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Size of Histone Gene Transcripts in Different Embryonic Stages of the Sea Urchin, *Strongylocentrotus purpuratus*†

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ABSTRACT: Histone genes in sea urchins are organized into a repeat unit of DNA with a modal length of 6–7 kilobases. Since all five histones are encoded in the unit, it is possible that a large initial transcript containing the sequences of the complete repeat is cleaved into 9S histone mRNAs. We prepared total RNA from embryos labeled for 90 min or 3 h with ^{14}C-labeled nucleosides and for the last 10 min of this period with [^{3}H]uridine. The RNA was sedimented in 85% formamide gradients and the position of the histone transcripts was then determined by hybridization with the histone DNA recombinant, pCO1. Unless care was taken to denature the RNA, considerable aggregation of the histone transcripts was observed. Using denaturing conditions, no high molecular weight histone transcripts were identified in RNA from the late cleavage embryo; all hybridizable RNA was in the 7–12S region of the gradient. Treatment of the embryos with toyocamycin or proflavin, or growth at 9 °C did not alter the results. Transcripts from four-cell embryos were also devoid of high molecular weight histone RNA. However, RNA prepared from mesenchyme blastula embryos did contain high molecular weight transcripts which hybridized to the histone DNA. These results were obtained when the hybrids were not treated with RNase. The high molecular weight RNA was markedly enriched in newly synthesized transcripts as determined from the high $^{3}H/^{14}C$ ratio of hybridized counts. These transcripts are further differentiated from the 7–12S RNA in that they are twice as sensitive to RNase when in hybrid form and can be recycled through another round of 85% formamide gradients retaining, for the most part, their large size.

The histone genes of the sea urchin are organized into a repeating structure which contains coding sequences for all five major histones (Weinberg et al., 1975; Birnstiel et al., 1975; Kedes et al., 1975b; Cohn et al., 1976; Wu et al., 1976; Schaffner et al., 1976; Portmann et al., 1976; Holmes et al., 1977). Superficially, at least, this structure resembles a prokaryotic operon in that related proteins are encoded on adjacent stretches of DNA. The structure might serve as a template for a multigene transcript which would then be processed into the mRNAs. The availability of cloned histone gene fragments (Kedes, 1975; Clarkson et al., 1976; Overton & Weinberg, 1978) provides a pure hybridization probe to identify newly synthesized histone transcripts. Hybridization of labeled RNA to the probe, present in DNA excess, should indicate the amount of newly synthesized RNA in the fraction tested. This procedure allows a direct measurement of the labeled RNA and not merely a determination of the total complementary RNA concentration. It is particularly important to design a method to distinguish old and new transcripts (Curtis & Weissmann, 1976; Ross, 1976), especially since aggregation is of great concern in these experiments (Nemer et al., 1974). In our experiments, we have distinguished the newly made RNA from older transcripts by labeling embryos for a long period with one isotope and for a short period with a second isotope.

A multi-gene histone transcript would be a convenient means of coordinate regulation of protein synthesis. However, as we report here, we have not been able to identify a large transcript in the late cleavage stage where there is a high level of histone mRNA synthesis. We have, on the other hand, demonstrated that part of the newly synthesized histone transcript in the mesenchyme blastula is greater than 18S in size. This developmental stage is characterized by a shift in the synthesis of particular H2A, H2B, and H1 subtypes (Cohen et al., 1975) made on different mRNAs which are encoded by different genes (Weinberg et al., 1978; Newrock et al., 1978; Kunkel & Weinberg, 1978). A fundamental change in the expression of histone genes appears to occur at this embryonic stage.

Materials and Methods

Embryo Development and Preparation of Labeled RNA. Eggs of *S. purpuratus* (Pacific Biomarine Supply Co.) were fertilized and cultured at 17 °C. Embryos which were cultured for 9 h at 17 °C developed to the 128 cell stage, just starting to form a blastocoel. These embryos are designated to be in the "late cleavage" stage. Embryos cultured for 22.5 h at 17 °C

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are designated "mesenchyme blastula." The primary mesenchyme was seen to have migrated into the blastocoel. The embryos were labeled with [^{3}H]uridine (26–29 Ci/mM, Amersham/Searle) for 10 min at 25–50 μCi/mL at the designated stage. In those cases in which the RNA was labeled with two different isotopes, [^{14}C]adenosine (549 mCi/mM, Amersham/Searle) was added for a 90-min period to late cleavage embryos or [^{14}C]uridine (462 mCi/mM, Amersham/Searle) was added for a 3-h period to mesenchyme blastula embryos, at the end of which [^{3}H]uridine was added for 10 min. After the labeling period, embryos were centrifuged at low speed and were lysed in a Dounce homogenizer with a tight fitting pestle, at room temperature in 20 volumes of a buffer described by Holmes & Bonner (1973): 0.35 M NaCl, 1 mM EDTA,[1] 10 mM Tris (pH 8.0), 7 M urea (Schwarz/Mann, UltraPure), 2% sodium dodecyl sulfate ($NaDodSO_4$). Glassware was treated with 0.1% diethylpyrocarbinate and dried before use. The homogenate was extracted with a phenol (water saturated)–chloroform–isoamyl alcohol (24:24:1, v/v) solution, the phases were separated by low speed centrifugation, and the aqueous phase and interphase were reextracted twice with an equal volume of the phenol–chlorofom–isoamyl alcohol mixture. The aqueous phase was then extracted two times with chloroform–isoamyl alcohol (24:1) and then precipitated with 2 volumes of ethanol and 0.1 volume of 3 M NaCl at −20 °C.

