Nucleic Acid Polymerizing Enzymes in Developing *Strongylocentrotus franciscanus* Embryos†

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ABSTRACT: DNA-dependent RNA polymerase, DNA-dependent DNA polymerase, and terminal riboadenylate transferase (TRT) activities have been measured after DEAE-Sephadex chromatography of whole cell extracts prepared from eggs and staged embryos of the urchin, *Strongylocentrotus franciscanus*. Activity of each of these three polymerase classes is present in the egg, and the total activity per embryo is constant throughout embryogenesis to the pluteus stage (~1000 cells). Thus the egg appears to contain sufficient DNA polymerase, RNA polymerase, and TRT for embryogenesis. The increases in the synthesis of DNA, RNA, and polyadenylated RNA tracts observed after fertilization must be due to the activation of the preexisting egg enzymes. Separation of the egg into nucleate and anucleate halves demonstrates that the RNA polymerases are not restricted to the egg nucleus. During development, the enzymes become progressively more associated with the cell nucleus. The egg extracts contain low activities (~6% total) of RNA polymerase II as measured by sensitivity to α-amanitin. This is confirmed by resolution of the RNA polymerase forms I, II, and III by gradient sievorptive elution on DEAE-Sephadex. Later stage embryos contain more nearly equal activities of RNA polymerase I, II, and III, although the total RNA polymerase activity per embryo is not changed. Additionally, two chromatographically distinct species of RNA polymerase III are detected, one of which is observed only in later stages. Thus interconversion of enzymes via addition of new subunits or coordinate synthesis and loss of enzyme species must occur.

The sea urchin has been extensively used in research on the biochemistry of developing cell systems (see Guidice, 1973, for a full review). Fertilization of the urchin oocyte characteristically results in the activation of several metabolic pathways including DNA, RNA, and protein synthesis. A mechanistic explanation of the activation of these synthetic processes is not now possible. In this communication, we examine the correlation between the observed activation of nucleic acid synthesis in vivo and the extractable activity of the nucleic acid polymerases.

DNA accumulates in the embryo following fertilization (Brachet, 1933) and DNA synthesis in the cleavage stage embryo occurs during discrete (~15 min) periods in the cell cycle (Hinegardner et al., 1964). The extent of DNA synthesis during each synthesis phase is proportional to the number of replicating cells in the embryo (Hinegardner et al., 1964; Nemer, 1962; Gross and Cousineau, 1964; Fansler and Loeb, 1969). In the urchin, *Strongylocentrotus purpuratus*, the first cell division occurs at 100–120 min postfertilization; and the first DNA replication has occurred prior to this time. DNA synthesis and cell division continue until there are about 1000 cells/embryo (Hinegardner et al., 1964). Thus, fertilization causes an extensive activation of replicative DNA synthesis, and the synthesis is regulated with respect to time and extent. The synthesis of several classes of RNA molecules is known to be activated in the urchin embryo. Polyadenylation of RNA molecules of both oogenic and embryogenic origin increases rapidly after fertilization (Slater and Slater, 1974; Wu and Wilt, 1973; Slater et al., 1973; Slater et al., 1972). Stage specific syntheses of DNA-like polydisperse RNA (Wilt, 1963; Gross et al., 1965), ribosomal RNA (Sconzo et al., 1970), and histone mRNA (Farquhar and McCarthy, 1973; Skoultchi and Gross, 1973) are observed during urchin embryogenesis.

Among various postulates on the mechanism for control of nucleic acid synthesis in the embryonic system is one in which the synthetic rate is limited by the total number of polymerase molecules. Regulation would then be effected by control of the number of polymerases. A converse postulate is that the total number of polymerase molecules are in excess and that regulation is effected by activation-deactivation of the pathway. The nucleic acid enzymes have been previously studied in sea urchin. Loeb and colleagues (Loeb et al., 1969; Fansler and Loeb, 1969) examined the occurrence and distribution of DNA polymerase in the urchin egg and found that DNA polymerase is present in the egg cytoplasm and fractionates into nuclei as replication proceeds. Further, the DNA polymerase activity in dialyzed homogenates was nearly constant from the egg to the gastrula stage embryo. Roeder and Rutter (1970a) examined nuclei from various stages of urchin embryos and found increasingly more RNA polymerase activity per embryo in the later development stages. Further, the RNA polymerase I activity in the isolated nuclei increased at the stage corresponding to the presumed time of in vivo ribosomal RNA synthesis activation. The terminal riboadenylate transferase[1] has previously been reported in the urchin (Hyatt, 1967a,b) but not quantitated during various development stages.

Using techniques designed to partially purify total cellular polymerase while avoiding loss of enzyme activity, we have quantitated the DNA polymerase, RNA polymerase, and the terminal riboadenylate transferase activities during embryogenesis of the west coast urchin, *Strongylocentrotus francis-*

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[1] Abbreviations used: TRT, terminal riboadenylate transferase; DEAE, diethylaminoethyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

canus. For each of these three polymerase systems, our present data support the postulate that polymerase activity is in excess and that regulation is not primarily affected by control of the number of polymerase molecules.

Materials and Methods

Urchin Collection and Embryo Development. *Strongylocentrotus purpuratus* and *S. franciscanus* were collected along the California coast between San Francisco and Point Arena. Urchins were kept in temperature-controlled, synthetic sea water (Instant Ocean, Aquarium Systems, Inc.) until needed. Collection of eggs and development of embryos were also carried out in synthetic sea water. Induction of spawning, washing of eggs, fertilization, and culturing of the embryos were by the previously described procedure (Roeder and Rutter, 1970a); *Strongylocentrotus* embryos were cultured at 13.5–14.5 °C. No cultures were used unless fertilization was 95% and the embryo morphology normal. At appropriate stages during development, embryos were collected by settling in an ice bath, decanting the artificial sea water, and washing three times in 0.55 M KCl by sedimenting at 600*g* for 10 min. In some cases the embryos were collected by filtration over a 54-μm nylon monofilament mesh, washed with 0.55 M KCl while on the mesh, aspirated, and finally sedimented (600*g*, 10 min) from 0.55 M KCl. When used with care, there was no significant breakage of embryos or eggs with either method and the polymerase profiles obtained were comparable.

