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Comparison of the Early Histone H4 Gene Sequence of *Strongylocentrotus purpuratus* with Maternal, Early, and Late Histone H4 mRNA Sequences†

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ABSTRACT: A shift occurs in the utilization of histone mRNAs during early embryogenesis of the sea urchin. Maternal histone mRNAs are present in the unfertilized egg and can be utilized during early embryogenesis until the blastula stage. Soon after fertilization of the egg, a family of several hundred (early) histone genes are activated. During blastulation the mRNA products of these genes gradually disappear from the polysomes and are replaced by a new class of (late) histone mRNAs that differ in size and sequence and code for a new group of histone proteins. A sequence comparison of these three classes of histone H4 mRNAs to the cloned early H4 gene sequence is described. Early H4 gene fragments were used as primers for dideoxynucleotide sequence analysis of maternal and early H4 mRNAs. Portions of both translated and untranslated regions were compared. It was shown that these segments, comprising 26% of maternal and early H4 mRNAs, have the same sequence. The same early H4 gene fragments did not serve as primers for dideoxynucleotide sequencing of late H4 mRNA under the conditions of hybridization. Therefore late H4 mRNAs would not have been detected in the egg even if they were present there. Early and late H4 mRNAs were labeled in vivo and analyzed by using ribonuclease T_1 and a two-dimensional "fingerprint" separation. These oligonucleotides were compared to the early H4 gene sequence. Divergence between early and late H4 mRNAs was shown to be extensive. Within a selected portion of the gene there is a minimum of 9.4% divergence. This divergence has affected both translated and untranslated regions. It is concluded that an early H4 mRNA sequence is synthesized prior to fertilization and is then stored in the egg. A similar if not identical sequence is synthesized after fertilization until the mesenchyme blastula stage. It is then replaced on polysomes by a highly divergent late H4 messenger RNA.

Histone genes of the sea urchin are repeated several hundred times per haploid genome (Kedes & Birnstiel, 1971; Weinberg et al., 1972; Grunstein et al., 1973a; Grunstein & Schedl, 1976). The basic repeat unit is a DNA sequence, approximately 6500 bases in length, which contains the genes for histones H1, H4, H2B, H3 and H2A, in that order (Schaffner et al., 1976; Wu et al., 1976; Cohn et al., 1976). These repeats are tandemly arranged in the genome in an undetermined number of clusters (Kedes & Birnstiel, 1971; Birnstiel et al., 1974). However, the histone gene family is

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not homogeneous. The multiple repeats active in embryogenesis are subdivided into two main functional groups, the early and late histone genes (Grunstein, 1978; Kunkel & Weinberg, 1978). Early genes, the major class, are active before, and the late class after, the mesenchyme blastula stage. Other heterogeneity manifests itself in the presence of sperm-specific histone genes (Strickland et al., 1977; Schaffner et al., 1978) and multiple early (Grunstein et al., 1976) and multiple late histone genes (Childs et al., 1979).

Histone messenger RNAs are also present in the unfertilized egg. Their translation products are similar to those of the early histone messenger RNAs (Arceci et al., 1976). However, the translation products of early and late H4 mRNAs may also be identical, and yet we know that these RNAs are coded for by divergent genes. Therefore it is not known whether the maternal histone mRNAs are the products of a gene class that is very different from the early class. Alternatively it may be a subset of the early histone genes, being similar but not identical in sequence.

In this paper we have used the histone H4 DNA and mRNA sequences as a means of comparing developmentally specific maternal, early, and late histones genes. This was done by (a) determining the complete sequence of the early H4 gene, (b) using the early H4 gene to isolate and sequence homologous maternal histone mRNAs, and (c) fingerprinting RNAse T_1 digests of ^{32}P-labeled early and late histone mRNAs and comparing their sequences to those of the early histone gene.

Materials and Methods

a. Bacteria and Plasmids. The plasmids used conferred colicin E1 resistance and were amplified in the *Escherichia coli* strain HB101 (hsm^-, hrs^-, $recA^-$, gal^-, pro^+, str^r (Boyer & Roulland-Dussoix, 1969) by using chloramphenicol at 150 μg/mL of M9 broth supplemented with glucose (Clewell, 1972).

The procedure used for plasmid DNA isolation is a modification of that described by Katz et al. (1973). A 1-L cell culture was grown to log phase (OD_{590} = 0.6) when chloramphenicol was added. After 12–18 h the cells were harvested by centrifugation and resuspended in 40 mL of 0.01 M Tris-HCl, pH 8.0, and 0.001 M EDTA (ethylenedinitrilotetraacetic acid, disodium salt) at 0 °C. All subsequent steps were done at 0–2 °C. The cells were pelleted and resuspended in 5 mL of 25% sucrose and 0.05 M Tris-HCl, pH 8.0; 1 mL (10 mg/mL) of lysozyme was added. After 5 min, 1 mL of 0.5 M EDTA, pH 8.0, was added, and this was followed by the addition 5 min later of 8 mL of Triton lysis solution (Triton lysis solution = 1 mL of 10% Triton X-100, 12.5 mL of 0.5 M EDTA, pH 8.0, 5 mL of 1 M Tris-HCl, pH 8.0, and 80 mL of H_2O). The lysate was centrifuged for 30 min in a Beckman SW27 rotor, 25 000 rpm, 2 °C, to remove cell debris. Technical grade CsCl (11.4 g) and 1.2 mL of ethidium bromide (10 mg/mL) were added to 12 mL of the cleared supernatant. CsCl gradients were generated in a 60 Ti rotor (Beckman Instruments), 42 000 rpm, 24 h, at 23 °C. The lower, plasmid-containing band was removed by side puncture and recentrifuged for 24 h. Ethidium bromide was removed from the DNA by chromatography of the plasmid in CsCl on Dowex 50W-X8. The DNA was dialyzed against 0.01 M Tris-HCl, pH 8.0, and 0.001 M EDTA and precipitated by addition of 0.1 volume of 3 M NaCl and 2 volumes of ethanol. After the solution was cooled for 30 min at −70 °C, the DNA was pelleted by centrifugation and was then dissolved in 0.01 M Tris-HCl, pH 8.0, and 0.001 M EDTA. The yield obtained was 2–4 mg of DNA/L of nutrient broth.