In an alternative extraction procedure the pelleted embryos were resuspended in 0.05 M sodium acetate (pH 6.0), 1 mM EDTA, and 0.5% $NaDodSO_4$ and homogenized in a Dounce tissue grinder with a tight fitting pestle. The homogenate was extracted with an equal volume of phenol–cresol–water (70:10:20, v/v) containing 0.1% hydroxyquinoline (Parish & Kirby, 1966). The mixture was incubated for 10 min at 55 °C and then centrifuged at low speed to separate the phases. The aqueous phase and interphase were extracted an additional two to four times until very little interphase remained. The aqueous phase was then extracted with chloroform–isoamyl alcohol (24:1, v/v) and the RNA precipitated with 2 volumes of ethanol and 0.1 volume of 3 M NaCl.

The RNA was centrifuged out of ethanol and allowed to dry in a stream of N_2. The pellet was resuspended in 10 mM sodium acetate (pH 5.0), 5 mM $MgCl_2$, 10 μg/mL poly(vinyl sulfate) (Brown & Suzuki, 1974) containing 10 μg/mL DNase (Worthington, electrophoretically pure). The DNase was tested for RNase activity and had no effect on the absorbance profile or regions of hybridization in the gradients. After 10 min at room temperature, the solution was made 0.1% in $NaDodSO_4$ and extracted with the phenol–chloroform–isoamyl alcohol mixture and precipitated with ethanol and NaCl.

Conditions of Centrifugation and Denaturation. The 15–30% nondenaturing sucrose gradients were prepared in 0.01 M Tris (pH 7.4), 1 mM EDTA, 0.1 M NaCl, 0.5% $NaDodSO_4$. RNA dissolved in this buffer were centrifuged through these gradients in a Spinco SW27 rotor at 25 000 rpm for 20 h at 20 °C. Fractions were collected and monitored with an Isco density gradient fractionator.

The formamide gradients were formed using 5–20% sucrose dissolved in an 85% formamide buffer (85% formamide, 2 mM EDTA, 10 mM Tris, pH 7.5). The formamide (Fisher, reagent grade) was pretreated with Bio-Rad Chelex 100 which was then removed by filtration. Before loading, the RNA was dissolved in the 85% formamide buffer and heated at 65 °C for 10 min. No more than 200 μg of RNA was loaded on each gradient prepared in Spinco SW50.1 rotor tubes. The gradients were centrifuged at 40 000 rpm for 20 h at 20 °C. Fractions were collected with an Isco density gradient fractionator and were pooled as indicated in the figure legends. For determination of radioactivity in each fraction, an aliquot was removed into a tube containing 0.1 mg of bovine serum albumin and 2 mL of ice-cold 10% trichloroacetic acid was then added. Precipitates were collected on glass fiber filters, washed, dried, and scintillation counted.

Preparation of Plasmid DNA. Hybridization was performed with pCO1 DNA, a plasmid containing a full length histone repeat inserted into pBR313 (Overton & Weinberg, 1978). The plasmids were extracted from *E. coli* K12 strain HB101 (Boyer & Roulland-Dussoix, 1969) which was grown in M9 medium plus casamino acids (Tanaka & Weisblum, 1975) supplemented with 10 μg/mL ampicillin. The yield of plasmid was amplified in the presence of chloramphenicol according to the Tanaka & Weisblum (1975) modification of the procedure of Clewell (1972).

Hybridization of RNA Fractions to pCO1 DNA. Fractions from the formamide gradients were pooled as indicated in the figure legends and to each pooled sample, 30 μL of 10× Denhardt's buffer was added [Denhardt, 1966; 1× Denhardt's buffer: 0.02% Ficoll (Pharmacia, average mol wt 400 000), 0.02% poly(vinylpyrrolidine) (Sigma, average mol wt 360 000), and 0.02% bovine serum albumin (Armour, fraction V)]. The RNA in this solution was precipitated with 2 volumes of ethanol, 0.1 volume of 3 M NaCl, at −20 °C for at least 10 h. Denhardt's buffer was not added as a carrier to the fractions from nondenaturing gradients as there was sufficient RNA to obtain good pellets. The RNA precipitates were centrifuged in a Sorvall HB4 rotor at 8000 rpm for 15 min. Pellets were resuspended in 0.3 mL 3 × SSC, 0.1% $NaDodSO_4$ (1 × SSC: 0.15 M sodium citrate, 0.015 M NaCl, pH 7.0).

The filter hybridization procedure of Gillespie & Spiegelman (1965) as adapted by Birnstiel et al. (1972) and Weinberg & Overton (1977) was used. Nitrocellulose filters 13 mm in diameter, containing 10 μg of pCO1 DNA or *E. coli* DNA were suspended in the RNA-containing solution. To the 3 × SSC, 0.1% $NaDodSO_4$ solution, Denhardt's buffer (if not previously used in the precipitation step) was added to achieve a final concentration of 1× Denhardt's buffer. The hybridization reaction was carried out at 65 °C for 18 h. Filters were washed twice for 1 h with 1 L of 2 × SSC, 0.1% $NaDodSO_4$ at 55 °C. In the case of filters which were treated with RNase before counting, the filters were first washed with two 1-L changes of 2 × SSC at room temperature, then once with 2 × SSC containing 10 μg/mL RNase A (Sigma), and finally once again in 2 × SSC. Filters were dried at 80 °C for 30 min and were scintillation counted. The average radioactivity on two *E. coli* DNA filters (usually 15–20 cpm) was subtracted from that on two pCO1 DNA filters.

Hybrid Melting Profiles. DNA filters were removed from scintillation fluid, washed several times with chloroform. They were placed for 30 min in a 0.1 × SSC solution which had been treated with 0.1% diethylpyrocarbinate which was evaporated away. The filters were heated in 1-mL aliquots of 0.1 × SSC which were incubated at increasingly higher temperatures. After 5 min at each temperature, the 1-mL solution was removed from the filter and placed on ice and a new 1-mL aliquot of 0.1 × SSC was added to the filter at a higher temperature. Each aliquot was then precipitated with 10% Cl_3CCOOH and 100 μg of yeast RNA. Melting curves were constructed by plotting accumulated radioactivity eluted from the filter as a

[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; SSC, a buffer consisting of 0.15 M sodium citrate, 0.015 M NaCl, pH 7.0; RNase, ribonuclease.

