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Histone Synthesis. Assignment to a Special Class of Polyribosomes in Sea Urchin Embryos*

Boaz Moav† and Martin Nemer‡

ABSTRACT: In the early sea urchin blastula the s-polysomes (3 to 7 ribosomal aggregates) constitute more than 70% of the polysomal ribosomes. The function of this predominant class of polyribosomes was pursued through an examination of associated nascent proteins. Histone-like proteins were purified from the nascent proteins of s-polysomes by successive acid extraction, cation-exchange chromatography, and acrylamide gel electrophoresis. A similar procedure with the nascent proteins from the larger polyribosomes yielded proteins that did not appreciably resemble histones. We conclude that the s-polysomes are the site of chromosomal histone synthesis. The nascent proteins of these polyribosomes have a substantially enhanced incorporation of arginine and lysine relative to tryptophan, as compared to these relative incorporations in the larger polyribosomes. This enhancement reflects the unique overall amino acid composition of histones.

The extent of this enhancement changes during embryonic development in a manner parallel to changes in DNA synthesis.

A class of polyribosomes containing 3–7 ribosomes/mRNA plays a prominent role in the early development of the sea urchin embryo (Infante and Nemer, 1967). These polyribosomes, designated "s-polysomes," increase in concentration from barely perceptible amounts in the early cleaving embryo to over one-third of the total ribosomes in the 10-hr, 200-cell early blastula of *Stronglyocentrotus purpuratus*. The formation of s-polysomes is largely dependent on RNA newly synthesized by the early stage embryo, rather than upon RNA preexisting in the egg (Infante and Nemer, 1967). This new polysomal RNA is predominantly of a 9–10S class (Nemer and Infante, 1965; Kedes and Gross, 1969). The significance of the very extensive synthesis of this narrow class of mRNAs and the accumulation of these polyribosomes in early sea urchin blastulae has been the subject of several recent studies. Nemer and Lindsay (1969) have reported that the incorporation ratio of tryptophan/arginine in nascent protein was substantially less in the s-polysomes than in the rest of the polysomal population. The absence of tryptophan in chromosomal histones (Mirsky and Pollister, 1946; Hnilica, 1967) suggests that the tryptophan-deficient nascent proteins of the s-polysomes may include nascent histones, in accordance with the rationale of Borun *et al.* (1967), bearing on their observations with mammalian tissue culture cells. A similar tryptophan/lysine asymmetry in s-polysomes has been noted recently by Kedes *et al.* (1969), together with the observation through autoradiography that preponderantly nuclear protein is synthesized in the early sea urchin embryo.

In order to assign a specific function to the newly accumulating class of s-polysomes of the early stage embryo, we have attempted to analyze the polysomal protein products

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directly. Our approach has not been to characterize the nascent proteins *in toto*, whose vastly heterogeneous population might present a morass for analysis, but instead we have submitted this material to purification procedures that might reasonably be applied to completed histones. The result was indeed the selection from this heterogeneous population of a small fraction resembling completed histones. Purification of histone-like protein was conducted by first obtaining the material freed from polyribosomes that was soluble in 0.4 N H_2SO_4. This acid-soluble protein was fractionated by cation-exchange chromatography. A striking result was that approximately 70% of this nascent protein became irreversibly bound to the cation-exchange resin. The rest could be fractionated into nonhistone and histone-like proteins. The histone-like proteins were then submitted to acrylamide gel electrophoresis, as a final step in purification. We conclude that a major portion of the proteins of the s-polysomes that are susceptible to fractionation closely resemble histones. The rest of the polysomal population yields considerably less histone-like protein.

Materials and Methods

(*a*) *Incubation of Embryos and Extraction of Polyribosomes.* Sources of sea urchins, conditions for fertilization and development, and incubation have been documented previously (Nemer and Infante, 1967). Embryos were incubated with 5 μM [^{3}H]L-leucine (750 Ci/mole), 0.26 μM [^{3}H]DL-tryptophan (24 Ci/mmole), 0.7 μM [^{14}C]L-arginine (312 Ci/mole), 3 μM [^{14}C]L-lysine (312 Ci/mole), and 0.016 μM 2,3-[^{3}H]L-alanine (50 Ci/mmole) Schwarz BioResearch, Orangeburg, N. Y. Also, a reconstituted protein hydrolysate (40 Ci/atom of carbon, Schwarz BioResearch), consisting of Ala, Arg, Asp, Glu, Ilu, Leu, Lys, Phe, Pro, Ser, Thr, Tyr, and Val, was incubated at 2 μCi/ml.

The standard medium in which cells were disrupted and cell-free components were examined consisted of 240 mM KCl, 5 mM $MgCl_2$, and 50 mM triethylamine·HCl, at pH 7.8. Cell disruption was effected by a modification of the method of Hinegardner (1962), involving a single passage of embryos through a narrow gauge hypodermic needle (No. 20 or 25), with bentonite added at a concentration of 1 mg/ml. Embryos of *S. purpuratus* were demembranized by treatment with hatching enzyme in sea water from just-hatched blastulae. Embryos of *Lytechinus pictus* could be demembranized at any stage by rapid passage through bolting silk (No. 16 standard). They were washed twice in ice-cold 1 M dextrose and once in standard medium, suspended in medium (1:5 volumes) in a hypodermic syringe, and then passed through a hypodermic needle. Only a single passage could be made without some nuclear lysis also occurring (Fromson and Nemer, 1970). The use of this method insures that the system under study is present in the cytoplasm and not leaked from the nucleus.

The embryo lysate was centrifuged for 10 min at 15,000*g*, and the supernatant fluid (S15) was layered onto 15–30% (w/w) sucrose gradients, prepared in standard medium. Centrifugation was at 50,000 rpm for 27 min in the Spinco rotor SW50 or SW50.1. Gradient fractions were collected after passage through a recording spectrophotometer. Incorporation was assayed after plating fractions on membrane filters (Infante and Nemer, 1968).

