Posttranscriptional regulation of ectoderm-specific gene expression in early sea urchin embryos

MICHAEL L. GAGNON, LYNNE M. ANGERER and ROBERT C. ANGERER

Department of Biology, University of Rochester, Rochester, NY 14627, USA

Corresponding author

Summary

During development of the sea urchin *Strongylocentrotus purpuratus* embryo, transcription of the Spec1 and actin *Cyllla* genes is activated and the corresponding mRNAs accumulate specifically in ectoderm cells. We show that in gastrulae this tissue specificity of mRNA accumulation is regulated largely if not entirely at a posttranscriptional level. We used RNAase protection assays with intron and exon probes to measure the levels of nuclear precursors and mature message, respectively, in total RNA from embryo fractions enriched for ectoderm (Ect) or endoderm + mesenchyme (E/M) cells. These measurements demonstrate that E/M cells, which do not accumulate Spec1 and actin *Cyllla* mRNAs, contain high levels of intron transcripts, indicating that cells of the E/M tissues transcribe these genes. At later stages, transcripts containing intron sequences are restricted to ectoderm cells. These results indicate that there is a transition from posttranscriptional to transcriptional regulation of tissue-specific mRNA accumulation during the gastrula stage. Measurements of transcription rate by nuclear run-on assays substantiate this conclusion for Spec1 and extend it to two other genes, *SpEGFl* and *Spec2c*, which also encode ectoderm-specific mRNAs. Posttranscriptional regulation was not observed for the SM50 gene whose mRNA accumulates only in primary mesenchyme cells, or for actin *Cyl* which is expressed predominantly in E/M cells of gastrulae.

Key words: sea urchin, posttranscriptional regulation, ectoderm, tissue specificity.

Introduction

During the cleavage stage of sea urchin development, a geometrically regular pattern of cell division progressively divides the early embryo into blastomeres that provide the founder cells for lineages and sublineages of six major tissue territories (reviewed by Davidson, 1989). By the time the embryo reaches the blastula stage, all of these major territories can be identified by distinct patterns of accumulation of different messenger RNAs (reviewed by Angerer and Davidson, 1984; Davidson, 1989; Angerer et al., 1990). In recent years, several laboratories have focused on determination and differentiation of one of these tissues, the aboral ectoderm, and have identified a half dozen mRNAs that accumulate exclusively in aboral ectoderm of pluteus larvae (Lynn et al., 1983; Hardin et al., 1988). *Cyllla* mRNA, like *Sped* mRNA, is absent from adult tissues (Shott et al., 1984) and during embryogenesis it accumulates only in aboral ectoderm (Angerer and Davidson, 1984; Cox et al., 1986). Although these messages are detectable in mesenchyme blastulae, it is not until mid-gastrula stage that aboral ectoderm cells begin to differentiate histologically to form the expanded squamous epithelium that covers approximately three-quarters of the unfed pluteus larva.

The prototype aboral ectoderm-specific genes are the Spec gene family (Bruskin et al., 1981) and the cytoplasmic actin gene, *Cyllla* (Cox et al., 1986). The Spec family includes a set of approximately six genes that encode peptides bearing Ca\(^{2+}\)-binding domains. Although the explicit function of these proteins has not yet been determined, one likely possibility is that they are involved in transport of Ca\(^{2+}\) across the ectodermal wall for the construction of the calcite spicules (reviewed by Klein et al., 1990). *Spec1*, the most abundant of these mRNAs (Bruskin et al., 1981) and the less abundant *Spec2a, 2c and 2d* messages accumulate exclusively in aboral ectoderm of pluteus larvae (Lynn et al., 1983; Hardin et al., 1988). *Cyllla* is one of five cytoplasmic actin genes in this species (Lee et al., 1984). *Cyllla* mRNA, like *Spec1* mRNA, is absent from adult tissues (Shott et al., 1984) and during embryogenesis it accumulates only in aboral ectoderm (Angerer and Davidson, 1984; Cox et al., 1986). *Spec1* and *Cyllla* genes are regulated temporally at the transcriptional level. Nuclear run-on transcription assays indicate that both genes are active by 12 hours of development, or about 180-cell stage (Hickey et al., 1987; Tomlinson and Klein, 1990). Transcriptional activation has also been demonstrated for *Cyllla* (Flytzanis et al., 1987) and *Spec1* (Gan et al., 1990).
promoters driving reporter genes on microinjected constructs. Endogenous Sped (Hardin et al., 1988) and Cyl1la mRNAs (Cox et al., 1986) are already spatially restricted at hatching blastula stage (18 hours) when they can first be detected by in situ hybridization. At gastrula and later stages, when different regions of the ectoderm can be distinguished, these mRNAs are confined to aboral ectoderm. Results of recent nuclear run-on assays of transcription using tissue fractions enriched for ectoderm, or endoderm + mesenchyme, are consistent with the idea that ectoderm is the major site of transcription of the Sped gene between late gastrula (48 hour) and pluteus stages (Tolminson and Klein, 1990). Extensive studies of the regulation of the Cyl1la gene using microinjected constructs also have shown that transcription is confined to ectoderm in the pluteus larva (Hough-Evans et al., 1987, 1988, 1990). All of these data have supported the proposition (Davidson, 1989) that founder cells for aboral ectoderm establish a unique transcriptional territory in the early sea urchin embryo.