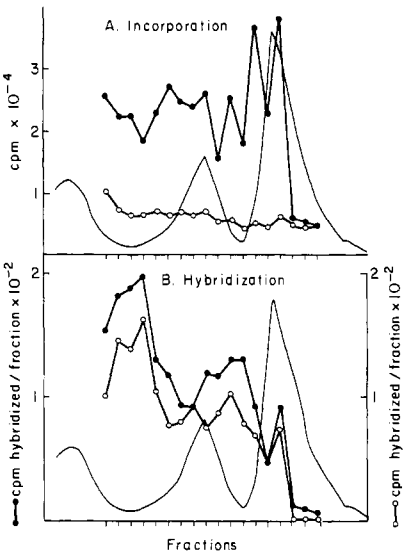

FIGURE 1: Size of early blastula histone transcripts in nondenaturing gradients. Sucrose gradients (15–30%) in an aqueous buffer were used to separate RNA made from 9-h embryos labeled with [^{14}C]adenosine for 90 min and for the last 10 min of this period with [^{3}H]uridine. RNA was extracted using urea–NaDodSO$_4$ buffer (Holmes & Bonner, 1973). (A) Distribution of [^{3}H]uridine and [^{14}C]adenosine in Cl_3CCOOH insoluble material; 5-μL aliquots of 1.2-mL fractions were counted. (B) Hybridization of fractions to pCO1 DNA immobilized on filters. The total fraction was used in the reaction. Hybrids were treated with RNase. (●) [^{3}H]Uridine-labeled material; (○) [^{14}C]adenosine-labeled material; (—) relative A_{254nm}. Top of gradient to the left.

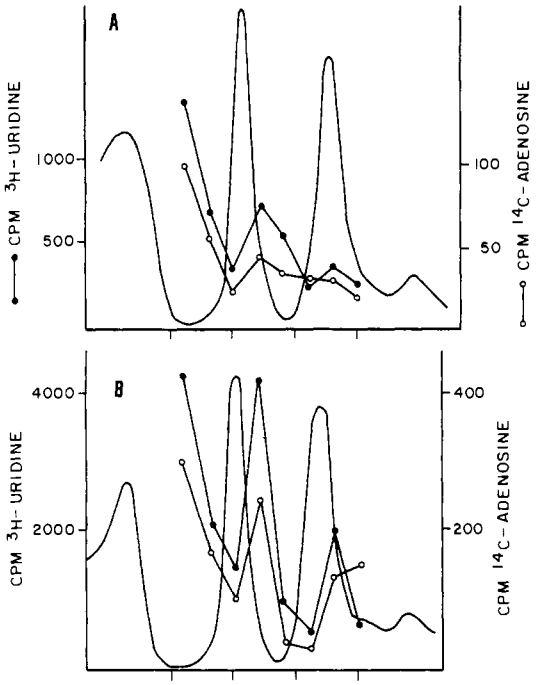

FIGURE 2: Size of denatured early blastula histone transcripts subsequently centrifuged in nondenaturing gradients. Gradients identical with those of Figure 1 were used to fractionate the same RNA preparation, except that the RNA was first heated for 10 min in 85% formamide at 65 °C. Each panel shows a different gradient centrifuged on a different day. Two adjacent fractions were pooled and the RNA then hybridized to pCO1 DNA. Hybrids were treated with RNase. (●) [^{3}H]Uridine-labeled material; (○) [^{14}C]adenosine-labeled material.

function of temperature (Birnstiel, 1972). Immersion of the filters in scintillation fluid and washing with chloroform had no effect on the T_m of the hybrids.

Results

Effect of Denaturing Conditions on Histone Transcript Size of the Late Cleavage Embryo. To test for the size of newly synthesized histone transcripts, equal volumes of sucrose gradient fractions were hybridized to an excess of pCO1 DNA. As long as the DNA is in excess, the amount of label which hybridizes to the DNA is a measure of the level of radioactive complementary RNA in that gradient fraction. It may be difficult, however, to achieve DNA excess in the 9S region of the gradient since the amount of histone mRNA would be greatest here. The competition for sites on the pCO1 DNA would lead to an underestimate of the amount of labeled histone RNA in the fraction. In any case, as has been previously discussed (Melli et al., 1977), we would not be underestimating the amount of labeled histone transcript in the high molecular weight regions of the gradients since competition for sites on the DNA would be least in this case.

Labeled RNA prepared from late cleavage embryos was centrifuged under a variety of conditions to test whether aggregation would be a problem in the determination of the size of histone gene transcripts. Embryos were labeled from 7.5 to 9 h after fertilization with [^{14}C]adenosine and for the last 10 min of this period, with [^{3}H]uridine. The RNA was applied to 15–30% sucrose gradients containing no denaturing agent and centrifuged. The profile of the incorporated counts is shown in Figure 1A. The [^{3}H]uridine and [^{14}C]adenosine are incorporated into all sizes of RNA, as expected for the total RNA population. The RNA fractions were tested for histone transcript content by hybridization to the histone recombinant, pCO1. As illustrated in Figure 1B, when equal aliquots of each fraction are used in the hybridization reaction, histone gene transcripts are found throughout the gradient, although there is a peak in the 7–12S region. There is no enrichment for the newly synthesized [^{3}H]histone RNA in any of the regions of the gradient. These results are not surprising in the light of other experiments indicating the high aggregation affinity of histone mRNA (Nemer et al., 1974).

