Peters, T. (1977) *Clin. Chem. (Winston-Salem, N.C.) 23*, 5–12.
Peters, E. H., Nishi, S., & Tamaoki, T. (1978) *Biochem. Biophys. Res. Commun. 83*, 75–82.
Peterson, J. A. (1976) *Nucleic Acids Res. 3*, 1427–1436.
Rosen, J. M., Wood, J. L. C., & Comstock, J. P. (1975) *Biochemistry 14*, 2895–2903.
Ruoslahti, E., & Terry, W. D. (1976) *Nature (London) 260*, 804–805.
Ruoslahti, E., & Engvall, E. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4641–4644.
Sala-Trepat, J. M., Savage, M., & Bonner, J. (1978) *Biochim. Biophys. Acta 519*, 173–193.
Sala-Trepat, J. M., Sargent, T. D., Sell, S., & Bonner, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 695–699.
Savage, M. J., Sala-Trepat, J. M., & Bonner, J. (1978) *Biochemistry 17*, 462–467.
Schechter, I. (1974) *Biochemistry 13*, 1875–1885.
Schimke, R. T., Palacios, R., Sullivan, O., Kiely, M. L., Gonzalez, C., & Taylor, J. M. (1974) *Methods Enzymol. 30*, 631–648.
Schreiber, G., Rotermund, H. M., Maeno, H., Weigand, K., & Lesch, R. (1969) *Eur. J. Biochem. 10*, 355–361.
Schreiber, G., Urban, J., Zahringer, J., Reutles, W., & Frosch, W. (1971) *J. Biol. Chem. 246*, 4531–4538.
Sell, S., & Gord, D. (1973) *Immunochemistry 10*, 439–442.
Sell, S., & Skelly, H. (1976) *J. Natl. Cancer Inst. 56*, 645–648.
Sell, S., Jalowayski, I., Bellone, C., & Wepsic, H. T. (1972) *Cancer Res. 32*, 1181–1189.
Sell, S., Wepsic, H. T., Nickel, R., & Nichols, M. (1974a) *J. Natl. Cancer Inst. 52*, 133–137.
Sell, S., Nichols, M., Becker, F., & Leffert, H. (1974b) *Cancer Res. 34*, 865–871.
Sell, S., Becker, F., Leffert, H., & Watabe, H. (1976) *Cancer Res. 36*, 4239–4249.
Shapiro, D. J., & Schimke, R. T. (1975) *J. Biol. Chem. 250*, 1759–1964.
Shapiro, D. J., Taylor, J. M., McKnight, G. S., Palacios, R., Gonzalez, C., Kiely, M., & Schimke, R. T. (1974) *J. Biol. Chem. 249*, 3665–3671.
Sheiness, D., & Darnell, J. E. (1973) *Nature (London), New Biol. 241*, 265–268.
Strair, R. K., Yap, S. H., & Shafritz, D. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4346–4350.
Strauss, A. W., Bennett, C. D., Donohue, A. M., Rodkey, J. A., & Alberts, A. W. (1977) *J. Biol. Chem. 252*, 6846–6855.
Studier, W. F. (1965) *J. Mol. Biol. 11*, 373–390.
Taylor, J. M., & Shimke, R. T. (1973) *J. Biol. Chem. 248*, 7664–7668.
Taylor, J. M., & Tse, T. P. H. (1976) *J. Biol. Chem. 251*, 7461–7467.
Tse, T. P. H., Morris, H. P., & Taylor, J. M. (1978) *Biochemistry 17*, 2121–2128.
Van Furth, R., & Adinolfi, M. (1969) *Nature (London) 222*, 1296–1299.
Watabe, H. (1971) *Cancer Res. 31*, 1192–1994.
Watanabe, A., Taketa, K., & Kosaka, K. (1975) *Ann. N.Y. Acad. Sci. 295*, 95–108.

Ribosomal Ribonucleic Acid Synthesis and Processing in Embryos of the Hawaiian Sea Urchin *Tripneustes gratilla*†

Jeffrey K. Griffith and Tom D. Humphreys*

ABSTRACT: Pulse-labeled RNA from plutei of the sea urchin *Tripneustes gratilla* was fractionated in sucrose gradients and sodium dodecyl sulfate–polyacrylamide gels and hybridized to recombinant plasmid DNA containing a portion of the *T. gratilla* ribosomal gene to identify a large, ribosomal RNA (rRNA) precursor of 2.5×10^6 daltons. Both pulse-chase and incorporation experiments indicate that the 2.5×10^6 dalton moiety is sequentially processed to 1.6×10^6 and 1.0×10^6 dalton intermediates and then to mature 1.45×10^6 (26 S) and 0.7×10^6 (18 S) dalton species. As judged by the respective molecular weights of the precursor and mature rRNA species, processing is nonconservative with approximately 15% of the precursor mass degraded and 85% contributing mature 26S and 18S rRNA. The absolute rate of synthesis of the rRNA precursor was measured by quantitating the flow of radioactivity through the embryos' GTP pool and into each of the rRNA species identified above. The precursor is synthesized at a rate of 3100 molecules h^{-1} $nucleus^{-1}$ and is present at a steady-state concentration of 1450 molecules/nucleus. Its half-life is 19 min. Comparison of the rate of synthesis of the precursor with the rate of accumulation of mature 26S and 18S rRNA shows that most precursor molecules yield mature rRNA. The number of copies of the ribosomal genes in the *T. gratilla* genome was measured by comparing the rates of reassociation of *T. gratilla* ribosomal DNA (rDNA) and single copy DNA with total *T. gratilla* DNA. These results indicate that the ribosomal genes are repeated 50 times per haploid genome. The ribosomal genes in pluteus cells are thus transcribed at an average rate of about 31 copies $sequence^{-1}$ h^{-1}.

The quantitative aspects of RNA metabolism and its developmental regulation have been extensively studied in sea urchin embryos (Emerson & Humphreys, 1970; Brandhorst & Humphreys, 1971, 1972; Dolecki et al., 1977; Galau et al., 1977; Brandhorst & Bannet, 1978; Dworkin & Infante, 1978). However, no unified picture of the synthesis, processing, and regulation of sea urchin embryo rRNA has emerged.

The paucity of information concerning sea urchin rRNA metabolism stems from its low rate of transcription during

† From the University of Hawaii, Pacific Biomedical Research Center, Kewalo Marine Laboratory, Honolulu, Hawaii 96813. *Received December 21, 1978.* This work was supported by grants from the National Institutes of Health. Jeffrey K. Griffith was a National Institutes of Health Postdoctoral Fellow.

0006-2960/79/0418-2178$01.00/0

embryonic and larval stages. In *Strongylocentrotus purpuratus* gastrulae and plutei, for example, the measured rates of rRNA synthesis are only 300–600 transcripts per cell per h or less than 1% of the total RNA synthesis (Emerson & Humphreys, 1970; Galau et al., 1977). Theoretically, the ribosomal genes could be transcribed more than 100-fold faster. The low rate of rRNA synthesis probably represents residual transcription of highly repressed genes, which are not activated until plutei begin to feed (Humphreys, 1973).

