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Localization of a family of mRNAs in a single cell type and its precursors in sea urchin embryos

(*in situ* hybridization/differentiation/recombinant DNA/*in vitro* RNA synthesis/autoradiography)

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ABSTRACT **Spec 1 mRNAs increase 100-fold in abundance per embryo during early sea urchin development. Previous studies indicated an enrichment of this mRNA in ectoderm fractions of gastrulae and plutei. We have determined the precise localization of this mRNA by *in situ* hybridization techniques. In pluteus larvae, the mRNA is highly restricted to a set of morphologically uniform ectoderm cells in the dorsal part of the embryo. The mRNA is not detectable in other regions of ectoderm or in endoderm and mesoderm. The pattern of localization is already established at the gastrula stage, before these cells are distinguishable by morphological criteria. This pattern of distribution of Spec 1 mRNA is distinct from that of bulk poly(A)$^+$ mRNA. Measurements of the amount of Spec 1 mRNA per embryo and the number of cells containing this RNA indicate that there are about 500 Spec 1 mRNA molecules per cell at the pluteus stage and probably twice as many at the gastrula stage. These results indicate that the sensitivity of the *in situ* hybridization method allows detection of sequences that comprise ≈0.05% of the embryo mRNA nucleotides.**

The sea urchin embryo is perhaps the best-characterized system with respect to developmental modulation in gene expression (1–4). Recent analysis of a large number of different mRNAs indicates that few undergo greater than 10-fold modulation in abundance per embryo during early development (5). It is likely that such measurements of whole embryo concentration underestimate larger cell lineage-specific differences in gene activity that either reflect or effect determination and differentiation. To date, no set of gene products has been shown to be cell type specific during sea urchin embryogenesis. More accurate analyses of determination and the onset of differentiation require studies of expression of individual genes in different cell lineages of developing embryos.

Recently, cell fractionation techniques have been used to identify a set of mRNAs and their associated proteins that are enriched in ectoderm of plutei (1, 6). These studies showed that mRNAs complementary to the cDNA clone pSpec1 increase markedly in concentration during embryogenesis beginning at early blastula stage. *In vitro* translation of mRNAs hybridizing to pSpec1 revealed a family of similar small acidic proteins whose quantitative regulation during development parallels that of their mRNAs.

In the experiments reported here, we used an improved *in situ* hybridization technique to map the distribution of Spec 1 mRNA precisely and to quantitate its concentrations in individual cells of developing embryos.

MATERIALS AND METHODS

Embryo Culture and Tissue Preparation. *Strongylocentrotus purpuratus* were obtained from Pacific Biomarine (Venice, CA) or from Patrick Leahy (Kerckhoff Marine Laboratory, California Institute of Technology). Embryos were cultured by standard techniques, fixed in 1% glutaraldehyde, embedded in paraffin, and sectioned and the sections were treated with proteinase K and acetic anhydride as described (7, 8).

Preparation of Probes for *in Situ* Hybridization. The pSpec1 sequence, originally inserted by A·T tailing in a partially deleted pBR322 vector (9), was transferred to a transcription vector, RVIIΔ7, as shown in Fig. 1. RVIIΔ7 was constructed in the laboratory of T. Maniatis by replacement of the shorter *Eco*RI/*Bam*HI fragment of pBR322 with a 580-base-pair (bp) sequence containing a promoter for the *Salmonella* phage Sp6 RNA polymerase (10) and a downstream multiple cloning site. The pSpec1 sequence with adjacent A·T tails (totaling 470 and 180 bp, respectively) was excised via the adjacent *Hha* I sites in the original vector and inserted at the *Hin*dIII site of RVIIΔ7 by blunt-end ligation of nuclease S1-trimmed fragments. After transformation of *Escherichia coli* K802, recombinants with pSpec1 inserts in opposite orientations were identified by restriction enzyme analysis and hybridization of RNA transcripts to total pluteus RNA. The recombinant yielding transcripts complementary to sea urchin RNA was designated pSpec1R$^+$ and that yielding the other strand, pSpec1R$^-$. Similarly, pCO2R$^-$ was constructed by transferring the early histone repeat unit (pCO2; ref. 11) to RVIIΔ7 in the R$^-$ orientation.

