Article

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Monocistronic Transcription Is the Physiological Mechanism of Sea Urchin Embryonic Histone Gene Expression

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We have examined histone gene expression during the early stages of sea urchin embryogenesis. The five histone genes expressed at that time are contained in tandem repetitive segments. It has been suggested that adjacent coding regions and their intervening spacer sequences are transcribed into large polycistronic messenger ribonucleic acid (RNA) precursors. We have subcloned into pBR322 deoxyribonucleic acid (DNA) sequences mapping either in the coding region, the 5′ spacer, or the 3′ spacer of the H2B histone gene. These clones were used to produce radioiodinated hybridization probes. We measured the steady-state quantity of H2B messenger RNA as well as spacer-specific RNA in the total RNA from embryos taken at various stages of development from fertilization to hatching of blastulae (0 to 22 h post-fertilization). Small amounts of RNA hybridizing to both spacer probes could be found. However, we show that these RNAs form mismatched hybrids with the spacer DNA and therefore cannot originate from the spacers present in the histone genes. We conclude that there is no detectable transcription of the spacer regions on either side of the H2B histone gene. The detection limit for RNA complementary to the 5′ spacer sequence corresponds to a maximum of about three RNA molecules per cell, an amount shown to be far less than the projected steady-state pool size of a putative polycistronic transcript, if such a precursor were to be the obligatory transcript of the histone genes. (This conclusion was derived by using the known rates of production of H2B mRNA throughout early development [R. E. Maxson and F. H. Wilt, Dev. Biol., in press].) The physiologically relevant transcript of the histone genes in early development is therefore monocistronic and probably identical to the messenger RNA itself.

The histone genes transcribed during the rapid cleavage stage of echinoderm embryogenesis are members of a moderately repetitive family of tandemly arrayed genetic units each of which contains, on the same deoxyribonucleic acid (DNA) strand, coding sequences for the five classes of histone messenger ribonucleic acid (mRNA) interspersed with spacer DNA (see Fig. 1; reviewed in reference 19). The DNA sequence of nearly the entire repeating unit is known for three species of sea urchin (3, 33, 38, 39; R. Cohn and L. H. Kedes, manuscript in preparation), and the 5′ and 3′ ends of the mRNA's have been determined and mapped on the DNA (17, 22, 37).

The nature of the primary transcripts of these genes is unknown. On one hand, it is evident that their organization might easily lend itself to polycistronic transcription. However, in other invertebrates, histone genes do not have the same organization: in *Drosophila melanogaster* they are clustered but are not all the same DNA strand (23). In yeasts, histone protein coding regions exist in unlinked pairs that are oppositely oriented (18). Furthermore, ultraviolet mapping experiments in HeLa cells measured the size of histone gene primary transcripts (16) as only being able to accommodate single mRNA's. Thus, polycistronic transcription of histone genes cannot be a universal strategy for their expression.

Several reports have shown that large nucleic acid molecules synthesized by sea urchin embryos contain sequences that form hybrids with histone genes (20, 35). The latter report holds that such molecules contain sequences that hybridize with more than one histone gene and suggests that polycistronic transcription does take place. Evidence to the contrary however

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was obtained in our previous experiments (5): although we could detect high-molecular-weight RNA that hybridized to histone genes immobilized on a cellulose matrix, such RNAs were found to be cross-hybridizing species rather than authentic transcripts of those genes.

Independent evidence against polycistronic transcription of sea urchin histone genes came from ultraviolet-inactivation studies (G. Childs, unpublished data). If polycistronic transcription started at a single location in the histone repeat unit, one should expect the appearance of individual histone mRNA's that are differentially ultraviolet sensitive, depending on their position in the transcription unit (16 and references therein). We were unable to detect such a gradient of histone mRNA ultraviolet sensitivity, whereas in the same experiments, the polarity of 18S and 28S rRNA could easily be demonstrated. This result argues against a single promoter per repeat unit but it cannot exclude the existence of overlapping polycistronic transcription units starting in front of each of the five genes. The work reported in this paper was designed to define the nature of the histone gene transcript in the sea urchin and to resolve the discrepancies of previous reports. First, we present qualitative evidence against polycistronic transcription obtained by separating embryo RNA on denaturing gels, blotting onto diazobenzyloxymethyl (DBM)-paper (1), and hybridizing to histone-specific probes. The main thrust of the present paper is, however, of a quantitative nature. If polycistronic or high-molecular-weight precursor transcription or both do take place on sea urchin histone genes, then it must cross or extend into the spacer segments flanking each of the mRNA coding regions. Furthermore, if transcription from the flanking regions occurs, RNA molecules containing these sequences should be detectable, even if the RNA molecules are short-lived precursors. In fact, such RNA species ought to be present in amounts compatible with the massive rate of histone mRNA production occurring in early embryos, at least if such transcripts are to be of any physiological significance. The evidence we present here demonstrates that there is no significant transcription of the spacer regions between the H4, H2B, and H3 histone genes. We conclude that the primary transcript of the H2B gene and, by inference, all five early histone genes are monocistronic and probably identical with the histone mRNA.