The same RNA preparation was then denatured in 85% formamide at 65 °C for 10 min and subsequently centrifuged in nondenaturing sucrose gradients as above. The labeled RNA is still broadly distributed across the gradient, similar to that shown in Figure 1A. The hybridization results of two such gradients are presented in Figures 2A and 2B. Although there seem to be two distinct peaks at 7–12S and at about 20S, there is no enrichment for the newly synthesized [^{3}H]RNA in any area of the gradient. When labeled polysomal RNA is prepared from similar embryos, a 20S peak is usually evident (Nemer & Infante, 1965; Kedes & Gross, 1969; Kedes & Birnstiel, 1971; Nemer et al., 1974) but it has been shown that, if a 9S RNA is first isolated and then run on nondenaturing sucrose gradients, even after a treatment with formamide, a certain amount of a 20S sedimenting RNA will result (Nemer et al., 1974).

A different result was obtained, as shown in Figure 3, when the RNA which was treated with formamide was then centrifuged in gradients containing 85% formamide. Although the incorporation of [^{3}H]uridine is still shown to occur in all size classes of RNA, now only the 7–12S region contains transcripts which hybridize to the histone DNA. Only a very small amount of large molecular weight histone transcript forms RNase resistant hybrid, and the hybrids in all regions are not enriched in newly synthesized ^{3}H-labeled sequences. Gradients containing 70% and 99% formamide yield hybridization profiles identical to the 85% formamide gradients (data not shown).

Effect of an Alternative Method of RNA Preparation. The RNA preparations used in the above experiments were pre-

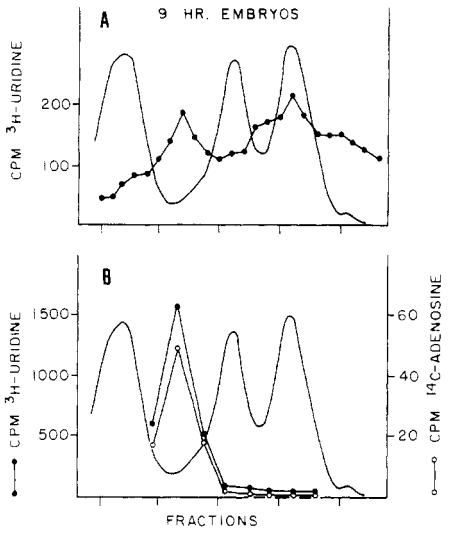

FIGURE 3: Size of early blastula histone transcripts in 85% formamide gradients. Gradients of 5–20% sucrose in 85% formamide were used to fractionate RNA made from 9-h embryos labeled with [^{14}C]adenosine for 90 min and for the last 10 min of this period with [^{3}H]uridine. The same RNA preparation was used as in Figures 1 and 2. RNA was extracted using urea–NaDodSO$_4$ buffers (Holmes & Bonner, 1973). (A, top panel) Distribution of [^{3}H]uridine into Cl_3CCOOH-insoluble material. Aliquots (10-μL) of 0.2-mL fractions were counted. (●) counts/min. (—) A_{254nm}. (B, bottom panel) Hybridization of RNA from two adjacent fractions obtained from two identical gradients, to pCO1 DNA immobilized on filters. Hybrids were treated with RNase. (●) [^{3}H]Uridine labeled material. (○) [^{14}C]adenosine-labeled material. (—) Relative A_{254nm}. Top of gradient is to the left.

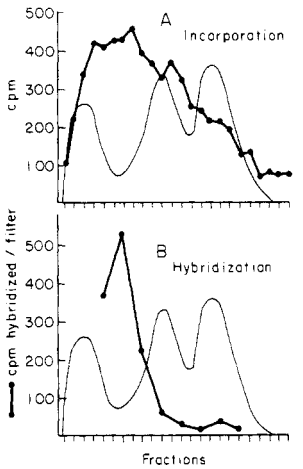

FIGURE 4: Transcript size of total RNA prepared by hot phenol extraction. Nine-hour embryos, labeled with [^{3}H]uridine for 10 min, were homogenized in an NaDodSO$_4$ buffer (0.05 M sodium acetate, pH 6.0, 1 mM EDTA, 0.5% NaDodSO$_4$), and the RNA extracted by deproteinization with hot phenol. The RNA was centrifuged in 85% formamide gradients. (A) Distribution of [^{3}H]uridine in Cl_3CCOOH-insoluble material. Aliquots (10-μL) of 0.2-mL fractions were counted. (B) Hybridization of RNA from two gradients to excess pCO1 DNA, immobilized on filters. Fractions from each gradient were pooled into groups of two and combined with corresponding fractions of the other gradient. Hybrids were treated with RNase. (●) [^{3}H]Uridine-labeled material (—) Relative A_{254nm}. Top of gradient is to the left.

pared by homogenization in a urea–NaDodSO$_4$ buffer (Holmes & Bonner, 1973) effective in the isolation of precursors to globin mRNA (Kwan et al., 1977). We also used an alternative approach of homogenization in a NaDodSO$_4$ buffer followed by extraction with hot phenol. As is shown in Figure 4, when this RNA, also made from 9-h embryos, was centrifuged in 85% formamide, the incorporation profile and hybridization profile were essentially the same as that obtained with the urea–NaDodSO$_4$ method.

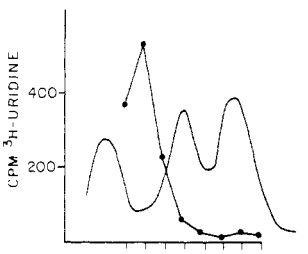

FIGURE 5: Transcript size in cold-treated embryos. Hybrids were formed with pCO1 DNA and RNA made from 9-h embryos grown at 9 °C, instead of 17 °C. The RNA was labeled for 10 min with [^{3}H]uridine and was extracted using the urea–NaDodSO$_4$ buffer (Holmes & Bonner, 1973) and centrifuged in 85% formamide gradients. Hybridization of RNA from three gradients to pCO1 DNA immobilized on filters. Fractions from each gradient were pooled into groups of two and combined with corresponding fractions of the other gradients. Hybrids were treated with RNase. (●) [^{3}H]uridine-labeled material. (—) Relative A_{254nm}. Top of gradient is to the left.