Preparation of Homogenates and Enzyme Assay. Extracts were prepared by mixing 1 volume of washed embryos or eggs with 1–4 volumes of buffer A (50 mM Tris-HCl, pH 7.9 at 4 °C, 5 mM $MgCl_2$, 25% glycerol, 0.5 mM EDTA, and 0.1 mM dithiothreitol) with added $(NH_4)_2SO_4$ as indicated. The cells were broken by six passes of a Teflon–glass homogenizer or by 30 s at half speed on a tissue homogenizer (TEKMAR). Extracts of this type were used for all of the various polymerase assays. For the assay of cytochrome oxidase, eggs were homogenized in 8–10 volumes of buffer A from which the dithiothreitol was deleted. Cytochrome oxidase was assayed by the procedure of Smith (1955). Reduced cytochrome *c* was prepared for this assay by catalytic reduction with H_2 and Pt on charcoal. RNA polymerase was assayed by incubating 30 μl of RNA polymerase reaction mixture with 20 μl of enzyme preparation, DNA polymerase was assayed by incubating 25 μl of DNA polymerase reaction mixture with 25 μl of enzyme preparation, and terminal riboadenylate transferase (TRT) by incubating 25 μl of TRT reaction mixture with 5 μl of enzyme preparation. All polymerase assays were carried out by incubation for 10 min at 30 °C. The reactions were initiated by adding the enzyme to the reaction mixture and were terminated by the addition of 30 μl of 0.5% sodium dodecyl sulfate–50 mM sodium pyrophosphate; 70-μl (50 μl for TRT only) aliquots of each polymerase assay were spotted on 2.3-cm DE-81 (Whatman) discs and all the discs washed several times (5–10 ml/disc) in 5% $Na_2HPO_4 \cdot 7H_2O$ (the first three washes contained 20 mM sodium pyrophosphate also) to remove the radioactive substrate. After six washes of 5 min each, excess phosphate was removed from the discs by two rinses (30 s each) in deionized H_2O, and the discs were dehydrated in 95% ethanol and then in diethyl ether. The radioactivity on the DE-81 discs was then determined by scintillation spectrometry after standing in scintillation fluid (3.8 l. of Toluene, 100 ml of NCS solubilizer (Amersham/Searle), 16.5 ml of H_2O, and 15.2 g of Omnifluor) for 10–14 h.

Constituents of the Polymerase Reaction Mixtures. RNA polymerase assays were carried out as described previously (Roeder and Rutter, 1970a). The 30-μl reaction mixture contributed the following final concentrations to the 50-μl assay volumes: 48 mM Tris-HCl (pH 7.9 at 30 °C), 6 mM NaF, 1.6 mM $MnCl_2$, 0.6 mM each of GTP, ATP, and CTP, 0.01 mM UTP containing [^{3}H]UTP (New England Nuclear) at 1000 cpm/pmol (50% counting efficiency), 0.02% 2-mercaptoethanol, and 320 μg/ml of calf thymus DNA (Sigma, type 1). DNA was either native or single stranded (alkali denatured and neutralized). α-Amanitin was used at a concentration of 3.4 μg/ml, except for the few cases noted. DNA polymerase assays were adapted from Loeb (1969) and Chang and Bollum (1971); the 25-μl reaction mixture contributed these final concentrations to the 50-μl assay volume: 48 mM Tris-HCl (pH 7.9 at 30 °C), 15 mM $MgCl_2$, 0.02% 2-mercaptoethanol, 0.1 mM each of dCTP, dATP, and dGTP, 0.01 mM dTTP containing 1000 cpm/pmol of [^{3}H]dTTP (New England Nuclear), and 240 μg/ml pancreatic DNase-activated (Aposhian and Kornberg, 1962) calf thymus DNA. TRT assays were performed with all ingredients as described by Tsiapalis et al. (1973). The $rA(pA)_4$ primer was a generous gift of Dr. Fred Bollum. Each TRT assay contained 15.6 nmol (12 cpm/pmol) of [^{3}H]ATP.

Ion-Exchange Chromatography. Pre-swollen DEAE-Sephadex A-25 (Pharmacia) was equilibrated with buffer A containing 0.095 M $(NH_4)_2SO_4$ and packed into 20 × 1.1 cm i.d. columns. After further equilibration of the packed column with 70 ml of 0.095 M $(NH_4)_2SO_4$–buffer A, urchin extracts were chromatographed by the ion-filtration procedure (Kirkegaard et al., 1972) or by gradient sievorptive elution (Kirkegaard, 1973). In ion-filtration chromatography, 1.0 ml of urchin extract at 0.35 M $(NH_4)_2SO_4$ in buffer A was loaded onto the column and eluted in a downward flow mode with 0.45 M $(NH_4)_2SO_4$ in buffer A. The flow rate was gravity controlled at 0.4–0.5 ml/min. This ion-filtration chromatography lends itself well to rapid separation of these enzymes; such a column typically is eluted in 35–45 min. In gradient sievorptive elution, a 5.8-ml gradient (constructed from 2.9 ml each of 0.095 and 0.35 M $(NH_4)_2SO_4$ in buffer A) was applied to the DEAE-Sephadex column in the upward flow mode with the aid of a peristaltic pump at 0.20 ml/min (low ionic strength followed by higher ionic strength regions of the gradient). Subsequently, 1.0 ml of urchin extract was applied to this column and followed with one bed volume of 0.45 M $(NH_4)_2SO_4$ in buffer A. Care was taken to assure that the sample density was intermediate between the high salt end of the gradient and the 0.45 M $(NH_4)_2SO_4$ elution buffer.

Results

Determination and Characterization of RNA Polymerase in Unfractionated Egg and Embryo Homogenates. In early experiments showing multiple RNA polymerase species in *S. purpuratus*, this laboratory reported considerable soluble (up to 27 000*g*, 30 min) [^{3}H]UTP-polymerizing activity in cleavage stage homogenates prepared in buffer A or in buffers more customarily used to prepare nuclei fractions (0.05 M Tris-HCl, pH 7.5 at 4 °C, 1 mM EDTA, 0.25 M sucrose or in this buffer diluted with 2.3 M sucrose, 0.01 M $MgCl_2$, 0.025 M KCl, and 0.01 M Tris-HCl, pH 7.5, after homogenization) (Roeder and Rutter, 1970a). These experiments suggested that the sea urchin egg contained a nonnuclear complement of the RNA polymerases as has been shown for DNA polymerase (Fansler and Loeb, 1969). Direct verification by DEAE-Sephadex chromatography of the RNA polymerase(s) in unfractionated egg or in extracts of early embryos was not feasible by the procedure of Roeder and Rutter (1970a) since the en-

TABLE I: [^{3}H]UTP Polymerization in *Strongylocentrotus purpuratus* Homogenates.

Embryo Stage	Assay Conditions	cpm Incorporated/assay[a] − Exogenous DNA	cpm Incorporated/assay[a] + Exogenous DNA
Egg	Control	748	3060
	+ amanitin	880	2620
	− GTP, ATP, and CTP	850	2090
Prehatching blastula (20 h)	Control	500	2020
	+ amanitin	378	1610
	− GTP, ATP, and CTP	510	1060

[a] Results expressed are the average of duplicate assays minus the background. In the egg homogenate, background = 64 cpm, n = 8. In the prehatching blastula homogenate, background = 135 cpm, n = 8.