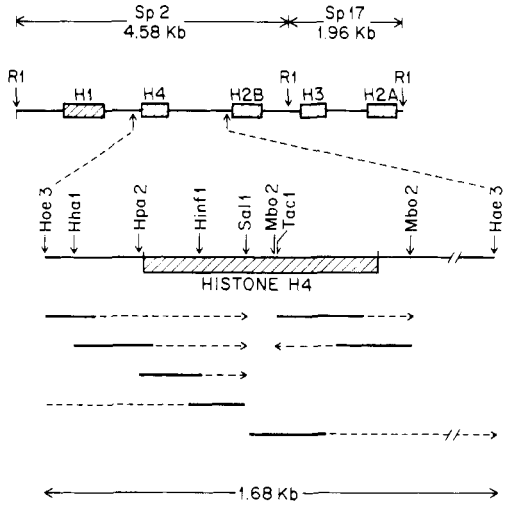

FIGURE 1: Schematic diagram of the early histone gene repeat unit of *S. purpuratus*. The region analyzed by DNA sequencing is represented by the heavy lines beneath the expanded histone H4 gene segment. The arrows from these lines represent unlabeled (3′) ends of the single-stranded DNA molecules, each of which is labeled at the 5′ end.

b. DNA sequencing was carried out as described by Maxam and Gilbert (1977).

c. Isolation of Early and Late Histone mRNAs. The procedures used in isolation of early blastula polysomal RNAs labeled with ^{32}P were as described by Grunstein & Schedl (1976). Later stage polysomal RNAs were isolated in a similar manner with the exception that the labeling time with ^{32}P was only 2 h. Electrophoresis, autoradiography of the slab gel, and elution of the RNA from acrylamide were described previously (Grunstein & Schedl, 1976; Grunstein, 1978).

d. Fingerprinting ^{32}P-Labeled Histone H4 mRNA. The procedures used for RNAse T1 digestion, high-voltage electrophoresis (pH 3.5), and homochromatography on poly(ethyleneimine) thin-layer sheets are those of Sanger and his colleagues and have been described by Brownlee (1972) and Grunstein (1978).

e. Sequencing Maternal H4 mRNA by Primer Extension.

(i) RNA Isolation and Hybridization. The starting material was a 4-L seawater culture containing 20 mL of eggs (packed volume). Messenger RNA was isolated as described above from 500-mL aliquots of this culture containing unfertilized eggs, 0.5, 8-, 24-, 30-, 48-, and 72-h embryos. RNA was isolated from eggs exactly as described for embryos with the exception that total RNA was used instead of polysomal RNA for the sequencing reaction. The RNA (approximately 1 mg) from each time point of development was extracted with phenol, precipitated with ethanol three times, and resuspended in 0.3 mL of 0.3 M NaOAc. To 0.075 mL of this solution was added 100 ng of primer DNA in 0.05 mL of TE (TE = 0.01 M Tris-HCl, pH 7.5, and 0.001 M EDTA). The RNA–DNA mix was then precipitated with ethanol and dried, and to it was added 0.01 mL of TE and 0.01 mL of 5 × Hinf buffer (1 × Hinf buffer = 6.6 mM NaCl, 6.6 mM Tris HCl, pH 7.5, 6.6 mM $MgCl_2$, and 6.6 mM DTT). The primer–template mix was then heated for 3 min in a boiling water bath and was allowed to reassociate for 90 min at 60 °C; 0.280 mL of [α-^{32}P]TTP (Amersham, 1 mCi/mL, 400 Ci/mmol) was dried under vacuum and resuspended in 0.035 mL of AGC mix (0.125 mM dATP, 0.125 mM dGTP, 0.125 mM dCTP, and 2.5 × Hinf buffer) for use in the sequencing reaction below.

(ii) Primer Extension and Sequencing. The procedure used was essentially that of Sanger et al. (1977). Each reaction contained 2 μL of primer–template mix (above), 0.001 mL