In addition to the low rate of rRNA synthesis, the study of the developmental regulation of rRNA synthesis during sea urchin embryogenesis is further complicated by the small number of nuclei present at early embryonic stages and their significantly higher rates of nonribosomal RNA synthesis (Emerson & Humphreys, 1970). Accordingly, qualitative studies have failed to detect rRNA synthesis before the late blastula stage (Hogan & Gross, 1972), although quantitative analysis of purified 26S RNA indicates that the per nucleus rate of rRNA synthesis may actually be quite constant between early blastula and pluteus stages, and possibly, during all of cleavage as well (Emerson & Humphreys, 1970, 1971).

Preliminary data from embryos of the Hawaiian species *T. gratilla* suggested that their rate of rRNA synthesis was considerably higher than that measured in *S. purpuratus* embryos and, thus, would be better suited for studies on sea urchin embryo rRNA metabolism. In this paper we identify the rRNA precursor and its processing intermediates, measure the rates of synthesis and processing of these molecules, and determine the reiteration of the rRNA coding sequences in order to calculate the average rates of expression of the ribosomal DNA sequences.

Materials and Methods

Culture and Labeling of Embryos. *Tripneustes gratilla* embryos were reared at 23–24 °C as described by Kleene & Humphreys (1977). Under these conditions, the embryos reach hatching blastula and early pluteus stages at approximately 16 and 48 h after fertilization, respectively, and remain viable for at least 90 h.

Plutei were harvested at the desired stages and resuspended to a final concentration of approximately 10^5 larvae/mL, and [^{3}H]guanosine (ICN, 14.5 Ci/mM) was added to a final concentration of either 10 or 20 μCi/mL. At the times noted, aliquots were removed from the culture and divided in half, and the plutei were washed successively through acid sea water (sea water containing 0.02 M sodium acetate, pH 4.5, 4 °C) and 1.5 M dextrose (4 °C). The plutei were then used for the RNA and GTP pool analyses described below.

RNA Extraction. The washed plutei were immediately lysed by the successive addition of 5.0 mL of urea extraction buffer (7 M urea, 0.35 M NaCl, 0.01 M Tris, 5 mM EGTA, 0.5% sodium dodecyl sulfate ($NaDodSO_4$),[1] pH 8.3) and 10 mL of water-saturated phenol–chloroform (1:1) with 0.1% hydroxyquinoline. The aqueous phase was extracted twice with the phenol–chloroform mix and then twice with chloroform alone. The interface was discarded at each step. The final RNA containing solution was precipitated overnight at −20 °C by the addition of 2.5 volumes of 95% ethanol. In some experiments, the RNA was redissolved in $NaDodSO_4$ buffer (0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris, 0.5% $NaDodSO_4$, pH 7.4) and analyzed directly on 15–35% (w/w) sucrose gradients (see below). In other experiments, the coextracted DNA was digested wtih DNase (Worthington, ribonuclease free, purified by digestion with proteinase K in the presence of Ca^{2+}; Tullis, in preparation) and reextracted with phenol–chloroform as described above. To remove polyadenylated RNA, the RNA (either before or after DNase digestion) was ethanol precipitated, dissolved in 0.5 M NaCl, 0.01 M Tris, 0.5% $NaDodSO_4$ (pH 7.4), and passed over an oligo(dT)-cellulose column (T-3, Collaborative Research).

Resolution of RNA. Extracted RNA was resolved in $NaDodSO_4$–sucrose gradients or $NaDodSO_4$–polyacrylamide gels. RNA samples were dissolved in $NaDodSO_4$ buffer and resolved in linear sucrose gradients (15–35%, w/w) made up in the same buffer. Centrifugation was for 5 h at 40 000 rpm (22 °C) in a Beckman SW 41 rotor unless otherwise stated. Sea urchin, insect, and rabbit rRNA UV absorbance markers were used to calibrate the gradients. Following centrifugation, the gradients were pumped through a Gilford spectrophotometer and scanned at 260 nm. For pulse-labeled RNA, the effluent was collected dropwise, acid precipitated, and collected on filters prior to counting.

Alternatively, RNA was dissolved in layering buffer (30 mM Tris, 36 mM NaH_2PO_4, 1 mM EDTA, 0.2% $NaDodSO_4$, 10% glycerol) and resolved in 7-cm 2.2% polyacrylamide gels for 3 h at 36 V (Loening, 1967). Following electrophoresis, the gels were soaked in distilled water for at least 60 min, scanned at 260 nm in a Gilford recording spectrophotometer, sliced, and incubated overnight in 5% NCS (Amersham/Searle), and 0.5% 2,5-diphenyloxazole in toluene prior to counting. Sea urchin, insect, and rabbit rRNA UV absorbance markers were used to calibrate the gels. In other experiments, the pulse-labeled RNA was denatured at 65 °C for 5 min in 40 μL of low salt buffer (0.01 M Tris, 1 mM EDTA, 0.5% $NaDodSO_4$, pH 7.5) prior to electrophoresis. The sample was then quickly cooled and 10 μL of 5× strength running buffer was added and the sample was electrophoresed as described above. This treatment caused a shift in the ratio of 26S to 18S UV absorbance and a reduction in the apparent molecular weight of the pulse-labeled poly(A^+) RNA but did not affect the rate of migration of any of the pulse-labeled rRNA species.

In some experiments, specific gel resolved peaks were excised from the gel, placed in a second gel tube, and overlaid with an additional segment of preelectrophoresed gel. The bottom of the tube was sealed with a dialysis bag. The RNA was electrophoresed out of the gel, collected in the dialysis bag, and then passed over a Sephadex G-25 column in $NaDodSO_4$ buffer to change buffers. Carrier RNA was added to the excluded fractions and the mixture was precipitated with ethanol as described above.

Analysis of GTP Specific Radioactivity. After the dextrose wash, the plutei pellets were routinely frozen in liquid N_2 and stored at −20 °C. The frozen plutei pellets were thawed in 5% trichloroacetic acid (4 °C) and dispersed with a Pasteur pipet. The resulting suspensions were placed on ice for 30 min and cleared by centrifugation at 10000*g* (4 °C) for 5 min. The supernatants were drawn off, extracted three times with 3 volumes of ethyl ether, neutralized with 1.0 M NH_4OH, and added to approximately 0.5 mL of prewashed Dowex-1 (HCO_3^-). The resin was then extensively washed with distilled water and the bound nucleotides were eluted with two 1.0-mL aliquots of 1.0 M NH_4HCO_3. The nucleotide containing solutions were evaporated to dryness under a gentle airstream, resuspended in 0.5 mL of distilled water and evaporated a second time to remove any remaining salt. The dried residues were then resuspended in distilled H_2O, applied to prewashed polyethylenimine plates (Brinkmann), resolved by ascending

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; PB, phosphate buffer; HAP, hydroxylapatite; NT, nucleotide.

KH_2PO_4 chromatography, and eluted into paper wicks as described previously (Griffith, 1978a). The specific radioactivity of the GTP pool was measured by using the luciferase reaction as described by Dolecki et al. (1976).

DNA Preparation. DNA was extracted from *Tripneustes* sperm as described by Kedes et al. (1975), winding the DNA out of ethanol at the final step. Following redissolution, the DNA was sheared to a length of 300 nucleotides in a Virtis homogenizer (Britten et al., 1974). The size of the sheared DNA was determined by electrophoresis in 99% formamide gels (Maniatis et al., 1975) or sedimentation in 5–20% alkaline sucrose gradients.