In the experiments reported here, we have used RNAase protection of intron probes and nuclear run-on assays to monitor the transcriptional activity of the Sped and Cyl1la genes. In agreement with other reports (Hickey et al., 1987; Tolminson and Klein, 1990; Lee et al., 1992), the intron RNAase protection measurements reveal very high transcription rates per gene at early stages relative to those observed in the pluteus larva. Surprisingly, assays on RNA from tissue fractions enriched for ectoderm and endoderm + mesenchyme cell types show that, during gastrula stages, these genes are transcribed at similar rates in cell types other than ectoderm. We conclude that spatial regulation of ectoderm-specific mRNA accumulation at early stages must include posttranscriptional mechanisms.

Materials and methods

Embryo culture and enrichment for specific cell types

S. purpuratus adults were obtained from Marinus Co. (Westchester, CA), and embryos cultured as described previously (Angerer and Angerer, 1981). Each embryo culture was the result of a single pair mating.

Nominal times of development for S. purpuratus embryos at 15°C are: 5 hour (16-cell), 7 hour (32-cell), 9 hour (very early blastula, ~128 cells), 12 hour (very early blastula, ~180 cells), 15 hour (early blastula), 24 hour (mesenchyme blastula), 36 hour (early gastrula), 48 hour (late gastrula), 60 hour (prism), 72 hour (pluteus larva). However, relative times for different cultures are not directly comparable, because developmental rates were variable.

Embryo fractions enriched for ectoderm (Ect) and endoderm + mesenchyme (EM) cells were prepared by a modification of the procedure of Harkey and Whiteley (1980). The gastrulae used in the experiment shown in Fig. 2A, B and D were harvested 43, 46 and 46 hours post-fertilization, respectively, and the plutei (Fig. 2C) at 72 hours. In all three of these cultures, the gastrulae had completed extension of the archenteron to the oral surface. All fractionation procedures were performed on ice or at 4°C. Embryos from individual cultures were collected by brief centrifugation at 1,000 g. Ectoderm cells were loosened from each other and the underlying basal lamina by 4-6 cycles of centrifugation and resuspension in calcium/magnesium-free sea water (CMFSW). The final pellet was resuspended in 1 M glycine, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, held on ice for 1-5 minutes, and centrifuged to pellet the endoderm + mesenchyme (EM) fraction cells contained within the basal lamina bags. Cells in this supernatant, predominantly ectoderm, were collected by centrifugation, combined with cells in the last 1-3 CMFSW supernatants and stored on ice while the ectoderm-depleted fraction was prepared. This latter fraction was washed 3-4 times in 0.4 M dextrose in 40% CMFSW, pH 8.0 (bag isolation medium, BIM; Harkey and Whiteley, 1980) to remove residual ectoderm. Both this final pellet, consisting of tissues enriched in endoderm and mesenchyme cells, and the pooled ectoderm cells were collected by brief centrifugation at 6,000 g.

Late mesenchyme blastulae were fractionated for tissues enriched and depleted in PMCs following a modification of a method of Harkey and Whiteley (1980). Ectoderm cells were dissociated from 27 hour embryos with 3 washes in CMFSW, a 1 minute incubation in 1 M glycine, 2 mM EDTA, and 3 washes in BIM. The bag pellets were resuspended in BIM and adjusted to final concentrations of 30% cells and 19% Percoll by addition of 100% Percoll and BIM. 9 ml aliquots were centrifuged between a 3 ml sucrose cushion and 3 ml BIM overlay for 20 minutes at 650 g. PMC-rich bags, along with some ectoderm cells, banded at the BIM/Percoll interface. A second fraction containing fewer PMCs and similar levels of ectoderm sedimented to the Percoll/sucrose interface. We refer to these fractions as M+ (mesenchyme-enriched) and M~ (mesenchyme-deficient).

RNA isolation

RNAs from tissue fractions were isolated by either the guanidinium method described by Kingston (1988) or the SDS-urea method of Nemer et al. (1984). In the first method, tissue lysates (1.6 ml) in guanidinium isothiocyanate were centrifuged over a 5.7 M CsCl (0.6 ml) cushion 3-5 hours at 30,000 revs/minute, 20°C, in a Beckman TLS55 rotor in a TL100 ultracentrifuge. After precipitation with ethanol, RNA pellets were redissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. For the preparation of 27 hour embryo RNA, residual Percoll was removed by centrifugation. In some preparations made by the second method, RNAs were further purified by centrifugation through CsCl or by treatment with RNAase I to remove residual traces of DNA that might have been present. However, neither of these treatments had any effect on the results. Furthermore, RNAase protection assays with sense strand controls were negative indicating that DNA did not contaminate these RNA preparations. All RNA preparations were checked for quality and concentration by spectrophotometry and gel electrophoresis.

Description of probe sequences

pGEM-2 (Promega Biotech) and the following recombinant clones were used to analyze transcription in nuclear run-on assays. Sped In 1 is an 800 nt single copy sequence of Sped intron 1; 5'Sped is the sequence between –720 and –420. The Spedc In 1 sequence is a 1.1 kb single copy sequence in the first intron of this gene. All the Spedc sequences were generously provided by Dr W. Klein. SpEGFl is a 1760 nt cDNA clone described in Grimwade et al. (1991). Cyl1 is a gene-specific 720 nt sequence in the 3’ untranslated region of the cyttoplasmic actin Cyl gene, as described by Cox et al. (1986).
For RNAase protection assays, the following probes were used. The *Cylla* exon probe was a 131 nt single copy sequence from the 3' untranslated region of the *Cylla* gene, as described by Cox et al. (1986). The *Cylla* intron is a 172 nt sequence from the first intron; the parent clone was kindly provided by Dr. C. Fitzanis. The *Sped* exon probe is a single copy sequence of approximately 180 nt derived from a cDNA clone that contains 134 nt of exon 3 and about 40 to 50 nt of exon 2. The *Sped* intron probes include shorter sequences (about 170 and 160 nts) from the 800 nt intron 1 sequence described above. The *Sm50* exon probe is a 190 nt sequence from exon 2, that was subcloned from a parent cDNA clone (from pHSH2; Sucov et al., 1987). The *Sm50* intron probe was a 300 nt sequence subcloned from a genomic subclone of BG305 (Sucov et al., 1987) and starts 517 nt downstream of the 5' splice site of intron 1. Both *Sm50* parent clones were kindly provided by Dr. F. Wilt.