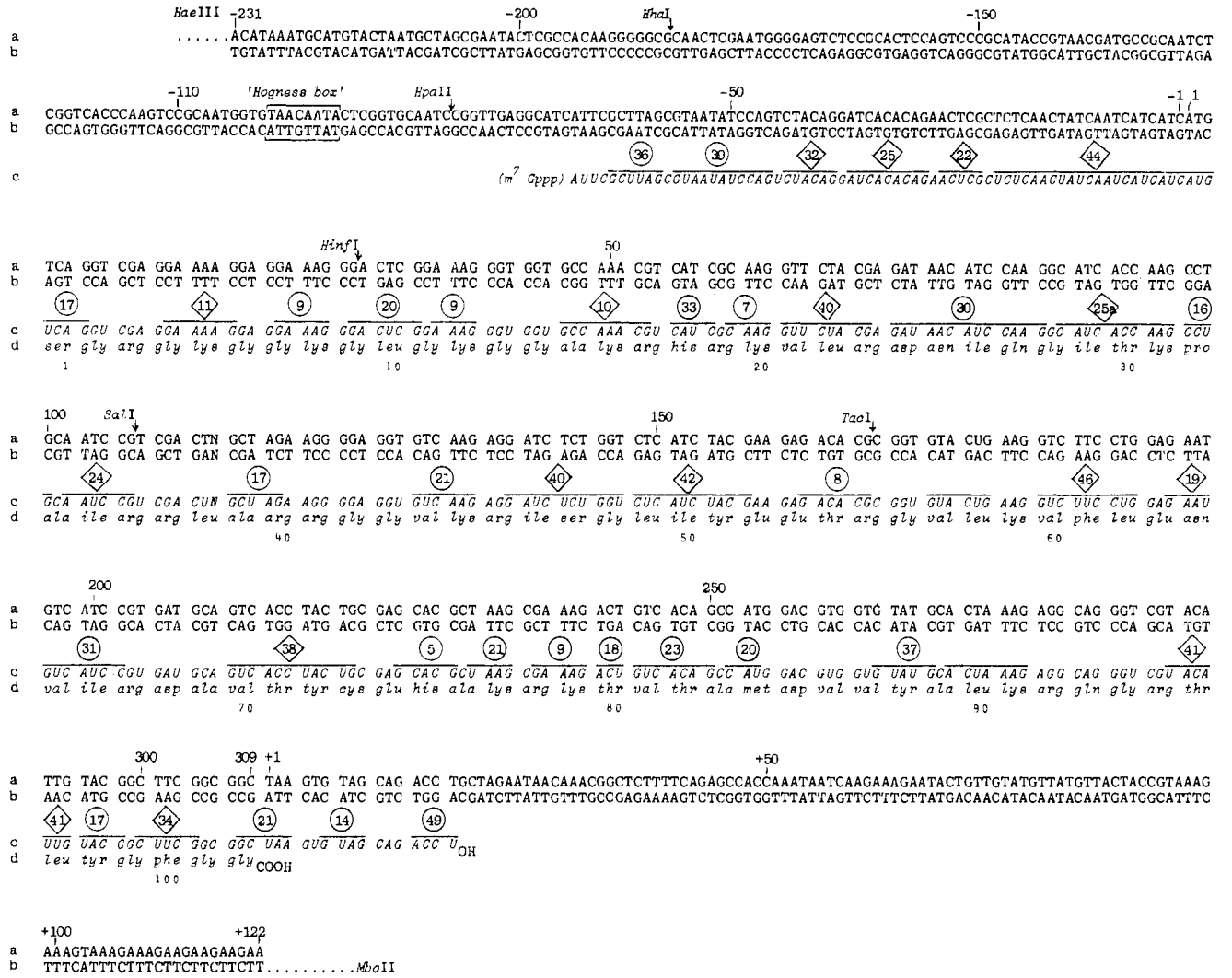

FIGURE 2: Sequence of the histone H4 gene of *S. purpuratus* and collinear early H4 mRNA oligonucleotides to illustrate positions of divergence between early and late histone H4 mRNAs. Lanes a + b = H4 DNA sequence; lane c = mRNA sequence; lane d = amino acid sequence. The numbers enclosed by a circle or diamond designate longer RNAse T_1 oligonucleotides; the circle designates an oligonucleotide which is present and the diamond an oligonucleotide which is not present in the late H4 mRNA as well. A note regarding the start of the H4 messenger RNA: both our oligonucleotide sequences and primer extension sequence analysis (Sures et al., 1980) suggest that the start of the H4 mRNA is approximately at base −67. In experiments not shown here we have sequenced the H4 mRNA after labeling the 5′ end using polynucleotide kinase and γ-^{32}P-ATP. RNAse partials utilizing pancreatic RNAse (cleaves at pyrimidines), U2 RNAse (cleaves at A), and T_1 RNAse (cleaves to G) (Donis-Keller et al., 1977) were used to create a sequencing ladder that was separated on polyacrylamide gels. The sequence obtained was XXXXG^{5}XXPyAG^{10}PyGPyXA^{15}PyAXXP^{20}yAG. We abandoned this sequence approach as a means of obtaining longer mRNA sequences due to the large number of bases (X) whose identity could not be determined. Nevertheless this sequence may be aligned with the DNA sequence and confirms the start of the H4 mRNA at base −67.

[α-^{32}P]TTP in AGC mix (above), and 0.001 mL of either 1000 μM ddATP, 50 μM ddTTP, 300 μM ddGTP, 200 μM ddCTP, or H_2O for each of the reactions; 0.001 mL (5 units) of avian myeloblastosis virus reverse transcriptase was then added, and this solution was indubated for 15 min at 42 °C. Chase solution (0.001 mL: 2.5 mL of dATP, 2.5 mM dTTP, 2.5 mM dGTP, and 2.5 mM dCTP) was then added and incubation at 42 °C continued for 15 min. To each of these reactions was added 0.009 mL of formamide loading buffer (this was obtained by filtering 10 mL of formamide through Amberlite MB1 and adding 30 μg of xylene cyanol FF, 30 μg of bromophenol blue, and 0.2 mL of 0.5 M Na_3EDTA, pH 7.0). The extended primer templates were heated at 90 °C for 3 min, and 0.005 mL was loaded onto a 0.4 mm thick gel (Sanger et al., 1977) containing 8% acrylamide, 0.4% bis(acrylamide), and 7 M urea. Electrophoresis took place at 30 mA and 1000 V.