MATERIALS AND METHODS

Cloning of restriction fragments. To obtain pure DNA probes, we generated a series of subclones from segments of the previously characterized sea urchin *Strongylocentrotus purpuratus* clones pSp102 and pSp117 (8). The *Hha*I-*Bam*HI fragment of pSp102 constituting the 5′ spacer probe was cloned, using standard recombinant DNA technology, and the resultant clone was called pSp2b-1. This restriction fragment lies entirely within that spacer region: it starts in the middle of the H4-H2B spacer and ends 37 nucleotides upstream of the H2B mRNA start (Fig. 1). The DNA sequence of this fragment was determined by the method of Maxam and Gilbert (24) (Fig. 2). In cloning this fragment, we followed the procedures of Ullrich et al. (40) except when mentioned otherwise. Briefly, the ends of this fragment were converted to blunt ends, using reverse transcription [conditions: 0.5 mM of each of the four deoxynucleotide triphosphates, 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.3), 6 mM $MgCl_2$, 40 mM KCl, and 0.2 mM primer-template incubated for 1 h at 37°C]. ^{32}P-end-labeled *Hin*dIII linkers were then ligated onto the fragment with T4 ligase, and the linked fragment was treated with *Hin*dIII and ligated into the corresponding site of *Hin*dIII-cut pBR322. Ligation of nonrecombinant circles was prevented by removing the 5′ phosphates from the linearized vector with calf intestinal phosphatase [in 100 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5]. Transformants of *E. coli* HB101 were screened, using the nitrocellulose filter colony assay of Grunstein and Hogness (14). The probe used in this step was the insert of pSp102 labeled with ^{32}P by nick translation (32).

The eucaryotic fragment of pGC1 (the 3′ spacer probe) is 167 base pairs long and derives from a region between the H2B and H3 genes from base 1131 to base 1297 in our published sequence (39). Unlike the other subclones, it was not created with *Hin*dIII linkers, but since it is an *Eco*RI-*Bam*HI fragment, it was substituted for the *Eco*RI-*Bam*HI fragment of pBR322. This fragment starts at a position approximately 467 bases downstream of the terminus of the H2B mRNA coding region and ends 43 nucleotides 5′ to the H3 mRNA capping site (Fig. 1).

Isolation, labeling, and strand separation of probe DNA. The techniques for growing plasmid-

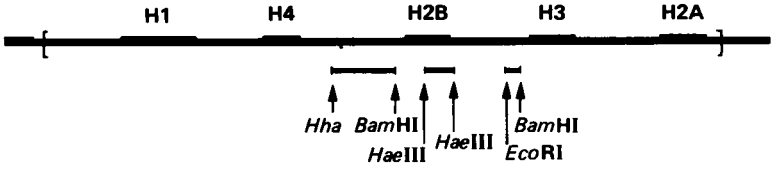

FIG. 1. *Map of probe fragments. The thick lines represent protein coding regions. The direction of transcription is left to right. The three restriction fragments shown were subcloned into pBR322 and used as hybridization probes for the H2B coding region, the 5′, and the 3′ spacers, respectively.*

```
      10         20         30         40         50         60         70         80         90        100
gcgCCTTTAG ATATATTATA TAGGTTCCCT TTTTCATTTG CTGTAACCAA ATAATTTTGA AATACAATGT CAAAGATATT TCATTGCTCA TTTCTGTTAT
cGCGGAAATC TATATAATAT ATCCAAGGGA AAAAGTAAAC GACATTGGTT TATTAAAACT TTATGTTACA GTTTCTATAA AGTAACGAGT AAAGACAATA

     110        120        130        140        150        160        170        180        190        200
ATCATTACGT TTATTATTAT TGTCTATCAT TGTGTATATT GTGTGAGAAG AAATGAAAAA TAAATTCGCT CTTTACATAT AGAGAGAGCT ACTACTACTA
TAGTAATGCA AATAATAATA ACAGATAGTA ACACATATAA CACACTCTTC TTTACTTTTT ATTTAAGCGA GAAATGTATA TCTCTCTCGA TGATGATGAT

     210        220        230        240        250        260        270        280        290        300
CTATATAATT ATATATCTCC GAGATAATGT CAAAAGTAGG AGTGANNNNN NNNNNNNNNN TGCAAGTATG TCTTGGATAA AAAGTCTCGA CATGTTCCAT
GATATATTAA TATATAGAGG CTCTATTACA GTTTTCATCC TCACTNNNNN NNNNNNNNNN ACGTTCATAC AGAACCTATT TTTCAGAGCT GTACAAGGTA

     310        320        330        340        350        360        370        380        390        400
ATTCCCATCA AAATTCATCG TCTTCTTCAA CTTTTTCACA TTTCTCATTC TTTGGGGATT GAATTGAACA ATGCAGACAG ANNNNNNNNN NTGCCCGTAT
TAAGGGTAGT TTTAAGTAGC AGAAGAAGTT GAAAAAGTGT AAAGAGTAAG AAACCCCTAA CTTAACTTGT TACGTCTGTC TNNNNNNNNN NACGGGCATA

     410        420        430        440        450        460        470        480        490        500
GATCACTATG TCGCCATCTC TAGGCAGGGG ATGGAACAGG CACTAANCTG CGACGCCTAA GACCAATGAA AGGATCGAGA CCGAGGCTCA TTTGCATACG
CTAGTGATAC AGCGGTAGAG ATCCGTCCCC TACCTTGTCC GTGATTNGAC GCTGCGGATT CTGGTTACTT TCCTAGCTCT GGCTCCGAGT AAACGTATGC

     510        520        530        540        550        560        570        580        590        600
GACCGCAGCA TACGgatccg gccccgtgta taaaaaggaa aggttctcgc tggccattca cagtatccaa agaatatttg cttgacatac tcgtttcgct
CTGGCGTCGT ATGCCTAGgc cggggcacat atttttcctt tccaagagcg accggtaagt gtcataggtt tcttataaac gaactgtatg agcaaagcga

     610        620        630    MET.....
gcatctttac agaccagaaa acctcaattc atcatg
cgtagaaatg tctggtcttt tggagttaag tagtac
```