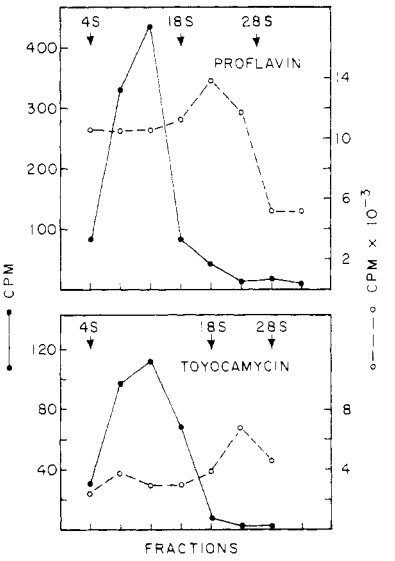

FIGURE 6: Histone transcripts in drug-treated embryos. Nine-hour embryos were treated with proflavin and toyocamycin. During a 10-min pulse of [^{3}H]uridine, 10 μg/mL toyocamycin was added concurrently or 50 mM proflavin was added during the last 2 min. The RNA was centrifuged in 85% formamide gradients and fractions were hybridized to pCO1 DNA. (--○--) Ethanol-precipitable counts from the indicated fractions which served as the input in the hybridization reaction. Hybrids were treated with RNase. (●) Hybridization to pCO1 DNA. Top of gradient is to the left. Toyocamycin was obtained from the National Cancer Institute, Drug Research and Development, Chemotherapy section.

Different Physiological Conditions. One possible explanation of the results is that the histone transcripts are made as large molecules but are virtually instantaneously processed. Embryos treated at low temperature might have a slower processing rate. Embryos in late cleavage were equilibrated at 9 °C before a 10-min incubation in [^{3}H]uridine. The profile of hybridization to histone DNA is once again limited to the lower sized transcripts, as shown in Figure 5.

An attempt was made to prevent any potential processing with drugs thought to have some effect on this event (Brinker et al., 1973; Weiss & Pitot, 1974; Venkov et al., 1977). When proflavin or toyocamycin was used, however, neither the incorporation profile nor the size of histone transcripts changed markedly, as illustrated in Figure 6. The effect seemed mostly to be an inhibition of transcription.

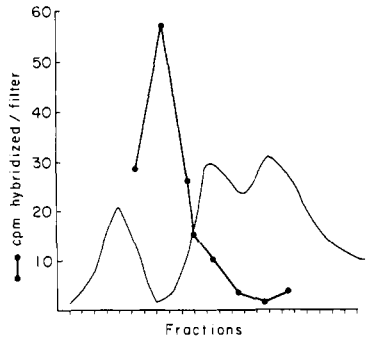

FIGURE 7: Histone transcripts in four cell embryos. RNA from four cell embryos was labeled for 10 min with [^{3}H]uridine and was extracted with urea-NaDodSO$_4$ buffer (Holmes & Bonner, 1973). The RNA was centrifugated in 85% formamide gradients and fractions from each of three identical gradients were pooled into groups of two and combined with the corresponding fractions of the other gradients. Hybrids were formed from the RNA and pCO1 DNA immobilized on filters. Hybrids were treated with RNase. (●) [^{3}H]uridine-labeled material. (--) Relative A_{254nm}. Top of gradient is to the left.

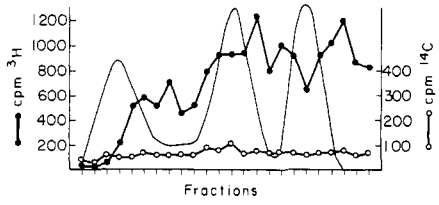

FIGURE 8: Transcript size of total RNA prepared from mesenchyme blastula. Embryos were labeled from 19.5 to 22.5 h after fertilization with [^{14}C]uridine and for the last 10 min of this period with [^{3}H]uridine. RNA was prepared using the Holmes-Bonner method and centrifuged in 85% formamide gradients. Distribution of [^{14}C]- and [^{3}H]uridine incorporated into Cl_3CCOOH-insoluble material is shown. Ten microliters of 0.2-mL fractions was counted. (●—●) [^{3}H]Uridine; (O—O) [^{14}C]uridine.

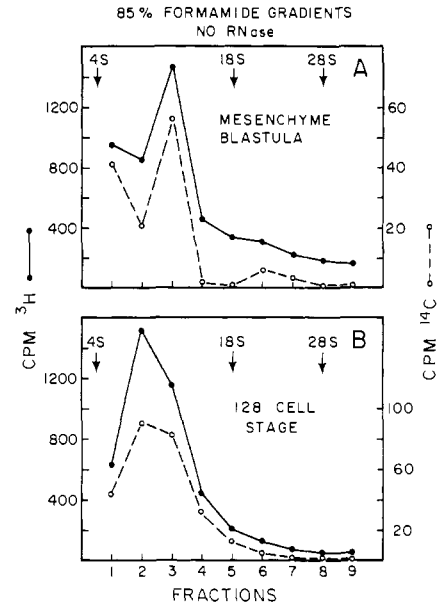

FIGURE 9: Comparison of histone transcripts made in early blastula and mesenchyme blastula. Hybrids were formed with pCO1 DNA and RNA made from 22.5-h mesenchyme blastula (A) or 9-h late cleavage (B) embryos. The late cleavage embryos were labeled from 7.5 to 9 h with [^{14}C]adenosine and for the last 10 min with [^{3}H]uridine. The mesenchyme blastula embryos were labeled from 19.5 to 22.5 h with [^{14}C]uridine and for the last 10 min with [^{3}H]uridine. RNA was extracted with urea-NaDodSO$_4$ buffer (Holmes & Bonner, 1973) and centrifuged in 85% formamide gradients. Adjacent fractions from each of four gradients containing mesenchyme blastula RNA were pooled into groups of two and combined with the corresponding fractions of other gradients. Similar pools were made from two gradients of early blastula RNA. (—●—) [^{3}H]-Uridine hybridization. (--O--) [^{14}C]Adenosine or [^{14}C]uridine hybridization. Hybrids were not treated with RNase. Top of the gradient on left.