Asymmetric RNA transcript probes (1.8×10^8 dpm/μg) were synthesized from R$^+$ and R$^-$ templates by using Sp6 RNA polymerase (fraction 5) purified according to Butler and Chamberlin (12) essentially as described. The reaction mixture (10 μl) was 40 mM Tris·HCl, pH 8.0/6 mM $MgCl_2$/10 mM dithiothreitol/200 μM CTP/200 μM GTP/100 μM [^{3}H]ATP (52.5 Ci/mmol; 1 Ci = 37 GBq; Amersham)/100 μM [^{3}H]UTP (46 Ci/mmol; Amersham), 2 mM spermidine containing 100 μg of DNA/ml and 1,800 units of polymerase/ml, and it was incubated at 37°C for 30 min. The R$^+$ and R$^-$ templates were cleaved with *Hin*dII (New England BioLabs) and *Bgl* II (Bethesda Research Laboratories), respectively, and then dialyzed against 5 mM Tris·HCl (pH 8.0). The length of the transcripts was determined by electrophoresis through and fluorography of 2.0% agarose gels containing 5 mM CH_3HgOH.

RNA was purified by extraction with phenol/chloroform (1:1), chloroform, and ether and precipitation with ethanol. Nucleic acids were dissolved in 50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$ and incubated with RNase-free DNase I (50 μg/ml; Worthington) at 37°C for 30 min. After deproteinization as described above, yeast tRNA (10 μg/ml; Bethesda Research Laboratories) was added and the RNA was ethanol precipitated. Fragment lengths of the probes were reduced by limited alkaline hydrolysis [0.1 M $NaHCO_3/Na_2CO_3$ (pH 10.2) 60°C for 20 min] to approximately

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Abbreviation: bp, base pair(s).

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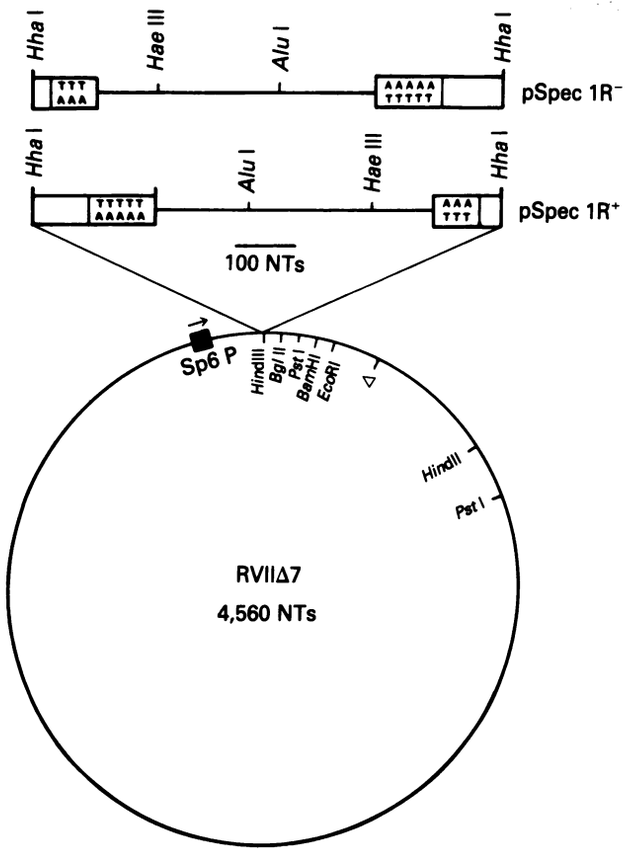

FIG. 1. Construction of pSpec1-RVIIΔ7 recombinants. The pSpec1 restriction map is taken from Bruskin *et al.* (1). During construction of the pSpec1R$^+$ recombinants, ≈350 bp of the vector, extending from the *Hin*dIII site to Δ, were lost. For *in vitro* transcription, pSpec1R$^+$ and pSpec1R$^-$ templates were truncated with *Hin*dII and *Bgl* II, respectively. NT, nucleotide.

150 bases as determined by electrophoresis at 60°C through 8% polyacrylamide/10 M urea gels. [^{3}H]Poly(U) (1.7×10^7 dpm/μg) was synthesized and partially hydrolyzed as described (7).