(*b*) *Characterization of Nascent Protein by Gel Filtration.* Polysomes were prepared from embryos incubated for 15 min with [^{3}H]L-arginine and [^{3}H]L-lysine or [^{14}C]amino acid mixture. The polyribosomes, collected from sucrose gradients as distinct sedimentation classes, were incubated with previously boiled pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N. J.) at 0.1 mg/ml for 30 min at 37°. The nuclease digest of polysomes was made 0.5 M with urea, 1% with sodium dodecyl sulfate, and 0.1% with 2-mercaptoethanol and dialyzed against phosphate buffer (0.01 M, pH 7.4) containing 0.1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol. The dialyzed extracts (1–2 ml) were mixed and applied to a Sephadex G-100 column (100 cm × 2 cm), previously equilibrated with the same phosphate buffer with sodium dodecyl sulfate and 2-mercaptoethanol. Fractions were collected at room temperature and a portion of each (0.2–0.5 ml) was used for radioactive assay in scintillation fluid (Bray, 1960).

(*c*) *Incubation of Polysomes in Vitro.* Ten-hour embryos were incubated for 20 min with tritiated amino acids and their polyribosomes fractionated on sucrose gradients. The s-polysomes and r-polysomes were pooled from the gradients and incubated separately *in vitro* for 1 hr. The incubation mixture at 25° contained the following substituents in a total of 15 ml: s-polysomes (2 mg) with nascent proteins labeled *in vivo* with [^{3}H]arginine, [^{3}H]lysine, [^{3}H]alanine, and [^{3}H]-leucine (or similarly labeled r-polysomes); 100,000*g* supernatant fluid (15 mg of protein); ATP (1 mM); phosphoenolpyruvate (5 mM), GTP (0.06 mM); 2-mercaptoethanol (5 mM), phosphoenolpyruvate kinase (5 μg/ml); a mixture of 20 unlabeled amino acids (1 mM each); KCl (240 mM), $MgCl_2$ (5 mM); and triethanolamine·HCl buffer (50 mM at pH 7.8). After this incubation the polyribosomes were pelleted by ultracentrifugation. The resuspended polyribosomes were incubated for 1 hr at 37° with pancreatic ribonuclease (100 μg/ml), then sonicated 30 sec (Bronson Sonifier, setting 4). The solution was made 0.4 N with H_2SO_4, and after 2 hr at 4°, an acid-insoluble residue was removed by centrifugation at 20,000*g* for 20 min. The acid-soluble extract was dialyzed against 8% guanidine phosphate buffer, pH 6.8, then concentrated by ultrafiltration, and layered on a column for ion-exchange chromatography. Fractions were collected and precipitated with 20% trichloroacetic acid. The precipitates were plated on glass fiber filter disks and assayed for radioactivity by scintillation spectrometry.

(*d*) *Extraction of Nuclear Histones.* Demembranized embryos were washed twice with 1 M dextrose, then diluted with 5 volumes of 2 mM $MgCl_2$ and passed through a No. 20 needle 2–4 times. The cell lysate was layered on a discontinuous sucrose gradient, consisting of 6 ml of 2.0 M; 6 ml of 1.75 M, 4 ml of 1.5 M, and 4 ml of 1.25 M sucrose in standard medium. Gradients were centrifuged for 45 min in the Spinco SW25.1 rotor at 20,000 rpm. Nuclei were pelleted under these conditions and were then washed twice with diluted (1:5) standard buffer. Purified nuclei were extracted with 0.25 N H_2SO_4. In several preparations 0.05 M $NaHSO_3$ was present both in the wash buffer and in the sulfuric acid, which could also be used at 0.4 N. Extraction was complete after 2 hr of constant stirring at 4°. A second extraction yielded only 5% more material. The extract was centrifuged at 42,000 rpm in the Spinco rotor 50 for 17 hr, to remove residual nucleoprotein and nucleic acid. The supernatant fluid was dialyzed against 8% guanidine phosphate, pH 6.8, in preparation for chromatography. In some cases the acid extract was dialyzed against 5% acetic acid, lyophilized, and kept at −70°. Comparative studies were done with preparations of calf thymus total histones from Sigma Chemical Company, St. Louis, Mo.

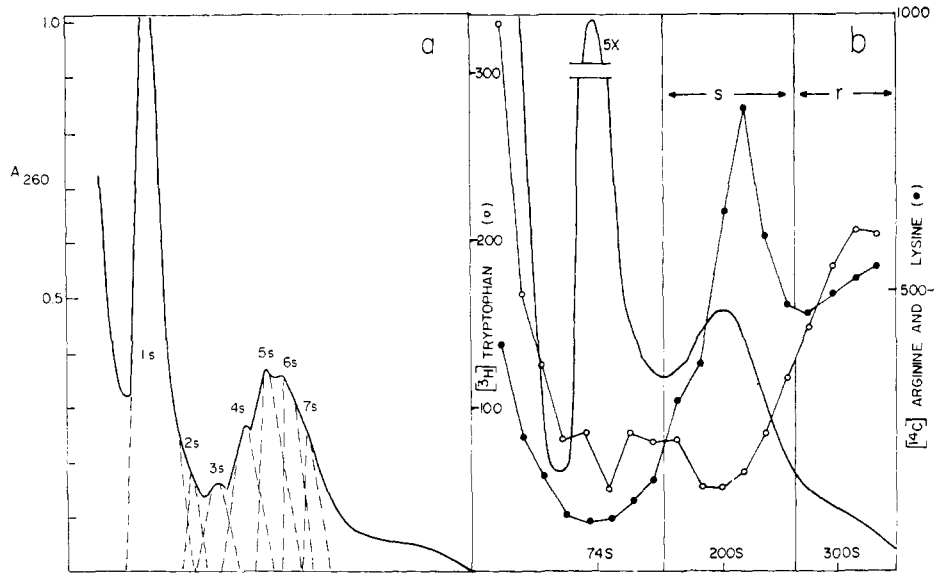

FIGURE 1: Polysomal classes in early sea urchin blastulae. (a) Eight-hour embryos of *S. purpuratus* were used without labeling. (b) Embryos of the same stage were incubated for 10 min (Methods) with [^{3}H]DL-tryptophan, [^{14}C]L-lysine, and [^{14}C]L-arginine. In both cases cells were disrupted by a single passage through a No. 20 gauge hypodermic needle and S15 supernatant was prepared. The S15 preparation was centrifuged through a 15–30% (w/w) sucrose gradient in the Spinco rotor SW50.1 at 50,000 rpm for 27 min.

