The skeleton of the sea urchin embryo is synthesized by primary mesenchyme cells (PMCs), descendants of the micromeres of the 16-cell stage embryo (for reviews see Gustafson and Wolpert, 1967; Okazaki, 1975a; Ettensohn et al., 1997). During gastrulation, the PMCs gradually become arranged in a characteristic ring pattern, composed of two aggregates of cells (the ventrolateral clusters) connected by cell chains extending circumferentially on the ventral and dorsal surfaces of the blastocoel (the ventral and dorsal chains, respectively). As the ring is forming, filopodial protrusions of the PMCs fuse, linking the cells in a syncytial network (Hodor and Ettensohn, 1995). Skeletogenesis begins with the formation of one triradiate spicule rudiment in each of the two ventrolateral clusters. Subsequently, the spicule rudiments elongate and branch in a stereotyped fashion to give rise to the embryonic skeleton.

Several PMC-specific gene products that participate in the synthesis of the skeleton have been identified. Msp130 is a sulfated cell-surface glycoprotein that has been implicated in calcium uptake or deposition (Carson et al., 1985; Leaf et al., 1987). Three proteins of the spicule matrix, SM50, SM30 and PM27, have also been analyzed in detail. SM50 and PM27 are predicted to be structurally similar, nonglycosylated, basic proteins (Sucov et al., 1987; Livingston et al., 1991; Katoh-Fukui et al., 1991, 1992; Harkey et al., 1995), whereas SM30 is an acidic glycoprotein (George et al., 1991). The specific roles of these matrix proteins have not yet been elucidated, although they may function in the nucleation or orientation of crystal growth (Wilt and Benson, 1988; Harkey et al., 1995).

Previous studies have shown that the ectoderm plays an important role in PMC guidance, skeletal patterning and skeletal rod elongation (Ettensohn, 1990; Hardin et al., 1992; Armstrong et al., 1993; Ettensohn and Malinda, 1993; Ettensohn et al., 1997). One means by which the ectoderm might influence skeletal morphogenesis is by controlling the expression of PMC-specific gene products involved in spicule biosynthesis. Harkey et al. (1992) found that the msp130 gene exhibits a complex pattern of spatial regulation within the PMC syncytium. At ingression, msp130 mRNA is expressed at high levels by all PMCs, but later in development this gene is down-regulated by PMCs that are localized in specific regions of the developing skeletal system. This suggests that at later developmental stages, the expression of msp130 is regulated by extrinsic cues. In support of this view, cell transplantation studies have shown that all PMCs initially have the capacity to migrate to any region of the sub-equatorial ring pattern and, therefore to contribute to any part of the developing skeletal system (Ettensohn, 1990). Molecular
differences among PMCs that arise after the formation of the ring pattern must, be a consequence of different stimuli that act on the cells.

In this study, we have further examined the regulation of skeletogenesis by the ectoderm. We provide both cellular and molecular evidence for local, ectodermal control of skeletal rod growth. Our results suggest that the ectoderm may control skeletal morphogenesis by regulating the expression of PMC-specific gene products involved in spicule biogenesis.

MATERIALS AND METHODS

Embryo culture

Adult *L. variegatus* were maintained and embryos cultured according to Ettensohn and Malinda (1993). The rate of development was controlled by incubating the embryo cultures at 18-25°C in constant-temperature water baths.

Calcein labelling

Because the rate of skeletal rod elongation varies with temperature, all embryos used for calcein labelling were cultured at 23°C. Calcein was prepared as a 1.25 mg/ml stock solution in Instant Ocean (IO), pH 8.3, and stored at 4°C. At selected stages, embryos were incubated for 1 hour in IO containing 50 µg/ml calcein, then washed and transferred to fresh IO for 2 hours. Labelled embryos were then fixed in 4% paraformaldehyde in IO at room temperature (RT) for 2 hours, followed by post-fixation in 100% methanol at −20°C for 20 minutes. Embryos were examined with epifluorescence optics using a standard fluorescence filter set. The length of the unlabelled spicule material at the tip of each skeletal rod was measured with an ocular micrometer, and this value was divided by 2 hours to give an average rate of rod growth (in µm/hour) during the postlabeling period.

RNA isolation and northern blot analysis

Total RNA was isolated from embryos using the method of Chomczynski and Sacchi (1987), with an additional precipitation of RNA with 8 M lithium chloride. Poly(A)+ RNA was isolated by subjecting total RNA to affinity chromatography on oligo(dT)-cellulose (Sambrook et al., 1989).