**RNAase protection**

Accumulation of both exon and intron RNA species synthesized from the *Sped*, *Cylla*, and *Sm50* genes was monitored using the RNAase protection assay described previously (Yang et al., 1989). For each gene, the antisense riboprobe complementary to the intron was synthesized at 10- or 20-fold higher specific activity than that of the corresponding exon probe in order to allow comparison of signals on the same autoradiographic exposure by laser densitometric scanning. Intron- and exon-specific activities for the *Sped* probes were 10^8 and 5 x 10^6, for *Cylla* were 10^8 and 10^7, and for *Sm50* were 2 x 10^8 and 10^7 disintegrations/minute/µg probe, respectively. For analysis of the culture in Fig. 2C, these specific activities were slightly different; for *Sped* intron and exon probes these were 3 x 10^8 and 1.5 x 10^8, and for *Cylla* they were 3 x 10^8 and 3 x 10^7 disintegrations/minute/µg, respectively. Each riboprobe was purified as previously described (Yang et al., 1989). Full-length as well as shorter riboprobe species caused by premature RNA polymerase stop sites were selected for elution and subsequent use in RNAase protections. In all reactions, excess intron and exon riboprobes were denatured 5 minutes at 85°C and cohybridized at 50°C with 2-15 µg total cellular RNA to kinetic termination, between 17.5 and 28 hours, in 20 µl of a solution containing 50% formamide, 0.5 M NaCl, 40 mM Pipes, pH 6.7, and 1 mM EDTA. Unhybridized RNA was digested at room temperature for 1 hour with 40 µg RNAase A and 0.75 U RNAase T1/µl in 0.3 ml of buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, and 5 mM EDTA). RNAase-resistant hybrids were purified and analyzed by electrophoresis through polyacrylamide gels as described previously (Yang et al., 1989).

**Nuclear run-on transcription assays**

Nuclei were purified from tissue fractions of embryos from the same cultures analyzed by RNAase protection that are shown in Fig. 2A and 2B. A portion of each of the fractionated tissues was used for RNA isolation and nuclei were isolated from the remainder as described by Hickey et al. (1987) and stored at -70°C for up to 6 months. In vitro transcription from these nuclei was performed as described previously (Grimwade et al., 1991) with the following minor modifications. Reactions contained larger volumes of nuclei (100 or 300 µl) and 200 µCi of [32P]-UTP (3000 Ci/mmol; Dupont-NEN). They were carried out for 1.5 hours at 15°C and terminated by adding an equal volume of a solution containing 20 µg DNAase I/ml, 1 M NaCl, 0.1 M MgCl2, 20 mM Tris-HCl, pH 7.5, 1 U RNasin/µl and incubated 3 minutes at 37°C. Sodium acetate, Tris-HCl and SDS were added to 50 mM, 10 mM and 0.2%, respectively, followed by the addition of proteinase K to 15-33 µg/ml and incubation at 37°C for 15 minutes. The total volume was brought to 1.6 ml with diethylypyrocarbonate (DEPC)-treated deionized H2O, and the mix was sheared with a 22-gauge needle. RNA was centrifuged through a 0.5 ml cushion of 5.7 M CsCl, at 30,000 revs/minute for 4-5 hours at 20°C in a TLS55 rotor. To eliminate possible effects of differences in fragment length of Ect and E/M probes, they were reduced to 300-400 nt by partial alkaline hydrolysis for 8 minutes at 60°C as described by Cox et al. (1984).

Radioactively labeled RNAs transcribed in vitro from nuclei of Ect and E/M fractions were hybridized to slot blots containing 2.5 µg of pGem-2 vector and selected recombinant DNAs. These included sequences from *Sped* intron 1, *Sped* upstream of the transcription initiation site, *Sped* exon 1, *Sped* intron 1, *Sped* exon 1, and *Sped* exon 2, that was subcloned from a genomic subclone of BG305 (Sucov et al., 1987) and starts 517 nt downstream of the 5' splice site of intron 1. Both *Sm50* parent clones were kindly provided by Dr. F. Wilt.

**Results**

**RNAase protection with intron probes accurately monitors temporal regulation of transcription in the embryo**

We used RNAase protection assays with mixtures of intron and exon probes to monitor the transcriptional activity of the *Sped* and actin *Cylla* genes and the corresponding mRNA levels during development of *S. purpuratus* embryos. This approach is based on the fact that differences in intron RNA concentration are proportional to differences in transcription rate, if the half-life of the intron RNA sequence remains constant. The steady-state concentration of introns is not a direct measure of instantaneous transcription rate because some time is required for intron sequences to achieve a new equilibrium concentration after a change in transcription rate. However, since the half-life of nuclear RNA is about 20 minutes in sea urchin embryos (Grainger and Wilt, 1976), equilibration of the typical intron to 90% of a new steady-state value takes only about 1 hour, which is rapid compared to the time

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**Posttranscriptional regulation**