(*e*) *Ion-Exchange Chromatography and Acrylamide Gel Electrophoresis.* The cation-exchange resin, Amberlite CG-50 (200–400 mesh, Mallinckrodt, New York, N. Y.) was regenerated by treatment successviely with 2 N HCl, water, 2 N NaOH, water, 2 N HCl, water, and then 2 N NaCl, before finally equilibrating it with 8% (w/w) guanidine chloride (Mann Research Laboratories, New York, N. Y.), containing 0.1 M Na_2HPO_4, pH 6.8. A 17 cm × 0.78 cm^2 column was packed with the resin by air pressure and washed with the 8% guanidine phosphate buffer. The sample protein solution was applied to the column and developed with 6 ml of 8% guanidine phosphate buffer, followed by a linear gradient of 8–13% (21 ml of each). The strongly bound material (histone fractions III and IV) was eluted by addition of 12 ml of 40% guanidine phosphate buffer. This was followed by 12 ml of 8% buffer solution. Radioactivity of fractions was assayed either after precipitation with 20% trichloroacetic acid in the presence of 200 μg of albumin carrier, followed by plating on glass fiber filter disks (grade 934AH, Reeve Angel, Clifton, N. J.) which were dried and counted in toluene scintillation fluid, or the fractions from the column were added directly to Bray's solution that was acidified by the addition of 0.1 ml of 2 N H_2SO_4 to 15 ml, and counted by scintillation spectrometry.

Acrylamide gel electrophoresis (Panyim and Chalkley, 1969) was preformed on preparations that had been lyophilized, then dissolved in 0.9 N acetic acid.

Results

(*a*) *Polysomal Classes and Their Associated Nascent Proteins.* In tracing the changes in the polysomal population during early sea urchin development, Infante and Nemer (1967) noted that the slowly sedimenting s-polysomes reached a maximum concentration at the 200-cell, 10-hr blastula stage. Sedimentation diagrams of good resolution, including Figure 1a, reveal that the approximate proportions of the component polyribosomes of this class do not change substantially during development from 7 to 10 hr. The weighted average size of this class holds constant at 4.5 ribosomes/polyribosome during this period, while the concentration of this class increases as a whole. The average size of protein produced by such a size class of polyribosomes might be expected to be 17,000, from the assumption that a ribosome occupies template equivalent to 30 amino acids (Warner *et al.*, 1963; Becker and Rich, 1966; Williamson and Askonas, 1967). Chromosomal histones might be expected to be synthesized on such a size class of polyribosomes, since the molecular weights of histones from several species range between 11,000 and 25,000 (Haydon and Peacocke, 1968; Butler *et al.*, 1968; Fambrough and Bonner, 1968; DeLange *et al.*, 1968).

Various lines of evidence (Robbins and Borun, 1967; Borun *et al.*, 1967; D. T. Lindsay, submitted for publication) have led us to postulate that the s-polysomes synthesize a special class of protein, namely the chromosomal histones. Nemer and Lindsay (1969) have reported that relatively less tryptophan compared to arginine was incorporated into the s-polysomes than into the more rapidly sedimenting "r-polysomes," and have suggested that the trytophan-deficient nascent protein population of the s-polysomes might include histones. The same asymmetric distribution was noted by Kedes *et al.* (1969) with lysine and tryptophan. The results with arginine and lysine, in combination (Figure 1b), are not readily distinguishable from the results obtained with these amino acids incubated separately. Such information as delineated in Figure 1a,b indicates only the possibility that histones may be synthesized by s-polysomes. A more direct analysis of the nascent proteins associated with these polyribosomes will be needed in order to identify the proteins they are synthesizing.

Nascent proteins derived from s-polysomes of 10-hr embryos were resolved into size classes by passage through Sephadex G-100 in the presence of 0.1% sodium dodecyl sulfate and 0.1% mercaptoethanol. Proteins of known molecular weights were included and used as markers (Figure 2). As shown, the elution position of the markers were linearly related to the logarithms of their molecular weights, so that

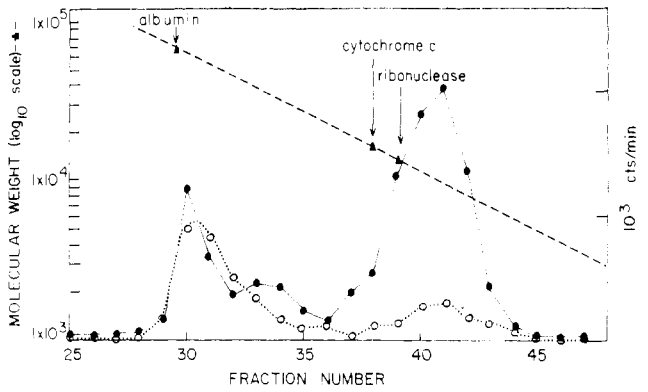

FIGURE 2: Gel filtration of nascent proteins. Nascent proteins were extracted from the s-polysomes of 10-hr early blastulae, labeled with [^{3}H]arginine and [^{3}H]lysine and from the r-polysomes of the same stage labeled with a [^{14}C]amino acid mixture. The preparations were mixed and passed through a column of Sephadex G-100, as described in Methods. Nascent protein of s-polysomes (●) and r-polysomes (○).