Early Cleavage Stage Transcripts. The possibility exists that the histone transcript size of the processing rate of a hypothetical precursor might change at different developmental stages. As diagrammed in Figure 7, however, the newly synthesized histone gene transcripts made at the four-cell stage (3-4 h) are of identical size to those of the 9-h embryo.

Mesenchyme Blastula Transcripts. Since the histone transcripts made in the mesenchyme blastula differ in sequence to some extent from those of the earlier embryo (Kunkel & Weinberg, 1978), it was particularly interesting to compare the size of the histone RNA in these stages. The mesenchyme blastula RNA forms hybrids with pCO1 DNA that are less stable and are more sensitive to RNase than late cleavage RNA (Kunkel & Weinberg, 1978). The assay for hybridization to pCO1 therefore was first done without treatment of the filters with RNase. The embryos were labeled with [^{14}C]uridine for 3 h and for the last 10 min of this period with [^{3}H]-uridine. The newly synthesized RNA sediments in the 85% formamide gradients as shown in Figure 8. Both the long term and short term labeled sequences are heterogeneous in size. The hybridization to pCO1 of fractions from this gradient is illustrated in Figure 9A. Although much of the histone transcript is of 7-12S size, at least one-fourth of the hybridizable [^{3}H]RNA is found in fractions of 18S and larger. These high molecular weight fractions do not contain significant amounts of [^{14}C]RNA which hybridize to the pCO1 DNA. The complementary RNA in these fractions is therefore not an aggregate of the lower molecular weight RNA. In a parallel experiment in which the filters were also untreated with RNase, late cleavage RNA, made from embryos labeled for 3 h with [^{14}C]uridine and for 10 min with [^{3}H]uridine, was hybridized to pCO1 DNA. The results illustrated in Figure 9B show that the vast part of the hybridizable RNA is in the low molecular weight fractions and that the minor amounts in the high molecular weight region which are complementary are not enriched for the newly synthesized RNA.

These very hybrid filters, made with RNAs from the two stages, were then treated with RNase. The profiles, shown in Figure 10, are now much harder to distinguish from each other. In the case of mesenchyme blastula the only significant ^{14}C counts were found in fractions 1 and 3 (20-30 cpm above background, not shown) and the high molecular weight fractions were, therefore, still enriched for newly synthesized [^{3}H]RNA. The late cleavage stage RNA in hybrid was affected much less by the RNase, and the ratio of ^{3}H to ^{14}C counts in the hybrids was constant. In both Figures 9A and 10A there is some hybridization of low molecular weight RNA in fraction 1. This could conceivably be due to a small amount of degradation or to the possible product of processing of a high molecular weight transcript.

The RNase sensitivity of the various fractions is plotted in Figure 11. Both ^{14}C and ^{3}H early cleavage RNA in hybrid form is about 80% resistant to the enzyme at all sizes tested. On the other hand, the mesenchyme blastula RNA is much less resistant and, importantly, the sensitivity increases in the higher molecular weight regions. This is additional evidence that the high molecular weight complementary RNA is not merely an aggregate of the smaller transcripts. By the criterion of RNase resistance, the high molecular weight RNA of the mesenchyme blastula shares less sequence homology with pCO1 DNA than does the low molecular weight RNA. When

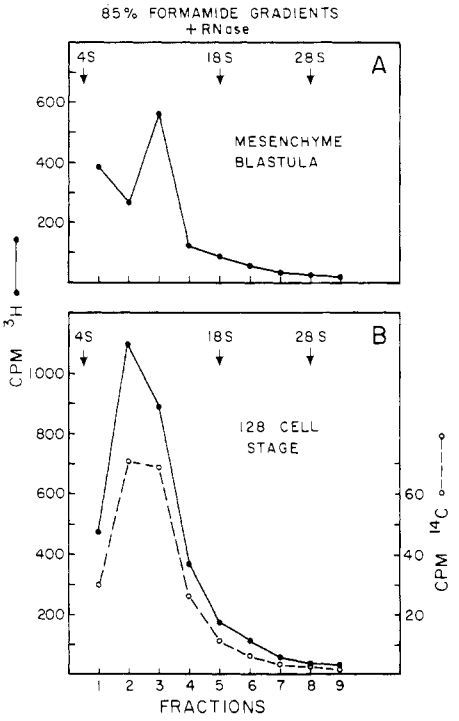

FIGURE 10: Comparison of histone transcripts in early blastula and mesenchyme blastula. The exact same filters used in Figure 8 containing hybrids formed with pCO1 DNA and total RNA from 9-h or 22.5-h embryos were treated with 10 μg/mL RNase A for 30 min. The filters were then recounted. (—●—) [^{3}H]uridine hybridization. (--○--) [^{14}C]-Adenosine or [^{14}C]uridine hybridization. Top of gradient is on left.