FIG. 2. *Sequence of pSp2b-1, the 5′ spacer probe. The eucaryotic insert of plasmid pSp2b-1 is the HhaI-BamHI fragment on the 5′ side of the H2B gene (Fig. 1). Its sequence has been determined by the Maxam-Gilbert technique (24). Beyond nucleotide 391, the sequence has been previously published (nucleotide 392 of this figure corresponds to nucleotide 1 in Fig. 4 of Sures et al. [39]). The nucleotides in upper-case letters belong to the HhaI-BamHI fragment. The TATA box and the capping signal sequence are underlined. The position of the initiation codon is indicated by MET.*

harboring bacteria and purifying plasmid DNA have been described previously (8). Plasmid DNA was digested with the appropriate restriction enzyme to liberate the eucaryotic insert. The vector insert mixture was extracted several times with phenol-chloroform, concentrated by ethanol precipitation, and labeled with ^{125}I according to Lev et al. (21). Removal of unreacted iodide was also done as described by Lev et al., and the strand separation (and at the same time, the separation of insert and plasmid) was performed on a large-pore polyacrylamide gel (25). Finally, residual contamination by the opposite strand was eliminated by allowing it to reassociate and separating the single-stranded fraction on hydroxyapatite. This material was used as a probe. The reactivity of such probes was 60 to 90%, and the background of self-annealing was less than 4%.

Hybridization conditions. Hybridization and hydroxyapatite chromatography, including the low-salt ribonuclease (RNase) control, were done essentially as described by Scheller et al. (34). Briefly, 500 to 2,000 cpm of [^{125}I]DNA and varying amounts of RNA were hybridized in 0.4 M phosphate buffer (PB)–0.05% sodium dodecyl sulfate–2 mM ethylenediaminetetraacetic acid at 68°C for 18 to 48 h, i.e., at a C_0t 10 or more times higher than the DNA $C_0t_{1/2}$. The hybridization mixture was flushed into 2 ml of 0.02 M PB and divided into two parts: 1 ml was adjusted to 0.12 M PB–0.05% sodium dodecyl sulfate and analyzed over hydroxyapatite (measure of total duplex); the other was incubated with RNase A (50 μg/ml) at 37°C for 1 h and then adjusted to 0.12 M PB–0.05% sodium dodecyl sulfate and passed over hydroxyapatite. This RNase treatment hydrolyzes RNA engaged in RNA-DNA hybrids as well as RNA in the free state. The difference in hybridization value found with and without RNase is indicative of the quantity of authentic RNA-DNA hybrids, as opposed to DNA-DNA hybrids which might form if the RNA preparations had been contaminated with genomic DNA. With all of the RNA preparations, however, RNase treatment reduced hybridization values to the low background levels, demonstrating that there is little contamination of the single-stranded probe by DNA of the opposite polarity. We conclude that all of our RNA preparations were free from measurable DNA contamination. Hydroxyapatite columns were loaded and eluted at 60°C (single-stranded material) and then shifted to 95°C (double-stranded material). Fractions (4 ml) were scintillation counted directly by addition of Aquasol (New England Nuclear Corp.).

The quantitative treatment of titration data has been presented by Scheller et al. (34). We defined T/T_0 as the fraction of reactive probe hybridized when a mixture of RNA/probe ratio equal to R is incubated to termination. We defined $1/a$ as the fraction of the RNA complementary to the probe. At low R values, the plot of T/T_0 versus R is linear:

$$\frac{T}{T_0} = \frac{R}{a} \quad (1)$$

To carry out such titrations, three constants must be known: the length of the DNA probe, the concentration of RNA, and the concentration of DNA in the reaction mixtures. The molecular length of the probe in base pairs is known directly from the DNA sequence, whereas the RNA concentrations are determined spectrophotometrically. Concentrations of the radioiodinated probe DNAs could only be determined by careful measurement of probe specific activities as discussed in the next section.

Specific activity determination. We used two different methods to determine the critical specific activity values. The first was the kinetic method described by Scheller et al. (34). The specific activities of the two 5′ spacer probes (the coding and noncoding strands) were determined by reannealing an equal radiochemical amount of each strand and determining the kinetics of the reaction, using S1 nuclease. The half-time of the reaction allowed us to calculate the amount of DNA present, knowing the value of $C_0t_{1/2}$. This latter quantity is directly related to the known sequence complexity of the DNA and was calculated by using the known relationship between complexity and the reassociation kinetics of a standard reaction (in this case, the reassociation of φX174 DNA sheared to 300 base pairs) (11).

The specific activity of the pGC1-derived ^{125}I probe was determined by titration instead of by hybridization kinetics. One of the separated strands, labeled L, was incubated with increasing amounts of cold, *Eco*RI-*Bam*HI double-digested pGC1 DNA. Therefore, the excess cold component of the reaction was a mixture of linear plasmid and insert. The titration curve was expressed as T/T_0 versus the ratio of micrograms of RNA/counts per minute of probe, R' (Fig. 3). Therefore, $R' = R/S$, where R is the mass ratio of driver/probe, as in equation 1.