459
A. Ex — In

Fig. 1. Transcription and mRNA accumulation patterns from the Sped and Cylla genes during early development. Total RNA isolated from two different cultures was analyzed by RNAase protection with probes complementary to intron sequence to monitor transcription, and with probes complementary to exon sequence to measure mRNA accumulation. Assays for cultures 1 and 2 are shown (A,C and B,D respectively). Times of development, in hours, are indicated above the lanes and defined in Materials and methods. P, probes before hybridization and RNAase treatment, with positions of exon (Ex) and intron (In) probes indicated at left; E, total RNA from unfertilized eggs; y, yeast tRNA used in place of sea urchin RNA; 48Ex and 48In, Sped exon probe and intron probes, respectively, hybridized individually to RNA from 48 hour embryos. In and Ex, at right mark the positions of RNAase-protected fragments hybridized to intron and exon probes, respectively. Specific activities of intron probes were 20- and 10-fold greater than exon probes for Sped and Cylla genes, respectively, in both developmental series.

course of development. In each of these assays, large sequence excesses of radioactively labeled intron and exon antisense RNA probes were combined and hybridized to kinetic termination with the same sample of total RNA isolated from whole embryos. The specific activities of intron probes were 10- to 20-fold higher than those of exon probes to compensate for target abundance differences and thus to allow direct comparisons on the same autoradiographic exposure. Both intron and exon Sped probes and the Cylla exon probe detect single copy sequences. The Cylla intron probe detects multiple bands in blots of genomic DNA (data not shown). However, RNAase protection of this probe monitors only Cylla gene transcripts because only a single full-length RNAase-resistant hybrid is ever observed. Transcripts complementary, but not identical, to this probe would produce multiple fragments since RNAase protection is very sensitive to sequence mismatch. The specificity of the Cylla intron probe is also supported by the observation that it does not detect any complementary sequence in RNA from unfertilized eggs or from an adult urchin (our unpublished observations). Furthermore, the temporal pattern of transcription of the Cylla gene, as determined by both nuclear run-on assays (Hickey et al., 1987) and in vivo labeling studies (Lee et al., 1992) is very similar to that indicated by intron abundance (see Fig. 1C, D). This correspondence also supports the assumption that intron half-lives are essentially unchanged throughout early development.

Fig. 1A and B show the developmental pattern of transcription and message accumulation for Sped in two different cultures. The exon probe detects traces of this message in RNA from unfertilized eggs. This maternal message decays during the period just after fertilization, and new zygotic transcripts accumulate until gastrula stage as previously reported (Bruskin et al., 1981; Hardin et al., 1988). The hybridizability of RNA from all stages was verified by the fact that the same series of samples yielded the expected temporal patterns with probes for a variety of different messages (e.g., Grimwade et al., 1991). No sequence complementary to the first intron is detectable in maternal RNA; calibration of similar analyses by solution titration indicates that this assay can detect as few as 100 transcripts per egg. The first new intron transcripts are detected between 5 and 7 hours, or by the 32-cell stage. The intron sequence content of embryo RNA increases significantly between 9 and 15 hours at a rate that exceeds the increase in cell number, which is less than two-fold. After 15 hours (250 cells), the level of introns/embryo decreases 5- to 10-fold, despite a 7-fold increase in cell number by pluteus stage. Thus, the transcription rate per gene is highest in the very early blastula stage and would be about 50-fold lower in pluteus larvae, if the fraction of nuclei that transcribe the Sped gene is similar at both stages. However, the data presented below imply that most, if not all, nuclei transcribe this gene at early stages while only the one third that are in aboral ectoderm do so in the pluteus larva. Therefore, the actual decrease in the transcription rate/gene is probably closer to about 15- to 20-fold. Using nuclear run-on assays, Tomlinson and Klein (1990) also showed an increase in rate of transcription of Sped mRNA between cleavage and blastula stage, and a large decrease in transcription rate during the
bystula-gastrula period. Despite a large decrease in transcription rate per embryo for the Spec1 gene at late stages, the concentration of mRNA remains virtually constant in the gastrula to pluteus period, indicating that these transcripts are quite stable. This result is in good agreement with the in vivo measurements of Cabrera et al. (1984), which showed that the half-life of Spec1 mRNA is longer than could be measured with their labeling times, and is at least 11.5 hours.

Similar developmental modulations of expression were observed for the Cyllla actin gene in embryos of these two cultures using a gene-specific exon probe and a probe for the first intron (Fig. 1C and D). The time course of accumulation of Cyllla mRNA (exons) is similar to those reported previously (Shott et al., 1984; Lee et al., 1986; Angerer et al., 1989). Intron transcripts are first detectable between 5 and 7 hours after fertilization, slightly earlier than observed in previous studies which used nuclear run-on assays (Hickey et al., 1987). Transcription of the Cyllla gene, like that of Spec1, is strikingly downregulated between early blastula and pluteus stages. This downregulation has also been observed both by run-on assays (Hickey et al., 1987) and by in vivo labeling studies (Lee et al., 1992). The kinetics of downregulation differ for embryos of the two cultures for the Cyllla gene and, to a lesser extent, for the Spec1 gene (cf. Fig. 1A with 1B and 1C with 1D). Furthermore, within the same culture, the temporal modulations of intron levels of Cyllla sometimes differ from those of Spec1. For example, in the culture shown in Fig. 1B and D, peak concentrations of Cyllla and Spec1 introns are achieved at 12 and 15 hours, respectively. In addition, the decrease in Cyllla intron sequences is more gradual than that observed for Spec1. Thus, although the two genes are coordinately expressed in the broad sense, their temporal patterns of transcription are not precisely regulated.