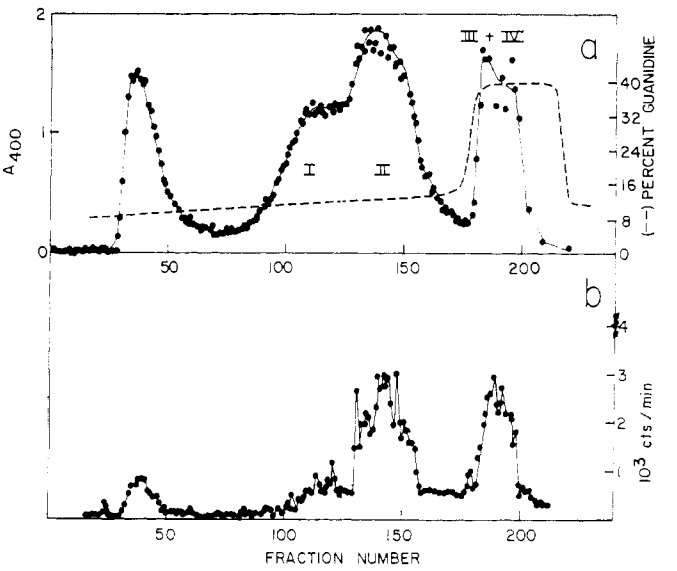

FIGURE 3: Cation-exchange chromatography of nuclear histones. Histone preparations were applied to columns of Amberlite CG-50 and eluted differentially with guanidine chloride in phosphate buffer. The per cent guanidine was monitored by measuring the refractive indices of fractions. (a) Commercial preparation of calf thymus histones. Aliquots of fractions were adjusted to 1.1 M trichloroacetic acid and the quantity of protein indicated by readings of turbidity at 400 mμ. Numerals I, II, III, and IV refer to the approximate locations of the histone classes according to Rasmussen *et al.* (1962). (b) Ten-hour embryos of *S. purpuratus* were incubated with [^{3}H]L-arginine and [^{3}H]L-lysine for 1 hr, then submitted to cellular fractionation and extraction of nuclear histones. Aliquots were taken for assay of radioactivity (Methods).

estimation of the molecular weights of the nascent proteins could be made. The major portion of r-polysomal nascent protein resided in a component of 57,500 average molecular weight. The major portion of the s-polysomal nascent protein (70%) was distributed in a peak with an average molecular weight of 9350. The remaining 30% of radioactivity appeared in material of higher molecular weight, which could have arisen from trailing of the r-polysomes into the segment taken for s-polysome analysis or from the formation of aggregates. The value for the average molecular weight for the nascent protein agrees favorably with the average nascent protein molecular weight (8500) derived from the average ribosome number in the s-polysomes given in Figure 1a.

The sizes of the polypeptides synthesized by the two polysomal classes differ by a factor of 6.15. If the rates of synthesis of these polypeptides were equivalent and their amino acid compositions similar, the incorporations of amino acid in nascent polypeptide per ribosome (counts/min per A_{260}) would reflect these size differences. Using the data of Figure 1b, we may test this relationship for tryptophan and for the combined arginine and lysine incorporation. The specific activity for tryptophan in r-polysomes is 8.6 times that in s-polysomes. This ratio is 1.4 times the ratio of nascent protein sizes, and may be indicative of a relatively greater content of tryptophan in r-polysomal nascent protein. The specific activity for arginine and lysine in r-polysomes is 3.2 times that in s-polysomes. This ratio, being less than the ratio of nascent protein sizes, indicates that the combined arginine and lysine content in s-polysomal nascent protein is 1.9 times that of the r-polysomes. We do not know whether in either case the deviation from the ratio of nascent protein sizes is due, at least partly, to a difference in translation rate between the two polysomal classes. Irrespective of such a difference in rates, the relative amino acid compositions of s- and r-polysomal nascent proteins can be quantitated on a per residue of tryptophan basis. Thus the ratio of the amounts of arginine and lysine per tryptophan in s-polysomes compared to r-polysomes is obtained from $(Arg,Lys)_s/(Trp)_s/(Arg,Lys)_r/(Trp)_r$, where each term refers to the specific incorporation in the respective polysomal classes, and is equivalent to the product $1.9 \times 1.4 = 2.6$. This value indicates the extent to which the s-polysomes are enriched by a class of proteins of relatively high arginine and lysine and low tryptophan, as compared to the r-polysomes. The extent of this asymmetry will be shown later to be a function of developmental stage.

(*b*) *Extraction of Histone-Like Proteins from Nascent Proteins Associated with the s-Polysomes.* The broad array of polypeptides among the nascent proteins associated with polyribosomes may not be characterized in any precise manner. One has two approaches toward an examination of the function of the polyribosomes in question. (i) Employ a purification applicable to known proteins which these polyribosomes supposedly synthesize; (ii) allow completion of proteins in reconstituted *in vitro* systems, preferably under conditions which afford release of finished proteins. We have pursued both approaches with informative results.