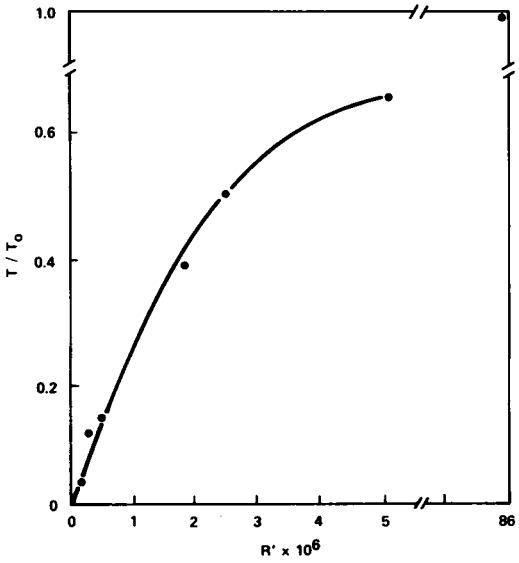

FIG. 3. *Specific activity of the 3′ probe. The specific activity of the 3′ probe was determined by titration of unlabeled, BamHI-EcoRI double-digested pGC1 with single-stranded iodinated DNA, as explained in the text.*

If we consider the initial, linear portion of this curve, we can again apply equation 1:

$$\frac{T}{T_0} = \frac{R}{a} = \frac{R' \times S}{a} = \frac{R'}{a'}$$

where $1/a' = 1/a \times S$ is the initial slope of the titration curve when the abscissa represents micrograms of cold nucleic acid over counts per minute of probe. We estimate that this initial slope $1/a' = 2.86 \times 10^5$ cpm/μg. On the other hand, $1/a$ is the mass fraction of the reacting DNA (one strand of the insert) in the mixture. The nonreacting components are therefore the other strand of the insert and both pBR322 strands. We computed $1/a =$

$$\frac{165}{165 + (2 \times 4{,}000)} = 2.02 \times 10^{-2}$$

Finally, the specific activity was calculated as:

$$S = \frac{1/a'}{1/a} = 1.4 \times 10^7 \text{ cpm}/\mu\text{g}$$

Determination of polarity of 5' spacer probe. To determine the polarity of the U and L probes relative to the messenger coding strand, we used a *Hha*I-*Eco*RI fragment which encompasses the 5' spacers and the H2B coding sequence (Fig. 1). This fragment had been previously strand separated, and the relationship of the single strands to the mRNA was determined. We used that strand of the *Hha*I-*Eco*RI fragment which we knew to be identical in polarity to the mRNA and reacted it with the U and L probes. The fraction of probe taken into hybrid at terminal C_0t was determined by the S1 nuclease assay performed as described by Lev et al. (21).

Determination of polarity of 3' spacer probe. The polarity of the U and L strands relative to the coding strand was determined by using a DNA fragment encompassing both the 3' spacer and a coding sequence, as was done with the 5' spacer. In this case, however, we had a clone derived from pSp117 in which the eucaryotic piece has been recloned into the vector M13mp2 (13), a single-stranded phage. The phage used contained the sea urchin sequence identical to the H3 and H2A messenger sequences as well as the sequence in pGC1. Since the coding strand is the same for all five mRNA's, the pGC1 strand hybridizing to this single-stranded DNA probes for spacer transcripts of the same polarity as the mRNA.We hybridized the U and L strand of the pGC1 probe to this DNA under the usual conditions and passed both hybridizations over hydroxyapatite. The result shows that L hybridized to the cloned single-stranded DNA, whereas U did not.

Preparation of embryo RNA. Sea urchin embryos were grown as previously described (15). Total RNA was prepared, using the guanidine hydrochloride method of Strohman et al. (36) as previously described (6). In addition, residual contamination of the RNA by DNA was eliminated by treating with pancreatic deoxyribonuclease, proteinase K, and phenol-chloroform, as described by Wold et al. (41). For this purpose, RNase-free deoxyribonuclease (Worthington Diagnostics) was further purified by affinity chromatography on agarose 5'-(*p*-aminophenylphosphoryl)uridine-2'(3')-phosphate (Miles-Yeda) (27).

RESULTS

Detection of histone-specific RNAs on Northern blots. We separated histone-specific RNA sequences by electrophoresis to detect any high-molecular-weight species differing in length from histone mRNA's. We prepared nuclear as well as polysomal RNA from 7-h-old embryos and separated them by electrophoresis on denaturing agarose gels (28). After we blotted the gel onto DBM-paper (1), we searched for histone-specific RNAs by hybridizing the blot with histone gene probes labeled by nick translation. Figures 4A to D show the autoradiography of such blots hybridized to pSp102 and pSp117, which contain the H4, H1, and H2B genes and the H3 and H2A genes, respectively, and which together encompass an entire histone gene repeat unit. The only reproducible and specific RNA bands apparent on these deliberately overexposed autoradiographs are messenger sized, both in nuclear and in polysomal RNA. No high-molecular-weight histone-specific RNA was detected.

Very small amounts of a polycistronic transcript, which must also include spacer sequences, might be revealed with greater sensitivity with a spacer-specific probe. We therefore probed similar RNA blots with nick-translated DNA from pGC1, a subclone of pSp117 specific for part of the spacer between H2B and H3 (see Fig. 1). No high-molecular-weight RNA was detectable (Fig. 4, lanes E and F). There is faint

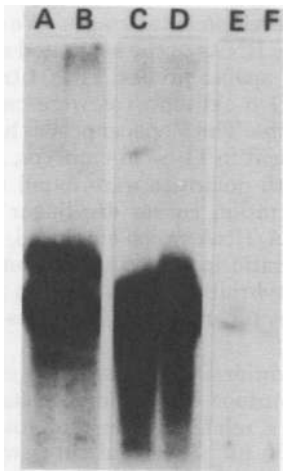

FIG. 4. *Northern blot hybridization of histone-specific RNAs. Nuclear and polysomal RNA from early embryos was separated on a denaturing gel, blotted onto DBM-paper and hybridized with histone-specific, ^{32}P-labeled probes. (A, C, and E) Polysomal RNA. (B, D, and F) Nuclear RNA. The probe for A and B was pSp102; for C and D, it was pSp117, and for E and F, it was the spacer-specific probe pGC1.*

hybridization at a position corresponding to a histone mRNA. This signal is more intense in polysomal than in nuclear RNA, and we believe that it is due to cross-hybridization between the probe and H4 mRNA since they have a short base pair sequence in common. We conclude that the qualitative RNA blot experiments demonstrate no specific, high-molecular-weight histone gene transcripts. Nevertheless, to assess further the validity of this negative result, we turned to the quantitative approach described below.