Results

a. Early Histone H4 DNA Sequences. In *S. purpuratus* the early histone gene repeat unit contains five histone genes (Kedes et al., 1975) as shown in Figure 1. The *Eco*RI generated Sp2 fragment contains the genes for histones H1, H4, and H2B. This DNA was cleaved with the restriction enzymes shown in Figure 1 for sequence analysis by the procedure of Maxam & Gilbert (1977) and the thin gel system of Sanger et al. (1977). A 662 bp fragment bounded by a *Hae*III enzyme cleavage site at one end and an *Mbo*II site at the other end was sequenced. The result is shown in Figure 2. Some pertinent observations regarding the H4 gene sequence and its contiguous regions are described under Discussion.

b. Early H4 mRNA Sequence. In previous publications we described the oligonucleotide fingerprinting and sequencing of the early *S. purpuratus* H4 mRNA (Grunstein et al., 1976; Grunstein & Grunstein, 1977). These data (Table I) which produced a catalogue of RNAse T_1 generated sequences has been used here in order to align the early H4 mRNA with the DNA sequence (Figure 2). The approximate DNA boundaries from which the mRNA is generated may be established in this way.

Table I: Sequences of Oligonucleotides Generated by Ribonuclease T1 Digestion of *S. purpuratus* Early Histone H4 mRNA and Their Presence or Absence in Late H4 mRNA[a]

no.	early (RNAse T_1) oligonucleotide sequence	molar yield	present (+) or absent (–) in late histone H4 mRNA
1	(G)G	24.5	+
2	(G)CG	3.8	+
3	(G)AG	7.4	+
4	(G)CAG+(G)ACG	3.9	+
5	(G)CACG	0.5	+
6	(G)AAG	2.8	+
7	(G)CAAG	0.7	+
8	(G)CCAAG+(G)ACACG	1.1	+
9	(G)AAAG	2.5	+
10	(G)CCAAACG	0.7	–
11	(G)AAAAG	0.7	–
12	(G)UG	10.2	+
13	(G)UCG+(G)CUG	3.2	+
14	(G)UAG	0.7	+
15	(G)AUG	1.3	+
16	(G)CCUG	1.2	+
17	(G)CUAG+(G)UACG+(G)UCAG	3.5	+
18	(G)ACUG	1.2	–
19	(G)AAUG	1.3	–
20	(G)CCAUG+(G)ACUCG	2.4	+
20a	(G)CCUAG	0.7	+
21	(G)CUAAG+(G)UCAAG	1.9	+
22	(G)AACUCG	0.8	–
23	(G)UCACAG	0.9	+
24	(G)CAAUCCG	1.0	–
25	(G)AUCACACAG	0.8	–
25a	(G)CAUCACCAAG	0.3	–
30	(G)AUAACAUCCAAG	0.8	–
31	(G)UCAUCCG	1.2	+
32	(G)UCUACAG	1.1	–
33	(G)CUUCG	1.0	+
34	(G)CUUCG	1.3	–
36	(G)UACUG+(G)ACUUG+(G)UCUAG+CUUAG	3.8	+
37	(G)UAUG	1.2	+
38	(G)UCACCUACUG	0.9	–
39	(G)UAAUAUCCAG	0.8	+
40	(G)AUCUCUG+(G)UUCUACG	2.2	–
41	(G)UACAUUG+?	1.4	–
42	(G)UCUCAUCUACG	1.4	–
44	(G)CUCUCAACUAUCAAUCAUCAUCAUG	0.4	–
46	(G)UCUUCCUG	1.3	–
49	(G)AC,C,X_{OH}	0.6	?

[a] (+) serves to designate the presence of a spot in the same location on the late histone H4 mRNA fingerprint. (–) designates the absence of that spot on the late H4 mRNA fingerprint. The oligonucleotides represented here were taken from fingerprints of four separate RNA preparations. This was necessary since occasional overdigestion with T_1 ribonuclease and inadequate transfer of longer oligonucleotides from cellulose acetate to poly(ethylenimine) thin layers causes these oligonucleotides (e.g., no. 30, 44) to be present in variable yield.

For example, we find no RNA sequence which can be aligned continuously upstream of oligonucleotide no. 36, suggesting that the 5′ terminus is within the oligonucleotide directly upstream from no. 36. Primer extension sequencing [below, and Sures et al. (1980)] also establishes the 5′ end as shown in Figure 2 (see also legend to Figure 2 for additional sequence corroboration). Similarly, oligonucleotide no. 49 (AC,C,X_{OH}) is used to define the 3′ end, and no sequences predictable from the region directly downstream of this point are found in the T1 sequence catalogue. On the basis of these data, the total length of the H4 mRNA is 392 bases, which corresponds closely to the measurements (400–410) previously reported when both quantitation of labeled phosphates (Grunstein et al., 1973b) and gel electrophoresis (Grunstein et al., 1976) were used. Of the 392 bases, 67 are untranslated at the 5′ end, 16 are untranslated at the 3′ end (including two termination signals, UAA and UAG), and 309 bases code for the 102 amino acids of the H4 protein and the initiator methionine.

c. Maternal and Early Histone H4 mRNAs. Maternal histone mRNAs cannot be labeled with ^{32}P to high specific activity since the eggs are not highly active metabolically. Therefore it is difficult to obtain fingerprints of maternal mRNAs for comparison to early H4 mRNA sequences. An alternative approach, the primer-extension sequencing procedure, is that developed by Sanger et al. (1977) and used by Sures et al. (1980) for sequencing early histone mRNAs. Consequently a fragment of early cloned H4 gene was hybridized to total maternal RNA and polysomal RNA of embryos at various stages of early development. Reverse transcriptase was used to extend the primer by using the histone H4 mRNA as a template and each of the four dideoxynucleotides for random chain termination in order to generate sequence "ladders". [α-^{32}P]TTP was also included in each case to label the extended primer sequences for autoradiography.