The purification of histone-like proteins from nascent proteins was performed in a manner similar to the extraction and characterization of chromosomal histones from purified sea urchin nuclei. Nuclei of early blastulae were acid extracted [Methods (d)], and the acid-soluble proteins were characterized by successive chromatography and electrophoresis. Ion-exchange chromatography was used to separate the nuclear histones into the major classes, similar to those reported for calf thymus histones (Rasmussen *et al.*, 1962). A commercial preparation of calf thymus histones has been chromatographed in Figure 3a. In Figure 3b, labeled chromosomal histones, derived from 10-hr early blastuale of *S. purpuratus*, were chromatographed under the same conditions. The histones from the sea urchin embryos display the same general classes as that of the calf thymus histones, but apparently in different proportions. In this early blastula class I, corresponding to lysine-rich histones, is comparatively under-

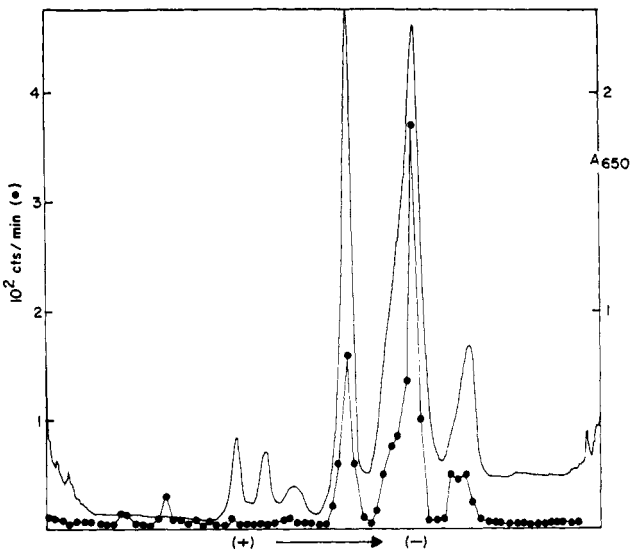

FIGURE 4: Acrylamide gel electrophoresis of nuclear histones of sea urchin embryos. A nuclear histone preparation from 7-hr embryos of *L. pictus* together with a trace amount of a similar preparation from 10-hr embryos of *S. purpuratus*, labeled as in Figure 3, was applied to a 15% gel (4.5 × 85 mm) and run for 2.5 hr at 2 mA/gel. Protein bands were stained with Amido Black. All procedures were according to Panyim and Chalkley (1969). Bands of stained protein were detected by scanning of gels with a recording spectrophotometer at 650 mμ (—).

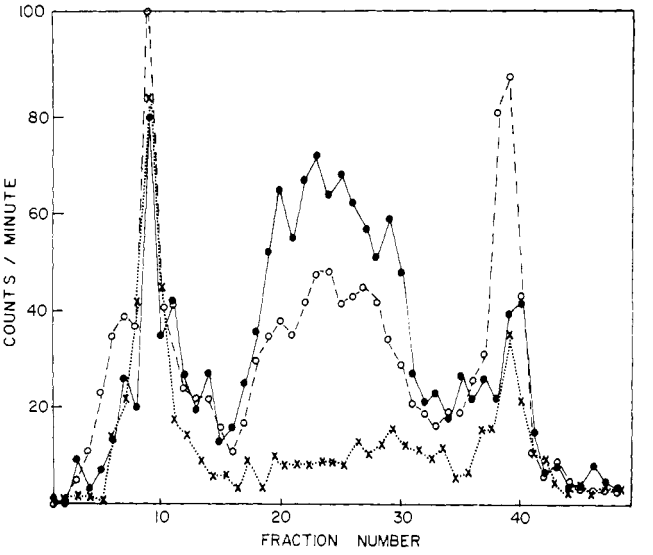

FIGURE 5: Cation-exchange chromatography of acid-soluble nascent proteins from s-polysomes and r-polysomes. Ten-hour embryos of *S. purpuratus* were incubated for 20 min either with a [^{14}C]amino acid mixture or a mixture of tritiated amino acids [as in Methods (c)]. The s-polysomes, labeled with ^{14}C, were isolated on sucrose gradients and their nascent proteins extracted by RNase treatment and subsequent acidification. The s- and r-polysomes, labeled with tritium, were separated and each incubated *in vitro* [Methods (c)]. After pelleting the polyribosomes, the labeled proteins were extracted as above. The ^{14}C-labeled nascent proteins of the s-polysomes (○) were mixed with the tritiated proteins of the s-polysomes, that had been subjected to *in vitro* incubation (●), and these preparations were then cochromatographed through a column of CG-50 resin. In a parallel column the tritiated proteins of the r-polysomes that had been similarly incubated *in vitro* were chromatographed through CG-50 resin (×).

represented. This observation is in agreement with that of Thaler *et al.* (1970). A subsequent characterization (Figure 4) was by means of high-resolution acrylamide gel electrophoresis (Panyim and Chalkley, 1969). Unlabeled nuclear histones of *L. pictus* blastulae were submitted to gel electrophoresis together with the labeled histones of blastulae of *S. purpuratus*, analyzed in Figure 3b. Six major bands were revealed by the dye binding assay. An exact coincidence of labeled histones with unlabeled bands was evident, except that little label was associated with the less-pronounced bands, which correspond to lysine-rich histones. It appears then that the histones of the blastulae of these two species are not readily distinguishable.

Nascent proteins were extracted from polyribosomes [Methods (b)] of early blastulae labeled *in vivo* with amino acids. The fraction of this material that was soluble in 0.40 N sulfuric acid (approximately 20%) was submitted to cation-exchange chromatography (Figure 5). Whereas all of the acid-soluble chromosomal protein so treated (Figure 3) was recovered, approximately 70% of the acid-soluble nascent proteins became irreversibly bound to the cation-exchange resin, defying elution even with 60% guanidine and with 1 N sulfuric acid. In other experiments, nascent proteins obtained from total polyribosomes of several embryonic stages (5, 10, 15, and 20 hr) were examined in the same way, and found to be irreversibly bound to the resin in the same high proportion. We cannot offer an explanation for this effect, but may suggest that it is the result of both the charge and the peculiarities of nascent polypeptide configuration. The phenomenon allows, at least, the isolation of those proteins that resemble histones from the large and diverse population of nascent proteins. It is likely that only those nascent histones that are comparatively near completion exhibit histone-like behavior under these circumstances. The proteins derived from the s-polysomes, that can be eluted and thus fractionated, fall into two categories (Figure 5). The class which does not adhere to the resin can be designated as nonhistone. It comprises approximately 30% of the eluted material. The next proteins are eluted at the positions of the histone classes (see Figure 3). These histone-like nascent proteins constitute 70% of the total eluted material. Ribosomal proteins would have been eluted with the initial, nonhistone-like proteins (Bonner *et al.*, 1968). However, the synthesis of ribosomal protein in this early stage embryo would not be expected, since ribosomal RNA synthesis is not detectable (Nemer, 1963), and since the two syntheses are apparently linked (Hallberg and Brown, 1969).