For northern blot analysis, total RNA (20-25 µg/stage) was separated in 1% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes (Sambrook et al., 1989). Blots were hybridized overnight at 42°C in the presence of 50% formamide, with [α-32P]dCTP-labelled DNA probes synthesized with the Boehringer Mannheim random-primed DNA labelling kit. Blots were washed at a final stringency of 0.2× SSC, 0.1% SDS at 42°C. Radioactive signal was quantitated using an AMBIS 4000 radioanalytic imaging detector (AMBIS, Inc.).

*L. variegatus* homologues of SM50, SM30 and *msp130*

An oligo(dT)-primed *L. variegatus* mesenchyme blastula stage cDNA library was constructed in AZAP (Stratagene) using poly(A)+ RNA. To isolate a cDNA encoding the *L. variegatus* homologue of SM50, this library was screened with LSM34, a cDNA that encodes the *L. pictus* homologue of SM50 (Livinston et al., 1991). Filters were hybridized overnight in the presence of 50% formamide at 42°C, and washed at a final stringency of 0.1× SSC, 0.1% SDS at 65°C. A 0.9 kb cDNA was identified, and digested at an internal StuI site located 145 nucleotides from the 3′ end to eliminate poly(A)+ sequences. This subclone, referred to as *LvSM50-StuI*, was used as template for synthesis of DNA and RNA probes for *L. variegatus* SM50 (*LvSM50*) transcripts.

A cDNA encoding the *L. variegatus* homologue of SM30 was cloned by screening a *L. variegatus* prism stage cDNA library (Wessel et al., 1989) with pNG7, a cDNA that encodes SM30 from *S. purpuratus* (George et al., 1991). Hybridization was performed as described above, and filters were washed at a final stringency of 0.2× SSC, 0.1% SDS at 50°C. One 1.6 kb clone, *LvSM30* (P2C), appeared to be nearly full-length and was used for synthesis of DNA and RNA probes for *L. variegatus* SM30 (*LvSM30*) transcripts.

Positive clones were excised in vivo as directed by Stratagene to generate pBluescript SK(−) plasmids, and sequencing was performed using Sequenase 2.0 (United States Biochemical). Nucleic acid sequence similarity searches and alignments were performed using the BLASTN program (Altschul et al., 1990) and MacVector (International Biotechnologies, Inc.).

A cDNA clone encoding a portion of the *L. variegatus* homologue of *msp130* was the gift of C. Marshall (UCLA) and R. Raff (Indiana University), and was used to generate DNA and RNA probes for *L. variegatus* *msp130* (*Lvmsp130*) transcripts.

Whole-mount in situ hybridization

Whole-mount in situ hybridizations were performed as described by Harkey et al. (1992) with the modifications of Ransick et al. (1993, 1995). To reduce background and extraneous binding of probes to pigment cells (Fang and Brandhorst, 1996), some samples were treated with 50 µg/ml ribonuclease A and 50 U/ml ribonuclease T1 following hybridization (Frudakis and Wilt, 1995). Digoxigenin-11-labelled antisense and sense RNA probes were synthesized with the MEGAscript in vitro transcription kit (Ambion) under the conditions described by Ransick et al. (1993), and alkaline hydrolyzed. Bulk processing of fixed embryos was carried out entirely in 1.5 ml microfuge tubes (approx. 50 µl packed embryos/tube). Small numbers of embryos (fewer than 300) were fixed and processed in nylon mesh-bottomed baskets placed in flat-bottomed 24-well plates. For hybridization, embryos resuspended in hybridization buffer were drawn into the narrow portion of a 9 inch pasteur pipette, which was then sealed at both ends and submerged in a water bath. Embryos were returned to the baskets for post-hybridization steps. Appropriate control embryos were processed in parallel with microsurgically manipulated embryos. Hybridizations with sense probes were performed for *Lvmsp130* and *LvSM30* and showed no specific signal above background (not shown).

Embryo microsurgery

Depletions and transplantsations of PMCs were carried out as described by Ettensohn (1990). For heterochronic cell transplantations, PMCs isolated from mesenchyme blastula-stage donor embryos were transplanted into the blastocoel of very early (pre-hatching) blastula-stage hosts (20-70 PMCs/host). Host embryos were allowed to develop for 5-5½ hours at 24°C, and fixed for whole-mount in situ hybridization. By this time, control embryos from the donor and host batches had developed to the mid-late gastrula and mesenchyme blastula stages, respectively. For isochronic (control) transplantations, PMCs isolated from mesenchyme blastula-stage donor embryos were transplanted into the blastocoel of same-stage recipients from which the endogenous PMCs had been removed.