TABLE II: Centrifugal Fractionation of RNA Polymerase Activity.

Embryo Stage	Total pmol of UMP Incorporated: Soluble[a]	Total pmol of UMP Incorporated: Pellet	Ratio Soluble/Pellet
Egg	705	92.5	7.6
Gastrula, early	511	608	0.84
Prism	115	1180	0.097

[a] With 27 000g supernatant.

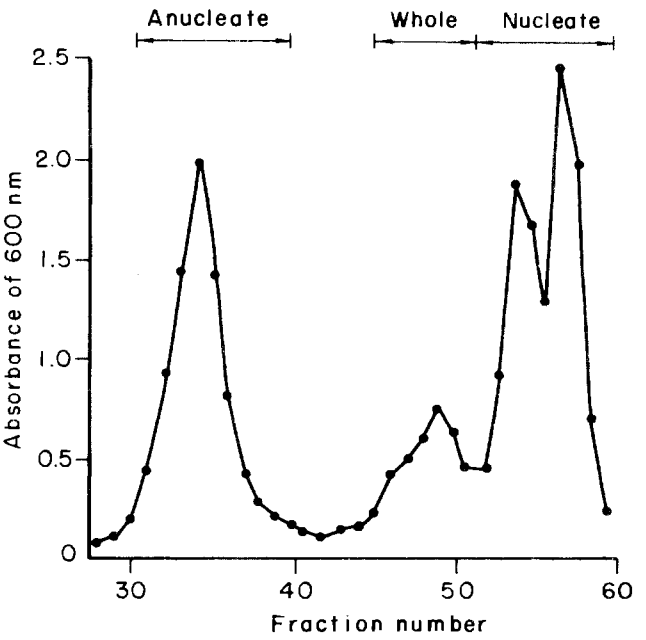

FIGURE 1: Isopycnic banding of nucleate and anucleate halves of *S. franciscanus* eggs. The procedure was carried out in general according to Fansler and Loeb (1969). A 2-ml aliquot of egg suspension (1 volume of eggs in 4 volumes of Ca^{2+}-free, artificial sea water) was layered on a linear sucrose-artificial sea water gradient and centrifuged for 30 min at 12 000g (8500 rpm, Sorvall HB-4 rotor). The gradient was prepared with 23 ml each of 1.0 M sucrose in distilled H_2O and 1:1 mixture of 1.0 M sucrose and Ca^{2+}-free, artificial sea water. Fractions 18–26, 33–38, and 41–48 were pooled for anucleate, whole, and nucleate cell fractions, respectively. Fractions from two gradients were used for the experiments described in Table III. Cell fractions were localized in the gradient by light scattering at 600 nm. Localization of the nucleus in the nucleate fragment was easily demonstrated under the phase microscope; no anucleate fragments were found in the nucleate fraction.

zyme activities eluted in a broad peak with a maximum at 0.07–0.10 M $(NH_4)_2SO_4$ and trailing across the salt gradient to approximately 0.35 M $(NH_4)_2SO_4$. At the low salt concentrations (0.05 M $(NH_4)_2SO_4$) used in loading the whole cell extracts onto DEAE-Sephadex, the RNA polymerases appear to preferentially complex with other components of the extract and thus fail to interact strongly with the ion-exchange resin. We have now been able to circumvent this problem by chromatography at higher ionic strengths and further characterize the polymerizing activity.

Characterization of the [^{3}H]UTP polymerizing activity in unfractionated homogenates of eggs and early embryonic stages reveals an activity indicative of RNA polymerase. As shown in Table I, this activity in the egg or the 20-h blastula is stimulated by exogenous DNA as could be expected for a DNA-dependent RNA polymerizing enzyme. The activity is, however, not dependent on the addition of all four nucleoside triphosphates in contrast to what is expected from an enzyme transcribing a native DNA template. This may be due to endogenous substrates since this embryological system carries the nutrients required for 3 days of development. In fact, the requirement for exogenous nucleoside triphosphates becomes greater as development progresses; an homogenate prepared from pluteus stage embryos shows a fourfold reduction in [^{3}H]UTP polymerization when ATP, GTP, and CTP are deleted from the assay reaction. Finally, in egg extracts the substitution of [^{3}H]uridine for [^{3}H]UTP resulted in 56% of the molar incorporation provided by [^{3}H]UTP. Thus a significant and metabolically active endogenous pool of nucleoside precursors exists in these whole cell extracts.

The solubility characteristics of the RNA polymerases are summarized in Table II. Embryos (1 volume) were homogenized with a Teflon–glass homogenizer in buffer A (3 volumes) and centrifuged (27 000g, 15 min), and the pellet was resuspended in buffer A. Significant amounts of RNA polymerase activity are found in the soluble fraction from the egg with decreasing amounts found there as development proceeds. The corresponding activity in the pellet fraction from eggs is low and increases with development. Similar results are obtained when the homogenates are prepared in 0.05 M Tris-HCl (pH 7.5), 1 mM EDTA, 0.25 M sucrose (data not shown). These data for RNA polymerase are reminiscent of DNA polymerase (Fansler and Loeb, 1969; Loeb et al., 1969) which is soluble in the egg and progressively partitions into the nuclear fraction during embryogenesis.

Subcellular Distribution of Egg RNA Polymerase. The readily soluble RNA polymerase activity of the egg (Table II) could be derived from the nuclei during homogenization, it could be mitochondrial, or it could be truly cytoplasmic. To distinguish among these possibilities, *S. purpuratus* and *S. franciscanus* eggs were fractionated into nucleate and anucleate halves by the isopycnic centrifugation procedure of Fansler and Loeb (1969) (Figure 1). The egg halves as well as intact eggs were assayed for RNA polymerase and for cytochrome oxidase (a mitochondrial marker enzyme; Harvey, 1965) and the results for *S. franciscanus* are expressed in Table III (similar results were obtained with *S. purpuratus*). The presence of substantial RNA polymerase activity in the anu-

TABLE III: Distribution of RNA Polymerase and Cytochrome Oxidase in *Strongylocentrotus franciscanus* Eggs.

	RNA Polymerase[a,b] (pmol of UMP/ml of egg)[c]	Cytochrome Oxidase[c] (s^{-1}/ml of egg)	Ratio of RNA Polymerase to Cytochrome Oxidase
Nucleate half	211	161	1.31
Whole egg	182	304	0.60
Anucleate half	266	379	0.70

[a] Results expressed are the mean of two experiments. [b] RNA polymerase was assayed as outlined in Materials and Methods using the total homogenate as the enzyme source; in these assays the homogenate was diluted 1:12, egg fraction:homogenization buffer. [c] One milliliter of eggs = 0.6×10^6 eggs.

