyzed on a 10% polyacrylamide slab gel (Figure 4), and the RNA was detected by fluorography. The same components were present at each stage of development. To quantitate the RNA in each band, the fluorographs were scanned in a densitometer and the area under each peak was determined by planimetry. Results were expressed relative

Table II: Stability of the RNA during a Chase[a]

sea urchin species	RNA components	proportion of each RNA (relative to 5 S) labeling period	chase
		Blastula	Pluteus
Stronglyocentrotus purpuratus	5S	1	1
	N1	1.9	1.6
	N2	0.82	0.75
	C3	0.6	0.5
		Morula	Pluteus
Lytechinus vareigatus	5S	1	1
	N1	1.5	1.6
	N2	0.85	0.75
	C3	0.7	0.6

[a] Embryos were labeled for 6 h with [^{3}H]uridine, starting in morula or in blastula, washed, and allowed to develop until gastrula or pluteus. Samples were taken from each stage and the RNA was analyzed. There was no detectable turnover of RNA. This result is not due to reutilization of the label as the final labeling pattern after the chase reflects the stage at which the embryos were labeled and not the stage at which they were harvested. The numbers represent arbitrary units with 5S RNA normalized to 1. The total counts in the low molecular weight RNA decreased 5–10% during the chase.

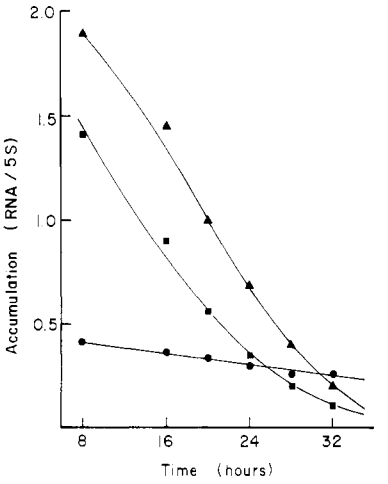

FIGURE 5: Quantitation of RNA synthesis. Embryos were labeled for 4 h with [^{3}H]uridine at various times after fertilization, and the RNA was analyzed as shown in Figure 5. The times refer to the end of the labeling period. The film was scanned in a densitometer, and the areas under the peaks were determined by planimetry. The embryos were in hatching blastula at 12 h and at gastrula in 24–28 h. (▲) RNA N1; (●) RNA C3; (■) RNA N2.

to 5S rRNA. The nRNAs, N1 and N2, were predominant in morula and early blastula but their accumulation decreased 10-fold relative to 5S RNA in later stages (Figure 5). In contrast, RNA C3 behaved differently, showing a nearly constant rate of accumulation relative to 5S RNA.

The 4.5S RNA synthesis paralleled that of the nRNAs (Figure 4). Because of the difficulty in completely separating this RNA from tRNA, it could not be quantitated exactly.

Is Low Molecular Weight RNA Synthesized in Early Stages? The synthesis of low molecular weight RNA was clearly apparent in morula (Figures 2A and 4) (labeled from about 60–200 cells prior to ciliation), and, as in blastula, the nuclear RNAs N1 and N2 were dominant, accumulating at a rate about twice that of 5S RNA. RNA C3 accumulated at a lower rate. However, in early cleavage, 1–32 cells, there were no detectable low molecular weight RNAs formed other than a trace of label incorporated into tRNA (Figure 4). We estimate that, if the rate of synthesis of RNA N1 and N2 in early stages (1–32 cells) was 5% of that found in morula, they would have been detected. Thus the synthesis of these RNAs is probably initiated in late cleavage.

Stability of Low Molecular Weight RNA. The stability of the low molecular weight RNAs was determined by a chase experiment, in which the embryos were labeled during cleavage or early blastula, washed with sea water, and allowed to develop to gastrula and pluetus, respectively. In each case, there was less than 10% loss of label from the low molecular weight RNAs. In addition, the relative amounts of the different low molecular weight RNAs remained constant, reflecting the stage at which they were labeled and not the stage at which they were harvested (Table II). Thus the retention of label was due to the stability of the RNA and not to turnover and reutilization of the nucleotide precursors during the chase period (Galau et al., 1977). The molecules synthesized in morula were still present in the pluteus embryo.