In one set of experiments, the DNA fragment (60 bp, 107 → 167), generated by cleaving the H4 DNA with *Sal*1 and *Tac*1 endonucleases, was used as a primer. It was hybridized to mRNAs taken from eggs, representing mRNP bound histone mRNA, 0.5-h embryos, representing histone mRNA assembled onto polysomes prior to synthesis of early histone mRNAs, and 8-h embryos, representing the newly synthesized early histone messenger RNA. An example of such a com-

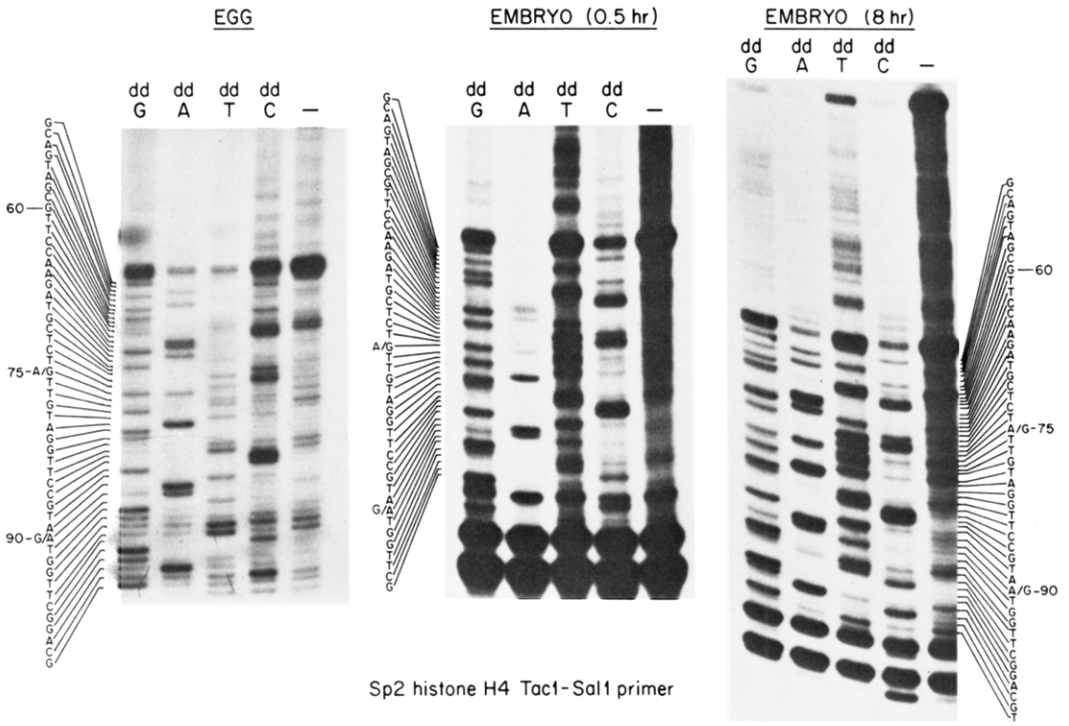

FIGURE 3: Primer-extension sequencing using histone mRNAs as templates and the *Tac*1-*Sal*1 histone H4 DNA fragment. RNAs were isolated (as described under Materials and Methods) from eggs or embryos at various stages after fertilization, as shown. It is evident from this comparison that histone mRNAs generate the identical sequence from bases 52 to 101. These sequences show heterogeneity at two bases (75 and 90) which has probably been generated due to heterogeneity in early histone H4 genes as evidenced by the presence of at least two early H4 mRNAs (Grunstein et al., 1973a,b) and a small number of T_1 oligonucleotides (e.g., no. 25a) that are present in minor yield.

parison is shown in Figure 3. In each case the 49 bases (52 → 101) generated from the H4 mRNA template are complementary to a message that codes for H4 amino acids 17-34. We see no difference between maternal and early mRNA bases. Similarly, the *Sal*1-*Hin*f1 restriction endonuclease generated primer (78 bp; 30 → 107) was used to hybridize to each of these mRNAs and generate a 51-base sequence between bases −22 → 29 (data not shown). No differences were detected between egg and 8-h H4 mRNAs. We conclude that maternal and early H4 mRNAs detected by the use of the early H4 gene primers must be very similar to if not identical with each other.

d. Early and Late Histone H4 mRNAs. (*i*) *Timing of Utilization in S. purpuratus.* We have previously shown in the sea urchin *Lytechinus pictus* that the switch between early and late histone mRNAs occurs prior to the mesenchyme blastula stage and coincident with hatching from the fertilization membrane (11–12 h of development) (Grunstein, 1978). Since *S. purpuratus* embryos develop more slowly than those of *L. pictus*, we also ezamined the timing of the early to late switch in this sea urchin.

Polysomal mRNAs were isolated from embryos at 2-h intervals of development surrounding the time of embryonic hatching. The results are shown in Figure 4. There is a gradual transition from the early to the late pattern of histone mRNAs. We first observe the late class of histone mRNAs at approximately 12 h of development, some 7 h *prior* to the hatching stage. With increasing time the late mRNAs gradually replace the early histone messages until at 20 h of development the transition is complete. Therefore, the histone gene switch occurs at an earlier developmental stage in *S. purpuratus*, although the ages of the embryos are approximately the same when the switch occurs. Similar results have been reported independently by Childs et al. (1979).

(*ii*) *Localizing Sequence Differences.* Our sequence analysis of the early and late H4 mRNAs of *L. pictus* (Grunstein, 1978) and the hybridization experiments of Kunkel & Weinberg (1978) with histone mRNAs of *S. purpuratus* have shown that these mRNAs are divergent from each other in sequence. In this paper we extend these observations by localizing many sequence differences between early and late H4 mRNAs. The locations of divergence were mapped by fingerprint comparisons of the two H4 mRNAs as described here.