TABLE IV: Relative Amounts of RNA Polymerase II during Embryogenesis.

Embryo Stage	Polymerase II % of Total Act.
I. Unfractionated Homogenates	
Egg	5
4 cell	5
16 cell	15
16-h blastula	36
46-H gastrula	41
II. DEAE Fractionated Homogenates	
Egg	7
16 cell	16
64 cell	27
20-h blastula	49
34-h gastrula	41

cleate half clearly demonstrates that in situ the enzyme is not localized solely within the nucleus; how much of the RNA polymerase is localized in the egg nucleus cannot be determined by these experiments. The distribution of the polymerase appears not to coincide with mitochondria as evidenced by the lack of congruity between the distribution of cytochrome oxidase and the polymerase activity: The RNA polymerase to cytochrome oxidase ratio is 1.31 in the nucleate half but only 0.70 in the anucleate half (Table III).

α-Amanitin Inhibition of RNA Polymerase during Embryogenesis. We have employed selective inhibition by α-amanitin to assay the polymerase II activity in homogenates of urchin eggs. At the concentration of α-amanitin used (3.4 μg/ml), RNA polymerase II is fully inhibited and III only slightly inhibited in the sea urchin (Morris, Masiarz, and Rutter, unpublished) as is the case in other eucaryotes (Weinmann and Roeder, 1974; Benson and Blatti, 1974; Schwartz et al., 1974). Preliminary experiments comparing the α-amanitin sensitivity between purified urchin RNA polymerase II (Lindell et al., 1970; Morris and Rutter, data not shown) and RNA polymerase II in whole cell homogenates of unhatched blastula show that a two- to fourfold higher α-amanitin concentration is required for complete inhibition of the crude RNA polymerase II. This requirement may be caused by α-amanitin binding to lipophilic or other components of the whole embryo homogenates. Urchin eggs, washed two times in 0.55 M KCl, were homogenized in buffer A and assayed at 0.07 M $(NH_4)_2SO_4$ for RNA polymerase activity. In 14 different preparations, the average RNA polymerase II activity was equal to 6.2 ± 4% of the total RNA polymerase activity. A fourfold elevation of the α-amanitin concentration resulted in no further inhibition of RNA polymerase activity. This suggests that polymerase II represents approximately 6% of the total polymerase activity.

Experiments similar to the ones described above were performed with homogenates of embryos collected at intervals throughout embryogenesis (Table IV). The egg is notably deficient in assayable RNA polymerase II but following fertilization its activity increases dramatically, and by the gastrula stage, this enzyme approaches 50% of the total RNA polymerase activity. Under these experimental conditions, no distinction is made between polymerase I and III activities.

Quantitation of Nucleic Acid Polymerases during Embryogenesis. In order to assay the polymerases without interference due to intracellular substrate pools and templates, it is necessary to partially purify the enzymes. We have selected ion-filtration chromatography (Kirkegaard et al., 1972) which, under the salt concentration and pH parameters used in these experiments, efficiently removes the nucleic acids and nucleoside triphosphates from the RNA polymerase fractions (<5% activity in the absence of either exogenous DNA template or one of the four nucleoside triphosphates). The RNA polymerases (Figure 2A) elute after one column volume with the salt front, while the terminal riboadenylate transferase enzyme elutes in the void volume (Figure 2B), and the DNA polymerase activity exhibits two distinct peaks, one in the void volume and one in the sieving range (Figure 2C).

The RNA polymerase assays (Figure 2A) show a minor peak in addition to the major peak of activity. This minor peak elutes in the void volume and co-elutes with DNA polymerase; it is not a typical RNA polymerase since it has nearly imperceptible activity on denatured DNA and is stimulated by nuclease-activated DNA template (F. M. Racine and P. W. Morris, unpublished data). In contrast, the typical RNA polymerase activity eluting with the salt front is strongly inhibited (60%) by nuclease-activated DNA. The minor activity eluted in the void volume is quite sensitive to inhibition by various deoxynucleoside triphosphates (Figure 3), while the typical RNA polymerases are not. The activity might represent DNA polymerase (van de Sande et al., 1972) or some unknown polymerase activity.

At the present, the difference between the two peaks of DNA polymerase activity (Figure 2C) is unknown. They do not correspond to the 3.4S and 6–8S DNA polymerases described by Chang and Bollum (1971, 1972b); in the urchin blastula, the 3.4S polymerase is a minor (<5%) constituent of the total DNA polymerase activity (F. R. Masiarz, personal communication). They could represent different complexes of the DNA polymerase with other proteins.

Figure 4 and Table V summarize the quantitative data for each of the three types of nucleic acid polymerases. There is no significant change in the level of these activities per embryo (Figure 4) from the unfertilized egg to the pluteus; the standard error of the mean in the determinations is about ±10% for each enzymatic activity (Table V). The enzyme activity profile during development is remarkable when contrasted to the 1000-fold increase in cell number and hence the 1000-fold increase in nuclei per embryo. This constant activity of each of these three polymerases demonstrates that regulation of

