Three *Strongylocentrotus purpuratus* actin genes show correct cell-specific expression in hybrid embryos of *S. purpuratus* and *Lytechinus pictus*

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Summary

The cell-specific expression of three actin genes from the sea urchin species *Strongylocentrotus purpuratus* was examined in hybrid embryos of *S. purpuratus* and another species, *Lytechinus pictus*, by *in situ* hybridization. The mRNAs from each of these genes displayed distinct spatial patterns of expression in late-stage hybrid embryos (constructed in either direction), being detected only in the cell lineages where they are normally found in *S. purpuratus* embryos (i.e. CyIIla, only in the aboral ectoderm lineage; Cyl, in the gut, oral ectoderm and some mesenchyme cells of plutei, and preferentially in the archenteron of gastrulae; M, only in two small clusters of cells near the esophagus in plutei). These results, together with our previous observation that expression of each of these genes is activated at the same stage in these hybrid embryos as in normal *S. purpuratus* embryos, demonstrate that the trans-acting factors which are necessary to regulate both the temporal and spatial expression of these genes are present in the hybrid embryos. Previous experiments have shown that the expression of a chimeric gene containing the CyIIla promoter fused to a bacterial chloramphenicol acetyltransferase (CAT) gene is not confined to the correct cell lineage (aboral ectoderm) when injected into *Lytechinus* embryos. The conclusion from these sets of data is that the factor(s) that regulate the spatial expression of at least one of the actin genes must derive from transcription of the zygotic genome.

Key words: actin genes, hybrid embryos, sea urchins, *in situ* hybridization, mRNA localization.

Introduction

We have demonstrated previously that the timing of accumulation of the mRNAs from at least four of the six functional actin genes of the sea urchin *S. purpuratus* is the same in hybrid embryos of *S. purpuratus* and *L. pictus* as it is in normal *S. purpuratus* embryos, and that these mRNAs accumulate to about normal levels in the hybrid embryos (Bullock et al. 1988). Several other studies have also shown that a wide variety of additional genes are expressed in hybrid embryos of different sea urchin species (Harding et al. 1954; Fedeka-Bruner et al. 1971; McClay & Hausman, 1975; Maxson & Egrie, 1980; Tufaro & Brandhorst, 1982; Crain & Bushman, 1983; Conlon et al. 1987). In most of the reported cases, it cannot be determined whether the expression of particular genes is regulated normally in the hybrid embryos, but the accurate timing and normal amounts of the *S. purpuratus* actin mRNAs in the *S. purpuratus* × *L. pictus* hybrids suggests that these genes are in these embryos. However, gene injection experiments have demonstrated that a chimeric gene containing the promoter of one of the *S. purpuratus* actin genes, CyIIla, fused to the bacterial CAT gene is not expressed in any particular cell lineage in plutei that develop from injected *Lytechinus variegatus* embryos (Franks et al. 1988), even though this gene construct contains the cis-regulatory elements necessary for aboral ectoderm-restricted expression when it is injected into *S. purpuratus* embryos (Hough-Evans et al. 1987). Furthermore, in these experiments the fusion gene was activated at the correct developmental stage in *L. variegatus* embryos despite its inaccurate spatial expression, suggesting that the temporal and spatial regulation of this gene are functionally separable and that the trans-acting elements present in the *Lytechinus* embryos are not capable of directing the correct spatial expression of this *S. purpuratus* promoter-driven gene. A question that emerges from the consideration of the hybrid embryo and gene injection experiments is whether the spatial expression of the CyIIla (and other) actin gene(s) is regulated correctly in hybrid embryos of *S. purpuratus* and *Lytechinus* species. If it is, then the trans-acting factors that are necessary for spatially restricted expression of this gene must derive from transcription of the *S. purpuratus* genome after fertiliz-
tion.

In this study, we have examined the cell-specific expression of the S. purpuratus CyIIa, CyI and M actin genes in hybrid embryos of S. purpuratus and L. pictus to determine whether their spatial expression is correctly regulated. We have found that the mRNA from each gene accumulates in hybrid embryos of S. purpuratus and L. pictus only in the same cells where they are normally found in S. purpuratus embryos when the hybrid embryos are constructed in either direction (i.e. S. purpuratus Q X L. pictus Q and L. pictus Q X S. purpuratus Q). It thus appears that all elements that are required for the correct temporal and spatial expression of these genes are present and active in these embryo hybrids, and that, at least for CyIIa, these elements result from transcription of the zygotic genome.