We have previously determined the sequences of many of the oligonucleotides resulting from RNAse T_1 digestion of the early histone H4 mRNA of *S. purpuratus* (Grunstein et al., 1976) and shown their collinearity with the early H4 gene (Figure 2). In the case of the late histone H4 mRNA we have not obtained sequences of the T_1 oligonucleotides since it is difficult to obtain large quantities of radiolabeled mRNA. However, we can obtain enough ^{32}P-labeled late H4 mRNA to compare fingerprints of the early and late H4 mRNAs (Grunstein, 1978). It can then be determined whether a T_1 oligonucleotide present in the early H4 mRNA is present in the late message. Furthermore, because we know the position of the T_1 oligonucleotides in the early H4 mRNA, we can determine the region by divergence between the two mRNAs.

Two-dimensional fingerprints were produced by using H4 mRNAs digested with T_1 ribonuclease (Figure 5). We show here autoradiograms of the early H4 message, the late H4 message, and a mixture of the two, allowing a superimposition of the two fingerprints. The results of this comparison are shown in Table I, in which we display the catalogue of the early T_1 oligonucleotides and their sequences. Of 33 oligonucleotides (whose position is designated) in the early mRNA, at least 15 are no longer present in the late H4 message. This is a minimum estimate since we are unable to detect (a) changes resulting in sequence isomers which would occupy the same position on the fingerprint, (b) changes removing only some of the multiple sequence isomers in a spot, (c) changes in short oligonucleotides present in several copies per molecule, since

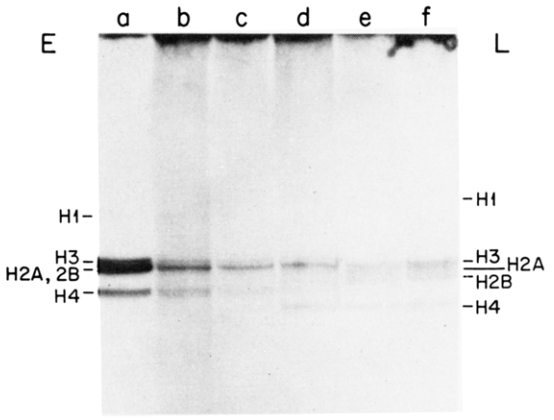

FIGURE 4: Developmental time course of the transition from early to late histone mRNA synthesis. Developmental times: (a) 10 h; (b) 12 h, 5 min; (c) 14 h, 15 min; (d) 17 h; (e) 20 h, 15 min; (f) 24 h. (a) RNAs were labeled continuously after fertilization; (b–f) labeling took place for the final 2 h of development only. The late histone mRNAs first become evident at ~12 h of development. Embryos were labeled at 15 °C with 20 μCi of inorganic ^{32}P/mL of phosphate-free seawater (Grunstein & Schedl, 1976) in a total volume of 100 mL containing 1 mL of embryos (packed volume). The deproteinized polysomal RNA was precipitated from ethanol, washed 3 times by centrifugation in 70% ethanol, and dried under vacuum. It was then dissolved in 0.1 mL of 7 M urea, 5 mM Tris-borate, pH 8.3, 0.01% xylene cyanol, and 0.01% bromophenol blue. 0.01 mL of this solution was heated for 3 min at 50 °C and fractionated on 6% acrylamide, 0.2% bis(acrylamide), 7 M urea, 50 mM Tris-borate, pH 8.3, and 1 mM EDTA slab gels (0.15 × 14 × 16 cm) (Donis-Keller et al., 1977). The gels were electrophoresed at 70 V for 20 h, dried under heat lamps and vacuum, and autoradiographed under a Cronex-4 Dupont intensifying screen and Kodak XR-1 X-ray film for 6 h at −70 °C. The sizes of the early and late histone mRNAs (in nucleotides) were determined by comparison to the *L. pictus* histone mRNAs (Grunstein, 1978) (data not shown) and are as follows:

mRNA	early	late
histone H1	620	650
H3	495	465
H2A	465	453
H2B	465	433
H4	410	370

neither the deletion nor addition of a single such sequence visibly alters the position or intensity of the spot on the fingerprint, and (d) more than one change per oligonucleotide.

Oligonucleotides which are no longer present in the late H4 message are marked with a diamond. There is divergence in both the coding and noncoding portion of the early and late H4 mRNA. Within the coding segment of the early mRNA molecule there are 10 large T_1 oligonucleotides which occur once per mRNA molecule. Nine of the ten are no longer present in the late H4 mRNA fingerprint. The 10 oligonucleotides encompass 96 bases. If we assume a minimum estimate of 1 base change per altered oligonucleotide, 9.4% divergence has occurred within this selected portion of the coding region of early and late H4 messenger RNAs.

Discussion

a. Histone H4 DNA Sequence. The DNA that was sequenced (662 bp) contains a region (392 bp) homologous to early H4 messenger RNA. It includes 164 bp directly upstream and 106 bp directly downstream of this sequence. Some features of the contiguous sequences bear mentioning from the standpoint of possible control regions and from an evolutionary point of view: 33 bases in front of the mRNA start is the canonical "Hogness box" TAACAATA which differs by two bases from the concensus sequence TATAAATA that is analogous to the *E. coli* promoter sequence or Pribnow box (Gannon et al., 1979). A computer analysis of the contiguous regions shows no obvious long regions of symmetry or repetition, although there is a polypyrimidine stretch at bases 632–662 which is known to occur at other regions of the early *S. purpuratus* histone gene repeat unit (Sures et al., 1978).