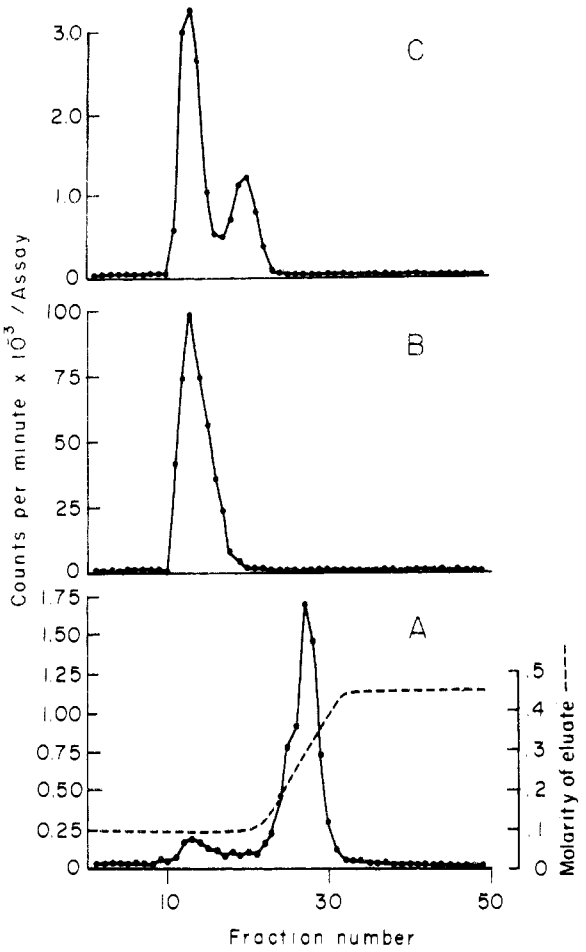

FIGURE 2: DEAE-Sephadex ion filtration of urchin polymerases. A 1.1 × 20 cm column of DEAE-Sephadex was prepared and equilibrated with buffer A containing 0.095 M $(NH_4)_2SO_4$. One milliliter of an embryo homogenate (1 volume of embryo to 2 volumes of buffer A, final $[(NH_4)_2SO_4]$ = 0.35 M) was layered onto the column and eluted with 0.45 M $(NH_4)_2SO_4$ in buffer A. Fractions (0.48 ml) were collected at 0.4–0.5 ml/min flow rate. Aliquots of each fraction were assayed by the standard method for TRT, DNA polymerase, and RNA polymerase (native DNA with and without α-amanitin and alkali denatured DNA without α-amanitin). $(NH_4)_2SO_4$ concentrations were determined by conductivity. (A) RNA polymerase activity profile assayed with native DNA template. The RNA polymerase species peak is in fraction 27. The minor peak of activity in fraction 13 is not inhibited by α-amanitin, is inhibited by thymidine triphosphate, and has nearly undetectable activity with denatured DNA template. (B) Terminal riboadenylate transferase activity profile. The column is identical with that shown in A. (C) DNA polymerase activity profile. The column is identical with that shown in A. Two distinct DNA polymerase peaks are found, one co-eluting with terminal riboadenylate transferase and one eluting between TRT and the RNA polymerase.

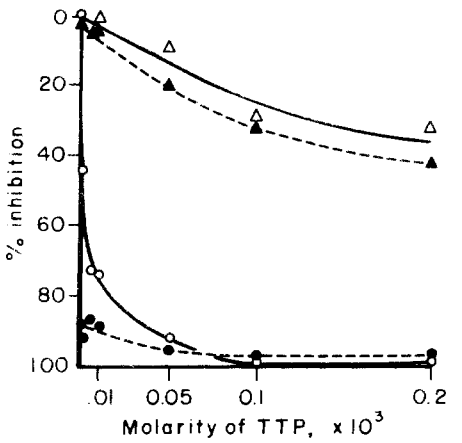

FIGURE 3: Differential inhibition of RNA polymerase and the DNA polymerase-associated ribonucleotide polymerization activity. Peak fractions (such as fractions 13 and 27, Figure 2A) of each polymerase activity from DEAE ion filtration were assayed with the RNA polymerase reaction mixture containing either varying concentrations of TTP alone (—) or varying concentrations of TTP plus 0.1 mM each of dGTP, dCTP, and dATP (- - -). Triangles: RNA polymerase. Circles: DNA polymerase-associated activity. No inhibition corresponded to 1050 cpm of [^{3}H]UTP incorporated/assay for the DNA polymerase-associated activity and 7800 cpm/assay for the RNA polymerase.

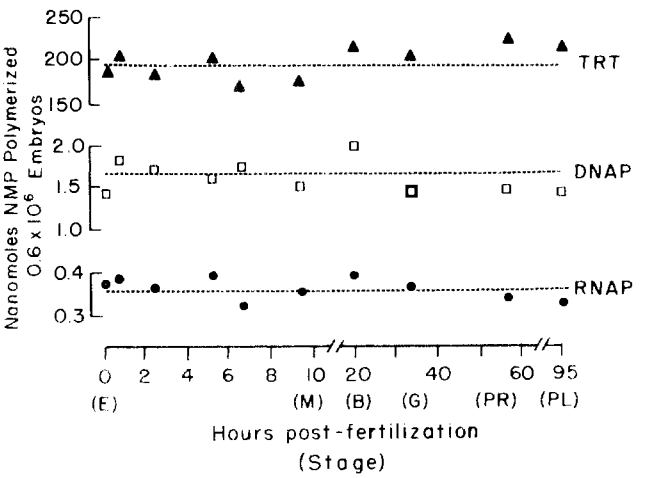

FIGURE 4: Polymerase activity profile during *S. franciscanus* embryogenesis. DEAE ion-filtration columns like that shown in Figure 3A–C were assayed for each of the polymerases and the data expressed as the nanomoles of nucleoside monophosphate incorporated/0.6 × 10^6 eggs/ml. Assay of the RNA polymerases with saturating concentrations of [^{3}H]-UTP gives activities 2.8-fold greater than the activities shown here. The DNA polymerase activities are the sum of both peaks shown in Figure 3C; there is no significant change in the relative proportion of the two peaks during embryogenesis. Optimally activated DNA template increases the DNA polymerase activity by 4.7-fold over the values shown. The embryonic stages are: E = egg; M = morula; B = blastula; G = gastrula; PR = prism; and PL = pluteus.

macromolecular synthesis in vivo (polyA, RNA, and DNA) is not primarily effected by the total activity of the respective enzymes.

The absolute level of the RNA polymerase activities in these embryos is 2.8-fold greater than the values shown. This correction is the ratio of enzyme velocity at saturating UTP to the enzyme velocity at 0.01 mM UTP used here. When the RNA polymerases are assayed with denatured, rather than native, DNA, the activity increases 1.3–1.8-fold (data not shown). From the one-cell to the blastula stage, this stimulation ranges from 1.3 to 1.4-fold. By the late gastrula, prism, and pluteus stages, this stimulation by denatured DNA increases to 1.7–1.8-fold. Since both polymerases I and II are stimulated by denatured DNA (II by 2-fold and I by 1.4-fold, Roeder and Rutter, 1970a), these stimulations are consistent with the observed increase in the proportion of RNA polymerases I and II in the latter stage embryos (Table IV).

Classes of RNA Polymerases in the S. franciscanus Egg. The alteration in the degree of α-amanitin inhibition (Table IV) and the constant amount of RNA polymerase per embryo (Figure 4) raise a question of the RNA polymerase class preexistent in the egg and the mechanism for its embryonic change. Prior to the gradient sievorptive elution (Kirkegaard, 1973), chromatographic techniques were not adequate to an-

TABLE V: Cell Number and Polymerase Activities during Embryogenesis.