^{14}C-labeled single copy DNA was prepared by culturing early cleavage stage embryos (10^5/mL) in 2 μCi/mL each of [^{14}C]thymidine and [^{14}C]adenosine (Schwarz/Mann, 57 and 48 Ci/mol, respectively). The embryos were harvested at the hatching blastula stage and their nuclei isolated by the method of Brandhorst & Humphreys (1971) except Triton N-101 was substituted for Triton X-100 and 5 mM EGTA was added to the lysis buffer. The nuclear pellet was subsequently lysed and extracted as described for sperm DNA, digested with RNase A (Worthington, 50 μg/mL, preboiled for 10 min at 1 mg/mL), reextracted with phenol–chloroform, and sheared to a single-strand length of approximately 300 nucleotides. Following precipitation and redissolving, the DNA was dialyzed exhaustively against 0.12 M phosphate buffer (pH 6.5) containing 1 mM EDTA, melted, and reannealed at 60 °C to an equivalent C_0t (EC_0t) of 680 (Britten et al., 1974). The single-strand fraction, representing primarily single copy DNA (see Kleene & Humphreys, 1977), was isolated by hydroxylapatite (HAP) chromatography. The final specific radioactivity of the single copy DNA was about 700 cpm/μg.

DNA complementary to rRNA (rDNA) was prepared from *Escherichia coli* cells carrying the *T. gratilla* DNA containing chimeric plasmid, pTg 5034. This pMB-9 derived plasmid contains a 4.5-kb *Eco*RI fragment complementary to a portion of the 26S rRNA moiety (Griffith, Fregien, Simmen, and Humphreys, unpublished results). The plasmid DNA was purified from cleared lysates (Clewell & Helinski, 1970) by CSCl–ethidium bromide centrifugation, linearized with restriction endonucleases, and either sheared to 300 nucleotides as described above or used as substrate for the synthesis of "nick repaired" rDNA tracer (see below).

High specific radioactivity DNA complementary to rRNA was prepared from the plasmid DNA by the method of Nonoyama & Pagano (1973). Following linearization, plasmid DNA was incubated for 40 h with *E. coli* DNA polymerase (Boehringer-Mannheim) and [^{3}H]TTP (Schwarz, 52 Ci/mM) at 10 °C. The mixture was then phenol–$CHCl_3$ extracted (see above) and dialyzed against 0.1 M sodium acetate, 0.01 M EDTA to remove unincorporated nucleotides. A 50-fold excess of DNA from the parent plasmid pMB-9 was added as a carrier. After precipitation and dissolution, the mixture was sheared to single-strand length of 300 bases (see above). After a second dialysis and precipitation, the radioactive DNA mixture was melted and reassociated to a DNA EC_0t of 0.55 as described above. The double-strand fraction, representing those sequences complementary to pMB-9, was collected on HAP. The single-strand fraction was mixed with 100 μg each of gradient purified 26S and 18S rRNA, melted, and reannealed to an ER_0t of 0.75 at 60 °C. The double-strand fraction was collected on HAP, eluted, digested with RNAse (10 μg/mL, 0.05 M phosphate buffer, 37 °C, 18 h), brought up to 0.12 M with phosphate buffer, and rechromatographed on HAP (60 °C). The single-strand fraction, representing the DNA sequences complementary to rRNA released from the RNA:DNA hybrids by RNase, was then dialyzed and precipitated with carrier DNA. The final specific radioactivity of the rDNA was in excess of 5×10^5 cpm/μg.

DNA:DNA and DNA:RNA Hybridization. ^{3}H-labeled ribosomal DNA, ^{14}C-labeled single copy DNA, and bulk sperm DNA (see above) were mixed in a constant ratio at two different DNA concentrations. The DNA mixtures were dialyzed exhaustively against 0.4 M phosphate buffer with 0.001 M EDTA, sealed in glass capillary pipettes, melted, and allowed to reanneal at 67 °C to the appropriate EC_0ts. After hybridization, samples were diluted into 0.12 M PB and assayed on HAP.

Pulse-labeled RNA was mixed with 300 nucleotide long total plasmid DNA. A minimum tenfold excess of plasmid rDNA sequences was added over the amount of *radioactive RNA* in each sample calculated from the specific radioactivity of the GTP pool. Samples were melted and allowed to reanneal in 0.12 M PB at 60 °C or in 0.4 M PB at 67 °C. Following hybridization, the samples were diluted into 0.25 M PB containing 50 μg of calf-thymus DNA and RNase (boiled at 1 mg/mL for 10 min) was added to a final concentration of 10 μg/mL. After 60–90 min at room temperature, the samples were diluted to 0.12 M PB and the RNase resistant radioactivity (i.e., that contained in hybrids) was assayed on HAP. Matched controls, containing no plasmid DNA or nonribosomal plasmid DNA, were coanalyzed with each experimental sample. The apparent RNase resistant radioactivity in each control hybridization ranged from 0 to 2% and was subtracted from the RNase resistant radioactivity in its paired experimental sample. In other controls, radioactive, gradient purified 18S and 26S rRNA was also hybridized to excess plasmid DNA. In these experiments, 5% and 35% of the respective rRNA radioactivities were rendered RNase resistant.

Results

When sea urchin embryos are reared from the mesenchyme blastula to early pluteus stage in the presence of [^{3}H]-guanosine, most of the accumulated radioactive RNA cosediments in $NaDodSO_4$–sucrose gradients with the 26S and 18S peaks of rRNA UV absorbance (Figure 1A). Although this result indicates that rRNA is being synthesized during this time, early pluteus stage larvae pulsed for only 10 min have no detectable peak(s) of rRNA radioactivity (Figure 1A). In order to identify the initial rRNA transcript, we hybridized the gradient fractionated, pulse-labeled RNA in Figure 1 (see legend for details) with a recombinant plasmid DNA driver containing sequences complementary to a portion of the 26S rRNA. Calculating from the specific radioactivity of the GTP pool, a sufficiency of rDNA was added to ensure a minimum tenfold excess over all of the *radioactive* RNA in each fraction, but insufficient to provide an excess over the nonradioactive RNA. Under these conditions, only rRNA sequences contained in a peak of rRNA precursor resolved from the bulk rRNA or sequences unique to a rRNA precursor will hybridize significantly. Other radioactive rRNA will be competed out by the excess, nonradioactive, bulk rRNA. This experiment revealed a single peak of hybridizing radioactivity at 34 S, indicating an apparent molecular weight of 2.5×10^6. No higher molecular weight peak of hybridizable radioactivity was observed, although these gradients should display molecules whose molecular weights are in excess of 10^7.