Spec1 and Cyllla intron probes detect transcription in non-ectodermal cells

The temporal regulation of the Spec1 and Cyllla genes at the transcriptional level, coupled with the accumulation of stable mRNAs only in aboral ectoderm, has led to the hypothesis that these genes are transcriptionally active only in aboral ectoderm and its precursors. To test this hypothesis, we hybridized simultaneously probes for intron and exon sequences of these genes in similar RNAase protection assays to total RNA prepared from tissue fractions enriched for ectoderm (Ect) or for endoderm + mesenchyme (E/M) as described by Harkey and Whiteley (1980). In this procedure embryos are washed in calcium/magnesium-free sea water, which leads to separation of ectoderm cells from the basal lamina bag containing endoderm and mesenchyme. The extent of contamination of the E/M fraction with Ect cells is measured by the abundance of Cyllla or Spec1 exon sequences which accumulate specifically in Ect RNA. At prism and pluteus stages of Strongylocentrotus purpuratus, this procedure results in a 5- to 20-fold depletion of ectoderm cells from the E/M fraction as assayed with Spec1 and Cyllla exon probes. However, at gastrula stage, separations are less effective resulting in a 3- to 5-fold difference in exon concentration between these two fractions for different preparations. Separation is less effective because the basal lamina does not mature or toughen sufficiently at earlier stages to allow efficient removal of contaminating ectoderm cells from E/M tissues. Nevertheless, because exon and intron probes are hybridized with embryo RNA in the same reaction, the hybridizations are internally controlled, the ratios are independent of RNA input or recovery and it is possible to determine intron/exon ratios quite precisely.

Using these assays, we observed the expected transcriptional regulation of cell-type-specific mRNA accumulation at pluteus stage, but posttranscriptional regulation at an earlier stage. Transcriptional and posttranscriptional regulatory mechanisms result in different ratios of intron to exon transcripts in RNA of E/M versus Ect fractions. For strict transcriptional control, relative levels of intron sequence in RNA of the E/M and Ect fractions should be the same as the relative levels of exon sequence. This is because contamination of the E/M fraction by Ect cells contributes intron and exon sequences in the same proportion as in the Ect fraction. Alternatively, if posttranscriptional mechanisms regulate mRNA accumulation, then the E/M fraction would contain more intron sequence than can be attributed to contamination by Ect cells. Posttranscriptional regulation is thus indicated by a larger ratio of intron to exon RNA sequence in the E/M fraction than in the Ect fraction.

Representative results for several cultures are shown in Fig. 2. In the culture of gastrulae analyzed in Fig. 2A, RNAase protection shows approximately five-fold higher content of both Spec1 and Cyllla exon sequences in the Ect fraction than in the E/M cells. A similar ratio is observed for intron transcripts, as expected for regulation at the transcriptional level. In contrast, in a second culture of gastrulae (Fig. 2B), the E/M fraction has approximately four-fold lower concentration of both Spec1 and Cyllla exon sequences but levels of intron sequences are similar in both fractions. This unexpectedly high level of intron transcripts in the E/M fraction strongly suggests that these cells transcribe both the Spec1 and Cyllla genes but that this transcription does not result in accumulation of stable mRNAs. The fact that intron levels are as high as those in RNA of the Ect fraction implies that most of the cells in the E/M fraction transcribe these genes, since it is unlikely that transcription in only a few cells could achieve this high intron concentration, if intron half-lives are similar in all cells. The fact that both posttranscriptional and transcriptional regulation were observed in different cultures of gastrulae suggested the existence of a switch in the mode of control around the time of gastrulation. This switch from posttranscriptional to transcriptional regulation is directly demonstrated for both genes in Fig. 2B and C, which compares results for gastrulae and plutei, respectively, from the same culture.

The timing of this transition varies among cultures for
Fig. 2. Spatial regulation of the *Spec1* and *Cyllla* genes occurs at both transcriptional and posttranscriptional levels. Relative amounts of intron and exon sequences in fractionated tissues were measured by RNAase protection after simultaneous hybridization to intron and exon probes. This analysis was carried out for both the *Spec1* (upper panels) and *Cyllla* (lower panels) genes. The *Spec1* intron probe used in B was the same sequence as used in other experiments, but ~10 nt shorter. Examples are shown for three gastrula cultures (A, B, D) and for plutei (C) from the same embryo batch as the gastrulae of B. The intron/exon ratios are consistent with transcriptional regulation for the *Spec1* gene and gastrula culture in A. The gastrula culture in B shows a higher intron/exon ratio in E/M than in Ect fractions, consistent with posttranscriptional regulation of both genes. The gastrula culture in D is mixed, with intron/exon ratios that indicate transcriptional control for the *Spec1* gene but posttranscriptional control for the *Cyllla* gene. P, In, and Ex, same as in Fig. 1; Ect, total RNA isolated from ectoderm-enriched fraction; E/M, total RNA isolated from ectoderm-depleted fraction, enriched in endoderm and PMCs. Hybridizations in which yeast tRNA was substituted for sea urchin RNA were free of self-protected probe bands (data not shown).

### Table 1. RNAase protection assays showing posttranscriptional and transcriptional spatial control

<table>
<thead>
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<th>Level</th>
<th><em>Cyllla</em></th>
<th><em>Spec1</em></th>
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<td></td>
<td>Gastrula</td>
<td>Pluteus</td>
</tr>
<tr>
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<tr>
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Both genes and may be gradual, as shown by the results of similar assays of 21 gastrula cultures and 3 pluteus cultures summarized in Table 1. Cultures of similar chronological age showed either type of regulation. The gastrulae in all cultures had complete archenterons and the timing of the transition does not appear to be linked to any identifiable morphological event during gastrulation. We also occasionally observed an intermediate pattern in which intron/exon ratios in E/M fractions were significantly elevated, but not as high as in the examples shown in Fig. 2B. An important observation that emerged from examination of this large number of cultures is that the switch from posttranscriptional to transcriptional regulation is made at a slightly later time for *Cyllla* than for *Spec1* (Table 1). Thus, in six cultures the *Cyllla* gene is transcribed in E/M cells, but the *Spec1* gene is not. No cases of the converse were observed. An example of one such mixed culture is shown in Fig. 2D. These results reinforce the conclusion that the temporal programs of expression of these genes during development are not precisely regulated. Furthermore, they provide compelling evidence that high intron levels in E/M fractions are not due to trivial artifacts, such as contamination of these fractions with ectoderm nuclei or differential extraction of nuclear versus cytoplasmic RNAs.