b. Maternal and Early Histone H4 mRNAs. The sea urchin egg contains histone messenger RNAs, as shown by the ability of egg mRNAs to compete with early histone mRNAs in hybridization experiments (Skoultchi & Gross, 1973), and to serve as templates in vitro for histone protein synthesis (Arceci et al., 1976). These proteins comigrate with the early class of histones. Furthermore, fertilized eggs may be treated with actinomycin D to inhibit transcription. These embryonic cells can still divide until the blastula stage [see review by Weinberg (1977)], suggesting that the embryo can use maternal histone mRNAs during the period when early histone messengers would be utilized. However, these data are still consistent with the maternal histone mRNAs being a class of separate RNAs that have diverged extensively from the early messengers as have early and late histone H4 mRNAs. Our data show that there are no sequence differences between maternal and early H4 mRNA in the regions analyzed (100/392 bases), and therefore the histone H4 mRNA in the egg is similar to if not identical with that synthesized in the pre-blastula embryo.

We stress, however, that these data do not prove that the early class of histone H4 messenger RNAs are the only class present in the egg. The early H4 gene primers do not, under our conditions, detect late H4 mRNAs, even at 24–30 h of development when late histone mRNA synthesis is extensive and late histone mRNAs have replaced early mRNAs on polysomes (data not shown). Therefore the possibility still exists that late histone mRNAs or possibly other divergent, egg-specific histone mRNAs are also present in the egg.

c. Divergence of Early and Late H4 mRNAs. (i) Divergence of Noncoding Regions. In Figure 2 the DNA sequence of the early histone H4 gene is aligned with oligonucleotides produced by digestion of early H4 mRNA with RNAse T_1. In this manner the approximate boundaries of mRNA on the DNA sequence may be determined since the contiguous oligonucleotides which would be produced by the further upstream and downstream DNA sequences are absent from the catalogue of mRNA sequences (Table I). The length of the early H4 mRNA obtained is 392 bases, and most of the untranslated mRNA is at the 5′ end of the mRNA molecule. The late H4 mRNA is approximately 40 bases shorter than the early mRNA (Grunstein, 1978). It is highly unlikely that this size difference manifests itself as a shorter late H4 protein because in vitro translation products, utilizing early and late H4 mRNA as templates, were identical in size (Newrock et al., 1977; Childs et al., 1979). Therefore, since there are only 13 bases present past the first termination signal at the 3′ end of the early H4 message (Figure 5), the late H4 mRNA must differ is size mostly at the 5′ end of the RNA. The significance of this size change is unclear, but suggests that late H4 mRNA does not require the extra bases at the 5′ end for translation into protein. However this does not mean that the size difference has no significance. Untranslated 5′ mRNA sequences are conserved for histone genes (Busslinger et al., 1980), and it may be that the additional sequences in the early mRNA are involved in the regulatory events of the shift in histone protein synthesis.