Consistent with these results, the greater resolution afforded by gel electrophoresis also reveals a discrete peak of newly synthesized pluteus RNA with an apparent molecular weight

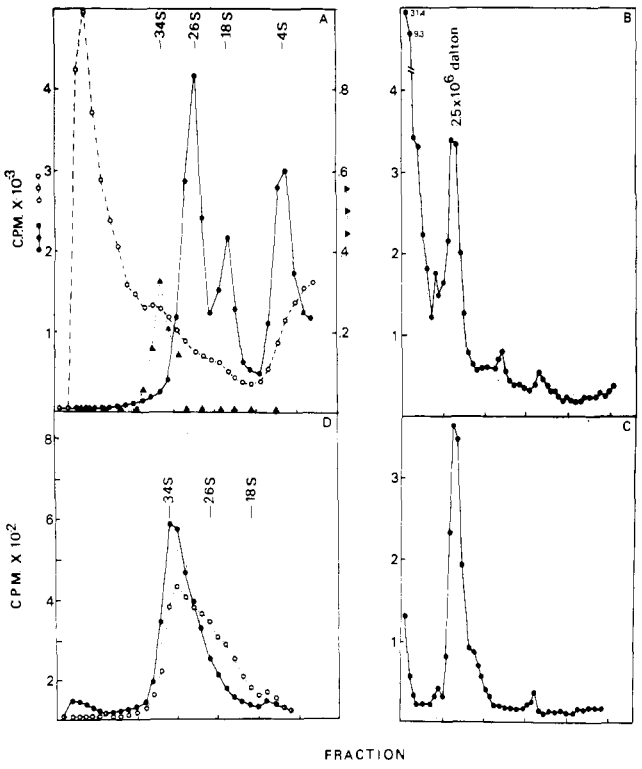


FIGURE 1: Identification of the primary rRNA transcript. (A) $NaDodSO_4$–sucrose gradients of RNA extracted from early pluteus stage larvae pulse-labeled with [^{3}H]guanosine for 24 h (●) or 10 min (○). In order to identify the newly synthesized RNA which was complementary to ribosomal DNA (▲), 10-min pulse-labeled pluteus RNA was resolved in $NaDodSO_4$–sucrose gradients, and groups of 4–5 adjacent fractions were pooled and ethanol precipitated. Each of the pooled samples was fractionated on oligo(dT)-cellulose, and the poly(A$^-$) RNA fraction was denatured by heating to 60 °C in $NaDodSO_4$ buffer and resolved on a second, identical gradient. Each gradient was then fractionated and that portion of each RNA sample which had resedimented to its original position in the first gradient was precipitated and hybridized with rDNA as described under Materials and Methods. (B) Extracted RNA from plutei pulse-labeled for 20 min with [^{3}H]guanosine was DNase digested, stripped of polyadenylated sequences, and electrophoresed for 3 h in 2.2% $NaDodSO_4$–polyacrylamide gels as described under Materials and Methods. (C) Twenty-minute pulse-labeled pluteus RNA was resolved in $NaDodSO_4$ gels as in B. The gel fraction containing the 2.5 × 10^6 dalton RNA was excised and electroeluted from the gel as described under Materials and Methods. The electroeluted RNA and egg RNA UV absorbance markers were then coelectrophoresed in $NaDodSO_4$ gels as in B. (D) Electroeluted 2.5 × 10^6 dalton RNA and egg rRNA UV absorbance markers were resuspended in 0.01 M Tris, 0.5% $NaDodSO_4$ (pH 7.5). One-half of the sample was denatured by heating at 60 °C for 5 min (○); the other half did not receive heat treatment (●). Both samples were then resolved in $NaDodSO_4$–sucrose gradients for 7 h at 40000. For additional details, see Materials and Methods.

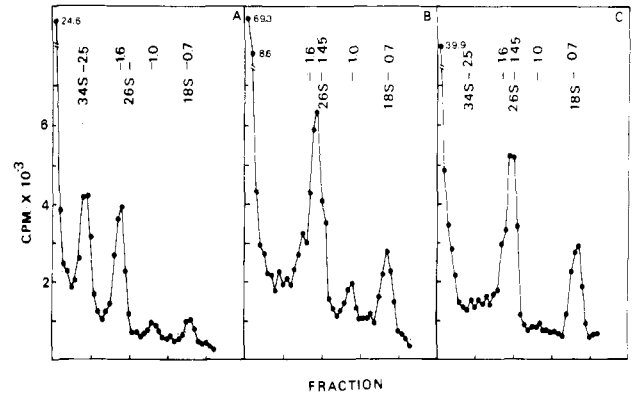


FIGURE 2: Processing of the 2.5 × 10^6 dalton precursor in the absence of transcription. Early pluteus stage embryos were incubated for 20 min in 20 μCi/mL [^{3}H]guanosine in sea water containing ca. 100 μg/mL gentamicin. After 20 min, actinomycin was added to a final concentration of 20 μg/mL. Samples were removed at 15 (A), 45 (B), and 120 min (C), and the RNA was prepared and electrophoresed as described in the legend to Figure 1B. Gel profiles of 20-min pulse-labeled pluteus RNA (i.e., "zero time" actinomycin chase) are shown in Figure 1B.

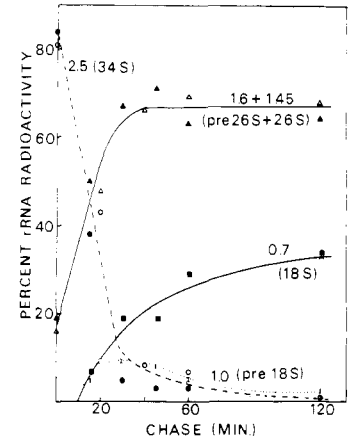


FIGURE 3: Summary of rRNA processing in actinomycin. Early pluteus stage larvae were pulse-labeled and transferred to actinomycin containing sea water as in Figure 2. After RNA extraction and gel resolution, the peaks of rRNA radioactivity were graphically delineated from the underlying heterodisperse RNA radioactivity by the method of Girard et al. (1965; see also Emerson & Humphreys, 1970; Anderson & Smith, 1977; Griffith, 1978b). The fractional radioactivity in each rRNA species was quantitated in two independent experiments after each of several chase lengths (open and closed symbols). In one of the two experiments, there was slight bacterial rRNA radioactivity obscuring the 1.0 × 10^6 dalton intermediate. Consequently, these data were not included. All other details are the same as in Figure 2. 34 S (○, ●); 26 S (△, ▲); 18 S (□, ■); pre 18 S (×).

of 2.5 × 10^6 (Figure 1B). This peak was electroeluted from the gels and analyzed more extensively. In one experiment, an aliquot of the electroeluted RNA was mixed with 26S and 18S UV absorbance markers and reelectrophoresed under the original conditions. The radioactive RNA migrated as a single sharp peak whose apparent molecular weight was again 2.5 × 10^6 (Figure 1C). A second aliquot was hybridized to the plasmid DNA containing a portion of the 26S rRNA sequence. Eighteen percent of the radioactivity was recovered as RNase resistant hybrids. A third aliquot was divided in half and mixed with 26S and 18S rRNA. One of the two samples was denatured by heating at 60 °C for 5 min in 0.01 M Tris–0.5% $NaDodSO_4$. The second sample, also dissolved in 0.01 M Tris–0.5% $NaDodSO_4$, was not heated and served as a control. Both samples were resolved in $NaDodSO_4$–sucrose gradients (Figure 1D). No appreciable change in the molecular weight of the radioactive 34S RNA was produced by this treatment, although the optical profiles of the added marker RNA indicated a change in the ratio of 18S to 26S UV absorbance, thereby demonstrating the efficacy of the denaturation (not shown). Thus, these experiments establish that a significant portion of the 2.5 × 10^6 dalton putative rRNA precursor contains rRNA sequences by the criterion of sequence complementarity and that this large species does not seem to arise via RNA aggregation.