***In Situ* Hybridization.** Details and optimization of the *in situ* hybridization methodology will be published elsewhere. Hybridizations with pSpec1R$^+$, pSpec1R$^-$, and pCO2R$^-$ transcripts were carried out at 0.1, 0.1, and 1 μg/ml, respectively, in 50% formamide/0.3 M NaCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin/tRNA (500 μg/ml; Bethesda Research Laboratories)/10% dextran sulfate (Sigma). Poly(A) (500 μg/ml) was added to pSpec1R$^+$ and R$^-$ hybridization mixtures to prevent hybridization of poly(U) in the probe sequence to poly(A) in sections. Hybridization mixtures (5 μl/cm^2) were applied to sections under silicon-treated coverslips, and slides were immersed in mineral oil. Transcripts of pSpec1R$^+$ and pCO2R$^-$ were hybridized for 16 hr at 45°C and 50°C, respectively, which is ≈25°C below the melting temperature of duplexes formed *in situ*. Poly(U) was hybridized as described (7); as a control for nonspecific binding, pCO2R$^-$ transcripts were hybridized for the same time both at comparable stringency (50°C, 25°C below the melting temperature) and at the same temperature (37°C) used for poly(U). No difference in backgrounds was observed for these two conditions. After hybridization, mineral oil was removed by washing three times with chloroform, and unhybridized probe was removed by brief washing with three 50-ml portions of 0.6 M NaCl/0.06 M trisodium citrate followed by digestion with RNase A (20 μg/ml; Worthington) in 0.5 M NaCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA at 37°C for 30 min. The slides were then washed with RNase digestion buffer at 37°C for 30 min, with 4 liters of 0.3 M NaCl/0.03 M trisodium citrate at room temperature for 30 min, and finally with 4 liters of 15 mM NaCl/1.5 mM trisodium citrate at room temperature for 30 min. Autoradiography, staining, photography, and grain density quantitation were done as described (7, 8). pSpecR$^+$ and -R$^-$ and poly(U) *in situ* hybridizations were autoradiographed at 4°C for 14 and 10 days, respectively.

Measurement of Spec 1 mRNA Content per Cell. Total RNA from 68-hr plutei was isolated by the guanidine hydrochloride procedure as described (1) and contaminating DNA was removed by sedimentation through CsCl (1.7 g/ml). Various amounts of RNA were hybridized with 0.3-ng aliquots of labeled pSpec1R$^+$ transcripts (1.8×10^8 dpm/μg) for 60 hr at 45°C in 50% formamide/0.3 M NaCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA, the same buffer used *in situ*. The total RNA concentration in each mixture was adjusted to 10 mg/ml by adding carrier tRNA. Unhybridized RNA was digested with RNase A as described above, the hybrids that precipitated with cold 10% trichloroacetic acid were collected on GF/C filters, and their radioactivity was determined in Betafluor (National Diagnostics). The fraction of probe resistant to RNase A after hybridization in the presence of heterologous RNA was equivalent to that at zero time.

The number of dorsal ectoderm cells in plutei was estimated from measurements made on stained whole mounts of the average area covered by a cell and of the approximate area of the dorsal ectoderm computed by assuming a simple tetrahedral or conical surface. Estimates of the number of gastrula cells containing Spec 1 mRNA were made from similar measurements of cell area, combined with the fraction of the surface area (≈0.5) in labeled cells determined directly from complete serial sections. These estimates are probably accurate within ≈20%.

RESULTS AND DISCUSSION

Spec 1 mRNAs Are Highly Localized in a Single Cell Type of Pluteus Larvae. As a probe for determining the sites of expression of Spec 1 mRNAs in sea urchin embryos, we have used the cDNA clone pSpec1 identified by Bruskin *et al.* (1). The sea urchin DNA sequence consists of 470 bp derived entirely from the 3′ noncoding region of Spec 1 mRNA. RNA blot analysis of embryo mRNAs has shown that, under conventional conditions, this probe reacts strongly with 1,500-base mRNA(s) and weakly with 2,200-base mRNA(s) (1). The 1,500-base mRNA appears to comprise ≈90% of the transcripts from this family of related genes (unpublished data). To gain the sensitivity required for detection of these messages by *in situ* hybridization, the pSpec1 sequence was transferred to the RNA transcription vector RVIIΔ7, and asymmetric radioactively labeled run-off transcripts were synthesized. The advantages of such probes for *in situ* hybridization will be detailed elsewhere.