Strategy for the measurement of histone spacer transcripts. Our overall strategy was to determine the absolute concentration of RNA molecules per cell or per embryo capable of forming RNA-DNA hybrids with histone gene spacer DNA fragments. The method we used for titrating single-stranded DNA probes against RNA-containing fractions was the probe excess titration technique described by Scheller et al. (34). Briefly, the fraction of probe annealed at hybridization termination was determined for increasing RNA concentrations but under conditions of probe excess relative to the hybridizing RNA sequences. The plot of this fraction versus the RNA/DNA ratio is linear if only one strand is represented in the RNA and is initially linear, even in cases of symmetrical representation (34). From the slope, the concentration of reacting RNA can be computed directly.

Hybridization of the spacer probes to sea urchin RNA. We prepared total cellular RNAs from mature eggs and from embryos harvested at various developmental stages. We then hybridized these RNAs to the separated strands of the 5′ and 3′ spacer probes. First, titrations of RNA from 11-h-old embryos were carried out with each probe. The 5′ spacer probes hybridized to RNA present in 11-h-old embryos, and transcripts of both polarities were found (Fig. 5A). Since the titration curves are linear over the range of DNA/RNA ratios employed, we could use a single ratio in this range to evaluate the amounts of hybridizing transcripts in the other RNA samples taken at various stages in development.

Single hybridization mixtures containing RNA from various developmental stages were prepared at a relatively constant total DNA/RNA ratio of 10^6. These mixtures were incubated in hybridization conditions, and the degree of hybridization was measured by hydroxyapatite chromatography with the appropriate controls, as explained above.

The results of these titration measurements are presented in Table 1 and Fig. 6. The values in Table 1 for numbers of molecules per egg or

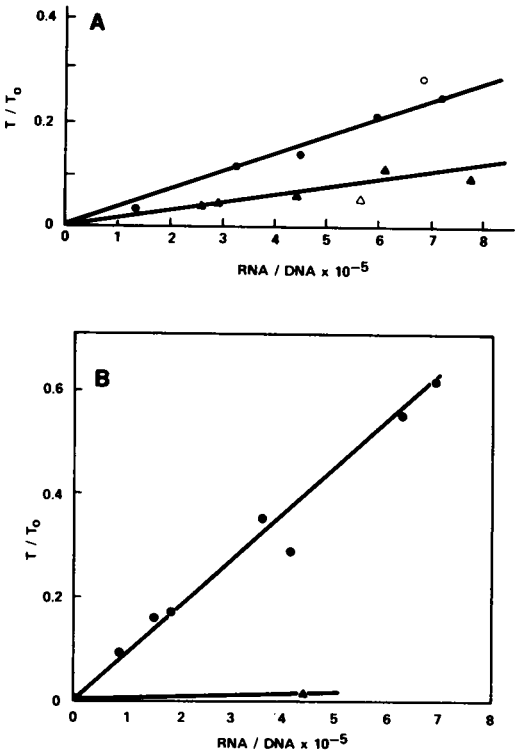

FIG. 5. *Titration of spacer sequences in 11-h-old embryo RNA. (A) Total RNA from 11-h-old embryo was hybridized to both strands of 5′ spacer-specific, iodinated DNA (pSp2b-1). The polarity of the probes relative to the coding strand has been determined as described in the text. Symbols: ●, titration with L strand (coding strand); ▲, titration with U strand; ○, L strand and △, U strand hybridized at 60°C (lower stringency). (B) Similar experiments were done using the 3′ spacer probes (pGC1). Symbols: ●, titration with L strand (coding strand); ▲, titration with U strand.*

embryo were calculated as indicated in the footnotes to Table 1. These data demonstrate the presence in embryos of RNA molecules able to hybridize to the spacer sequence flanking the H2B histone gene on the 5′ side. Moreover, RNAs of both polarities could be detected. Each of these two RNA classes exhibited a distinctive time course of accumulation. For the purpose of comparison, Fig. 6 also shows the time course of accumulation of H2B mRNA sequences. These data were obtained by hybridizing these same RNA samples to a radiolabeled probe specific for the H2B coding region (unpublished data). The quantities of the 5′ spacer RNAs are low, in the order of 1/2,000 the amounts of H2B mRNA present at the same developmental stage.