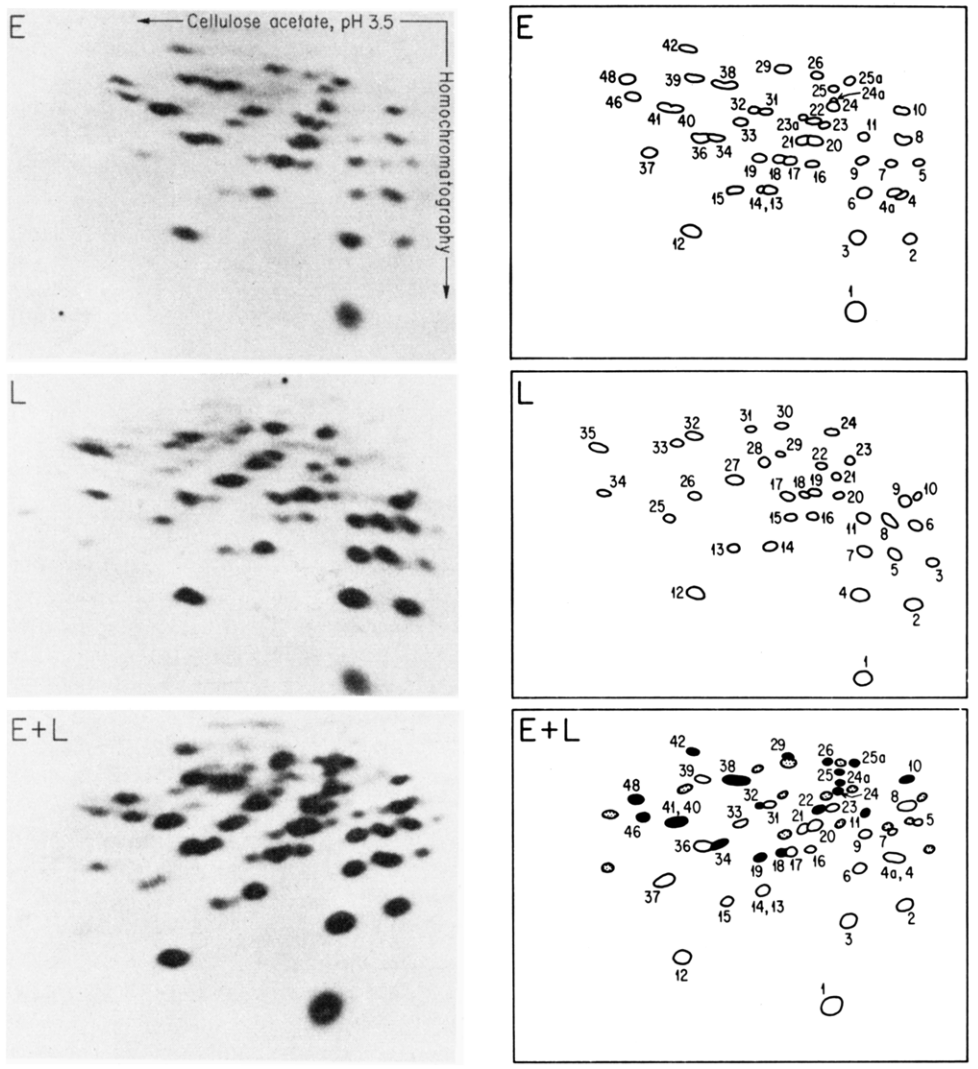

FIGURE 5: RNAse T_1 digest homochromatograms of early and late histone H4 mRNAs and a mixture of the two. (E) RNA from 8-h embryos; (L) RNA from 24-h embryos; (E + L) a mixture of approximately equal ^{32}P counts of both mRNAs. Only E spots are numbered. Oligonucleotides common to both E and L H4 mRNAs are unfilled; those present in E only are filled; those present in L only are stippled. RNA was prepared for fingerprinting by electrophoresis in 6% acrylamide and 7 M urea. After autoradiography the H4 mRNA band was excised and eluted into a dialysis bag by electrophoresis (Grunstein & Schedl, 1976). The RNA solution was shaken with phenol to remove acrylamide, precipitated with ethanol in the presence of 50 μg of *E. coli* tRNA, dried under vacuum, and digested with T_1 ribonuclease (Calbiochem) at an enzyme: RNA ratio of 1:20 for 1 h at 37 °C in 0.01 M Tris-HCl, pH 7.5, and 1 mM EDTA. The digested RNA in a volume of 4 μL was applied directly to a cellulose acetate Cellogel strip (Kalex Scientific) for electrophoresis at 5000 V for 20 min at pH 3.5 (5% acetic acid and 0.5% pyridine). The oligonucleotides between the blue and pink dyes were then transferred to poly(ethylenimine) (PEI) thin-layer plates (Macherey-Nagel) for homochromatography in Homomix (b) (Brownlee, 1972) for 6–8 h at 60 °C. E oligonucleotide no. 49 which is present directly under the origin was not transferred to the second dimension. There appears to have been slight overdigestion of the H4 mRNAs in this set of experiments. E oligonucleotide no. 44, a 25-base sequence normally present above oligo no. 42 and known to be absent from the L H4 mRNA (M. Grunstein, unpublished results), is not present on this particular fingerprint.

(*ii*) *Divergence of the Coding Region.* Despite the conservation of the histone H4 protein, the nucleic acid sequence of the H4 gene has diverged extensively. We have determined that of 96 bases represented in 10 coding oligonucleotides of the early H4 mRNA, there has occurred a minimum of 9 base changes, i.e., 9.4% divergence. Due to the extreme evolutionary conservation of histone H4 (Delange et al., 1969) and since no histone H4 variants have been detected in late embryos (Newrock et al., 1977; Childs et al., 1979), we believe that the nucleic acid divergence has occurred mainly in degenerate bases of the codons and in untranslated regions of the mRNA.

In conclusion, a comparison of the early H4 gene sequence with the mRNAs of the egg, early, and late embryos shows that an early-type histone H4 mRNA sequence is first synthesized prior to fertilization. The early class of histone mRNA sequences is then produced again after fertilization and is gradually replaced on polysomes at mesenchyme blastula stage by a late H4 mRNA sequence that is highly divergent.

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Concentration of Activated Intermediates of the Fructose-1,6-bisphosphate Aldolase and Triosephosphate Isomerase Reactions†

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ABSTRACT: As discovered by Grazi & Trombetta [Grazi, E., & Trombetta, G. (1978) *Biochem. J. 175*, 361], fructose-1,6-bisphosphate aldolase of rabbit muscle causes the slow formation of inorganic phosphate (P_i) and methylglyoxal when incubated with dihydroxyacetone phosphate (DHAP). In addition, these authors found an acid-labile intermediate in equilibrium with the aldolase–dihydroxyacetone phosphate complexes representing ~60% of the enzyme-bound DHAP species. Experiments are reported here which argue that this acid-labile species is the enzyme-bound enamine phosphate or its equivalent that decomposes by β elimination in acid. A similar mechanism involving an enediol phosphate is proposed to explain a phosphatase action of triosephosphate isomerase that produces methylglyoxal and P_i at the rate of ~0.1 s^{-1} at pH 5.5. When DHAP with excess isomerase is quenched in strong acid, the formation of P_i indicates that ~5% of bound reactant is in the form of enediol phosphate. The remainder of the substrate is about equally distributed between bound forms of DHAP and D-glyceraldehyde 3-phosphate. This equilibrium differs by 300-fold from the appropriate equilibrium in solution. Yeast aldolase, contrary to expectation, does not catalyze formation of inorganic phosphate and methylglyoxal when incubated with DHAP and gives no evidence for an enediol phosphate intermediate when quenched in acid.

Grazi & Trombetta (1978) observed the slow production of inorganic phosphate and methylglyoxal when rabbit muscle fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) and dihydroxyacetone phosphate (DHAP)[1] were incubated. This reaction occurred optimally in the pH range 5–6 with a rate ~10^{-4} that of the exchange of the C-3 hydrogen of DHAP with water. When the incubation was quenched with trichloroacetic acid (Cl_3CCOOH), an additional 0.6 equiv of inorganic phosphate (P_i) per equiv of enzyme was observed.

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[1] Abbreviations used: DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; FDP, fructose 1,6-bisphosphate; Cl_3CCOOH, trichloroacetic acid; TEA, triethanolamine; CP, carboxypeptidase; P_i, inorganic phosphate.

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