Stage	Cells/ Embryo	Polymerase Act.[a]/0.6 × 10^6 Embryos: RNA Polymerase	DNA Polymerase	TRT
Egg, unfertilized (0 time)	1	0.37	1.38	186
Egg, fertilized (+0.75 h)	1	0.38	1.83	206
Cleavage (+2.5 h)	2	0.36	1.70	179
Cleavage (+5.5 h)	16	0.39	1.63	199
Cleavage (+6.5 h)	32	0.31	1.79	169
Cleavage (+9.5 h)	90–140	0.37	1.50	173
Blastula (+20 h)	~400	0.40	1.99	215
Gastrula (+33.5 h)	~600	0.36	1.41	204
Prism (+57 h)	~800	0.33	1.48	228
Pluteus (+96 h)	~1000	0.33	1.38	211
Average ± SEM:		0.36 ± 0.027	1.61 ± 0.20	197 ± 18

[a] All polymerase activities are expressed as nanomoles of nucleotide incorporated. The RNA polymerase values are corrected to saturating levels (two- to eightfold). One milliliter of unfertilized eggs contains ~0.6 × 10^6 embryos.

swer this question. No combination of conventional extraction procedure and ion-exchange chromatography was found to yield adequate enzyme resolution from the yolk-laden embryo extracts. Ion-filtration chromatography purifies the RNA polymerases so that re-chromatography on DEAE with gradient elution resolves the enzyme classes. Yet the re-chromatography procedure was limited due to the 30–50% loss of RNA polymerase activity incurred (during dialysis or dilution of the enzyme). With gradient sievorptive elution, as in ion filtration, the enzyme sample is loaded onto the column at salt concentration where electrostatic interactions with other molecules are minimized. Sample preparation consists only of breaking the cells in buffer solution, adjusting the density of the homogenate, and applying to a DEAE-Sephadex column containing the pre-formed elution gradient. Thus it takes no longer than 2 h to go from the intact cell to the collected column fractions. Loss of enzyme activity in lengthy dialysis or ammonium sulfate fractionation is avoided.

Figures 5A and 5B exhibit the RNA polymerase profiles obtained from an egg and a hatching blastula. The elution profile from the egg shows that polymerase III is the dominant species with some polymerase I and a small proportion of polymerase II. The blastula (Figure 5B) has the three typical peaks of activity, eluting at 0.13, 0.22, and 0.37 M $(NH_4)_2SO_4$. In marked contrast, the egg exhibits a major peak of activity eluting at 0.28 M $(NH_4)_2SO_4$. Both RNA polymerases II and IIIA are shown to elute in this ammonium sulfate concentration from DEAE-Sephadex (Roeder and Rutter, 1970a; Schwartz et al., 1974). Assay of the fractions in the presence of 2.3 μg/ml α-amanitin shows the egg enzyme to be largely resistant to this concentration of the toxin while the blastula enzyme has a much greater sensitivity. This suggests that the egg contains mostly RNA polymerase IIIA with a small relative amount of RNA polymerase II. The RNA polymerase I also occurs in the egg but in relatively low proportions. During subsequent embryogenesis, the proportion of IIIA decreases and concomitantly, the proportions of I, II, and IIIB increase.

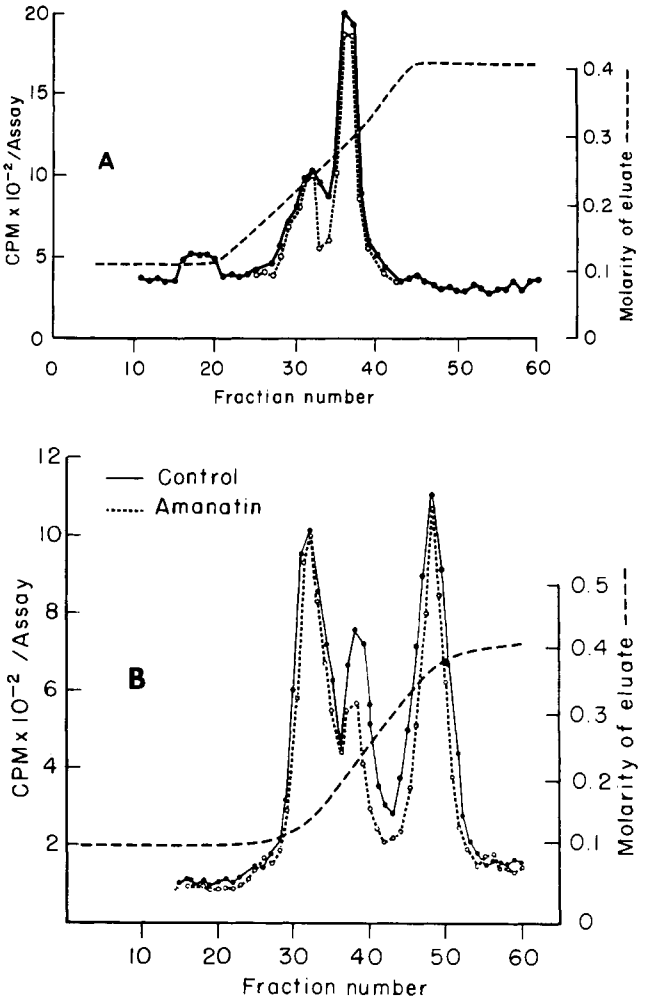

FIGURE 5: Resolution of the RNA polymerase classes in urchin extracts by gradient sievorptive elution over DEAE-Sephadex. Extracts were prepared and applied to DEAE columns as described in Materials and Methods. Aliquots of each fraction were assayed for RNA polymerase both with and without 2.3 μg/ml of α-amanitin. (A) Unfertilized egg; (B) 16-h post-fertilization blastula.

Discussion

The present studies extend the knowledge of nucleic acid polymerizing enzymes in sea urchin embryos. Terminal riboadenylate transferase previously was described by Hyatt (1967a,b) in extracts of the urchins, *S. purpuratus* and *S. franciscanus*. The substrate and primer requirements, Mn^{2+} stimulation, and inhibition by GTP, UTP, or CTP of the crude urchin enzyme resembled parameters for the Mn^{2+}-stimulated TRT from calf thymus (Tsiapalis et al., 1973) rather than the parameters for Mg^{2+}-activated poly(A) polymerase of calf thymus nuclei (Winters and Edmonds, 1973a,b). The enzyme described in the present work is Mn^{2+} stimulated, as was the enzyme described by Hyatt (1967a). From our data on the unchanging TRT activity profile during development, it is adequately clear that the post-fertilization increase in polyadenylylation is not linked to de novo synthesis of the TRT. Regulation of the TRT enzyme must be via substrate availability, RNA primer availability, or specific activators or inhibitors. Using the TRT activity of Figure 4, the maximal specific activity reported for the homogenous calf thymus enzyme, and an approximate molecular weight of 60 000 (Tsiapalis et al., 1973), we calculate that there are 4.5 × 10^7 molecules of TRT per embryo. The number of TRT mole-

cules/cell is reduced with cell division, as embryogenesis proceeds.