The results regarding the 3′ spacer probes are

TABLE 1. *Quantitation of 5′ spacer-specific RNA at various stages of development*

Time of embryo development (h)	Strand	RNA/DNA mass ratio[a]	T/T_0[b]	Reacting RNA		
				Fraction of total ribonucleotides[c]	Ribonucleotides (μg) per egg or embryo[d]	No. of molecules per egg or embryo
Mature egg	U	1.64×10^6	0.004	$<10^{-8}$	$<3 \times 10^{-11}$	<100
	L	1.89×10^6	0	$<10^{-8}$	$<3 \times 10^{-11}$	<100
5.7	U	4.00×10^5	0.070	1.75×10^{-7}	5.08×10^{-10}	1,780
	L	3.74×10^5	0.010	2.67×10^{-8}	7.74×10^{-11}	271
9	U	8.76×10^5	0.505	5.76×10^{-7}	1.67×10^{-9}	5,840
	L	8.26×10^5	0.108	1.31×10^{-7}	3.80×10^{-10}	1,330
11	U	1.42×10^5	0.272	1.92×10^{-7}	5.54×10^{-10}	1,940
	L	1.47×10^6	0.540	3.67×10^{-7}	1.06×10^{-9}	3,710
13	U	2.10×10^6	0.129	6.14×10^{-8}	1.78×10^{-10}	622
	L	3.03×10^6	0.544	1.80×10^{-7}	5.22×10^{-10}	1,830
16	U	1.53×10^6	0.055	3.59×10^{-8}	1.04×10^{-10}	364
	L	1.66×10^6	0.197	1.19×10^{-7}	3.45×10^{-10}	1,210
22	U	1.22×10^6	0.119	9.75×10^{-8}	2.83×10^{-10}	990
	L	1.20×10^6	0.161	1.34×10^{-7}	3.89×10^{-10}	1,360

[a] RNA quantity obtained from determination of absorbance at 260 nm. DNA quantity was computed from input counts per minute, and probe specific activity was measured kinetically as explained in the text.

[b] This measures authentic RNA-DNA hybrid as determined by the low-salt RNase test. The data are corrected for reactivity and self-annealing; therefore, the value range is 0 to 1.

[c] Calculated as $1/a = (T/T_0)/R$ or from the slope of the complete titration curve of RNA from 11-h-old embryos (Fig. 5A).

[d] Assumes 2.9×10^{-3} μg of total RNA per embryo throughout early development (L. Washburn, Ph.D. dissertation, University of California, Berkeley, 1971).

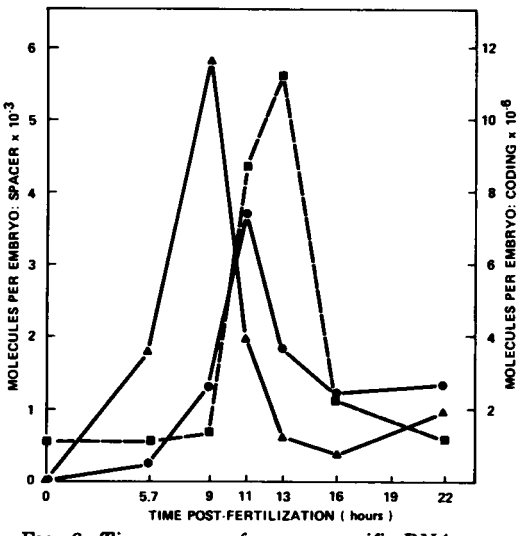

FIG. 6. *Time course of spacer-specific RNA accumulation. Data are taken from Table 1. Symbols: ●, RNA specific to L strand of 5′ spacer probe pSp2b-1; ▲, RNA specific to U strand; ■, accumulation curve of H2B mRNA, determined by titration hybridizations with an H2B coding region probe. Note the different scales for the two data sets.*

somewhat different: spacer transcription was asymmetric; i.e., only one of the two probe strands (corresponding to the strand from which the mRNA was transcribed) formed detectable hybrids. Typical titration data derived with RNA extracted with 11-h-old embryos are shown in Fig. 5B. The initial slope of the titration with the L strand corresponds to about 3×10^4 molecules per embryo. This is one order of magnitude greater than the concentration of the 5′ spacer transcripts at the same developmental stage. We also investigated the presence of RNA homologous to the 3′ spacer at other developmental stages. A small amount of such RNA was detectable in egg RNA, whereas it seemed to have entirely disappeared by the 22-h stage (data not shown).

Spacer RNAs are not authentic transcripts. Since *S. purpuratus* histone genes are asymmetrically transcribed (8), only RNA transcripts homologous to one or the other of the two spacer strands can be considered as evidence for a possible histone mRNA precursor. In the case of both 5′ and 3′ spacer probes, the RNAs complementary to the L strands are of the correct polarity; i.e., they are transcribed from the

same strand as the mRNA's. To investigate whether these RNAs are indeed transcribed from this region of the histone genes, we determined the melting profile of the hybrids. RNA-DNA hybrids were formed at 68°C in 0.4 M PB, using RNA from 11-h-old embryos and either the U or L strand of ^{125}I-labeled spacer DNA. As a control for the melts, these probes were similarly incubated with an excess of cold, homologous DNA. The melting profile of these preparations was determined by hydroxyapatite chromatography in 0.12 M PB, starting at 60°C (Fig. 7).

The melting curves show that all of the RNA-DNA hybrids melted over a broad range of temperatures and at temperatures significantly lower than those of the reassociated DNA. The differences in T_m's for the 5′ spacer probes were 15°C for both the U and the L strands. In addition, the RNA-DNA melting profile suggests significant heterogeneity of the hybrids, as evidenced by the shallower slopes. Although there is an intrinsic difference between the T_m of RNA-DNA and DNA-DNA melting, this difference is small in aqueous solutions. Furthermore, since the DNA sequences of the histone genes and spacers within the repeating cluster, and from individual to individual, are virtually identical to one another (unpublished data), the hybridizing RNAs cannot be homologous to any member of the principal histone gene repeat family. We conclude that the bulk of the hybridizing RNA is not an authentic transcript from the histone gene spacer DNA. Assuming that we might not detect perfectly matched hybrids that amounted to less than 10% of the total hybridizing material, the maximum number of authentic 5′ spacer transcripts of the correct polarity could be 370 molecules per 11-h-old embryo, corresponding to an average of three molecules per cell. Duplexes formed with the 3′ L strand were even less stable, with a T_m difference of 23°C. We estimate that no more than 5% of authentic transcript would have escaped detection in this melting curve. We can therefore set the maximum number of 3′ spacer transcripts present in 11-h-old embryos at 1,500 molecules per embryo.