To remove triradiate spicule rudiments, mid gastrula-stage embryos were immobilized in micromanipulation chambers (Ettensohn, 1990). A micropipette with a continuous flow of seawater was used to flush one of the two spicule rudiments and the associated PMCs out of the blastocoel. Manipulated embryos were allowed to develop until sibling embryos had reached the pluteus stage, when they were fixed as described for calcein-labelled embryos (see above).

Nickel experiments

Embryos were venterilized with NiCl2 as described by Hardin et al. (1992). Following fertilization, embryos were raised in 0.05-0.2 mM NiCl2 in IO until the desired stage and fixed for in situ hybridization. For cell transplantation, samples of sibling embryos were reared in parallel in the presence and absence of 0.1 mM NiCl2 until the mesenchyme blastula stage. At this stage, the nickel-treated embryos were
RESULTS

Analysis of skeletal rod elongation in vivo

In a previous study, elongation rates of the postoral and body rods were estimated by calculating changes in the mean lengths of these rods at different time intervals (Ettensohn and Malinda, 1993). Because this method involved comparing averages of measurements made from populations of embryos, we sought an alternative that would allow us to measure directly elongation rates in individual embryos. A simple and accurate method was to label spicules with calcein, a polyanionic derivative of fluorescein that binds Ca²⁺ and other divalent cations (Wallach et al., 1959) (Fig. 1A,B).

Based upon the results of calcein-labelling experiments, we constructed a 'rate map' of skeletal rod elongation (Fig. 1C). This map revealed several unexpected features of skeletal rod growth. The three arms of the triradiate spicule rudiments initially elongated at the same rate — about 6 μm/hour at 23°C. The three radii showed very different growth characteristics, however, as development proceeded. The radius that gave rise to the ventral transverse rod elongated for 8-10 hours (until the late prism stage), when its growth ceased. The radius that formed the dorso-ventral connecting rod extended toward the animal pole at a constant rate of 6 μm/hour, then branched to form the anterolateral and recurrent rods. Initially, the anterolateral rod continued to extend at about this same rate, although it elongated more rapidly late in embryogenesis. The third radius of the rudiment, the anonymous rod, extended only a short distance before branching to form the postoral and body rods. After branching, these rods more than doubled their rate of elongation. The body rod extended rapidly to the posterior apex of the prism stage embryo, slowing near the end of its growth as the branches of the scheitel began to form. The postoral rod continued to elongate rapidly throughout embryogenesis, reaching a maximum rate of 14-15 μm/hour.

The ventral transverse rods were the only branches of the skeleton that ceased to elongate during embryogenesis. The tips of these rods eventually meet at the ventral midline, suggesting that contact between the rods might restrict their growth. To test this hypothesis, we eliminated one of the two triradiate spicule rudiments (and the PMCs associated with it) at the mid-gastrula stage and examined the elongation of the remaining ventral transverse rod (Fig. 2). During the post-operative period, the half-skeleton on the operated side usually re-formed, at least partially. We excluded from our analysis embryos in which the regenerated ventral transverse rod came into contact with the ventral transverse rod on the unoperated side. In the remaining embryos (n=20), in three cases no skeleton re-formed on the operated side, and in the remaining 17 cases, the average separation between the tips of the ventral transverse rods at the late pluteus stage was 20.8 μm (s.d.=10.8 μm).

In these 20 embryos, the average length of the ventral transverse rods on the unoperated side was 1.2× the length of ventral transverse rods in control, sibling embryos (s.d.=0.2). The maximum length of the ventral transverse rods in control embryos is 55-60 μm; an additional 20% in length represents 11-12 μm of spicule. At an average growth rate of 5.6 μm/hour (Fig. 1C), about 2 hours would be required to deposit this much material. During normal development, however, other rods (e.g. the postoral rod) continued to elongate for at least 10 hours after the ventral transverse rods ceased their growth.