Hybridization *in situ* of pSpecR$^+$ transcripts to sections of 68-hr pluteus larvae at a temperature 25°C below the melting temperature shows that these mRNAs are restricted to a specific region of the ectoderm. Examples of the *in situ* hybridization pattern are shown in Fig. 2 (row 2) and grain densities over different regions of plutei are given in Table 1. Signals above background levels are observed over the conical epithelium of dorsal ectoderm cells, which appear to be uniformly labeled. This is shown in section 2C (cut perpendicular to the anterior-posterior and parallel to the dorsal-ventral axis) where dorsal ectoderm on both right and left sides of the embryo is labeled. In sections 2A and 2B (which approximately bisect the embryo), labeled cells form a continuous sheet from the anterior side through the apex (most dorsal region of the pluteus) and to the posterior side. (The ventral side of the embryo is the region around the mouth, lying between the growing arms of the larva. We define posterior as the

side on which the anus opens and anterior as directly opposite and adjacent to the oral lobe.)

Labeling over all other major regions of the embryo is not above background levels. To estimate this background, we used transcripts representing the mRNA strand, synthesized from pSpec1R$^-$ (Fig. 2, row 3). Regions that do not show detectable hybridization of the R$^+$ probe (row 2) include the tripartite gut, which is derived from endoderm, and mesodermal cells of the coelomic rudiment (data not shown). Most interestingly, all ectoderm except the dorsal cone is also unlabeled, including developing oral and anal arms (Fig. 2, sections 2A and 2C) and ventral ectoderm around the mouth (sections 2A and 2B).

The labeled dorsal ectoderm forms a continuous sheet of cells of distinctive and uniform morphology: These are squamous epithelial cells that stain lightly with eosin and are of similar size. The only exception is in the apical region, where the cells are less flattened and more irregularly packed. Borders between dorsal ectoderm and other cell types (e.g., Fig. 2, section 3B) appear to be congruent with borders between labeled and unlabeled regions of the embryo. We conclude that the Spec 1 mRNAs are highly restricted in plutei to cells that probably represent a single differentiated type.

Localization Is Established at the Gastrula Stage Before Morphological Differentiation. RNA blot analysis showed a large increase in Spec 1 mRNAs beginning at the early blastula stage and reaching a maximum at the late gastrula stage (1). *In situ* hybridization revealed that they are already sharply localized in gastrulae. In 40-hr gastrulae, cells of prospective dorsal ectoderm are not distinguishable by morphological criteria and the ectoderm is nearly radially symmetric about the animal–vegetal axis. The pattern of distribution of the Spec 1 mRNAs, determined from a large number of individual sections and several partial series of sections, is diagrammed in Fig. 3 and documented by the *in situ* hybridization patterns shown in Figs. 2 and 4. (While the general features of the pattern are clearly defined by our data, the dimensions of the labeled region shown in Fig. 3 are not known precisely.)

Table 1. *In situ* hybridization signals over defined regions of plutei and gastrulae

	Probe	Grains, no./ 100 μm^2	Sections, no.	Grains, no.	Signal/ noise*
Pluteus	pSpec1R$^+$				
Dorsal ectoderm		9.9 ± 2.8	19	3,408	6.4
Gut		2.8 ± 1.4	20	419	0
Arms		3.1 ± 2.1	19	142	0
Ventral ectoderm		2.4 ± 0.8	23	1,525	0
Pluteus	pSpec1R$^-$	3.5 ± 0.5	17	1,450	—
Gastrula	pSpec1R$^+$				
Labeled		21.4 ± 4.1	44	6,563	17.1
Unlabeled		3.7 ± 1.7	45	906	0
Archenteron		3.3 ± 1.6	23	249	0
Gastrula	pSpec1R$^-$	4.3 ± 0.8	16	924	—

Results represent mean ± SD.
* Grains per 100-μm^2 area observed over the indicated region with pSpec1R$^+$ minus grains per 100-μm^2 area over whole plutei or gastrulae with pSpec1R$^-$.