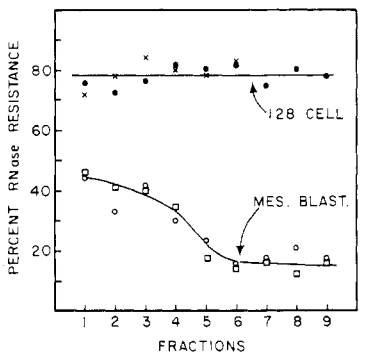

FIGURE 11: Sensitivity of hybrids to RNase. Resistance to digestion with 10 μg/mL RNase, for 30 min, of the fractions shown in Figures 9 and 10 is shown for each fraction of the gradients. (●) [^{3}H]RNA of 9-h embryos; (X) [^{14}C]RNA of 9-h embryos; (○) [^{3}H]RNA of mesenchyme blastula embryos; (□) [^{3}H]RNA of mesenchyme blastula embryos (from a repeat experiment).

the hybrids made with the 9S RNA and with the larger RNA were melted, no difference could be seen in their stabilities although both melted with a T_m about 10 °C lower than hybrids made with late cleavage RNA. These results, presented in Figure 12, indicate the homogeneous nature of the melts. The sequences which do hybridize to the pCO1 DNA in the two mesenchyme blastula RNA fractions appear to be very much alike. The complementary high molecular weight RNA is probably covalently attached to sequences, perhaps transcribed from spacer regions, which do not share homology with the pCO1 DNA and are therefore completely sensitive to RNase.

To further test for the possibility of aggregation, the high molecular weight mesenchyme blastula RNA was recycled through another 85% formamide gradient. The initial formamide gradient had the absorbance profile as shown in Figure 13A and was the same RNA preparation as illustrated in Figure 8. The fractions were pooled as illustrated and centrifuged in a second gradient. The distribution of radioactivity, shown in Figure 13B, is as expected. The fractions of the gradients containing the low (I) and high (II) molecular weight RNAs were hybridized to pCO1 DNA and the filters were washed without RNase treatment. The RNA of fraction I still contains most of the labeled histone transcripts in low molecular weight form with a peak at 9 S. On the other hand, the transcripts of fraction II are, on the average, much larger with a peak heavier than 18 S. Over 72% of the hybridizable RNA appears to be in fractions greater than 9 S. Some material is found also in the 9 S region. This RNA could result from some residual aggregation in fraction II or from some breakdown in the RNA during the treatment involved in recycling.

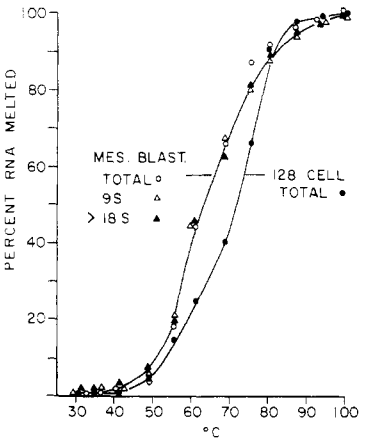

FIGURE 12: Thermal elution profiles of hybrids formed with [^{3}H]uridine labeled RNA from 9- and 22.5-h embryos. Total or fractionated RNA from 9- and 22.5-h embryos, labeled for 10 min with [^{3}H]uridine, was hybridized to pCO1 DNA immobilized on filters. The percent of the cumulative labeled RNA eluted from the filters at each temperature is shown. (●—●) Nine hour total RNA; (○—○) 22.5-h total RNA; (△—△) 22.5-h RNA fractionated on 85% formamide gradients as in Figure 13, 9S region used, as indicated; (▲—▲) same, high molecular weight region used, as indicated. Hybrids were not treated with RNase.

Discussion

The determination of size of primary transcripts is complicated by the property of aggregation of RNA molecules. That this can be a serious problem in the case of histone gene transcripts was known from previous studies (Nemer et al., 1974). To distinguish between true high molecular weight transcripts and aggregates of low molecular weight RNA we labeled the embryos for a long period with ^{14}C-labeled precursor and for only a short period with ^{3}H-labeled precursor. The transcripts which hybridize to the histone gene DNA can thus be characterized as newly synthesized if the ratio of ^{3}H/^{14}C is high. These methods show very clearly that, after a rather short pulse of 10 min, no histone gene transcripts larger than mRNA size can be identified in the early embryo. Although it is difficult to rule out the possibility of an almost instantaneous cleavage of a large molecule as it is synthesized, there is no indication of such a precursor from our studies.

In the mesenchyme blastula, in contrast, the situation is quite different. Here some large newly synthesized histone transcript can be recognized. This RNA is not thought to be an aggregate for the following reasons: (a) under the same conditions of RNA preparation and centrifugation, little high molecular weight material is found from late cleavage embryos; (b) the hybrids made with the mesenchyme blastula high

molecular weight fraction are greatly enriched in newly synthesized [^{3}H]- over older [^{14}C]RNA; (c) the high molecular weight mesenchyme blastula RNA in hybrid form is twice as sensitive to RNase as hybrids made with RNA from the 9S region of the same gradient; and (d) when recycled on a second formamide gradient, a broad high molecular weight mesenchyme blastula RNA fraction retains over 70% of its histone transcripts in sizes greater than 9–12 S.

The high molecular weight histone transcript is not of one discrete size. It extends well past the 28S absorbance peak in the gradients. If the large molecules were a precursor to the 9S RNA, one might expect a series of intermediates extending in size from that of the mRNAs to that of the primary transcripts and these might not be resolved on a gradient. Our results, however, do not distinguish between a precursor–product relationship and the simultaneous synthesis of two classes of histone transcripts—some small and some large.

The histone gene transcripts of mesenchyme blastula and gastrula can be distinguished in three ways. As has been shown here, high molecular weight newly synthesized histone RNA is detected in the later embryo but not in the late cleavage stage. The transcript of the mesenchyme blastula has been shown here and elsewhere (Kunkel & Weinberg, 1978) to form hybrids with pCO1 which are less stable to thermal dissociation and which are more sensitive to RNase. These three properties indicate a fundamental qualitative change in expression of the histone genes at the mesenchyme blastula stages. The 10 °C difference in the T_m of the two types of hybrids indicates the extensive divergence which has taken place between the genes coding for the early and late histone mRNAs. The H4 early mRNAs from two sea urchin species also may be quite diverged in sequence (Weinberg et al., 1972; Grunstein et al., 1976) due at least in part to third base substitutions in the coding region (Grunstein et al., 1976). But in the case discussed here, the divergence of the two classes of genes had to have taken place in the identical genome.