Absolute Rate of Synthesis. The above results are consistent with either an increase in the rate of 5S rRNA synthesis or a decrease in the rate of low molecular weight nRNA synthesis during development. To answer this question, embryos were labeled for a short time with [^{3}H]uridine and total RNA was prepared. The percent of the total RNA which each low molecular weight species represented was determined by sucrose gradient centrifugation and gel electrophoresis (Table III). The low molecular weight nRNAs were readily detected in the 30-min label (Figure 6), with the exception of the 4.5S RNA which was obscured by the tRNA precursor.

The absolute rate of RNA synthesis per cell has been estimated previously and shown to decrease during development (Emerson & Humphreys, 1970; Brandhorst & Humphreys, 1971; Roeder & Rutter, 1970; Wu & Wilt, 1974). Correcting for this decrease and knowing the molecular weight of each species, we can estimate the absolute rate of synthesis (Table III). The data clearly show that the change in relative rates of accumulation of these RNAs is due to a decrease in the rate of synthesis of the low molecular weight nRNAs while the 5S

Table III: Rates of RNA Synthesis during Development[a]

stage	5S RNA % of total	5S RNA (molecules/cell)/min	N1 RNA % of total	N1 RNA (molecules/cell)/min	N2 RNA % of total	N2 RNA (molecules/cell)/min
morula	0.03	60	0.09	140	0.08	110
blastula	0.18	230	0.21	220	0.20	180
gastrula	0.21	130	0.06	30	0.03	15
pluteus	0.37	150	0.10	33	0.07	20

[a] From 30-min pulse labels with [^{3}H]uridine, the proportion of newly made RNA which was N1, N2, and 5S was estimated by gel electrophoresis (left). Using the values for the absolute rate of RNA synthesis determined by Roeder & Rutter (1970) and Brandhorst & Humphreys (1971), we calculated the number of molecules of each RNA synthesized per minute. The results have been corrected with the assumption that the specific activity of the triphosphate pool increases linearly with time and that 90% of the RNA turns over with a half-life of 10 min. The rates of RNA synthesis were taken as 15×10^{-15} in morula, 9.7×10^{-15} in blastula, 4.8×10^{-15} in gastrula, and 3.1×10^{-15} (g/cell)/min in pluteus. Similar results were obtained with *S. purpuratus* (shown here) and *L. variegatus*.

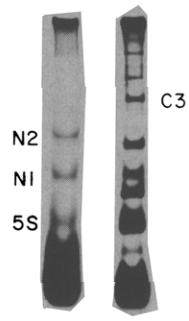

FIGURE 6: RNA synthesized in a short pulse. Embryos (*L. variegatus*) were labeled for 30 min in blastula. Total RNA was prepared by hot phenol extraction and analyzed as described in Figure 5. Mouse myeloma LMW RNA was analyzed in the same gel. Left: sea urchin blastula. Right: mouse myeloma.

Table IV: Amount of Low Molecular Weight RNA in the Egg and Embryo[a]

stage	RNA	% total RNA[b]	molecules/ embryo (or cell)	N RNA[c]/ 5S RNA
egg	N1	0.031	10^7	0.016
	N2	0.015	5×10^6	0.01
prism	N1	0.28	10^8 (10^5)	0.11
	N2	0.175	5×10^7 (5×10^4)	0.07

[a] The amount of LMW RNA in the egg was determined by hybridization competition (Figure 8) and by gel electrophoresis. The amount in the prism embryo was determined by gel electrophoresis. Results are expressed relative to 5S rRNA and as molecules per embryo (or cell), when we assume an RNA content of 3.3×10^{-9} g/embryo and 1000 cells in the prism stage. [b] Determined by competition hybridization (Figure 7) for the egg RNA and by gel electrophoresis for the prism embryo when we assumed that 5S rRNA is 2.5% of the total cell RNA. [c] Determined by gel electrophoresis.

rRNA synthesis remained relatively constant. Thus these RNAs are synthesized at a high rate in late cleavage and early blastula and at a much lower rate in gastrula and pluteus, while 5S rRNA synthesis increases from cleavage to blastula and then becomes relatively constant.