**Fig. 1.** The calcein-labelling method and a map of skeletal rod elongation rates. (A, B) Brightfield and epifluorescence micrographs of one postoral arm of an early pluteus larva that was incubated in calcein for 1 hour, followed by a 2 hour ‘chase’ in the absence of dye (see Materials and methods). Spicule elongation after the removal of calcein has created an unlabeled zone of material at the rod tip (indicated by arrowheads). Scale bar, 15 μm. (C) A map of skeletal rod elongation rates. The heavy black lines represent the skeletal rods of a two-armed pluteus larva. Numbers along the rods indicate the elongation rates (in μm/hour) of the rods at different stages of growth. Each value represents an independent measurement determined by calcein labelling, and data from six different batches of embryos were pooled. The position of each value (thin black lines) indicates the length of the rod at the mid-point of the corresponding chase period. Boxes show means and standard deviations of all rate measurements for each individual rod.
Even in the absence of its partner, therefore, the ventral transverse rod slowed or stopped its growth while other rods were still elongating rapidly. We concluded that although contact between the tips of the ventral transverse rods plays a limited role in restricting their length, other factors cause these rods to terminate at a relatively early stage of development.

Isolation of probes for molecular analysis: cloning of LvSM50 and LvSM30

In order to carefully compare the temporal and spatial expression patterns of several PMC markers in a species amenable to embryo micromanipulation, homologues of SM30 and SM50 were obtained in L. variegatus (Materials and Methods). Alignment of approx. 300 nucleotides of sequence information obtained from the L. variegatus homologue of SM50 with the L. pictus homologue (Livingston et al., 1991), and approx. 250 nucleotides with the S. purpuratus (Sucov et al., 1987; Katoh-Fukui et al., 1991) and H. pulcherrimus (Katoh-Fukui et al., 1992) homologues, showed nucleic acid sequence identities of approximately 90%, 70% and 70%, respectively. Alignment of approx. 300 nucleotides from the L. variegatus homologue of SM30 with the S. purpuratus homologue (George et al., 1991) showed nucleic acid sequence identities of approximately 70%. Based on these sequence similarities and the spatial and temporal expression patterns described below, we concluded that these cDNAs corresponded to transcripts encoded by the L. variegatus SM50 and SM30 genes, referred to as LvSM50 and LvSM30.

Expression of PMC-specific transcripts in L. variegatus

By northern blot analysis, the LvmSp130 probe recognized a transcript of approx. 4 kb, which was detectable by the hatched blastula stage (Fig. 3). Expression levels peaked at the mid gastrula stage and decreased through the late pluteus stage. By whole-mount in situ hybridization, expression was detectable in the vegetal plate of hatched blastula-stage embryos (Fig. 4A), prior to PMC ingress. All PMCs in the embryo appeared to express equal levels of LvmSp130 through the late gastrula stage (Figs 4C,D, 7). By the prism stage (Fig. 4E), however, a gradual reduction in transcript level had begun in

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**Fig. 2.** Microsurgical removal of one triradiate spicule rudiment. (A) Ventral view of a late gastrula-stage embryo immediately after microsurgery. One triradiate rudiment has been removed along with the associated PMCs (arrowhead), leaving a single spicule rudiment (arrow). Scale bar, 30 μm. (B) Pluteus larva derived from such an embryo. A half-skeleton, including a ventral transverse rod (arrowhead), has partially reformed on the operated side of the embryo. The ventral transverse rod on the unoperated side of the embryo (arrow) is only slightly longer than normal, even though the tips of the two ventral transverse rods remain well separated. Scale bar, 50 μm.

**Fig. 3.** (A) Northern blot hybridization showing expression of LvmSp130, LvSM50 and LvSM30 transcripts during development. A ubiquitin probe was used to determine the relative amounts of hybridizable RNA in different lanes (Gong et al., 1991). The bottom panel shows the 18S rRNA band on the ethidium bromide-stained gel. Size standards were based on the migration of rRNAs. HB, hatched blastula; MB, mesenchyme blastula; G, gastrula; PR, prism; PL, pluteus larva. (B) Quantitation of radioactive signals from blots shown in A. Values were corrected for relative amounts of hybridizable RNA/lane based on ubiquitin expression levels. Maximal values were normalized to developmental stages with the highest levels of expression of a particular mRNA. Error bars represent two standard deviations calculated based on counting efficiency during quantitation.
PMCs throughout the embryo except those located in the ventrolateral clusters. By the pluteus larva stage (Fig. 4I,J), transcript levels were highest in cells at the tips of the elongating anterolateral and postoral rods, while intermediate levels of expression were visible in cells in the scheitel. Expression of *Lvmsp130* transcript was no longer detectable in cells distributed along the shafts of the skeletal rods. Therefore, the decreased levels of expression at later stages apparent by RNA blot analysis appeared to be due to a reduction of transcript levels in only a subset of the PMCs.