In vivo labeled polyribosomes were incubated *in vitro* with unlabeled amino acids, 100,000*g* supernatant fluid, and energy sources, in an attempt to promote completion of nascent polypeptides. At the end of the incubation the polyribosomes were pelleted by centrifugation, and the soluble and sedimented proteins were analyzed separately. There was negligible release of labeled protein during this incubation. The labeled proteins associated with the s-polysomes were analyzed by ion-exchange chromatography, just as the unincubated nascent proteins had been, and the results were similar, except for slight quantitative shifts (Figure 5). Upon incubation the proportion of nonhistone protein decreased slightly, and the relative proportions of histones shifted with a decrease in arginine-rich classes and an increase in lysine-rich classes. Although these shifts proceeded in the direction of more nearly completed histones, a large proportion of acid-soluble nascent proteins remained again irreversibly bound to the resin. Thus the apparent shift is in the

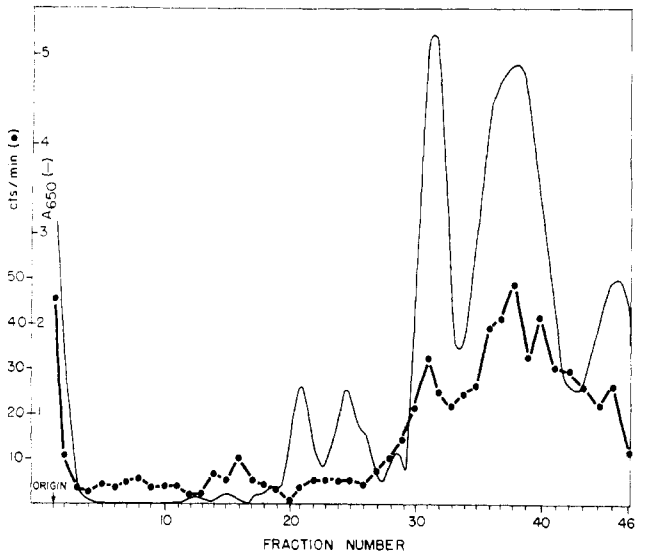

FIGURE 6: Acrylamide gel electrophoresis of nascent proteins of s-polysomes allowed to proceed toward completion of polypeptide chains *in vitro*. The s-polysomes were incubated *in vitro*, extracted with acid, and chromatographed as in Figure 4. The histone-like protein (fractions 18–45 of Figure 4) was dialyzed against 0.9 N acetic acid, lyophilized, and dissolved in 25 μl of 15% sucrose in 0.9 N acetic acid. This sample was mixed with 25 μl of the same unlabeled nuclear histones from *S. purpuratus* and electrophoresed as in Figure 5.

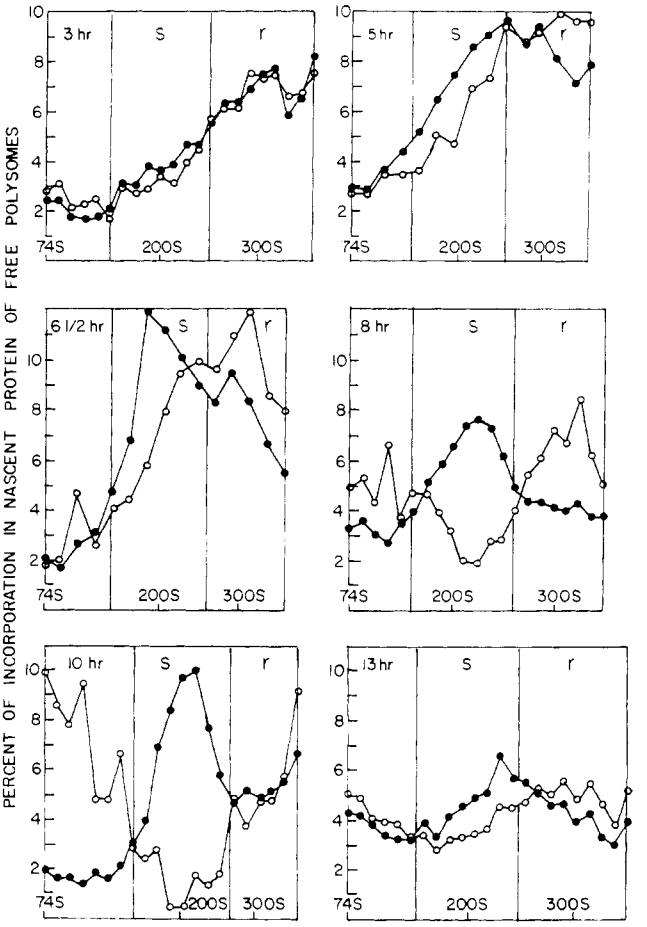

FIGURE 7: Differential incorporation of tryptophan compared to arginine and lysine in nascent proteins of polysomes at different embryonic stages. Embryos of *S. purpuratus* at the indicated stages of development were incubated as in Figure 1b. Incorporation in gradient fractions of sucrose gradients are indicated as the per cent of the total incorporation in the free polysomes, *i.e.*, material sedimenting more rapidly than the 74S monoribosomes: (○) [^{3}H]DL-tryptophan, (●) [^{14}C]L-arginine and lysine. The s-polysomal and r-polysomal regions are demarcated as indicated in Figure 1b.

nature of the isolated histone-like material. A comparison between the s-polysomes and r-polysomes was undertaken by incubating *in vivo* labeled r-polysomes under the same *in vitro* conditions applied to the labeled s-polysomes. The labeled protein associated with pelleted r-polysomes (again release was not detected) was analyzed in parallel with similar material from s-polysomes (Figure 5). There was a striking difference: considerably more chromatographed protein was nonhistone and very little corresponded to histone classes I and II. We may conclude that the nascent proteins of the s-polysomes have a much higher representation of histone-like proteins than those of the r-polysomes.