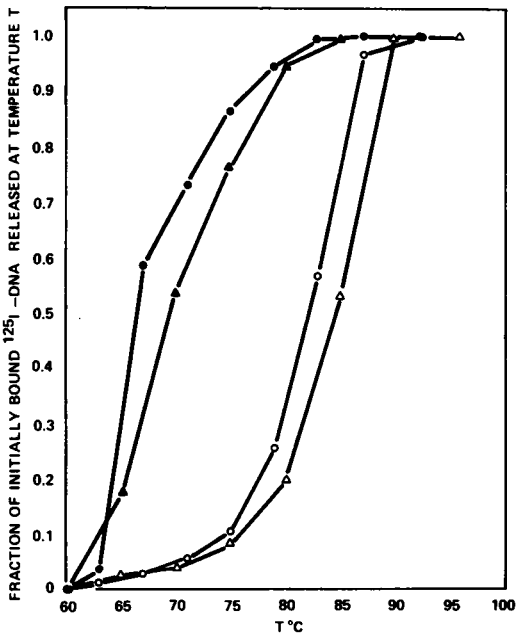

FIG. 7. *Thermal analysis of hybrid fidelity. RNA-DNA hybrids prepared using total, 11-h-old embryo RNA and 5′ spacer-specific [^{125}I]DNA were loaded on hydroxyapatite at 60°C in 0.12 M PB, and the melting profile was determined. In each case, reassociated [^{125}I]DNA was run on a parallel hydroxyapatite column. Symbols: ▲, RNA versus U strand hybrid; △, simultaneous DNA-DNA melt; ●, RNA versus L strand hybrid; ○, corresponding DNA-DNA melt. The reassociated probe DNA melts at a temperature consistent with its guanine plus cytosine content and intact fragment length.*

DISCUSSION

A polycistronic primary transcript is very unlikely. Our experiments were aimed at establishing the existence of spacer transcripts of the early histone genes, a necessary consequence of polycistronic transcription. Using two independent approaches, hybridization to RNA blots and titration-hybridization, we were unable to find any such transcripts. Obviously, it would be impossible to argue from these results that such transcripts never exist, since this negative finding is only as certain as the finite detection limit of our experiments. However, the purpose of this discussion is to argue a quite different conclusion, namely, that no physiologically significant transcription of spacer sequences of early histone genes takes place during sea urchin embryogenesis, at least through the first 22 h of development. We shall first examine the data pertaining to the 5′ spacer of the H2B gene and show that they constitute compelling evidence that a spacer transcript cannot be the physiologically obligatory transcript of most histone genes.

Let us consider the kinetics of the H2B gene expression process: if a colinear 5′ spacer-gene transcript (such as a polycistronic transcript) were part of the obligatory pathway of H2B gene expression in early development, such molecules would have to be present in amounts that are compatible with the rate of H2B synthesis during that time. More specifically, the smaller the pool size of such a transcript, the faster it would

have to turn over to provide a given rate of H2B mRNA production; it happens that we are in a position to know just how fast this has to be.

The maximum amount of 5′ spacer transcript present in 11-h-old embryos and which could have gone undetected in our experiments is equal to 370 molecules per embryo. Let us assume that all of these 370 molecules represent such spacer-containing precursors and that they are converted to functional H2B mRNA with 100% efficiency. Therefore, 370 molecules is the maximum pool size of the H2B mRNA precursor at a point in development at which synthesis of histone mRNA is maximal (26). These authors have determined that the rate of synthesis of H2A + H2B + H3 + H4 messenger RNAs peaks at approximately 83 fg/min per embryo at 10 h of development. We can assume that one-quarter of this value is H2B mRNA synthesis, i.e., 20.8 fg/min per embryo, or 75,000 molecules. This must also be the minimum rate of production of H2B primary transcripts, assuming 100% efficient precursor-product conversion. The following equation relates the pool size of a putative RNA precursor C, its rate of synthesis, k_s, and its rate of first order decay, k_d (10):

$$\frac{dC}{dt} = k_s - k_d C$$

The steady-state pool size C_0 is obtained for $dC/dt = 0$:

$$C_0 = \frac{k_s}{k_d}$$

Assuming that the precursor pool has reached its steady state in the 11-h-old embryo, we can calculate the half-life of such a precursor.

$$k_d = \frac{k_s}{C_0} = 75{,}000 \text{ molecules} \times \text{min}^{-1} \text{ per } 370 \text{ molecules} = 203 \text{ min}^{-1}$$

$$t_{1/2} = \frac{\ln 2}{k_d} = 3.4 \times 10^{-3} \text{ min} \simeq 0.2 \text{ s}$$

The rate of RNA polymerization in sea urchin embryos has been shown to be 5 to 8 nucleotides per second (2). Thus, our calculated precursor half-life of 0.2 s corresponds to the time it takes to polymerize only 1 to 2 nucleotides. This unrealistically low half-life simply shows that a precursor pool size of 370 molecules per embryo is utterly inadequate to sustain the high rate of H2B mRNA synthesis which is occurring in these embryos. Therefore, the true precursor must be of another type which does not include the 5′ spacer sequence.

One can, of course, argue that the half-life of the putative H2B precursor pool is longer than we calculate because the pool has not yet reached a steady-state level. But no reasonable correction for steady-state levels can stretch the half-life to values compatible with the existence of a polycistronic transcript.