DNA polymerase activity is present in the mature oocyte in confirmation of earlier reports (Loeb et al., 1969; Fansler and Loeb, 1969). This activity, like the TRT activity and total RNA polymerase activity, is constant per embryo up to the pluteus stage in embryogenesis. Neither the initiation of DNA replication following fertilization nor the decline in replication late in gastrulation appears to be effected by a regulation of the amount of DNA polymerase. The existence of a possible inhibitor controlling DNA polymerase activity has been suggested by Fansler and Loeb (1969) and a factor stimulating DNA polymerase described (Murakami and Mano, 1973). Whether either of these possibilities function in vivo remains to be seen. Although DNA synthesis declines in the late stage urchin embryo, our data show that the DNA polymerase is retained with the embryo. A biological explanation for this phenomenon is suggested by the growth which ensues in the pluteus after active feeding has begun.

The DNA polymerase assays performed in these experiments under strictly controlled conditions of solubilization, chromatography, and assay give a valid estimation of the change in DNA synthetic capability throughout the development stages, although the absolute activities reported can only be considered as approximate. Our estimate of the total units (1 unit corresponds to the polymerization of 1 nmol of TMP/10 min) of DNA polymerase per embryo does not agree with some of the available data (Fansler and Loeb, 1969; Loeb, 1970) but does agree with other data (Loeb, 1969). The discrepancy in the reports may reflect variations in the template as well as other assay conditions used by these authors. Since the first cycle of replication in the urchin is largely independent of protein and RNA synthesis (Young et al., 1969; Gross and Cousineau, 1964), we expect that all enzymes required in DNA replication are present in the egg. The effect of these enzymes on DNA polymerase activity in vitro is undefined at the present. An enzyme molecular weight of 150 000 (Loeb, 1969) and an estimated specific activity of 500 units/mg protein (Loeb, 1970) allow us to calculate that there are approximately 1.6×10^8 DNA polymerase molecules/*S. franciscanus* embryo.

Our results indicate that the egg contains 1.73 units of RNA polymerase/10^6 embryos and that this activity is constant throughout embryogenesis. This agrees with the value of 1.71 units/10^6 embryos reported previously (Table II, summed activity for polymerases I, II, and III in Roeder and Rutter, 1970a) for a prism stage *S. purpuratus* embryo. The presence of significant RNA polymerase activity in both anucleate and nucleate halves of the urchin egg argues that much of the RNA polymerase is localized in the cytoplasm. This cytoplasmic RNA polymerase may be partitioned into the nucleoplasm during nucleus formation in the cleavage stage embryo. This is consistent with the increase in nuclei-associated RNA polymerase as embryogenesis proceeds (Roeder and Rutter, 1970a, Table III), until at the prism stage all of the detectable RNA polymerase is present in the nuclei.

Although the amount of total RNA polymerase activity remains constant throughout embryogenesis, the relative amounts of RNA polymerase I, II, and III change significantly. This is demonstrated in Figures 5A and 5B in which the predominant (α-amanitin resistant) polymerase of the egg is replaced by the three, more typical RNA polymerases of the blastula. The α-amanitin resistant enzyme of the egg differs in chromatographic behavior from the α-amanitin resistant activity later in development. Its chromatographic properties resemble (Passo and Morris, unpublished data) RNA polymerase IIIA from mouse myeloma (Schwartz et al., 1974); the blastula shows activities similar to IIIA and IIIB. Interestingly, the *Xenopus* oocyte shows no detectable amount of RNA polymerase IIIA (Roeder, 1974a,b), although I, II, and IIIB are present.

The embryonic alteration of the RNA polymerase III complement in the urchin must be explained in the context of the cellular function of these enzymes. There are currently three hypotheses: (1) polymerases IIIA and IIIB have different transcriptive functions; (2) one is an inactive precursor to the active enzyme; and (3) one is a degradative product of the other. If RNA polymerase IIIA were biologically inactive, one can envision the activation and conversion to RNA polymerase IIIB proceeding through a number of mechanisms including subunit exchange or modification. The phenomenon of the embryonic alteration in RNA polymerase classes will be more explicable as the molecular architecture and function of each become defined. This area is currently under investigation.

Present experiments in one of our laboratories (Passo and Morris, unpublished data) show that these relative proportions of RNA polymerase I, II, and III are peculiar for *S. franciscanus* eggs and not typical of *S. purpuratus* or *Lytechinus pictus*. The eggs of the latter two species contain more nearly equal amounts of each of the RNA polymerase classes I, II, and III. Thus the skewed distribution of polymerases in *S. franciscanus* need not have significance to general functions important in the embryogenic process.

The major finding of these experiments pertains to the intracellular regulatory mechanisms for polynucleotide synthesis. Synthesis of polyadenylate tracts, DNA and RNA, is very low in the mature urchin egg and all are strongly activated following fertilization. Perhaps most interesting is the activation of DNA synthesis which shows brief (10–20 min) synchronized synthetic phases separated by longer (up to 50 min) inactive phases (Hinegardner et al., 1964). The biosynthetic enzymes for all three of these nucleic acid classes preexist in the egg and their overall concentration per embryo changes little during development. Clearly, gross alterations in nucleic acid synthetic rates are not effected by corresponding change in the respective enzyme concentrations as assayed in vitro. Previous work showing increasing RNA polymerase activities during urchin embryogenesis (Roeder and Rutter, 1970a) may not be understood in terms of increasing amounts of RNA polymerase tightly bound to the nuclei. Whereas the previous work was restricted to the nuclear polymerases, these present studies have examined the polymerases of the whole cell. Both RNA and DNA polymerases seem to partition into the nucleus as development proceeds and the synthetic rate of the respective nucleic acid increases. We view the egg as a repository for the enzymes of nucleic synthesis, thus as cleavage proceeds there is a reduction in the polymerase concentration within individual cells.

In addition to the embryonic system, other biological phenomena are known to result in stimulation of nucleic acid synthesis. Examples are hormone stimulation (Barry and Gorski, 1971; Mainwaring et al., 1971; Smuckler and Tata, 1971; Blatti et al., 1970), partial hepatectomy (Blatti et al., 1970; Drews and Brawerman, 1967), Jerusalem Artichoke germination (Rose et al., 1972), and heterokaryon formation (Harris et al., 1969). In some of these cases there is an increase in the nuclear polymerase activity (see also Yu and Feigelson, 1971). Based on our present observations in the urchin embryo and the low degree of RNA polymerase III associated with nuclei (Schwartz et al., 1974), we think it likely that such

stimulation of nucleic acid synthesis may occur through "activation" of preexistent polymerase molecules. The chromatographic procedures described in this paper may be employed to reexamine the polymerase activity in cells before and after the physiological transition in order to measure total cell as compared with nuclear activity. It is possible that the polymerase ability to react with the template is specifically regulated.