The size distribution of acid-soluble s-polysomal nascent protein was compared to that which was subsequently submitted to cation-exchange chromatography and eluted as histone-like protein. Gel filtration of these preparations was performed with Sephadex G-50, equilibrated with 8% guanidine phosphate buffer. The histone-like proteins displayed an appreciably higher average size (by an increment of 4000) than the unfractionated material (B. Moav and M. Nemer, unpublished). We may conclude that a selection of larger, presumably more complete, proteins occurs during fractionation of these proteins on the cation exchanger. The larger proportion of proteins that become irreversibly stuck to the resin probably represent the major portion of small, incomplete proteins.

The histone-like proteins eluted from the cation-exchange resin were concentrated and then submitted to acrylamide gel electrophoresis (Figure 6). The labeled nascent proteins were run together with unlabeled chromosomal histones from the same embryonic stage and found to coincide with the major chromosomal histone bands. The resolution of lysine-rich classes (the components in fractions 20–30) is superior to that obtained by chromatography. The ersult indicates that little radioactivity is associated with these histone classes, which are present in the least abundance (Thaler *et al.*, 1970). The chromatography and electrophoresis represent two independent characterizations. By either test the purified s-polysomal proteins under question were not distinguishable from chromosomal histones. This evidence greatly supports the proposal that the s-polysomes synthesize histones. However, more detailed resemblances would have to be shown before a firm conclusion could be drawn.

(*c*) *Developmental Changes.* The amino acid labeling pattern of polyribosomes, exemplified by the early blastula of Figure 1b, changes markedly during the course of embryonic development. In the early cleaving embryo the distribution of arginine and lysine and tryptophan incorporation appear to be uniform in the s- and r-polysomes (demarcated by vertical lines in Figure 7). However, a period of development (morula and early blastula) is reached during which the incorporation of arginine and lysine in s-polysomes, $(Arg,Lys)_s$, is proportionately high, and that of tryptophan, $(Trp)_s$, is relatively low, particularly when these are compared to their counterparts in the r-polysomes, $(Arg,Lys)_r$ and $(Trp)_r$. The ratio $[(Arg,Lys)_s/(Trp)_s]/[(Arg,Lys)_r/(Trp)_r]$ changes from 1.5 at 5 hr to 2.6 at 8 hr and 4.0 at 10 hr. The significance of the developmental increase in this value resides

in the probability that the s-polysomes contain increasing amounts of nascent protein enriched in arginine and lysine and deficient in tryptophan. Such a situation would be consistent with an increased representation of nascent histones.

If we assume that there does indeed exist a class of nascent proteins in the s-polysomes lacking tryptophan, and by this criterion "histone-like," we may estimate the relative output of these histone-like proteins for each developmental stage. We may subtract from $(Arg,Lys)_s$ that portion of the arginine and lysine incorporation in the s-polysomes which is present in the same proportion to $(Trp)_s$ as arginine and lysine is to tryptophan in the r-polysomes. Thus $(Trp)_s(Arg,Lys)_r/(Trp)_r$ is equivalent to arginine and lysine incorporation in those s-polysomal proteins that resemble the proteins of the r-polysomes in their arginine, lysine, and tryptophan composition. The difference resulting from this subtraction would yield the incorporation due to tryptophan-deficient, histone-like, s-polysomal proteins. The relative fractional output of these proteins would be represented by $[(Arg,Lys)_s - (Trp)_s(Arg,Lys)_r/(Trp)_r]/[(Arg,Lys)_r + (Arg,Lys)_r]$. This relative output by the polysomes has been plotted during the course of development up to the 20-hr late blastula (Figure 8). A striking feature of these developmental changes is that a maximum is reached at the 10-hr, 200-cell blastula stage. The value at this point is ten times that for the earlier, 3-hr cleaving embryo or the later, 16-hr swimming blastula. If we assume that the combined arginine and lysine content of the histone-like proteins is twice that of the rest of the protein population, then the values for relative output in Figure 8 may be divided by 2, to obtain values approximating the actual fraction of the total polysomal production representing histone synthesis. A positive correlation is afforded here by the inclusion in Figure 8 of the rate of cell formation per embryo, which may be regarded as indicative of the rate of DNA synthesis. In the 3-hr, 4-cell embryo the rate of cell formation is 4 cells/hr. This rate reaches 55 cells/hr at the 200-cell blastula stage, but declines thereafter. The changes in amino acid labeling pattern and in rate of cell formation occur in approximate parallel. A similar correlation between histone synthesis and DNA synthesis would be expected (Bonner, 1965).