The relatively high signal/noise ratio in these hybridizations shows a sharp division of the embryo into "labeled" and "unlabeled" domains. Quantitation of grain densities (Table 1) shows that signals obtained with the R$^+$ probe are about 4-fold higher than the nonspecific binding of R$^-$ transcripts and that grain densities over unlabeled regions of gastrulae are not distinguishable from background. Labeled cells are restricted to a continuous region, including about one-half of the ectoderm, that is separable from unlabeled cells by a single line drawn on the surface of the gastrula. Unlabeled regions include about one-half the ectoderm, the archenteron, and the primary mesenchyme cells (Fig. 2, sections 5A and 5C). While it is not possible to show

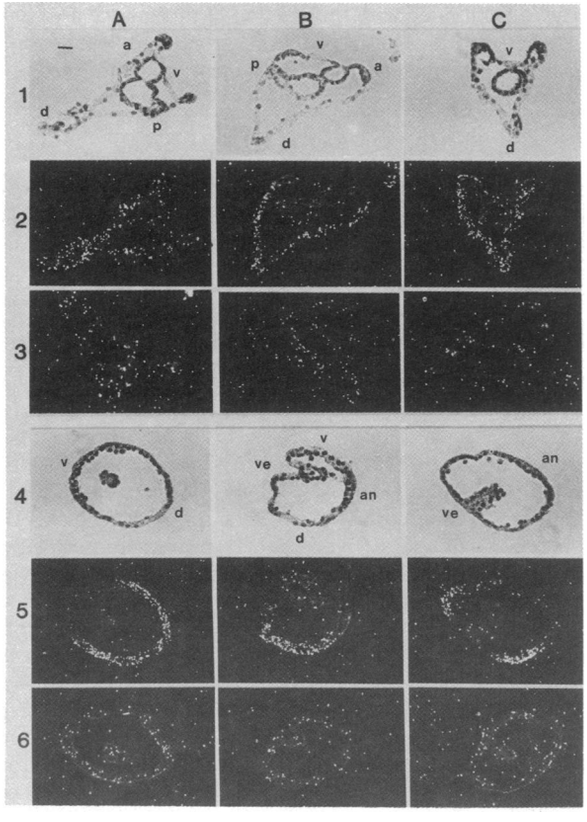

FIG. 2. *In situ* hybridization of pSpec1R$^+$ and R$^-$ probes. pSpec1R$^+$ transcript probes (rows 2 and 5) and control (nonhybridizing) pSpec1R$^-$ transcripts (rows 3 and 6) were hybridized *in situ* to sections of plutei (rows 1–3) and gastrulae (rows 4–6). Rows 2, 3, 5, and 6 were photographed under dark-field illumination; rows 1 and 3 are phase-contrast micrographs of the sections shown in rows 2 and 4, respectively. The orientation of the sections and the labeling patterns are described in the text. a, Anterior; p, posterior; d, dorsal; v, ventral; an, animal; ve, vegetal. (Bar = 10 μm.)

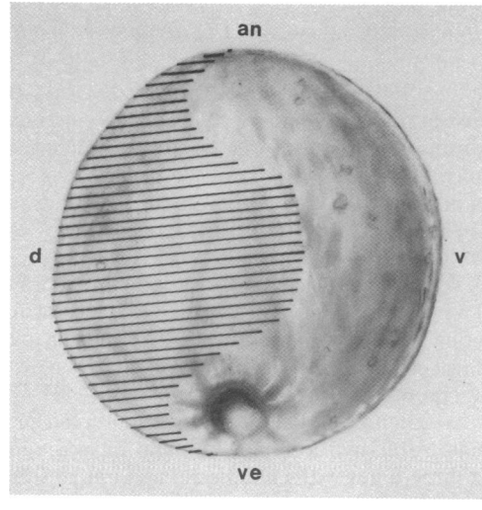

FIG. 3. Schematic diagram of Spec 1 mRNA localization in gastrulae. The shaded area indicates the region of ectoderm that is labeled by pSpec1R$^+$ transcript probes. an, Animal; ve, vegetal; d, dorsal; v, ventral.

that all labeled cells in gastrulae are precursors to dorsal ectoderm of plutei, this is the simplest interpretation and is consistent with the fate map of sea urchin embryos (13).