Materials and methods

Embryo culture

Normal S. purpuratus and L. pictus embryos were fertilized and cultured at 15°C in filtered sea water with gentle stirring according to standard procedures. S. purpuratus Q X L. pictus Q hybrid embryos were made by fertilizing S. purpuratus eggs that had been incubated overnight at 4°C in sea water with a 1:200 dilution of fresh L. pictus sperm. After fertilization, the embryos were washed three times with filtered sea water to remove excess sperm, and then cultured in sea water with 10 i.u. ml⁻¹ penicillin and gentle stirring at 15°C. The development of these embryos appeared normal up to the early gastrula stage, but arrested at that point. For the reciprocal cross, L. pictus Q X S. purpuratus Q, the L. pictus eggs were dejellied by gently centrifuging in a conical centrifuge tube. They were then resuspended in filtered sea water and solid trypsin was added to a concentration of 0.25 mg ml⁻¹ and the eggs allowed to settle. The eggs were then washed two cycles of settling and decanting, allowed to sit for one to two h at 15°C and were then fertilized and cultured as above. These embryos developed into morphologically distinct plutei.

Hybridization probes

The riboprobe vectors that were used to generate probes for CyI, CyIIa and M actin mRNAs were described previously (Cox et al. 1986) and were a gift from R. and L. Angerer. In all three cases, SP6 RNA polymerase was used to generate single-stranded RNA which contains a sequence that is complementary to a specific mRNA. Transcription of the CyI plasmid linearized with EcoRI yields a 1·1 kb RNA that is complementary to the last 517 nucleotides of the 3' untranslated portion of the CyI mRNA (Lee et al. 1986). Transcription of the CyIIa riboprobe vector linearized with EcoRI yields an RNA that is complementary to 131 nucleotides within the 3' untranslated portion of that mRNA (Lee et al. 1986). Transcription of M yields an approximately 1·7 kb RNA that is complementary to the last 250 nucleotides of the 3' untranslated region of that mRNA (Lee et al. 1986).

Riboprobe was synthesized at 40°C in a final volume of 0.020 ml containing 0.5 μg of linearized plasmid DNA, 0·5 mM-ATP, 0·5 mM-CTP, 0·5 mM-GTP, 150 μCi (9 μM) of 35S-UTP (Sp. act. 800 Ci mmol⁻¹, Amersham), 40 mM-Tris- HCl (pH 7·9), 6 mM-MgCl₂, 10 mM-dithiothreitol (DTT), 2 mM-spermidine, and 10 units of SP6 RNA polymerase. The template was removed by digestion with DNase RQ1 (Promega, 1 unit μg⁻¹ template) for 15 min at 37°C and the RNA was purified by phenol/chloroform extraction. Unincorporated nucleotides were removed by centrifugation through a 1 ml bed of Sephadex G-50, and the RNA was ethanol precipitated with 5 μg of tRNA as carrier. All transcripts were approximately the length of the DNA fragment inserted into their respective vectors. The riboprobes that were greater than 200 nucleotides in length were hydrolysed to approximately that size by limited alkaline hydrolysis (Cox et al. 1983). The 35S-labelled riboprobes were resuspended to 0·033 ng μl⁻¹ of solution per kilobase of sequence complexity in 0·6 M-NaCl, 10 mM-Tris pH 7·4, 0·5 mM-EDTA, 1 mg ml⁻¹ BSA, 0·02% PVP (w/v), 0·02% Ficoll (w/v), 150 μg ml⁻¹ yeast tRNA, 10% dextran sulphate (w/v), 20 mM-DTT, 50% formamide. The probes were heated at 90°C for 3 min before layering onto the slides.

In situ hybridization

In situ hybridization was performed using procedures developed in the laboratory of R. and L. Angerer (Angerer & Angerer, 1981; Cox et al. 1983) with modifications. The embryos were fixed in 1% glutaraldehyde for 20 min, and then embedded and sectioned as described by the Angerers except that tolueen was always used in place of xylene. After deparaffinization and hydration, the embryo sections were treated with 0·2 M-HCl followed by a 40°C 2 x SSC rinse. The sections were then digested with proteinase K (1 μg ml⁻¹) for 10 min and postfixed in 4% paraformaldehyde. The sections were then acetylated and dehydrated and overlayed with 60 μl of denatured probe (Specific activity = 1·3 × 10⁹ disintegrations min⁻¹ μg⁻¹) in the buffer described in the previous section for hybridization at 50°C. After hybridization, the slides were rinsed with four 20 min changes of 0·5 x SSC, 10 mM-dithiothreitol (DTT) at room temperature and two 60 min changes of 2 x SSC, 50% formamide, 10 mM-DTT at 50°C. The sections were then digested with 0·25 μg ml⁻¹ of RNase A in 0·9 M-NaCl, 10 mM-DTT for 30 min at 37°C and washed with the same buffer (without RNase A) for 15 min at 37°C, followed by washing with 2 x SSC, 50% formamide, 10 mM-DTT for 30 min at 50°C, and finally with 0·5 x SSC, 10 mM-DTT for 30 min at room temperature. The samples were then dehydrated in graded ethanol (2 min at 30%, 60%, 80%, 100%) and air dried before dipping the slides in autoradiography emulsion (Kodak NTB2) in complete darkness. The dipped slides were allowed to air dry for 2 h and were then stored at 4°C during autoradiographic exposure.