The multigene histone DNA repeat unit in the sea urchin would seem to be a good candidate to serve as a template for a multigene transcript. One of the simplest regulatory mechanisms to attain coordinate synthesis of histones would be the formation of a single repeat length transcript which would then be processed. We have not, however, found such an RNA in the early embryo, although its existence is not completely excluded. The bulk of the histone genes of *S. purpuratus* including virtually all those organized into the previously characterized repeat (Weinberg et al., 1975; Kedes et al., 1975; Cohn et al., 1976; Wu et al., 1976; Holmes et al., 1977; Weinberg & Overton, 1977; Overton & Weinberg, 1978) probably code for the early embryo histones (Kunkel & Weinberg, 1978). The genes coding for the mesenchyme blastula histones are of unknown structure. We cannot state at this time whether the units containing the histone genes expressed at mesenchyme blastula contain genes for all of the histone classes.

Recently, progress has been made in the characterization of histone transcripts in other species and cell types. In HeLa cells, Melli et al. (1977) have demonstrated a high molecular weight RNA which hybridizes to sea urchin histone gene DNA and has certain properties expected of a precursor. In *Drosophila melanogaster,* on the other hand, the histone repeat unit is organized with three genes on one strand and two on the other strand of the DNA duplex (Lifton et al., 1978). Such a unit could not be a template for a single large transcript which could be processed into all the histone mRNAs. Smaller multigene transcripts might, however, exist and be transcribed from both DNA strands. There are obvious differences in histone gene structure and transcription in different species. We have demonstrated here differences even within embryos of a single species.

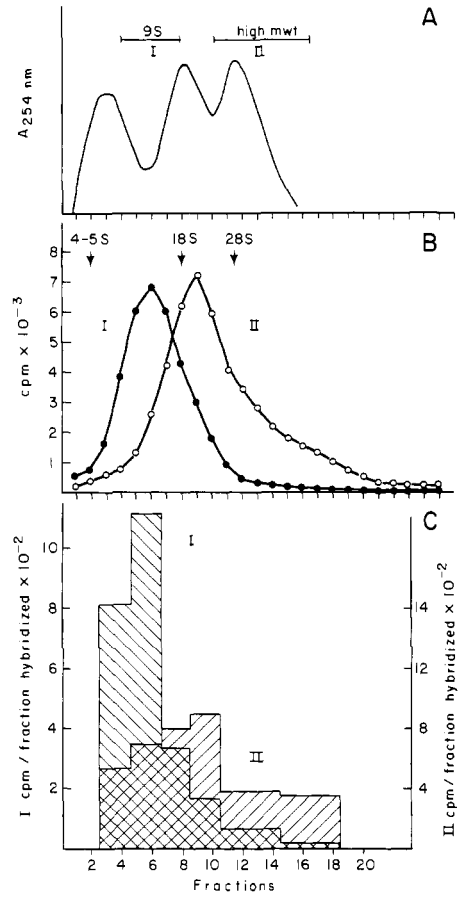

FIGURE 13: Recentrifugation of low and high molecular weight regions of 85% formamide gradients. (A) A preparation of RNA from 22.5-h embryos labeled for 10 min, as in Figure 8, was centrifuged in 85% formamide gradients. Fractions were pooled into two groups as shown and the RNA precipitated with ethanol and NaCl. (B) The two RNA samples were resuspended in 200 μL of 85% formamide buffer and heated at 65 °C for 10 min. Each sample was loaded on a 85% formamide sucrose gradient. After centrifugation, the position of the labeled RNA was determined by precipitating 10 μL of each 0.2-mL fraction with Cl_3CCOOH (●—●) Fraction I; (○—○) fraction II. (C) Fractions of each gradient were pooled in groups of two or four as indicated in the histogram and the RNA of each group hybridized to pCO1. The counts hybridized were normalized for the number of gradient fractions used to yield the RNA of each sample. Without this normalization the two largest samples, each containing four fractions, would appear to have more histone transcripts. Hybrids were not treated with RNase. (▥) frac I, (▨) frac II.

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Enzymatic Basis for the Ca^{2+}-Induced Cross-Linking of Membrane Proteins in Intact Human Erythrocytes†

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ABSTRACT: The accumulation of Ca^{2+} ions in intact human erythrocytes leads to the production of membrane protein polymers larger than spectrin. The polymer has a heterogeneous size distribution and is rich in γ-glutamyl-ε-lysine cross-links. Isolation of this isodipeptide, in amounts as high as 6 mol/10^5 g of protein, confirms the idea [Lorand, L., Weissmann, L. B., Epel, D. L., and Bruner-Lorand, J. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 4479] that the Ca^{2+}-induced membrane protein polymerization is mediated by transglutaminase. Formation of the polymer in the intact cells is inhibited by the addition of small, water-soluble primary amines. Inasmuch as these amines are known to prevent the Ca^{2+}-dependent loss of deformability of the membrane, it is suggested that transglutaminase-catalyzed cross-linking may be a biochemical cause of irreversible membrane stiffening.

The accumulation of Ca^{2+} ions in intact human erythrocytes leads to the formation of new high-molecular-weight protein polymers in the cell membrane which are produced by covalent bonds other than disulfides (Lorand et al., 1976a,b). Since formation of the polymer is accompanied by the disappearance of band 4.1 and also by a reduction in spectrin and band 3 materials (for nomenclature, see Fairbanks et al., 1971), at least three internal membrane proteins seem to be involved. The polymerization reaction could be induced by Ca^{2+} concentrations which are also capable of activating the intrinsic, but otherwise latent, transglutaminase of these cells, suggesting that, similarly to blood clotting (see Lorand, 1972), intermolecular γ-glutamyl-ε-lysine bridging might occur. This idea was further strengthened by the finding that polymer formation could be inhibited if, prior to and during loading with Ca^{2+}

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