The pattern of labeling is bilaterally symmetric. Cells at both the animal pole and around the blastopore are unlabeled. Blunt projections of labeled cells extend laterally on the future left and right sides of the embryo. Fig. 2 shows sections through gastrulae cut approximately in the three planes of symmetry. Section 5A (perpendicular to the archenteron, and hence to the animal–vegetal axis) shows that, in the region of the lateral projections, labeled cells encompass more than one-half the embryo circumference. Section 5B (bisecting the embryo along its midline) shows that labeled cells extend over less than one-half the circumference because of unlabeled areas at the animal and vegetal poles. Section 5C (through the animal–vegetal axis and perpendicular to the dorsal–ventral axis) shows two bands of labeled cells corresponding to the lateral projections on the left and right sides of the embryo; cells at both animal and vegetal poles are unlabeled.

The continuity of labeled and unlabeled regions is illustrated by two partial sets of serial sections (Fig. 4). Both series are cut approximately parallel to the animal–vegetal axis. In series B, section 5 enters on the side of unlabeled cells, sections 4 and 3 include the lateral projections, and sections 2 and 1 show that labeling becomes continuous on the vegetal side and progresses toward the animal pole as the plane of sections moves into the dorsal side of the embryo. In series A, sections 1–3 enter from the dorsal side and consist entirely of labeled cells. In sections 4 and 5, the plane of the sections enters the lateral projections, as evidenced by two bands of labeled cells separating unlabeled cells at the animal and vegetal poles. Note that, in series B, unlabeled cells are encountered at the vegetal pole before the archenteron is reached.

Grain density is largely uniform over the entire labeled region in gastrulae. Preliminary experiments indicate that the Spec 1 mRNAs are already localized in 23-hr blastulae, near the time when their concentration begins to increase markedly. At the gastrula stage, we have occasionally observed regions of intermediate labeling at the margin between labeled and unlabeled areas. In several cases, this pattern is observed in adjacent serial sections. Thus, it is possible that different cells of the presumptive dorsal ectoderm begin to express these mRNAs at slightly different times. It is expected that the morphogenetic movements of gastrulation will also alter the distribution of these cells on the surface of the embryo. Since the number of labeled cells does not change greatly between 40-hr gastrulae and 68-hr plutei (see below), it seems likely that at later stages the only changes in the spatial distribution of Spec 1 mRNAs are those associated with the remodeling of the dorsal ectoderm during morphogenesis.

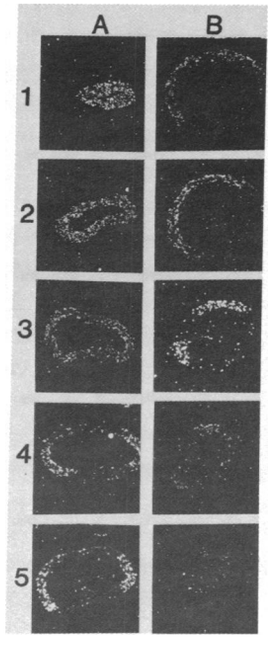

FIG. 4. *In situ* hybridization of the pSpec1R$^+$ probe to serial sections of gastrulae. Series of sections of two different gastrulae (A and B) were hybridized with pSpec1R$^+$ transcript probes. Alternate sections are shown in each series, beginning with the first section that encounters the embryo. The orientation of the sections and the labeling patterns are described in the text. (×180.)