Results

Aboral ectoderm expression of the cytoskeletal actin gene CyIIa in hybrid embryos

It has been shown that expression of the zygotic transcripts of the S. purpuratus cytoskeletal actin gene CyIIa is restricted to the aboral ectoderm lineage of developing S. purpuratus embryos (Angerer & Davidson, 1984; Cox et al. 1986). To ask whether expression of this gene is confined to the cells of this lineage in hybrid embryos, we hybridized a CyIIa-specific riboprobe in situ to sections of hybrid embryos of S. purpuratus and L. pictus (Fig. 1). Because this probe does not hybridize with any RNA from L. pictus embryos (Bullock et al. 1988), it will specifically detect the S. purpuratus CyIIa mRNA. As discussed previously (Materials and methods) the L. pictus Q X S. purpuratus Q embryos developed to the pluteus stage,
while development of the *S. purpuratus* ♀ × *L. pictus* ♂ embryos arrested at the early gastrula stage. Because the lineage-specific expression of the CyIIIa gene (and the Cyl and M genes) is already evident by the early gastrula stage (Cox *et al.* 1986) the early arrest of the *S. purpuratus* ♀ × *L. pictus* ♂ embryos will not prevent detection of the spatially regulated expression of these genes. As seen in Fig. 1A,B, the *S. purpuratus* CyIIIa mRNA is detected in plutei of *L. pictus* ♀ × *S. purpuratus* ♂ embryos and is confined to the cells of the aboral ectoderm, as it is in *S. purpuratus* plutei. In the reciprocal set of hybrid embryos, *S. purpuratus* ♀ × *L. pictus* ♂, the *S. purpuratus* CyIIIa mRNA is again restricted to the aboral ectoderm lineage, being present only in the presumptive aboral ectoderm cells of early-gastrula-stage embryos (Fig. 1C). These experiments therefore demonstrate that the cell-specific expression of this *S. purpuratus* gene is accurately regulated in

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**Fig. 1.** Cell-specific expression of the *S. purpuratus* cytoskeletal actin gene CyIIIa in normal and hybrid embryos. A single-stranded, 35S-labelled riboprobe which is complementary to 131 nucleotides within the 3'-untranslated region of the CyIIIa mRNA was hybridized to sections of normal *S. purpuratus* embryos and hybrid embryos of *S. purpuratus* and *L. pictus*. (A) Normal *S. purpuratus* plutei and (B) *L. pictus* ♀ × *S. purpuratus* ♂ hybrid plutei. The autoradiographic exposure for these sections was 14 h. (C) A gastrula-stage embryo from the *S. purpuratus* ♀ × *L. pictus* ♂ cross. The autoradiographic exposure was for 2 days. In each panel, both phase-contrast and dark-field images of the same embryos are shown. The signal over the hybrid embryo in B is lower than that over the normal *S. purpuratus* embryo in A. In general, this was the case, to some extent, for all three hybridization probes reported in this study. However, because of differences in signal intensity over different embryos on the same slide, which result from variations in thickness of sections and other factors, the sections shown here are meant to represent only the qualitative results (i.e. localized expression of the different mRNAs).
hybrid embryos regardless of whether it is contributed by the egg or the sperm.