Presence of the nRNAs in Unfertilized Eggs. Since these RNAs were not synthesized in early cleavage, the egg RNA was isolated and analyzed for these RNAs by gel electrophoresis and RNA–DNA hybridization. The egg RNA was fractionated by sucrose gradient centrifugation and gel filtration on Sephadex G-75. The low molecular weight nRNAs N1 and N2 were excluded from the column while the tRNA was included. 5S RNA was only slightly included and was found in both fractions. On gel electrophoresis, discrete small peaks were present at the position of the low molecular weight RNAs (not shown). There was about 1/10 as much of the nRNAs relative to 5S RNA as was found at gastrula (Table IV).

That this RNA was the low molecular weight nRNAs was confirmed by RNA–DNA hybridization. $^{32}PO_4$-labeled, purified low molecular weight RNAs (200 000 cpm/μg) were hybridized to sperm DNA in the presence of varying amounts of RNA excluded from the Sephadex G-75 column (Figure 7). The egg RNA effectively competed in the hybridization, indicating that low molecular weight nRNA sequences were present in the egg RNA. Knowing the amount of RNA per egg (3.3×10^{-9} g) (Whiteley, 1949), we could calculate the number of molecules per egg of N1 and N2 nRNAs. About 10^7 molecules of N1 nRNA and 5×10^6 molecules of N2 nRNA were present in the egg, and the amount increased

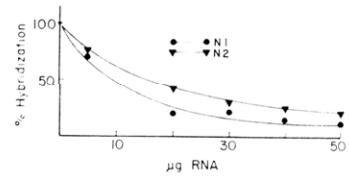

FIGURE 7: Presence of RNAs N1 and N2 in egg RNA. Purified $^{32}PO_4$-labeled RNAs N1 and N2 (0.04 μg/mL) were hybridized to sperm DNA immobilized on nitrocellulose filters in the presence of varying amounts of low molecular weight egg RNA, prepared by sucrose gradient centrifugation and Sephadex G-75 chromatography (representing 8% of the total RNA). All points were done in duplicate with less than 10% variation. The curves are theoretical, based on 0.04 μg of these RNA in 8.5 μg of RNA for N1 and in 17 μg of RNA for N2. (●) N1; (▼) N2.

about 10-fold in the 1000-cell gastrula (Table III). There was enough RNA in the egg to supply 100 cells at a level of 10^5 molecules/cell (Table III). The egg contained enough nRNAs to supply the early cleavage nuclei.

Discussion

The low molecular weight nRNAs present in the sea urchin share many characteristics with those of mammals. They contain capped 5′ termini with a modified guanosine in triphosphate linkage with 2′-methoxyadenosine. The cap I structure present in the sea urchin compared to the cap II structure in the mouse is consistent with the finding that only cap I structures are present in sea urchin mRNAs (Surrey & Nemer, 1976). Only two major capped LMW nRNAs are found in the urchin compared to four in the mouse (Brown & Marzluff, 1978). There is a 4.5S RNA present in mammals (Ro-Choi et al., 1970; Marzluff et al., 1975). It does not contain methyl groups and does not have a capped 5′ terminus. (Ro-Choi et al., 1970). In contrast, the major cytoplasmic RNA species was identical on electrophoresis in urchin and mouse and was not methylated.

The RNAs are present at levels of about 10^5 molecules/cell in the urchin embryo. These numbers are similar to that found in actively growing mammalian tissue culture cells (Weinberg & Penman, 1969; Marzluff et al., 1975), when we take into account the fourfold difference in genome size between mouse and urchin. The genes for these RNAs are present in multiple copies in the genome, as are the genes for other structural RNAs. Genes for RNAs N1 and N2 were reiterated about 50 times compared to about 200 times for 5S rRNA. These RNAs thus represent a slightly larger proportion of the genome in the urchin as compared to the mouse. The number of genes for 5S rRNA is similar to those found for 18S and 28S rRNA (Patterson & Stafford, 1971).