A prediction from these results is that the spatial regulation of *Spec1* and *Cyllla* mRNA accumulation should be posttranscriptional at all earlier stages. This prediction could not be tested because we were unable to separate different cell types with sufficient purity at
Evidence for posttranscriptional control from nuclear run-on assays

The results of the RNAase protection assays force the conclusion that Spec1 and CylIla intron transcripts are synthesized in some E/M cells. However, they cannot distinguish between the possibility that transcription rates are similar in cells of the Ect and E/M fractions and the alternative possibility that a low rate of transcription in E/M cells is coupled with unusual stability of the intron sequences. We considered the second alternative unlikely because the half lives of both introns tested would have to be manyfold greater in E/M cells and because intron RNAase protection assays agree well with other estimates of developmental changes in the transcription rate of these genes (Hickey et al., 1987; Tomlinson and Klein, 1990; Lee et al., 1992). Nevertheless, we used a second, independent measure of transcription. Nuclear run-on transcription assays were carried out for nuclei from the same Ect and E/M tissue fractions as shown in Fig. 2A and B. This approach also allowed us to analyze other genes with different known spatial patterns of mRNA accumulation in the embryo, using available clones.

Nuclear run-on assays shown in Fig. 3 confirm and extend the conclusions of RNAase protection. Because equal amounts of radioactivity in RNA from Ect and E/M nuclei were hybridized, the relative intensities of signals measure the relative fraction of nuclear RNA synthesis corresponding to an individual DNA target. Fig. 3A shows results of nuclear run-on assays for Ect and E/M fractions of embryos exhibiting transcriptional regulation as determined by RNAase protection assays (Fig. 2A). Transcription of the Spec1 gene is clearly higher for Ect nuclei than in E/M nuclei. Sequences of several other genes that encode ectoderm-specific mRNAs also show higher signals for RNA synthesized in Ect nuclei. These include the first intron of the Spec2c gene which encodes an mRNA that is less abundant than Spec1 message (Hardin et al., 1988) and SpEGFI cDNA, whose message is more abundant than Spec1 and accumulates both in aboral ectoderm and in a portion of oral ectoderm (Grimwade et al., 1991). Finally, hybridization to a sequence upstream of the Spec1 mRNA initiation site is similar to background levels measured with pGEM-2 vector.

Nuclear run-on analysis of the fractions from gastrulae exhibiting posttranscriptional regulation as determined by RNAase protection (Fig. 2B) is shown in Fig. 3B. In this case, signals for the Spec1 intron 1 are similar for nuclei from both tissue fractions. Signals for the Spec1 upstream probe appear to be only slightly above those observed with vector and much lower than signals with the intron probe. This low signal is probably due to cross-hybridization of a repetitive sequence in this probe to other transcripts (data not shown). In any case, the fact that this signal is much lower than that for the Spec1 sequence shows that transcription of the Spec1 gene in E/M cells is not due to readthrough from an upstream gene. Hybridization of nuclear run-on RNAs to a Spec2c intron 1 sequence or to an SpEGFI protein coding region sequence shows that the spatial expression of other genes encoding ectoderm-specific mRNAs is also regulated at a posttranscriptional level in embryos of this culture. These results cannot be attributed to variability among separate preparations of E/M nuclei since RNA synthesized by these nuclei always gave hybridization signals to actin Cyl mRNA sequences that were higher than those with Ect RNA (Fig. 3A, 3B). This is expected if the spatial regulation of Cyl mRNA accumulation is controlled transcriptionally in gastrulae, since at this stage Cyl message accumulates mainly in secondary mesenchyme and endoderm (Cox et al., 1986).

We carried out similar run-on transcription analyses for two additional cultures. In total, two cultures showed posttranscriptional control and two showed transcriptional control for the Spec1 gene by this assay as well as by RNAase protection. We were unable to analyze transcription of the CylIla gene by nuclear run-on assays because all available probes either hybridize to multiple sequences that cannot be discriminated in this assay (as they are in RNAase protection), or are too short to hybridize with a detectable amount of RNA.
at gastrula stage, at which time transcription of the *CyIIla* gene is markedly downregulated (cf. Fig. 1C, D).