**Localized expression of the cytoskeletal actin gene Cyl in hybrid embryos**

The zygotic mRNA from the cytoskeletal actin gene Cyl begins to accumulate in early-blastula-stage embryos of *S. purpuratus* (Crain et al. 1981; Shott et al. 1984; Lee et al. 1986; Hickey et al. 1987; Bullock et al. 1988), and is distributed nonuniformly in the developing cell lineages; in late blastulae and early gastrulae, this mRNA is preferentially localized in cells of the vegetal pole and the invaginating archenteron and, in plutei, it is located in the gut, oral ectoderm and some mesenchyme cells (Angerer & Davidson, 1984; Cox et al. 1986). In situ hybridization with a riboprobe that distinguishes the *S. purpuratus* Cyl mRNA from those of other family members was used to determine the spatial expression of the *S. purpuratus* Cyl mRNA in hybrid embryos and in normal *S. purpuratus* embryos (Fig. 2). In plutei of *L. pictus♀ × S. purpuratus♂ embryos, the autoradiographic signal is located over regions of the gut and over the cells of the oral ectoderm (Fig. 2B). Although not evident in this particular embryo, we also sometimes see labelling over a small number of cells near the apex of the embryo, which Cox et al. (1986) concluded are probably secondary mesenchyme. The CyI mRNA is thus restricted to the same cell types in this hybrid as it is in normal *S. purpuratus* embryos (Fig. 2A) at this stage. In early gastrulae of the reciprocal cross, *S. purpuratus♀ × L. pictus♂*, the strongest signal is present over the cells of the invaginating archenteron (Fig. 2D), also as is seen in normal *S. purpuratus* embryos.

The CyI hybridization probe used in these experiments contains sequence which is complementary to the last 520 nucleotides of 3’-untranslated portion of CyI mRNA. In a previous study, we showed that this probe hybridizes to a related, but nonidentical, RNA present in *L. pictus* embryos which is probably the homologue of this cytoskeletal actin mRNA (Bullock et al. 1988). However, RNase protection experiments demonstrated that the amount of the *L. pictus* CyI-like mRNA is substantially depressed relative to the *S. purpuratus* CyI mRNA in both sets of hybrid embryos. While the relative abundance of these two RNAs was not quantified, inspection of the band intensities indicates that the difference is roughly in the range of five- to ten-fold. It is clear then that the majority of the in situ hybridization signal which is detected in the hybrid embryos is due to hybridization of the probe to the *S. purpuratus* Cyl mRNA.

**Cell-specific expression of the muscle actin gene in hybrid embryos**

The single muscle actin gene (M) in the *S. purpuratus* genome is expressed late in embryogenesis (Garcia et al. 1984; Shott et al. 1984; Lee et al. 1986), with its mRNA accumulating only in two small clusters of cells in the vicinity of the esophagus in plutei and in the archenteron wall in late gastrulae (Cox et al. 1986). These cells are probably part of the first differentiated muscle structures of the embryo. In situ hybridization with the *S. purpuratus* M gene-specific probe (which does not hybridize with *L. pictus* RNA, Bullock et al. 1988) yielded two tight clusters of grains on either side of the esophagus in plutei of *L. pictus♀ × S. purpuratus♂ embryos, the same as is seen in *S. purpuratus* embryos (Fig. 3). The in situ hybridization analysis of the early gastrulae of the reciprocal cross, *S. purpuratus♀ × L. pictus♂*, showed no signal above background anywhere within the embryo (not shown). This was also the case in normal *S. purpuratus* embryos at a similar early gastrula stage (not shown). Although we were unable to see cell-specific expression of the muscle actin gene in the *S. purpuratus♀ × L. pictus♂* embryos because they arrested before the gene is normally expressed, the lack of signal at the stage examined is the expected result if the mRNA is correctly expressed in the hybrid embryos. The localized expression of the muscle actin mRNA in *L. pictus♀ × S. purpuratus♂* plutei indicates that the cell-specific expression of this gene is correctly regulated at least in the hybrid embryos constructed in this direction.

**Discussion**

Previously we have shown that four different *S. purpuratus* actin mRNAs accumulate at the same stages in hybrid embryos of *S. purpuratus* and *L. pictus* as they do in normal *S. purpuratus* embryos (Bullock et al. 1988). Here we report further that at least three of these mRNAs are expressed in the same cell lineages of the hybrid embryos as they are in *S. purpuratus* embryos. Taken together these results demonstrate that the regulation of expression of these genes is entirely normal in the hybrid embryos regardless of whether they are contributed by the egg or by the sperm. The obvious implication of this conclusion is that the factors that are required to activate these genes and to restrict their expression to specific cells are present in the hybrid embryos. What is not obvious is where these factors come from. Two possibilities should be considered. The first is that there are homologous factors in the two species and that those from either species will regulate the expression of the *S. purpuratus* genes. The second is that only *S. purpuratus* factors will regulate expression of the *S. purpuratus* genes and they must therefore derive from transcription of the *S. purpuratus* genome after fertilization, at least in the *L. pictus♀ × S. purpuratus♂* hybrids. The results of recent gene injection experiments are important to consider when trying to discriminate between these two possibilities (Hough-Evans et al. 1987; Franks et al. 1988). In those studies, the promoter of the cytoskeletal actin gene CyIIa was attached to the bacterial CAT gene and the chimeric gene was injected into fertilized eggs of *S. purpuratus* or *Lytechinus variegatus*, which were then allowed to develop. In situ hybridization showed that the fusion gene was expressed only in the aboral ectoderm cells of the injected *S. purpuratus* plutei.
Actin gene expression in hybrid embryos