The synthesis of RNA in sea urchin embryos has been studied extensively in recent years. These studies have led to the following general conclusions.

(1) Until the gastrula stage, accumulation of hetrogenous RNA predominates with little readily detectable rRNA (reviewed by Weinberg, 1977). This hRNA which accumulates represents primarily mRNA (and in early development, primarily histone mRNA).

(2) Most of the RNA synthesized at a given instant is hnRNA which has a short half-life of 5–15 min (Brandhorst & Humphreys, 1971; Grainger & Wilt, 1976).

(3) During development, there is a large decrease in the rate of RNA synthesis per cell (Emerson & Humphreys, 1970; Roeder & Rutter, 1970; Wu & Wilt, 1974; Brandhorst & Humphreys, 1971).

It has been postulated that the synthesis of structural RNAs, particularly rRNA, takes place at a constant rate throughout

development but their accumulation only becomes detectable later in development as the rate of hnRNA synthesis slows down (Emerson & Humphreys, 1970). The structural nRNAs represent a potentially different class of molecules, however, since the number of nuclei increases over 1000-fold during development. Thus there may well be a concomitant demand for increased synthesis of these RNAs.

Two studies of low molecular weight RNA synthesis in early embryos have been reported. O'Melia & Villee (1972) showed that there was some synthesis of 5S rRNA in early cleavage. No information was provided as to the stage reached by these embryos or to the amount of 5S synthesis (percent of total RNA) detected. Frederikson & Hellung-Larsen (1974) studied the accumulation of low molecular weight RNAs during development, and their results were in general agreement with ours, although no attempt to compare absolute rates of synthesis was made. They also reported (Frederikson & Hellung-Larsen, 1972) the presence of RNAs with a similar electrophoretic mobility to the LMW nRNAs in sea urchin eggs.

Major low molecular weight RNAs present in sea urchin embryos do not change as development progresses from morula to pluteus. Thus it is likely that these RNAs play a fundamental structural role in nuclear activity. It is apparent that their synthesis is under stringent control. During the first few rapid cleavages (up to about 64 cells), the synthesis of these RNAs is not detectable. This period of rapid cleavage is followed by a period of somewhat slower cleavage, leading to the blastula embryo of about 450 cells (Galau et al., 1976). During the morula stage the synthesis of the low molecular weight nRNAs becomes significant. As cell division slows and the embryos develop from blastula to gastrula, the rate of low molecular weight nRNAs synthesis drops abruptly.

After the early cleavage stages when these RNAs are not synthesized, their rate of synthesis is parallel to cell division. However, in mammalian cells (Rein & Penman, 1969; Marzluff, unpublished experiments) the synthesis of these RNAs is not correlated with the cell cycle, but their synthesis continues constantly. Thus, while their rate of synthesis follows the rate of cell division, it may well be controlled independently of DNA synthesis. In contrast, the rate of 5S RNA synthesis increased rapidly after early cleavage to a relatively constant rate.

In six experiments in both *L. variegatus* and *S. purpuratus*, we have not detected the synthesis of these RNAs prior to the 64-cell stage. This is the case even though the same amount of total RNA (cpm) has been analyzed in both 200–400-cell and 1–32-cell stages and exposure times of the gel have been lengthened 10-fold for the early RNAs. Since the absolute rate of RNA synthesis per cell is similar in both stages (Roeder & Rutter, 1970), we conclude that these RNAs are not synthesized early in development (or, if synthesized, are made at a rate less than 5–10% of that found in the later stage).