**Transcription of a primary mesenchyme-specific gene is not detectable in other tissues**

Recently, several laboratories have made the surprising observation that separated mesomere pairs from the animal hemisphere of the 16-cell embryo, which in normal development give rise only to ectoderm, can under certain conditions express genes and produce rudimentary structures characteristic of primary mesenchyme cells and endoderm (Henry et al., 1989; Livingston and Wilt, 1989, 1990; Khaner and Wilt, 1990). This result stands in contrast to the classical observations that only cells at the vegetal pole of the embryo (presumptive primary and some presumptive secondary mesenchyme) can give rise to skeletogenic cells (reviewed by Hörstadius, 1973) and indicates that presumptive ectoderm has a latent potential to express PMC-specific genes. Such genes might be transcribed in presumptive ectoderm of normal embryos but, like the *Spec1* and *CyIIla* genes in E/M cells, this transcription might not lead to accumulation of stable mRNA. We tested this hypothesis with a probe for transcripts from the prototype primary mesenchyme-specific gene, *SM50*, which encodes a spicule matrix protein (Benson et al., 1987), and cell fractions from mesenchyme blastulae and gastrulae. Two kinds of evidence argue against posttranscriptional regulation of *SM50* gene expression. First, as shown in Fig. 4A, *SM50* intron and exon sequences show similar ratios of abundance in Ect and E/M fractions, indicating that this gene is not detectably transcribed in ectoderm cells. The RNAs used for this experiment were from a culture of gastrulae which exhibited posttranscriptional control for *CyIIla* expression (Fig. 2D). Similar results were obtained for three other gastrula cultures. Second, the level of transcription of *SM50* in the whole embryo is low compared to genes encoding ectoderm-specific RNAs, as estimated by the relatively low level of intron sequence detected by RNAass protection and the lack of signal in our nuclear run-on assays (data not shown). This is consistent with transcription only in PMCs which comprise a small fraction of the cells in the embryo.

To test the possibility that *SM50* mRNA accumulation might be controlled posttranscriptionally at earlier stages, we also carried out RNAase protection assays for 27 hour mesenchyme blastula fractions differentially enriched for PMCs. The M+ fraction is significantly more enriched than the M− fraction, as shown by the increased abundance of *SM50* exon sequence (Fig. 4B). Nevertheless, since the PMCs account for less than 10% of the embryo volume, both fractions are still heavily contaminated with other cell types, as shown by their similar content of *CyIIla* exon sequences. However, this circumstance provides an indirect test for transcription of *SM50* in non-mesenchyme cells. If a significant fraction of epithelial cells were transcribing the *SM50* gene, then enrichment for the minor PMC population would lead to little or no enrichment for intron sequence. Clearly this is not the case since levels of both intron and exon sequence are elevated to the same extent in the fraction more highly enriched for PMCs, as shown in Fig. 4B. We conclude that also at this earlier stage, a significant fraction of cells other than PMCs does not transcribe the *SM50* gene at rates similar to those in PMC cells. Thus, although the derivatives of mesomeres, which constitute approximately half the embryo, have the potential to transcribe mesenchyme-specific genes under some experimental conditions (cited above), the suppression of this capacity in the normal embryo appears to be regulated at the transcriptional rather than the posttranscriptional level. We cannot exclude the possibility that broader transcription might occur at even earlier stages, or that a minor cell population, such as presumptive secondary mesenchyme, transcribes the *SM50* gene in normal embryos.

**Discussion**

We have used both intron RNAase protection and nuclear run-on assays to show that accumulation of ectoderm-specific mRNAs is spatially regulated at a posttranscriptional level in the sea urchin embryo. This phenomenon applies to at least two classes of mRNAs expressed in ectoderm: those that accumulate only in aboral ectoderm, including *actin CyIIla, Spec1* and *Spec2c*, as well as one message, *SpEGFI*, that accumulates both in regions of oral and in aboral ectoderm. RNAase protection assays show that E/M fractions...
contain higher levels of intron sequence than can be accounted for by contamination of this fraction with ectoderm cells, which is measured by levels of ectoderm-specific exons. These results were confirmed by nuclear run-on assays. In other cultures of gastrulae and in plutei, regulation is at the transcriptional level. These results suggest that there is a transition from posttranscriptional to transcriptional control in late gastrulae which was directly demonstrated for both genes in one culture. The timing of this switch apparently varies among cultures and is not the same for different genes, as shown by the fact that, in about 30% of cultures of gastrulae, the Spec1 gene is regulated transcriptionally while CyIIa is regulated posttranscriptionally.

The remote possibilities that transcription of Spec1 and CyIIa in E/M cells represents promiscuous transcription during preparation of tissue fractions of some cultures and/or that high levels of transcription result from contamination of E/M fractions by Ect nuclei are ruled out by several results. First, transcription of two non-ectodermal genes, actin Cyl and SM50, is higher in the E/M fraction regardless of the levels of regulation of Spec1 and CyIIa. Second, these two ectoderm-specific genes have been shown to be regulated at different levels in the same cultures. Furthermore, this difference in regulation is nonrandom since in no case have we observed posttranscriptional regulation for Spec1 and transcriptional regulation for CyIIa. In the case of the Spec1 gene, we have also shown that transcription in the E/M fraction cannot be attributed to readthrough from an upstream gene.

Our experiments indicate that at least some cells of the E/M fraction, which include presumptive primary and secondary mesenchyme and endoderm, transcribe aboral ectoderm-specific genes at early stages. The fact that signals obtained with intron probes for E/M fractions are similar to those for Ect cells suggests that this transcription is not attributable to a minor fraction of E/M cells. We speculate that cells derived from the veg2 tier of blastomeres, which normally give rise to endoderm and secondary mesenchyme and constitute about 80% of the cells in the E/M fraction, transcribe Spec1 and CyIIa genes while primary mesenchyme cells do not. This speculation stems from the fact that the fate of veg2 derivatives is plastic, whereas that of cells of the PMC lineage is fixed (reviewed by Hörstadus, 1973). For example, endoderm-specific mRNAs are not detectable until early gastrula stage (Nocente-McGrath et al., 1989; Wessel et al., 1989) and secondary mesenchyme can be induced to convert to skeletogenic mesenchyme until late gastrula stage (Ettensohn and McClay, 1988). Our experiments do not address the possibility that in the early embryo aboral ectoderm-specific genes are also transcribed in presumptive oral ectoderm. This possibility is interesting because several identified mRNAs initially accumulate uniformly in presumptive ectoderm, and subsequently become restricted to either oral or aboral regions of the pluteus (reviewed by Angerer et al., 1990). We have attempted to identify more precisely which cells of the embryo transcribe the aboral ectoderm-specific genes during early development by hybridizing intron antisense transcripts in situ. These experiments have been unsuccessful for several reasons. We have determined that, at gastrula stage, intron transcripts of both CyIIa and Spec1, if distributed among all nuclei, are present at only about 3-5 copies/nucleus which is below our detection limits. If confined to aboral ectoderm nuclei, their amount/nucleus would be about 3-fold higher. In the 15 hour early blastula, when intron concentrations are maximal, we estimate that there would be about 30 transcripts/nucleus if all cells are transcribing these genes. While we have obtained signals slightly above background at this stage, they are insufficient to make reliable conclusions. We have observed that the efficiency of detection of other nuclear sequences is low compared to that of mRNAs, which is probably related to their accessibility to probe.