Processing of the rRNA Precursor. We investigated the processing of the 34S, 2.5 × 10^6 dalton rRNA by tracing the flow of radioactivity out of precursor peak during an actinomycin block of new RNA synthesis (Figure 2). Concomitant with the loss of radioactivity from the precursor peak, new peaks of radioactivity appeared sequentially at 1.6 × 10^6

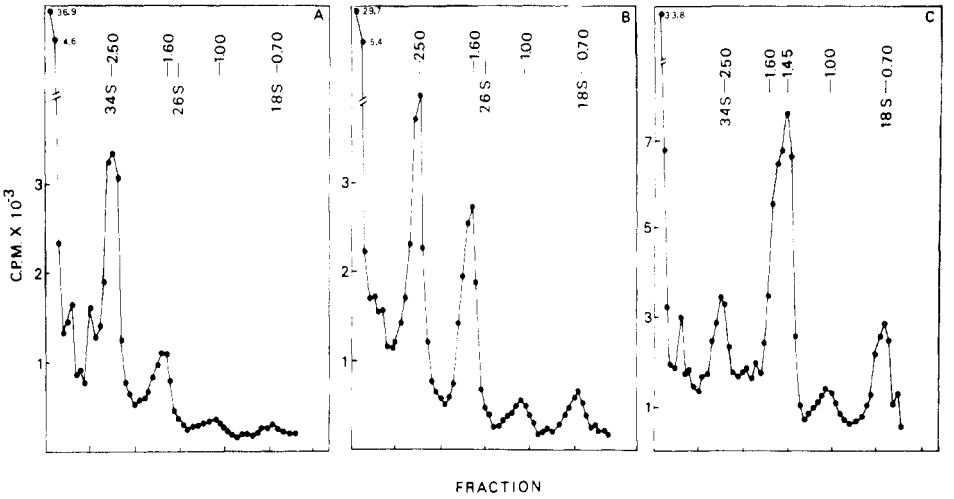


FIGURE 4: Time course of guanosine incorporation into rRNA. Early pluteus stage embryos were incubated for 5 min in sea water containing 10 μCi/mL of [^{3}H]guanosine and then diluted sixfold with fresh sea water. Samples were removed after 30 (A), 45 (B), and 120 min (C). RNA was extracted and electrophoresed as in Figure 2. Gel profiles of 20-min pulse-labeled pluteus RNA are shown in Figure 1B.

Table I: Time Course of [^{3}H]Guanosine Incorporation into GTP and rRNA

pulse length (min)	GTP pool sp radioact. (cpm/pmol of GTP)		ribosomal RNA incorp[a] (cpm/OD_{260})[b]			
	instantaneous[c]	av[d]	2.5 × 10^6	1.6 + 1.45 × 10^6	1.0 × 10^6	0.7 × 10^6
0	10					
10	294	147[e]	5 993	0	0	0
20	236	210	8 978	1 038	762	0
			7 800	750	708	0
30	206	212	22 794	6 173	1530	967
			15 700[f]	5 200[f]		
45	185	205	18 800	14 678	2525	3 025
60	157	195	16 032	27 314	3511	6 584
			11 300[f]	16 737[f]		
120	105	165	11 580	40 500	4000	12 000
			10 300[f]	36 000[f]		

[a] Calculated from gel resolved rRNA as in Figure 4. [b] The rRNA specific incorporation in each gel was normalized to the recovery of the rRNA UV absorbance by using a standard curve constructed by electrophoresing measured amounts of egg RNA and determining the area under the rRNA peaks in the resulting gel scans. [c] The measured GTP specific activity at each point in time. [d] The cumulative, average GTP specific radioactivity during the interval beginning at the addition of the guanosine and ending at the point indicated. [e] In other experiments, the GTP specific radioactivity was measured between the 0 and 10-min time points. The results (not shown) indicate that the pool specific radioactivity increases linearly during this initial phase. It is assumed that a comparable linear increase occurred in the present experiment. [f] The 2.5 × 10^6- and 1.45 + 1.6 × 10^6-dalton species were maximally resolved by electrophoresing these samples so that the 1.45 × 10^6-dalton species' peak of UV absorbance was at the bottom of the gel.

and 1.0 × 10^6 daltons and then comigrating with mature (i.e., 26S and 18S) rRNA at 1.45 × 10^6 and 0.7 × 10^6 daltons (Figure 2A–C). Figure 3 summarizes the proportion of radioactive rRNA in the various peaks with time for all actinomycin experiments. In a similar experiment, the kinetics of incorporation into each of these peaks was followed in the absence of actinomycin. These results (Figure 4A–C) verified the order of appearance of the individual peaks observed during the actinomycin chase. Identical results were obtained when the RNA was heat denatured in low salt prior to electrophoresis. In additional experiments (not shown), the pulse-labeled RNA was more fully resolved by electrophoresis for 10 h (see legend to Table I). This improved the separation of the 1.45 × 10^6 and 1.6 × 10^6 dalton species and confirmed the molecular weights and labeling kinetics observed in Figures 2, 3, 4, and 6. Finally, the subcellular localization of each of the rRNA species was examined. The mature 1.45 × 10^6 and 0.7 × 10^6 dalton peaks of radioactivity and UV absorbance were found only in the cytoplasm. The 2.5 × 10^6, 1.6 × 10^6, and 1.0 × 10^6 dalton peaks were recovered only in the nucleus (Figure 5). The results of these experiments are diagrammatically summarized in the Discussion.

Absolute Rate of Precursor Synthesis and Processing. Quantitative measurements of the absolute rate of precursor synthesis and processing were made by pulsing plutei with [^{3}H]guanosine and quantitating the flow of radioactivity through the GTP pool and each of the several rRNA species resolved on polyacrylamide gels. For this approach to be valid, it is essential that all of the radioactivity incorporated into RNA is derived from the GTP pool. Alkaline hydrolysis of the pulse-labeled RNA and subsequent chromatography of the resulting nucleotides produced no radioactive 2′,3′-AMP and demonstrates that there is no radioactive purine nucleotide interconversion. In addition, the difference in the maximum GTP specific radioactivity and that of the external guanosine indicates that the GTP pool has been expanded by less than 5%. Thus, the possibility of an artifactual pool expansion affecting the results is unlikely.