The similar RNAs in mouse myeloma cells are synthesized by RNA polymerase III (Brown, Pan, and Marzluff, unpublished experiments) as are 5S RNA and tRNA. Thus, transcription of genes transcribed by RNA polymerase III is not extensive in early embryos, even though the enzyme is present (Roeder & Rutter, 1970). The basis for the inactivity of this enzyme at early stages remains unknown. In addition, as development proceeds the enzyme changes the products it produces from largely low molecular weight nRNAs in morula to 5S RNA and tRNA in later stages. This is presumably related to a change in the structure of the chromatin template as development proceeds.

The nuclei in early embryos probably contain the same complement of low molecular weight nRNAs as are found in later stages. Stored in the egg are enough low molecular weight nRNAs to provide for 100 cells at a level per cell equivalent to that in the gastrula. Thus it is likely that these early embryos utilize RNA stored in the egg, although sufficient nuclei have not been isolated from these stages to unequivocally demonstrate their presence in the nuclei. During development the low molecular weight nRNAs are completely stable.

The absolute rate of synthesis of these RNAs has been estimated from their accumulation during a short incubation with [^{3}H]uridine. In the 30-min labeling period, there are several parameters which affect the accumulation of label in stable RNA. First, the specific activity of the triphosphate pool increases linearly with time (Galau et al., 1977) in the first hour. Second, a large proportion of the RNA turns over with a short half-life, 5–20 min (Brandhorst & Humphreys, 1971; Wu & Wilt, 1974). Thus the rate of stable RNA accumulation is overestimated due to the turnover of unstable RNA. This effect is reduced somewhat by the continual increase in specific activity of the triphosphate pool. We estimate that the measured rate of stable low molecular weight RNA accumulation is overestimated by a factor of 2, assuming a half-life of most of the RNA of 5–10 min and a linear increase in the triphosphate specific activity. The numbers in Table III have been corrected for this.

The relative rates of synthesis are probably an accurate reflection of the situation in vivo. The 5S and low molecular weight nRNAs are made at close to the same relative rate in 30-min as in 4-h labeling at each stage. The absolute rate of synthesis of each RNA can be estimated from the absolute rate of RNA synthesis previously measured by Roeder & Rutter (1970) and Brandhorst & Humphreys (1971). These measurements show clearly that the change in rates of low molecular weight RNA synthesis is due to a large decrease in the transcription rate between blastula and gastrula while 5S RNA transcription increases to a stable level between morula and blastula. The rates of transcription of the low molecular weight RNAs are very high during morula and early blastula, between 1 and 2 (molecules/gene)/min. For comparison, the histone mRNAs are synthesized at a rate comparable to this (Kunkel & Weinberg, 1978). They represent 10% of the synthesis on a weight basis and are about 12 times larger (2500 nucleotides as compared to 200), and the genes are present about eight times more per cell (Kedes, 1976). Thus, on a per gene basis, the low molecular weight nRNAs are made at an extremely high rate which decreased at least 10-fold [0.2 (molecule/gene)/min] in later development. At the same time 5S RNA is transcribed at a lower rate per gene [0.5 (molecule/gene)/min] which remains constant.

While much of the 5S rRNA accumulated is found in ribosomes, a large proportion is retained in the nucleus and is not found in ribosomes (Figure 1 and Nijhawan, 1978). A similar conclusion can be drawn from the data of Frederiksen et al. (1974) that the accumulation of 5S rRNA is much greater than the accumulation of 5.8S rRNA and N1 RNA combined during pluteus. Similar results have been found in mammalian cells (Marzluff et al., 1975; Brown & Marzluff, 1978). The 5S rRNA synthesis is known to be controlled independently of rRNA (Ford, 1971; Knight & Darnell, 1967; Liebowitz et al., 1973), and it may play a role in the nucleus as well as being a ribosomal component.

The failure to detect any changes in the small RNA components of the nucleus during development and the conservation of the RNA molecules from morula to pluteus support the idea that these RNAs play a fundamental structural role in nuclear activity. Their synthesis in large amounts in early stages suggests that they may be important in hnRNA synthesis and processing, rather than in rRNA synthesis.

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