A similar calculation can be made for the 3′ spacer sequences. We have seen that the maximum number of transcripts from the pGC1 region is 1,500 molecules per embryo at that same 11-h stage. The half-life calculated as described above is equal to 0.8 s.

In the case of the 3′ spacer, independent confirmation is available that its transcripts, if there are any, have an exceedingly short half-life. Experiments have been done in this laboratory (Childs et al., unpublished data) in which the nuclear RNA of embryos labeled for 5 min was hybridized to pGC1 DNA on nitrocellulose filters and the percentage of RNase-resistant radioactivity was determined. In these experiments, plasmids pSp2 and pRC9, the H1, H4, and H2B genes and the H4 gene, respectively, were used as hybridization controls. No values significantly above background were obtained, and the maximum proportion of total nuclear RNA synthesis related to pGC1-specific sequences was determined to be 0.002%. Again, if we assume that this maximum value represents sequences that are part of a primary transcript, then the half-life of the transcript must be considerably shorter than 1 min.

We have demonstrated the absence of any significant amounts of transcripts of spacer sequences present on either side of the H2B histone gene. The most straightforward interpretation of this evidence is that transcription of the H2B gene occurs as a monocistronic unit. The only model of polycistronic transcription compatible with our data would be one involving a tightly coupled transcription-processing complex that would degrade newly synthesized stretches of spacer transcripts before they obtained a significant length, i.e., more than a few nucleotides. Such a model would be at variance with what is known about other eucaryotic genes, in which primary transcripts are stable enough to be completed and then processed endonucleolytically.

There are additional arguments against a polycistronic mechanism of histone gene transcription. The 5′ end of the H2B mRNA has been mapped and located in relation to a sequence, PyCATTCPu, found next to it in the H2B gene (22). This is the "consensus" form of a sequence which is found 50 to 70 bases upstream of all of the other histone coding regions and nowhere else in the histone gene sequences (4, 37). Mapping experiments have pinpointed the 5′ ends of the histone mRNA's in or near this sequence. Furthermore, approximately 20 nucleotides upstream from the PyCATTCPu se-

quence there is a so-called "TATA box" (consensus sequence TATAAATA) which has been found in a similar position in most polymerase II-transcribed genes (12). This sequence is considered a major determinant of eucaryotic promoter function, leading to the conclusion that each of the five histone genes has its own promoter and its own transcription unit. Therefore, the positions of the 5′ ends of the mRNA suggest that the primary transcripts are identical or very similar to the final product. Also, when histone genes are used as templates for transcription by microinjection into *Xenopus laevis* oocytes (31) or in vitro, the synthesized 5′ ends have been synonymous with the 5′ ends of the natural mRNA's. In addition, the 5′ spacer probe that we used happens to end just a few nucleotides upstream of the TATA box (Fig. 2). If transcription started a significant distance upstream of the putative promoter, we should have detected the corresponding RNA. Our results therefore show that the physiological mechanism of histone gene expression conforms to the model suggested by structural and in vitro studies.

Significance of the RNAs partly homologous to the 5′ spacer. The RNA-DNA thermal dissociation profiles (Fig. 7) demonstrate that most or all of the RNA hybridizing to spacer probes is not transcribed from the H2B-flanking spacers. If the spacer-like RNAs are not legitimate histone precursors, what are they? The sequence homology of the RNAs hybridizing with pGC1 DNA is so poor that trivial explanations are likely to explain their detection, e.g., a random coincidence between pGC1 sequences and some totally unrelated RNA species. On the other hand, in the case of the 5′ spacer-like RNAs, the T_m of approximately 15°C is not so large that these findings can be dismissed a priori as artifacts of hybridization. It is therefore quite possible that there are, outside of the main histone gene cluster, sequences genuinely related to the 5′ spacer which are transcribed. As we have shown, these transcripts are partly symmetrical and exhibit a striking developmentally regulated time course. These properties are reminiscent of results obtained by Davidson and Britten and their colleagues. These authors have found that some sea urchin RNA transcripts from interspersed repetitive DNA typically exhibit the same properties of partial symmetry and developmental regulation (9, 29, 34). Our findings may be a further example of this phenomenon.

Conflicting reports exist as to whether large transcripts of histone genes exist. Evidence for their existence has been given by Kunkel et al. (20) and Spinelli et al. (35). In the first of these reports, large histone-specific RNAs were detected at a later stage in sea urchin development. The histone genes expressed then are transcribed from different genes altogether (6, 30), and therefore, results pertaining to these are probably not relevant to the early genes. In the second report, evidence for large polycistronic histone transcripts is given by blots and sequential hybridization to coding-region probes. This evidence is not necessarily contradictory with our results since, as we have emphasized before, we are not arguing that large transcripts are entirely absent. In fact, it is probably to be expected that an occasional RNA polymerase extends into forbidden areas of the gene region. On the other hand, our results could suggest alternative explanations for these findings. First, the presence of transcripts that hybridize to the 5′ spacer U (noncoding) strand indicates the difficulties that may be involved when assays for rare transcripts are employed, using techniques, such as hybridization against DBM-paper blots, that do not easily allow a direct analysis of hybridization fidelity (35). Second, a method has been developed in this laboratory to characterized histone gene variants that are not part of the main repeating cluster(s) (7; R. E. Maxson, G. Childs, T. Mohun, and L. H. Kedes, manuscript in preparation). The existence of H2B and H3 genes (or pseudogenes) lying outside the prevalent histone gene context has been established. At this point, it is quite possible that some of those variant clones will turn out to be transcribed and therefore to contribute small amounts of histone-specific RNAs of a distinct organization.

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