The *LvSM50* transcript was approx. 1.6 kb in length (Fig. 3). Although very faint signal was detectable at the hatched blastula stage by northern analysis, levels increased significantly at the early mesenchyme blastula stage. *LvSM50* transcripts increased in abundance as development proceeded. By whole-mount in situ hybridization, *LvSM50* transcripts were first detectable in PMCs at the mesenchyme blastula stage (Fig. 5B). Like *Lvmsp130*, this transcript was initially expressed at approximately equal levels in all PMCs but showed reproducible, regional variations in expression at later developmental stages. Specifically, a distinct increase in abundance was exhibited by PMCs in the scheitel by the late pluteus stage (Figs 5E,F, 7). Expression is no longer detectable in PMCs along the shafts of the skeletal rods.

that was first detectable at low levels at the mesenchyme blastula stage (Fig. 3). Expression increased during gastrulation and through the late pluteus larva stage. By whole-mount...
in situ hybridization, we observed that, unlike *Lvmsp130* and *LvSM50*, which appeared to initially accumulate equally in all PMCs, *LvSM30* expression commenced in a subset of the PMCs. During early gastrulation (Figs 6A,B, 7), *LvSM30* transcripts accumulated specifically in PMCs located in the ventrolateral clusters of the forming ring. By the late gastrula stage, a striking pattern of expression in different regions of the PMC ring was visible (Fig. 6C,D). Cells in the dorsal chain (bold arrow, D), and ventral chain (arrowhead, D) express intermediate and undetectable levels, respectively. (E,F) Prism stage. High expression is maintained by PMCs in the ventrolateral clusters (arrows), especially those along the anonymous rods. PMCs in the ventral chain and near the tips of the longitudinal chains (arrowhead, E) do not express detectable levels of *LvSM30*. (G,H) Early pluteus (two focal planes of one embryo). High levels of expression are maintained by cells at the tips of the postoral arms (arrow, G). Most PMCs except those along the ventral transverse rods (arrowhead, G) show increased levels of expression. (J) Pluteus larvae showing the anal surface (I) and oral hood (J). High expression is exhibited by all PMCs except those along the ventral transverse rods (arrow, I).

**LvSM30 expression in embryos with altered numbers of PMCs**

To determine if the activation of *LvSM30* expression in the ventrolateral clusters was a result of the aggregation of PMCs at these sites, we examined the pattern of expression in embryos in which the number of PMCs had been altered microsurgically (Ettensohn, 1990). In PMC-deficient embryos (containing 3-30 PMCs; *n*=30), the highest expression levels were seen in the ventrolateral clusters, despite the reduced numbers of cells at these sites (Fig. 8A). In some embryos with very few PMCs, the positions of the ventrolateral clusters could not be determined. Regardless, we observed *LvSM30* expression in

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**Fig. 6.** *LvSM30* transcript expression during embryogenesis. For each developmental stage except pluteus, a lateral view and a view along the animal-vegetal (A/V) axis are shown. (A,B) Early gastrula. Expression is initiated in cells that are forming the ventrolateral clusters (arrow, B). (C,D) Late gastrula. PMCs in the ventrolateral clusters (arrows) express the highest levels of transcript. Cells in the dorsal chain (bold arrow, D), and ventral chain (arrowhead, D) express intermediate and undetectable levels, respectively. (E,F) Prism stage. High expression is maintained by PMCs in the ventrolateral clusters (arrows), especially those along the anonymous rods. PMCs in the ventral chain and near the tips of the longitudinal chains (arrowhead, E) do not express detectable levels of *LvSM30*. (G,H) Early pluteus (two focal planes of one embryo). High levels of expression are maintained by cells at the tips of the postoral arms (arrow, G). Most PMCs except those along the ventral transverse rods (arrowhead, G) show increased levels of expression. (J) Pluteus larvae showing the anal surface (I) and oral hood (J). High expression is exhibited by all PMCs except those along the ventral transverse rods (arrow, I).