The measurements of the GTP pool specific radioactivity and rRNA incorporation kinetics are summarized in Table I and are used to calculate the absolute rRNA molar accumulation curves in Figures 6A and 6B. We have calculated the rate of rRNA synthesis separately from the 1.6 + 1.45 × 10^6 dalton peaks (Figure 6A) and also the 1.0 + 0.7 × 10^6

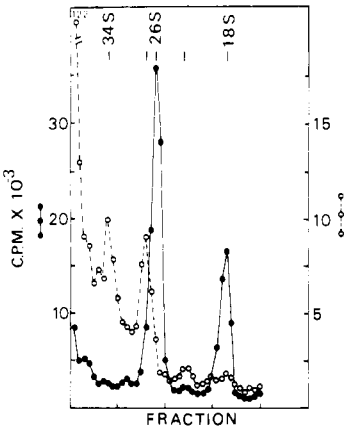


FIGURE 5: Subcellular localization of pulse-labeled rRNA. Early pluteus stage larvae were raised in sea water containing gentamicin and pulse-labeled with [^{3}H]guanosine as described in Figure 2. After 120 min of labeling, a second aliquot of [^{3}H]guanosine was added to the culture to ensure maximal labeling of unstable nuclear RNA species, and the incubation was continued for an additional 30 min. The larvae were then rinsed through acid sea water (4 °C) and dissociated by resuspension in 1.0 M glycine containing 5 mM EDTA as described by Kane (1973). After approximately 5 min, the cells were pelleted and then lysed by the addition of 2.0 mL of Triton N-101 containing homogenization buffer (Materials and Methods). The nuclei were spun out of the suspension at 5000 rpm in a Sorvall HB-4 rotor at 4 °C. The 2.0 mL of cytoplasmic supernatant was mixed with 8.0 mL of urea extraction buffer. The nuclear pellet was rinsed with homogenization buffer (4 °C) and then lysed with 10 mL of urea extraction buffer. Twenty milliliter volumes of phenol–chloroform mix were rapidly added. The samples were subsequently extracted, DNase digested, and stripped of polyadenylated RNA as described under Materials and Methods. Electrophoresis of the RNA was carried out as in Figure 2. Nuclear RNA (○); cytoplasmic RNA (●).

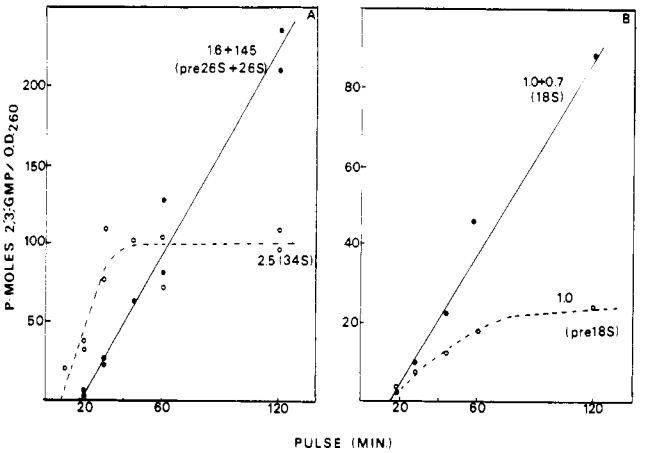


FIGURE 6: Molar accumulation of rRNA. (A) The molar accumulation of the 1.45 + 1.6 × 10^6 dalton rRNA species (i.e., 26S and pre-26S rRNA), and the 2.5 × 10^6 dalton precursor (i.e., 34S pre-rRNA) were computed from the data in Table I by using the cumulative average GTP pool specific radioactivities and instantaneous GTP pool specific radioactivities, respectively. (The 34S pre-rRNA is unstable and its synthesis is better defined by the instantaneous GTP pool value, while the stable, accumulating 26S species radioactivity is best reflected by the average GTP pool value over the entire labeling interval.) The molar accumulation curve of the 26S + pre-26S species has been corrected for the portion of the pre-26S rRNA mass which will be lost during nonconservative processing (i.e., 10%). Data points were fit by linear regression. (B) The molar accumulation of the 1.0 × 10^6 and 0.7 × 10^6 dalton rRNA (i.e., 18S and pre-18S rRNA, respectively) were each computed from the data of Table I by using the cumulative average GTP specific radioactivities. The 18S + pre-18S rRNA curve has also been corrected for the pre-18S rRNA mass eventually lost in processing (i.e., 30%). Data points were fit by linear regression.

dalton peaks (Figure 6B) after correcting for the RNA mass to be lost from the intermediate species during subsequent processing. Both determinations indicate an average initial rate of precursor synthesis of approximately 3100 molecules h^{-1} nucleus^{-1} (see Table II) based on a number of 750 nuclei per early pluteus larva (Kleene & Humphreys, 1977). Similarly, an estimation of the initial rate of rRNA synthesis computed from the kinetics of accumulation of the 2.5 × 10^6 dalton species alone (Table II) leads to a value which is virtually identical with that obtained from the accumulation of mature 26S and 18S rRNA species. Thus, there appears to be no significant precursor wastage at this stage.

The processing half-life of the 2.5 × 10^6 dalton precursor was determined from the incorporation kinetic measurements of the precursor's initial rate of synthesis and its steady-state concentration in the nucleus (see Table II). The relationship between these parameters is expressed in eq 1

$$K_s T_{1/2} (\ln 2)^{-1} = SS \qquad (1)$$

where SS is the steady-state concentration of the precursor, $T_{1/2}$ is its average first-order half-life, and K_s is its rate of synthesis (Griffith, 1978a). With these data, a half-life of 19 min was obtained. This value is consistent with the labeling kinetics of the processed rRNA species observed in both the incorporation and actinomycin pulse-chase experiments (Figures 2, 3, 4, and 6).

Reiteration of the Ribosomal DNA. To better define the quantitative parameters regulating rRNA synthesis, we have also measured the reiteration of the rRNA genes in *T. gratilla* sperm DNA. This was accomplished by driving a mixture of [^{3}H]DNA complementary to rRNA and ^{14}C-labeled single copy DNA with total *T. gratilla* sperm DNA and comparing their rates of reassociation (Figure 7).

Table II: Summary of Kinetic Parameters

	rRNA species		
	1.6 + 1.45 × 10^6	1.0 + 0.7 × 10^6	2.5 × 10^6
(I) Rate of rRNA Synthesis			
pmol of G min^{-1} A_{260}^{-1} [a]	2.2	0.9	3.9
pmol of NT min^{-1} A_{260}^{-1} [b]	6.3	2.8	11.5
total NT h^{-1} A_{260}^{-1}	2.26 × 10^{14}	1.0 × 10^{14}	4.15 × 10^{14}
molecules h^{-1} A_{260}^{-1} [c]	5.1 × 10^{10}	4.7 × 10^{10}	5.45 × 10^{10}
molecules h^{-1} nucleus^{-1} [d]	3183	2927	3400
molecules h^{-1} gene^{-1} [e]	31.8	29.7	34
(II) Steady-State Level of 2.5 × 10^6-Dalton rRNA			
pmol of G A_{260}^{-1} [a]	100		
pmol of total NT A_{260}^{-1} [b]	299		
molecules A_{260}^{-1} [c]	2.37 × 10^{10}		
molecules nucleus^{-1} [d]	1450		
(III) Processing Half-Life of 2.5 × 10^6-Dalton rRNA			
SS (molecules nucleus^{-1}) [f]	1450		
K_s (molecules min^{-1} nucleus^{-1}) [g]	51		
$T_{1/2}$ (min) [h]	19		

[a] From Figure 6. [b] Calculated assuming GMP represents 34.9% and 31.8% of the 26S and 18S rRNA moieties mass, respectively (Emerson & Humphreys, 1970). [c] Calculated assuming an "average" nucleotide molecular weight of 330 for rRNA and the molecular weights determined in the gel analyses. [d] Calculated assuming 750 nuclei/larva at the early pluteus stage (Kleene & Humphreys, 1977), 1.7 × 10^{-9} g of RNA/larva (Kleene & Humphreys, 1977) and 37 μg of rRNA/A_{260}. [e] Calculated assuming 100 genes/nucleus (Figure 7). See text for additional details. [f] From II. [g] From I. [h] Calculated from eq 1 in text.