The higher concentration of Spec 1 mRNAs in dorsal ectoderm cells (i.e., the mRNAs per volume of tissue) does not simply reflect higher mRNA concentrations *per se* in these cells. This is shown by hybridization to sections of the same cultures of plutei and gastrulae with [^{3}H]poly(U) to detect poly(A)$^+$ mRNA. Representative sections are shown in Fig. 5 (row 2). A control for nonspecific binding in this hybridization (row 3) was carried out using a mRNA (presumably noncomplementary) strand of the early histone gene cluster transcribed from pCO2R$^-$. Clearly, the distribution of poly(A) in plutei (sections 2C and 2D) is quite distinct from that of Spec 1 mRNAs. The pattern is, in fact, somewhat reciprocal since grain densities are slightly higher over the gut and in the tips of growing arms. While it is possible that these differences are weighted by different average poly(A) tract lengths in various regions of the embryo, the simplest interpretation is that the mRNA density is no higher, and probably significantly lower, in cells of the dorsal ectoderm than in other regions of the embryo. In addition, hybridization of a pCO2R$^+$ probe for histone mRNAs shows that they are significantly more concentrated in gut and arms than in dorsal ectoderm (data not shown). Hybridization of poly(U) to gastrulae yields uniform grain densities over all regions (Fig. 5, sections 2A and 2B), and the pattern of hybridization of a histone mRNA probe is quite distinct from that observed for the pSpec1R$^+$ probe (data not shown).

Spec 1 mRNA Molecules per Cell. We have measured the number of Spec 1 mRNA molecules per cell by determining the number of transcripts per embryo and combining the results with *in situ* hybridization estimates of the number of cells containing these mRNAs. In these calculations, we have assumed that essentially all Spec 1 mRNAs are contained in dorsal ectoderm cells,

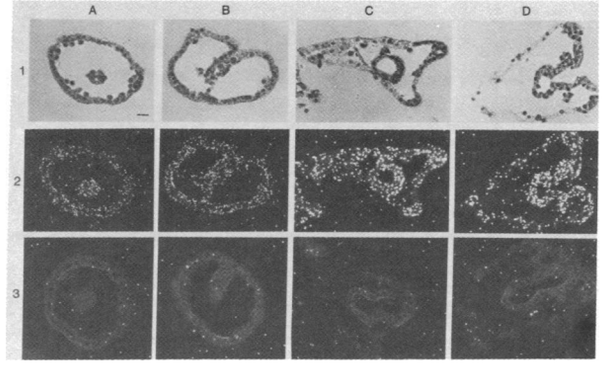

FIG. 5. *In situ* hybridization to poly(A)$^+$ mRNA. [^{3}H]Poly(U) was hybridized *in situ* to sections of gastrulae (2A and 2B) and plutei (2C and 2D). As a control for nonspecific background, transcripts of pCO2R$^-$ (representing the mRNA strand of the early histone repeat) were hybridized to gastrulae (sections 3A and 3B) and plutei (sections 3C and 3D). Photographs in rows 2 and 3 were taken under dark-field illumination; those in row 1 are phase-contrast micrographs of the sections shown in row 2. (Bar = 10 μm.)

as implied by the data in Table 1.

The fraction of embryo RNA complementary to the R^+ probe RNA was determined by solution titration using the same probes and conditions as for *in situ* hybridization. The labeled probe consisted predominantly of 1,280-base transcripts of which 660 bases consist of the pSpec1 sequence and adjacent poly(A) and poly(U). At saturating inputs of total RNA isolated from 68-hr plutei, about 56% of the probe was present as a RNase-resistant duplex. An unusual feature of the pSpec1 sequence is the large amount of intrastrand reassociation to form RNase-resistant duplexes at very low C_0t values. Both R^+ (Fig. 6) and R^- (data not shown) transcripts contain ≈350 bases of self-reassociating sequence that are not attributable to vector. About 160 bases of this are attributable to hybridization of poly(A) and poly(U) regions; the remainder presumably arises from (A+T)-rich regions in the mRNA sequence (unpublished data). The slope of the titration curve indicates that the remaining 310 bases of probe is complementary to 1.1×10^{-5} of total pluteus RNA. Since the pluteus contains ≈3.3 ng of total RNA, the probe detects ≈190,000 mRNA molecules per embryo. These are restricted to ≈400 dorsal ectoderm cells at pluteus, each containing ≈500 Spec 1 mRNA molecules. RNA blot analysis has shown that the content of these mRNAs is higher at gastrula (1). In agreement with this, the grain density is 2.7-fold higher over labeled cells in gastrula than in plutei, while the number of labeled cells at the gastrula stage is only slightly lower, ≈350.