**Fig. 7.** Summary of *LvSM30*, *LvSM50* and *Lvmsp130* transcript distributions at the late gastrula (left) and late pluteus (right) stages, based on whole-mount in situ hybridization data.
aggregates of as few as three cells. These observations indicate that clustering of PMCs is not necessary for the activation of \(LvSM30\) expression, at least to moderate levels. In PMC-supplemented embryos (containing 2-3 times the normal complement of PMCs, \(n=7\)), despite the greatly increased density of cells along the ventral chain, these cells did not express detectable levels of \(LvSM30\) (Fig. 8B,C). Therefore, high cell density is not sufficient to induce \(LvSM30\) expression.

**LvSM30 expression in embryos with ventralized ectoderm**

To test whether the spatial pattern of \(LvSM30\) expression was regulated by ectoderm-derived signals, we examined the expression of this gene in ventralized embryos. NiCl\(_2\) ventralizes the ectoderm; as a consequence, the arrangement of PMCs in the blastocoel is altered and the number of spicule rudiments is increased (Hardin et al., 1992; Armstrong et al., 1993). When embryos were raised continuously in NiCl\(_2\), both the patterning of the PMC ring and the expression of \(LvSM30\) were altered. In most NiCl\(_2\)-treated embryos, \(LvSM30\) expression was relatively uniform throughout the PMC ring (Fig. 9A). In some embryos, multiple, discrete clusters of cells expressing high levels of \(LvSM30\) were visible, with lower transcript levels exhibited by cells connecting these clusters (Fig. 9B). This pattern of expression was more common in embryos cultured in higher concentrations of nickel (e.g. 0.2 mM; not shown).

To determine whether the aberrant \(LvSM30\) expression pattern was due to an effect of nickel on the ectoderm or the PMCs, we examined \(LvSM30\) expression after transplanting nickel-treated PMCs into untreated, PMC-depleted host embryos. In such embryos, nickel-treated PMCs formed a ring and exhibited a pattern of \(LvSM30\) expression similar to that observed in control embryos (Fig. 9C-E). In 6/7 cases, the PMCs formed a ring in which two clusters and either the ventral and/or the dorsal chain were distinguishable. PMCs in the two ventrolateral clusters (Fig. 9C,D) exhibited the highest expression of \(LvSM30\). The dorsal chain (Fig. 9E), when present, exhibited faint to strong staining, whereas the ventral chain showed consistently low expression. As a control, a sample of nickel-treated donor embryos was allowed to continue development in IO in parallel with the experimental embryos. These donor embryos were ventralized and showed an aberrant pattern of \(LvSM30\) expression, as described above. These results indicate that nickel causes a misregulation of \(LvSM30\) expression by altering the ectoderm.

**Heterochronic cell transplantations**

Transplantation of PMCs into recipient embryos of different developmental stages has been used to provide evidence that aspects of PMC behavior are regulated by extrinsic cues (Ettensohn and McClay, 1986). We transferred PMCs from mesenchyme blastulae into younger embryos (pre-hatching blastulae) and examined \(LvSM30\) expression after 5-5½ hours, when sibling donor embryos had reached the mid-late gastrula stage and were expressing high levels of \(LvSM30\) mRNA. As a control for the transplantation procedure, PMCs were removed from mesenchyme blastulae and microinjected into recipient embryos of the same developmental stage from which all endogenous PMCs had been removed (isochronic controls).

In the combined results of three series of heterochronic transplants, in 23 of 40 experimental embryos, the PMCs
Table 1. Expression of LvSM30 by transplanted PMCs

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<th>Trial</th>
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<td>Time (hours)</td>
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**Notes:**
- Each trial denotes a set of experiments performed with a single batch of embryos.
- Period of incubation at 24°C following cell transplantation.
- Cases in which a PMC ring was clearly visible were additionally scored for the appearance of some degree of spatial regulation of LvSM30 expression (e.g. the highest level of expression was localized to at least one cluster).

showed no significant signal above background (Table 1, Fig. 10A). The remaining embryos contained 1-2 cells exhibiting signal above background (6/40) or more than two cells above background (11/40). We differentiated between these last two classes because the positively staining cells in the latter were clearly identifiable as PMCs and were usually in clumps, whereas the former were isolated and their identification as PMCs was ambiguous. Sibling donor embryos fixed at the same time exhibited a well-formed ring of PMCs that expressed high levels of LvSM30 (not shown). Sibling host embryos had reached the mesenchyme blastula stage, with PMCs that were not yet expressing detectable levels of LvSM30 expression (not shown).