(Hough-Evans et al. 1987), indicating that the cis-regulatory sequences that are necessary for correct spatial expression of the CyIIIA gene were present, but that in the injected L. variegatus plutei the expression was not confined to any particular cell lineage (Franks et al. 1988). The incorrect spatial expression of the fusion gene in the Lytechinus embryos must then indicate that they lack at least one trans-acting factor which is required for correct spatial expression of this gene. However, the experiments presented here demonstrate that, when the S. purpuratus CyIIIA gene (as well as the Cyl and M actin genes) is introduced into L. pictus eggs along with the remainder of the genome (i.e. in the L. pictus ♀ × S. purpuratus ♂ hybrids), its mRNA accumulates in the same cell lineage as in normal S. purpuratus embryos. Assuming that there is no significant difference in the trans-acting factors of the two closely related Lytechinus species (with respect to their interaction with the S. purpuratus genes), we conclude that the trans-acting factor(s) that control the spatial expression of at least the CyIIIA gene derive from the products of transcription of the S. purpuratus genome.

Fig. 2. Cell-specific expression of the S. purpuratus cytoskeletal actin gene Cyl in normal and hybrid embryos. A single-stranded 35S-labelled riboprobe which is complementary to the last 520 nucleotides of the 3'-untranslated portion of the Cyl mRNA, was hybridized to sections of normal S. purpuratus embryos and S. purpuratus × L. pictus hybrid embryos. (A) Sections of S. purpuratus and (B) L. pictus ♀ × S. purpuratus ♂ plutei. Autoradiographic exposure was for 14 h in both cases. (C) Sections of gastrulae of S. purpuratus and (D) S. purpuratus ♀ × L. pictus ♂. The developmental stage of the S. purpuratus embryo in C is more advanced than that of the S. purpuratus ♀ × L. pictus ♂ embryo in D accounting for the slightly different pattern of localized expression of the Cyl mRNA. In each case, the mRNA distribution is the same as has been reported previously for the equivalent stage. Autoradiographic exposure was for 1-5 days for the S. purpuratus embryo in C and 3-5 days for the S. purpuratus ♀ × L. pictus ♂ embryo in D.
Fig. 3. Cell-specific expression of the *S. purpuratus* muscle actin gene M in normal and hybrid embryos. An $^{35}$S-labelled riboprobe which is complementary to the last 250 nucleotides of the 3'-untranslated region of the muscle actin mRNA was hybridized to sections of plutei of *S. purpuratus* (A) and *L. pictus* ♀ × *S. purpuratus* ♂ hybrids (B). Autoradiographic exposure was for 2 days in each panel.

after fertilization. Similar experiments, using hybrid embryos of *S. purpuratus* and *L. variegatus*, have also shown that the CyIIIa transcript is confined to the aboral ectoderm lineage, indicating that hybrid embryos constructed with either *Lytechinus* species give similar results (B. R. Hough-Evans, R. J. Britten and E. H. Davidson, personal communication). In addition to the spatial distribution of these mRNAs, other arguments, which are based on the timing of expression of the *S. purpuratus* and *L. pictus* CyI genes and the transcribed repeated sequence SURF1 in hybrid embryos, have also suggested that zygotically produced factors play a role in regulating the expression of these genes in embryos (Bullock et al. 1988).

The implications of these conclusions are important for understanding the origin of the factors that regulate the expression of these particular genes in embryos. It seems probable that the cell-specific expression of those genes examined, which exhibit spatially distinct expression beginning at the early blastula stage or later, is controlled by zygotically produced factors. If this is the case then the question becomes – how is the expression of the zygotically produced factor genes regulated? It is possible that another set of factors that are highly conserved between *S. purpuratus* and *L. pictus* are present in earlier stage embryos, which activate the expression of the late factor genes whose products regulate the genes examined here. Another possibility is that there is a more general and less-specific mechanism for activating the transcription of certain regulatory factor genes which acts early in embryogenesis, possibly as early as fertilization. According to this hypothesis, transcription of a set of sequences would be essentially automatically activated very early (probably by some generalized physiological change in the embryo) and their products (RNAs or proteins) would then regulate the expression of specific additional genes, which in turn might regulate others and so on.

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