Intron RNAase protection is a simple, extremely sensitive way to measure changes in transcription rates, assuming intron half-lives are constant. Its accuracy is demonstrated for the cases presented here by the similarity of relative intron levels to relative transcription rates measured by nuclear run-on assays [CyIIa (Hickey et al., 1987); Spec1 (Tomlinson and Klein, 1990)] or by in vivo labeling (CyIIa, Lee et al., 1992). Using this method, we also observed that the transcription rates of Spec1 and CyIIa are very much lower in pluteus larvae than they are at very early stages (15 hour blastulae), long before the differentiation of ectoderm tissues. Between these two stages, the rate per embryo decreases 5- to 10-fold. Because the number of nuclei increases from about 250 cells to 1800 (about 7-fold), the per gene rate could decrease about 50-fold. If transcription occurs in almost all cells of blastulae but only in aboral ectoderm of plutei, as suggested from our experiments with gastrula and pluteus tissue fractions, then this rate decrease/nucleus would be about 15- to 20-fold. This is significantly more than the approximately threefold decrease that could be attributed to progressive restriction of transcription to aboral ectoderm. Thus, transcription must be markedly downregulated in aboral ectoderm cells as well, reflecting a shift from accumulation of these mRNAs and the proteins that they encode during early stages, to transcription at lower maintenance levels in differentiated cells.

Evidence for posttranscriptional regulation of gene expression in sea urchin embryos and adult tissues has been presented previously by Wold et al. (1978). Only about 40% of sequences of blastula polysomal RNA are found in polysomal RNA of gastrulae, and only 16-18% in cytoplasmic RNA of adult intestine or coelomocytes. However, all sequences were found in nuclear RNA of gastrulae, intestine and coelomocytes. While these results imply temporal regulation of a large number of mRNAs at a posttranscriptional level, this straightforward conclusion is confounded to some extent by the subsequent discovery of a large population of maternal transcripts that have characteristics of unprocessed or partially processed nuclear RNAs, some of which may persist in the embryo's cytoplasm to blastula stage.
(reviewed by Calzone et al., 1987). Such sequences might be represented in the polysomal RNA population examined by Wold and coworkers but restricted to nuclei at later stages of embryogenesis and in adult tissues. In this case, the phenomenon demonstrated by these workers could be persistence of incompletely processed maternal transcripts during embryogenesis, rather than posttranscriptional regulation in later embryos and adult tissues. No information is available on the spatial distributions of these blastula sequences or the sites of their transcription at later embryonic stages. However, assuming the original interpretation of Wold et al. is correct, we note that the Spcl and Cyllla genes are not members of the same set of sequences, because they are present as mRNAs at all stages of development examined and their regulation progresses from posttranscriptional to transcriptional rather than vice versa.

Our demonstration of posttranscriptional regulation of accumulation of ectoderm-specific mRNAs is consistent with all available information on the spatial expression of the Spcl and Cyllla genes. Cell-type-specific regulation of endogeneous gene transcription has been examined in only one previous study. Tomlinson and Klein (1990) used run-on transcription assays to analyze Ect and E/M fractions from a culture of late gastrula stage (48 hour) embryos, and showed that Spcl was regulated at the transcriptional level. Indirect evidence for spatially regulated transcription of endogenous genes comes from transient expression assays in which promoter constructs driving reporter genes are microinjected into eggs. Workers in Davidson's laboratory have extensively analyzed the regulatory region of the Cyllla gene and identified a region that drives reporter gene expression specifically in aboral ectoderm (Hough-Evans et al., 1990). However, all of these assays have been carried out at pluteus stages at which time all studies show that expression of the endogenous genes is transcriptionally regulated. Since the reporter CAT mRNA is short-lived, it is therefore possible that transcription from these exogenous templates could occur in other cell types at earlier stages.

The observation that different regions of the embryo establish specific patterns of mRNA accumulation by mesenchyme blastula stage (reviewed by Angerer and Davidson, 1984) has been interpreted to indicate that different early founder blastomeres define transcriptional territories, which are clonally inherited by their descendants (Davidson, 1989). However, analyses of spatial patterns of accumulation of a large number of mRNAs have shown that distributions of many mRNAs become progressively restricted during development (P. Kingsley, L. Angerer, R. Angerer, unpublished data), suggesting that individual transcriptional repertoires may necessarily be broader during early development when fates remain plastic. The results presented here show additional unsuspected examples of breadth of transcriptional activity of tissue-specific genes, coupled with posttranscriptional regulation of production of the gene products. We speculate that, in at least some lineages, the shift in regulation from posttranscriptional to transcriptional levels is mechanistically related to permanent restriction of cell fates.

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