In contrast to the low levels of LvSM30 expression observed following heterochronous transplantations, 26/27 isochronous control embryos exhibited more than two positive cells, although signal intensity ranged from just above background to very strong. In 17 of those embryos, a ring was distinguishable and the PMCs exhibited some degree of spatial regulation of LvSM30 expression, e.g., the highest degree of signal was localized to cells in at least one cluster (Fig. 10B). In the remaining cases, the presence of a ring could not be confirmed because of the orientation of the embryos (five cases), or the ring was incompletely formed and/or there were no differences in levels of staining in cells in different regions of the ring (four cases). We conclude that the delay in LvSM30 expression following heterochronous PMC transplantation cannot be attributed to the effects of microsurgery alone, since almost all of the isochronic controls showed detectable levels of LvSM30 expression after the same period of time.

**DISCUSSION**

**Local regulation of skeletal rod elongation rates and transcript expression patterns**

Our measurements using the calcein-labelling method show that rates of skeletal rod elongation are more tightly regulated during embryogenesis than was previously appreciated (e.g. Ettensohn and Malinda, 1993). At all stages of skeletogenesis, rates of spicule growth varied considerably in different regions of the PMC syncytium. These variations in growth rate are not simply attributable to differences in the numbers of PMCs associated with the various skeletal rods or to changes in PMC distribution during development (see Ettensohn, 1990; Ettensohn and Malinda, 1993). An alternative hypothesis is that growth rates reflect differences in the biosynthetic capacities of individual PMCs in different regions of the embryo. Consistent with this view, we found that three PMC-specific transcripts encoding gene products involved in skeletal synthesis showed dynamic, spatially regulated patterns of expression within the PMC syncytium.

We have described for the first time the spatial patterns of expression of the SM50 and SM30 mRNAs. Like msp130, these transcripts are expressed at the highest levels in regions of active skeletal rod growth, i.e. at the sites of the triradiate rudiments, the tips of the arm rods and/or the scheitel. When the LvSM30 expression pattern is compared with the map of skeletal rod growth, at least three additional correlations can be made. First, the initial formation of the spicule primordia within the two ventrolateral clusters is correlated with high levels of LvSM30 expression at these sites. Second, the lowest levels of LvSM30 mRNA expression are associated with the PMCs located along the ventral aspect of the subequatorial ring. Calcein labelling demonstrates that the ventral transverse rod, which extends along this chain of PMCs, is the only branch of the original triradiate spicule rudiment that ceases to elongate during embryogenesis. By removing one of the two triradiate spicule rudiments we have ruled out the possibility that the primary cause of this growth arrest is contact between the tips of the ventral transverse rods. Instead, it appears that the blastocoel wall at the ventral midline provides a relatively unfavorable local environment for spiculogenesis. Third, there is a substantial increase in the rate of elongation of the postoral

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**Fig. 10.** Heterochronic transplants. Expression of LvSM30 exhibited by PMCs in representative cases of embryos, classified in Table 1 as heterochronous transplants showing no positive cells (A) and isochronous transplants showing more than two positive cells (B). (A) In the majority of cases of heterochronous transplants, the cells did not exhibit any signal above background. (B) In nearly every case, isochronically transplanted PMCs were expressing LvSM30, frequently in a spatially regulated manner similar to their unperturbed late gastrula-stage siblings.
rod soon after it branches from the anonymous rod. At the same time, PMCs in this region of the syncytium show elevated levels of LvSM30 (as well as Lvmsp130) mRNA expression (Figs 4E,F, 6E).

It is not known whether the expression patterns of the proteins encoded by the LvSM30, LvSM50 and Lvmsp130 genes match those of the corresponding transcripts. Harkey et al. (1995) reported that in S. purpuratus, the msp130 protein is distributed evenly over the surfaces of all PMCs and spicules, in apparent contrast to the spatially regulated expression of the transcript (Harkey et al., 1992). It has recently been shown, however, that in S. purpuratus the SM30 protein is expressed at a reduced level along the ventral transverse rods, consistent with the pattern of mRNA expression we have described in L. variegatus (L. Urry and F. Wilt, personal communication). The PM27 and SM50 proteins also have nonuniform distributions in S. purpuratus, with relatively high levels of expression at the spicule tips (Harkey et al., 1995).