References

Aposhian, H. V., and Kornberg, A. (1962), *J. Biol. Chem. 237,* 519-525.

Barry, J., and Gorski, J. (1971), *Biochemistry 10,* 2384-2390.

Benson, R., and Blatti, S. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 791A.

Blatti, S. P., Ingles, C. J., Lindell, T. J., Morris, P. W., Weaver, R. F., Weinberg, F., and Rutter, W. J. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 649-657.

Bollum, F. J., Chang, L. M. S., Tsiapalis, C. M., and Dorson, J. W. (1974), *Methods Enzymol. 29,* 70-81.

Brachet, J. (1933), *Arch. Biol. 44,* 519-533.

Chang, L. M. S., and Bollum, F. J. (1971), *J. Biol. Chem. 246,* 5835-5837.

Chang, L. M. S., and Bollum, F. J. (1972a), *Biochemistry 11,* 1264-1272.

Chang, L. M. S., and Bollum, F. J. (1972b), *Science 175,* 1116-1117.

Drews, J., and Brawerman, G. (1967), *J. Biol. Chem. 242,* 801-808.

Fansler, B., and Loeb, L. A. (1969), *Exp. Cell Res. 57,* 305-310.

Farquhar, M. N., and McCarthy, B. J. (1973), *Biochem. Biophys. Res. Commun. 53,* 515-521.

Goldberg, M., Perriard, J. C., Hager, G., Hallick, R. B., and Rutter, W. J. (1974), in Control of Transcription, Biswas, B. B., Mandal, R. K., Stevens, A., and Cohen, W. E., Ed., New York, N.Y., Plenum Press, pp 241-256.

Gross, P. R., and Cousineau, G. H. (1964), *Exp. Cell Res. 33,* 368-395.

Gross, P. R., Kraemer, K., and Malkin, L. I. (1965), *Biochem. Biophys. Res. Commun. 18,* 569-575.

Guidice, G. (1973), Developmental Biology of the Sea Urchin Embryo, New York, N.Y., Academic Press.

Harris, H., Sidebottom, E., Grace, D. M., and Bramwell, M. E. (1969), *J. Cell Sci. 4,* 499-525.

Harvey, E. B. (1965), The American Arbacia and Other Sea Urchins, Princeton, N.J., Princeton University Press.

Hinegardner, R. T., Rao, B., and Feldman, D. E. (1964), *Exp. Cell Res. 36,* 53-61.

Hyatt, E. A. (1967a), *Biochim. Biophys. Acta 142,* 246-253.

Hyatt, E. A. (1967b), *Biochim. Biophys. Acta 142,* 254-262.

Kirkegaard, L. H. (1973), *Biochemistry 12,* 3627-3632.

Kirkegaard, L. H., Johnson, T. J. A., and Bock, R. M. (1972), *Anal. Biochem. 50,* 122-127.

Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G., and Rutter, W. J. (1970), *Science 170,* 447-449.

Loeb, L. A. (1969), *J. Biol. Chem. 244,* 1672-1681.

Loeb, L. A. (1970), *Nature (London) 226,* 448-449.

Loeb, L. A., Fansler, B., Williams, R., and Mazia, D. (1969), *Exp. Cell Res. 57,* 298-304.

Mainwaring, W. I. P., Mangan, F. R., and Peteckin, B. M. (1971), *Biochem. J. 123,* 619-629.

Murakami, K., and Mano, Y. (1973), *Biochem. Biophys. Res. Commun. 55,* 1125-1133.

Nemer, M. (1962), *J. Biol. Chem. 237,* 143-149.

Reeder, R. H., and Roeder, R. G. (1972), *J. Mol. Biol. 70,* 433-441.

Roeder, R. G. (1974a), *J. Biol. Chem. 249,* 241-248.

Roeder, R. G. (1974b), *J. Biol. Chem. 249,* 249-256.

Roeder, R. G., and Rutter, W. J. (1970a), *Biochemistry 9,* 2543-2553.

Roeder, R. G., and Rutter, W. J. (1970b), *Proc. Natl. Acad. Sci. U.S.A. 68,* 675-682.

Rose, R. J., Setterfield, G., and Fowke, L. C. (1972), *Exp. Cell Res. 71,* 1-16.

Rutter, W. J., Masiarz, F., and Morris, P. W. (1974), in Differentiation and Control of Malignancy of Tumor Cells, Nakahara, W., Ono, T., Sugimura, T., and Sugano, H., Ed., Tokyo, University of Tokyo Press, pp 55-65.

Schwartz, L. B., Sklar, V. E. F., Jaehning, J. A., Weinmann, R., and Roeder, R. G. (1974), *J. Biol. Chem. 249,* 5889-5897.

Sconzo, G., Pirrone, A. M., Mutolo, V., and Guidice, G. (1970), *Biochim. Biophys. Acta 199,* 441-446.

Skoultchi, A., and Gross, P. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2840-2844.

Slater, D. W., Slater, I., and Gillespie, D. (1972), *Nature (London) 240,* 333-337.

Slater, I., Gillespie, D., and Slater, D. W. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 406-411.

Slater, I., and Slater, D. W. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1103-1107.

Slater, I., and Slater, D. W. (1972), *Nature (London), New Biol. 237,* 81-85.

Smith, L. (1955), *Methods Enzymol. 2,* 732-740.

Smuckler, E. A., and Tata, J. R. (1971), *Nature (London) 234,* 37-39.

Tsiapalis, D. M., Dorson, J. W., DeSante, D. M., and Bollum, F. J. (1973), *Biochem. Biophys. Res. Commun. 50,* 737-743.

van de Sande, J. H., Loewen, P. C., and Khorana, H. G. (1972), *J. Biol. Chem. 247,* 6140-6156.

Weinmann, R., and Roeder, R. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1790-1794.

Wilt, F. H. (1963), *Dev. Biol. 9,* 299-313.

Winters, M. A., and Edmonds, M. (1973a), *J. Biol. Chem. 248,* 4756-4762.

Winters, M. A., and Edmonds, M. (1973b), *J. Biol. Chem. 248,* 4763-4768.

Wu, R. S., and Wilt, F. H. (1973), *Biochem. Biophys. Res. Commun. 54,* 704-714.

Young, C. W., Hendler, F. J., and Karnovsky, D. A. (1969), *Exp. Cell Res. 58,* 15-26.

Yu, F. L., and Feigelson, P. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2177-2180.