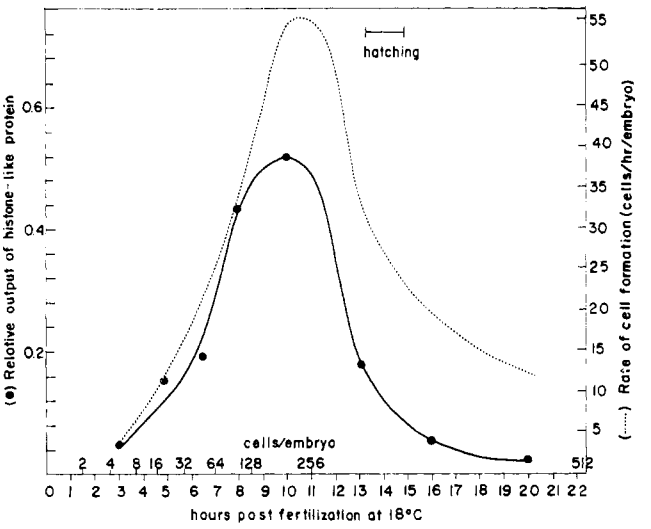

FIGURE 8: Relative output of histone-like protein by free polysomes during embryonic development. The data of Figure 7 are used together with similar sedimentation diagrams at other embryonic stages to estimate by the method described in the text the relative output of tryptophan-less proteins by the s-polysomes. The rates of cell formation were calculated from the data of Hinegardner (1967).

Discussion

The principal objective of this study has been to assign a function to the predominant class of polyribosomes in the early sea urchin blastula. These "s-polysomes" in the 200-cell blastula constitute over 70% of the polysomal ribosomes (Figure 1) and over 90% of the mRNA molecules, as estimated from the ribosomal content and the relative cistron sizes indicated by Figure 2. The newly synthesized RNA associated with these polyribosomes displays discrete size classes, the most prominant of which is 9–10 S (Nemer and Infante, 1965), recently confirmed by Kedes and Gross (1969). Another, intriguing property of this class of mRNA is its rapid annealing with DNA: Nemer and Infante (1965) observed greater than 40% hybridization of purified 9–10S RNA with 112 μg of DNA/ml in 24 hr. Thus the hybridized RNA transcripts could be estimated (Britten and Kohne, 1968) to correspond to highly "redundant" DNA. It is possible that the mRNAs of the s-polysomes are derived from genes present in multiple copies. The significance of the present study may therefore be viewed from several standpoints. The proteins synthesized by the s-polysomes are a sizeable portion of the total protein output during a brief and circumscribed period of early embryonic development. Their synthesis appears to be promoted almost entirely by newly synthesized mRNA (Infante and Nemer, 1967), as opposed to preexisting egg mRNA. And, a repository of multiple genes for their templates may exist, to serve, perhaps, some important developmental and evolutionary functions.

Our approach to the s-polysomal proteins involved separate qualitative and quantitative evaluations. Qualitatively, we analyzed the nascent proteins associated with these polyribosomes. An apparent limitation of this analysis is that the vast portion of the nascent polypeptides are not susceptible to meaningful characterization and cannot be expected to resemble the proteins to which they give rise. We have thus limited ourselves to the task of purifying from this large array of protein fragments those proteins resembling a narrow group of proteins, suspected of being synthesized by these polyribosomes. We were lead to suspect this group of proteins to be the chromosomal histones, because the relatively high arginine and low tryptophan incorporations in the nascent proteins associated with these polyribosomes tended to mimic the peculiar amino acid composition of histones (Nemer and Lindsay, 1969).

The purification of histone-like proteins involved three simple steps: acid extraction of labeled proteins removed from polyribosomes, cation-exchange chromatography, and, finally, acrylamide gel electrophoresis. Whereas preparations of chromosomal histones were recovered completely after passage through the cation-exchange resin, a considerable portion of the nascent polypeptides were irreversibly bound to the resin. The s-polysomes furnished a high proportion of the eluted proteins that were indistinguishable from histones, both according to their characteristic elution pattern from the resin and a coincidence with histone bands in acrylamide gel electrophoretograms. The recovered proteins can be presumed from our results to be complete or nearly completed. We have thus defined the function of the polyribosomes in question by virtue of the properties of the most nearly completed members of their nascent protein population. This

rationale may be implicit in the several studies directed at the assignment of specific enzyme synthesis to given classes of polyribosomes (Kiho and Rich, 1964, 1965; Bagdasarian *et al.* 1970). It is likely that only the completed or most nearly completed enzyme molecules are detected. Our conclusions are that the s-polysomes are responsible for the synthesis of histone-like protein and that such synthesis is restricted to these polyribosomes, since histone-like proteins are not significantly demonstrable in larger polyribosomes.

Developmental changes were examined on the basis of shifts in the incorporation in nascent protein of tryptophan relative to arginine and lysine. The output of histone-like protein (lacking tryptophan) by the s-polysomes changed in a manner similar to the changes both in the relative concentration of s-polysomes (Infante and Nemer, 1967) and in the rate of DNA synthesis, indicated by Figure 8. For all three a maximal value was reached in the 200-cell early blastula. The actual fraction of the total polysomal production representing histone synthesis at this embryonic stage could be calculated on the basis of assumptions that all of the tryptophan-less proteins are histones and as such contain twice as much arginine and lysine as the rest of the protein population. The value for this output might be estimated at 26% of the total. An absolute rate of protein synthesis of 4.7 to 8.7 pg per embryo per min at this embryonic stage has been measured by Fry and Gross (1970). According to this measurement the ouput of histone would be 1.2–2.3 pg/embryo per min. The formation of 50 diploid nuclei per hr in the 10-hr blastula (Figure 8) entails an average synthesis of 90 pg of DNA/hr or 1.5 pg/min per embryo. It appears then that equivalent weights of DNA and histone may be synthesized, in agreement with conclusions for other organisms (Marushige and Ozaki, 1967; Chalkley and Jensen, 1968). A correlation between the activity of the s-polysomes and DNA synthesis was shown previously through an inhibition of DNA synthesis (Boron *et al.*, 1967; Kedes and Gross, 1969). The present study indicates a quantitative coincidence between DNA synthesis and the production of histone-like proteins by the s-polysomes. We can conclude from several lines of evidence that these polyribosomes are responsible for the synthesis of chromosomal histones.

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