Non-uniform distributions of the LvSM30, LvSM50 and Lvmssp130 transcripts are established after the formation of the PMC syncytium (Hodor and Ettensohn, 1995). The patterns of mRNA localization we observed might, therefore, result from directional transport or trapping of mRNAs within the syncytium, in addition to possible local differences in rates of mRNA degradation and/or synthesis (see review by St Johnston, 1995). Our data indicate that the molecular mechanism responsible for the heterogeneous distribution of transcripts must be regulated by local cellular interactions.

Regulation of LvSM30 expression

Several features of LvSM30 expression suggest that this gene product may play an especially important role in skeletal morphogenesis. SM30 is the most abundant spicule matrix protein (F. Wilt, personal communication). As noted above, we found a close correlation between local levels of LvSM30 expression and skeletal rod growth in vitro. Other recent studies have shown that SM30 expression is also tightly coupled to spicule growth in vivo. In micromere cultures, cells that engage in overt synthesis of spicules express SM30 transcript and protein, while those cells that do not express this gene at detectable levels do not secrete spicule material (F. Wilt, personal communication). In contrast, SM50 is expressed by all cells in the same cultures.

Several lines of evidence suggest that the SM30 gene is regulated differently than SM50 and msp130. In both L. variegatus (this study) and S. purpuratus (George et al., 1991), SM30 mRNA accumulates distinctly later in development than the other two transcripts. Moreover, from the earliest stage that SM30 transcript can be detected by in situ hybridization, the gene appears to be expressed nonuniformly within the PMC population. In contrast, msp130 and SM50 transcripts are initially expressed at approximately equal levels in all PMCs, and show local modulations in expression only at later developmental stages. Lastly, SM30 expression in whole embryos is sensitive to treatment with β-aminopropionitrile (BAPN), an inhibitor of the post-translational processing of collagen, while SM50 expression is unaffected by this drug (Stebbins, 1994). This observation suggests that SM30 expression is regulated by extracellular matrix (ECM)-mediated cues. Recent studies using cultured micromeres have shown that expression of SM30, but not other PMC-specific markers, is highly sensitive to the presence of serum in the medium (X. Zhu and C. A. Ettensohn, unpublished observations). The cell transplantation experiments described in the present study provide direct evidence that short-range, ectoderm-derived cues regulate LvSM30 expression.

The regulation of skeletal morphogenesis

The morphogenesis of the skeletal system may be viewed as an interplay between intrinsic, developmentally programmed interactions between the PMCs and extracellular matrix, while those cells that do not express this gene at detectable levels do not secrete spicule material (F. Wilt, personal communication). The PM27 and SM50 proteins also have nonuniform distributions in S. purpuratus, with relatively high levels of expression at the spicule tips (Harkey et al., 1995).

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The regulation of skeletal morphogenesis

The morphogenesis of the skeletal system may be viewed as an interplay between intrinsic, developmental processes that regulate the molecular properties and behavior of the cells (Ettensohn et al., 1996). We propose that the early determinative processes that rigidly direct the progeny of the micromeres into a skeletogenic pathway of differentiation activate a basal, autonomous program of gene expression in this lineage. The basal program includes the initial activation of msp130 and SM50, genes encoding proteins involved in cell-cell fusion, and presumably other genes as well (Harkey et al., 1988; Stebbins, 1994; Hodor and Ettensohn, 1995). We suggest that the tight regulation of skeletal morphogenesis observed in vivo is achieved by coupling the expression of other genes, such as SM30, to extrinsic cues. A requirement of this model is that at least some of these signal-dependent genes encode proteins that are rate-limiting for skeletogenesis. The local, ectoderm-derived signals may be mediated by the ECM and may involve stimulatory factors analogous to those present in serum, EHS matrix or blastocoel fluid (Okazaki, 1975b; Benson and Chuppa, 1990; Kiyomoto and Tsukahara, 1991; Dolo et al., 1992; Page and Bénson, 1992). Later in embryogenesis, even those genes that are autonomously up-regulated by PMCs as part of the basal program come under local ectodermal influences, as indicated by the finding that all three PMC-specific markers show spatially regulated expression at later stages. According to this model, an important function of the basal, autonomous developmental program of the micromeres is to render the progeny of these cells receptive to external stimuli that confront them at later developmental stages. The layering of a program of signal-dependent gene activity onto an autonomous, lineage-based developmental program provides a means of coordinating the morphogenesis of the skeletal system with that of other tissues in the embryo.

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