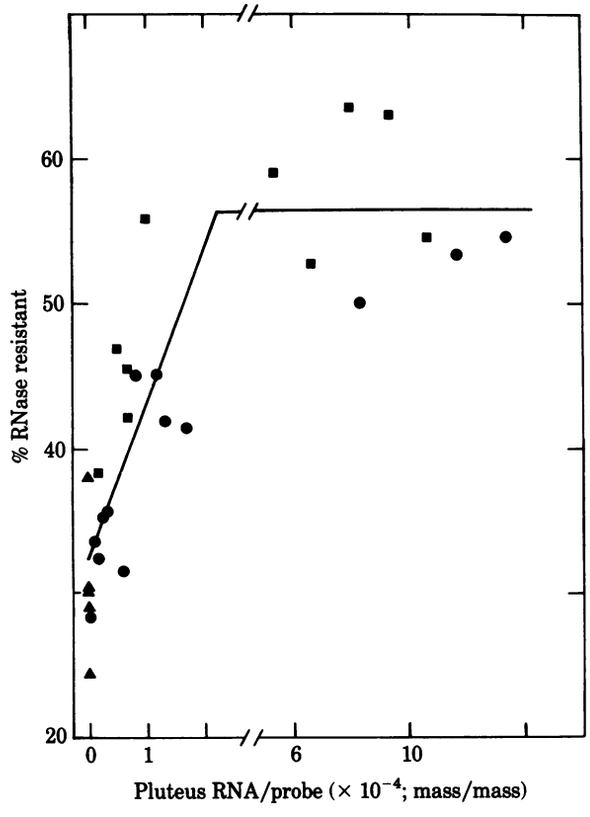

FIG. 6. Measurement of concentration of Spec 1 mRNA molecules per embryo. Radioactively labeled transcripts of pSpec1R$^+$ were titrated in solution with total RNA purified from two different cultures of plutei (●, 68 hr; ■, 73 hr), and the fraction of probe in RNase-resistant duplex was determined. Controls for probe self-reassociation were incubated with the same amount of heterologous RNA (▲). The line shown was fit to the combined data as follows. Linear regression analysis of data derived from RNA/probe ratios $>5 \times 10^4$ (mass/mass) gave a plateau value of 56% with a slope not significantly different from 0 (−0.10% per 1×10^4 mass ratio). Linear regression analysis of data for RNA/probe ratios $<2 \times 10^4$ (mass/mass) yielded a slope of 10.9% per 1×10^4 and a *y* intercept of 32.4%. The slope of the curve indicates that 310 bases of the pSpec1 mRNA probe reacts with 1.1×10^{-5} of total pluteus RNA.

In this regard, it is interesting to compare the localization and timing of Spec 1 mRNA expression with recent results of Cohen and co-workers (L. Cohen, personal communication). These workers labeled embryos continuously with [^{3}H]thymidine, starting at various times of development, and autoradiographed squashes of the embryos at the pluteus stage. Under these conditions, nuclei are unlabeled only if the cells have not gone through any part of an S phase after administration of label. It was observed that, when labeling was initiated at 18–19 hr, plutei contained numerous patches of labeled and unlabeled cells. In contrast, when the precursor was added at 21 hr, a region corresponding to the dorsal ectoderm consisted largely of unlabeled cells. Taken together, these results and ours imply that presumptive ectoderm cells cease to divide at around 21 hr and begin to express Spec 1 mRNAs at about the same time.

Our measurements indicate the current sensitivity of the *in situ* hybridization assay. Since the major mRNA(s) detected is 1,500 bases long, the mRNAs hybridized in solution, and presumably *in situ*, comprise 0.24% of the total embryo mRNA, assuming ≈70 pg of mRNA per embryo (14). However, only 310 nucleotides of the probe react with pluteus RNA in solution, and presumably also *in situ*. This corresponds to ≈0.05% of the total mRNA nucleotides. Estimates of the *in situ* hybridization efficiency using a histone mRNA probe indicate that at saturation close to 100% of target mRNAs are hybridized (unpublished data).

The Spec 1 mRNA is part of a family of mRNAs coding for a set of about 10 quite similar polypeptides (6). We do not yet know whether these proteins are functionally distinct or whether the individual mRNAs of this family have the same distribution in the embryo. Future studies with clones specific for the individual RNAs will help resolve these questions.

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