The reaction of the rDNA tracer is described by a single second-order transition with $EC_0t_{1/2}$ of 17. At termination, 94% of the rDNA tracer was in duplex.

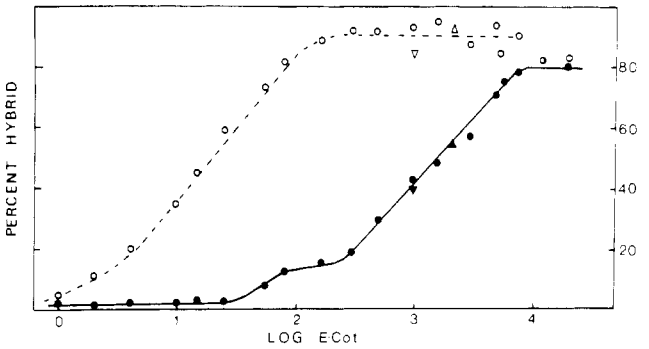


FIGURE 7: Reassociation of ^{3}H-labeled ribosomal DNA and ^{14}C-labeled single copy DNA with *T. gratilla* sperm DNA. ^{3}H-labeled ribosomal DNA (open symbols) and ^{14}C-labeled single copy DNA (closed symbols) were mixed with *T. gratilla* sperm DNA at two different concentrations as described under Materials and Methods. Triangles represent samples from the two DNA stocks which were incubated for the maximum time involved in obtaining the data herein and were then remelted and allowed to reanneal to the EC_0t indicated (see text and Materials and Methods for additional details).

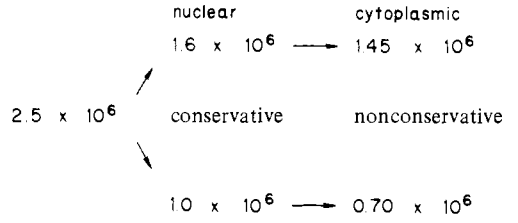
In contrast to the rDNA, the major transition in the ^{14}C-labeled single copy DNA reaction, amounting to a final value of 80% duplex, has an $EC_0t_{1/2}$ of 1300. This is in good agreement with the theroretical value of 910 calculated for the single copy *T. gratilla* genome (Kleene & Humphreys, 1977). The rapidly reassociating component, accounting for 15% duplex by an EC_0t of 100, represents the presence of a repetitive DNA contaminant.

The slightly lower reactivity of the ^{14}C-labeled single copy DNA tracer could indicate that the DNA was being degraded or shortened during the longer incubations required to hybridize the single copy DNA. To test this possibility, parallel hybridizations were set up with the concentrated and dilute DNA stocks and incubated for the maximum times involved in the reactions presented in Figure 7. They were then remelted and reannealed to the EC_0ts shown in Figure 7. In both instances, the preincubated controls reannealed with virtually the same kinetics as the untreated DNA. Thus, tracer reactivity does not decline measurably during the course of the incubations. We conclude, therefore, that the rDNA reacts 75 times faster than the single copy DNA.

The relative rates of reassociation of the two tracers are attributable to their respective sizes, base compositions, and abundance in the driver. All other factors are common to both tracers and thus cannot contribute to the different rates of reassociation. The single-strand lengths of the two DNA tracers and the DNA driver were determined by using alkaline sucrose gradients and 99% formamide gels (Maniatis et al., 1975). In both systems, the two tracers and the driver DNA had identical size distributions which were coincident with that of an added 300-nucleotide long DNA marker. Similarly, by using the migration of the tracking dye in the formamide gels as an independent sizing standard (Maniatis et al., 1975), all three DNA preparations had single-strand lengths of 270 nucleotides.

The GC contents of the DNA samples were determined by CsCl-actinomycin gradient centrifugation (Birnstiel et al., 1974). The main band DNA, which we assume contains the single-copy DNA, banded with a density indicating a GC content of 40%. On the other hand, hybridization of filter bound, CsCl gradient fractionated DNA to radioactive rRNA produced a peak of hybridizing radioactivity at a position indicating a GC content of 60% (not shown). This is identical with the GC content of rRNA (Emerson & Humphreys, 1970; Table II).

Scheme I



The quantitative relationship between DNA base composition and the rate of DNA reassociation has not been precisely defined. However, Wetmur & Davidson (1968) have shown that 60% GC DNA reacts 1.5 times more rapidly than 40% GC DNA under otherwise equivalent conditions. Assuming that a similar base composition dependent rate enhancement occurs here, the difference in the rates of reassociation of the single copy DNA and the rDNA is attributable to a 50-fold difference in their relative abundance in the sperm DNA driver.

Discussion

Identification of the rRNA Primary Transcript. We have identified by hybridization, a 34S, 2.5 × 10^6 dalton RNA species complementary to ribosomal DNA. This pulse-labeled rRNA species does not arise via RNA aggregation, is confined to the nuclear fraction, and has the expected labeling and pulse-chase kinetics of an rRNA precursor. This pre-rRNA species has the same molecular weight as a methionine pulse-labeled putative rRNA species isolated from gastrula RNA (Hogan & Gross, 1972), a uridine pulse-labeled putative rRNA species isolated from sea urchin oocyte nucleoli (Sconzo et al., 1972), and the ribosomal precursors of insect and amphibian cells (Edstrom & Daneholt, 1967; Hall & Cummings, 1975; Griffith, 1978b; Anderson & Smith, 1977). It is approximately 50% smaller than the rRNA precursors of mammalian cells (e.g., Loening et al., 1969; Perry et al., 1970).

Although the 2.5 × 10^6 dalton pre-rRNA species identified here could potentially be an intermediate species in the processing of a yet larger mammalian type rRNA precursor, we have been unable to obtain any evidence to support the existence of such a molecule. No higher molecular weight peak of radioactivity hybridizing to rDNA was found in the sucrose gradient resolved RNA, nor was there any higher molecular weight peak of radioactivity found in the polyacrylamide gels. Thus, any larger precursor would either have to be very short lived (a half-life less than 5 min) or be differentially lost during RNA extraction. The kinetic data argue against both possibilities.

Ribosomal RNA Processing. Our results suggest the pathway for processing of the 2.5 × 10^6 dalton precursor species given in Scheme I.

According to this outline, the initial 2.5 × 10^6 dalton precursor gives rise to two intermediate nuclear species via a single conservative scission. There is no indication of precursor wastage. Each of the two intermediates, in turn, undergoes additional nonconservative processing to yield mature, cytoplasmic rRNA. The combined molecular weights of the processing intermediates shown above are slightly greater than that measured for the precursor. However, these minor differences are within the experimental error inherent to these measurements. Comparably sized RNA moieties have also been found in sea urchin nucleoli (Sconzo et al., 1972) and methionine-labeled gastrulae (Hogan & Gross, 1972) and add further support for the pathway outlined above.

RNA Synthesis and Development. The measurements reported here for *T. gratilla* suggest that the quantitative aspects of rRNA metabolism are not comparable in all sea urchin species. For example, the number of ribosomal genes measured in *T. gratilla* DNA is about 3–4-fold lower than the estimates for *L. variegatus* (Patterson & Stafford, 1971; Wilson et al., 1976), while the per nucleus rate of rRNA synthesis in *T. gratilla* is 5–10-fold higher than those reported for either *S. purpuratus* or *L. pictus* (Emerson & Humphreys, 1970, 1971; Humphreys, 1973; Galau et al., 1977). There also appears to be significant differences in the degree of repression of the ribosomal genes in these species. Galau et al. (1977) have calculated that the rate of rRNA synthesis in *S. purpuratus* gastrulae is 0.1–1.0% of a calculated maximum based on the larger number of genes reported for *L. variegatus*. We estimate the rate of rRNA synthesis in *T. gratilla* plutei is approximately 10% of the theoretical maximum, assuming a nucleotide elongation rate of 10 nucleotides/s (Aronson & Chen, 1977) and an RNA polymerase packing of 1 polymerase molecule/100 base pairs (Miller & Beatty, 1969; Hamkalo et al., 1973). Since the per nucleus rate of rRNA synthesis in both *S. purpuratus* and *T. gratilla* changes very little between blastula and pluteus stages (Emerson & Humphreys, 1970; J. K. Griffith and T. Humphreys, in preparation), this difference most likely reflects physiological differences in the sea urchin species themselves.

The measurements of rRNA synthesis during embryogenesis are of particular interest in relation to the regulation of rRNA synthesis during oogenesis. Even at the higher per nucleus rate of rRNA synthesis measured in *T. gratilla* plutei, a tetraploid egg would require more than 6 years to accumulate the quantity of rRNA found in a mature *T. gratilla* egg (1.7×10^{-9} g, Kleene & Humphreys, 1977). Since sea urchin eggs are thought to mature in a few months, the actual rate of rRNA synthesis in the oocyte may be as much as 30–50 times higher than in the pluteus' nuclei. The accumulation of 1.7×10^{-9} g of rRNA in 2 months of oogenesis, for example, would require the maximal loading of all of the rRNA genes (assuming 1 polymerase molecule/100 nucleotides) and a nucleotide elongation rate of 40–50 nucleotides polymerized s^{-1} (polymerase molecule)$^{-1}$. On the other hand, the increase in RNA mass observed after feeding *L. pictus* plutei (approximately a threefold increase in 4 days) only represents a slightly higher rate of rRNA synthesis (about fivefold) than the per nucleus reported here for unfed *T. gratilla* plutei. Thus, if we assume that the rate of rRNA synthesis in fed pluteus cells is typical of that of somatic cells, then the rRNA genes of sea urchins may be fully activated only during oogenesis. At present, it is not evident whether all of a cell's ribosomal genes are equally active, or if there is a subset of rRNA genes which are active in tissue cells and another group which are expressed during oogenesis as reported for the 5S RNA genes in *Xenopus* (Brown & Sugimoto, 1973). On the one hand, the presence of apparently fully loaded ribosomal genes seen in electron micrographic images of spread sea urchin embryo cell chromatin (Busby and Bakken, in preparation) suggests that at least some ribosomal genes may be fully active in embryonic cells. However, if the rates of RNA chain elongation or termination are slow and thus regulate the reduced, average rate of expression of the ribosomal genes, one would expect to find fully loaded ribosomal genes.

Acknowledgments

We thank N. Fregien, F. Simmen, L. Perkocha, and Dr. M. Mandel for their help in preparing the recombinant plasmid DNA.

References

Anderson, D., & Smith, L. D. (1977) *Cell 11*, 663.
Aronson, A. I., & Chen, K. (1977) *Dev. Biol. 59*, 39.
Birnstiel, M., Telford, J., Weinberg, E., & Stafford, D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2900.
Brandhorst, B. P., & Humphreys, T. (1971) *Biochemistry 10*, 877.
Brandhorst, B. P., & Humphreys, T. (1972) *J. Cell Biol. 53*, 474.
Brandhorst, B. P., & Bannet, M. (1978) *Dev. Biol. 63*, 421.
Britten, R. J., Graham, D. E., & Neufeld, B. R. (1974) *Methods Enzymol. 29E*, 346.
Brown, D. D., & Sugimoto, K. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38*, 501.
Clewell, D. B., & Helinski, D. R. (1970) *Biochemistry 9*, 4428.
Dolecki, G. J., Anderson, D., & Smith, L. D. (1976) *Anal. Biochem. 71*, 37.
Dolecki, G. J., Duncan, R. F., & Humphreys, T. (1977) *Cell 11*, 339.
Dworkin, M., & Infante, A. (1978) *Dev. Biol. 62*, 247.
Edstrom, J. E., & Daneholt, B. (1967) *J. Mol. Biol. 28*, 331.
Emerson, C. P., & Humphreys, T. (1970) *Dev. Biol. 23*, 86.
Emerson, C. P., & Humphreys, T. (1971) *Science 171*, 898.
Galau, G. A., Lipson, E. D., Britten, R. J., & Davidson, E. H. (1977) *Cell 10*, 415.
Girard, M. H., Latham, M., Penman, S., & Darnell, J. E. (1965) *J. Mol. Biol. 11*, 187.
Griffith, J. K. (1978a) *Dev. Biol. 62*, 229.
Griffith, J. K. (1978b) *Dev. Biol. 65*, 353.
Hall, T. J., & Cummings, M. (1975) *Dev. Biol. 46*, 233.
Hamkalo, B., Miller, O., & Bakken, A. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38*, 915.
Hogan, B., & Gross, P. (1972) *Exp. Cell Res. 72*, 101.
Humphreys, T. (1973) in *Developmental Regulation, Aspects of Cell Differentiation* (Coward, S. J., Ed.) p 1, Academic Press, New York.
Kane, R. E. (1973) *Exp. Cell Res. 81*, 301.
Kedes, L. H., Cohn, R. H., Lowry, J. C., Chang, A. C. Y., & Cohen, S. N. (1975) *Cell 6*, 359.
Kleene, K. C., & Humphreys, T. (1977) *Cell 12*, 143.
Loening, U. (1967) *Biochem. J. 102*, 251.
Loening, U., Jones, K., & Birnstiel, M. (1969) *J. Mol. Biol. 45*, 353.
Maniatis, T., Jeffrey, A., & van de Sande, H. (1975) *Biochemistry 14*, 3787.
Miller, O., & Beatty, B. (1969) *Science 164*, 955.
Nonoyama, M., & Pagano, J. (1973) *Nature (London) 242*, 44.
Patterson, J. B., & Stafford, D. (1971) *Biochemistry 10*, 2775.
Perry, R., Cheng, F., Freed, J., Greenberg, J., Kelley, P., & Tartof, K. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 609.
Sconzo, G., Bono, A., Albanese, I., & Giudice, G. (1972) *Exp. Cell Res. 72*, 95.
Wetmur, J. G., & Davidson, N. (1968) *J. Mol. Biol. 31*, 349.
Wilson, F., Blin, N., & Stafford, D. (1976) *Chromosoma 58*, 247.