Isolation of pigment cell specific genes in the sea urchin embryo by differential macroarray screening

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Summary

New secondary mesenchyme specific genes, expressed exclusively in pigment cells, were isolated from sea urchin embryos using a differential screening of a macroarray cDNA library. The comparison was performed between mRNA populations of embryos having an expansion of the endo-mesodermal territory and embryos blocked in secondary mesenchyme specification. To be able to isolate transcripts with a prevalence down to five copies per cell, a subtractive hybridization procedure was employed. About 400 putative positive clones were identified and sequenced from the 5′ end. Gene expression analysis was carried out on a subset of 66 clones with real time quantitative PCR and 40 clones were positive. This group of clones contained sequences highly similar to: the transcription factor glial cells missing (*gcm*); the polyketide synthase gene cluster (*pks-gc*); three different members of the flavin-containing monooxygenase gene family (*fmo*); and a sulfotransferase gene (*sult*). Using whole mount in situ hybridization, it was shown that these genes are specifically expressed in pigment cells. A functional analysis of the *S. purpuratus pks* and of one *S. purpuratus fmo* was carried out using antisense technology and it was shown that their expression is necessary for the biosynthesis of the sea urchin pigment echinochrome. The results suggest that *S. purpuratus pks, fmo* and *sult* could belong to a differentiation gene battery of pigment cells.

Key words: Sea urchin, Pigment cells, Mesoderm specification, SMC, Notch signaling, Macroarray

Introduction

A detailed understanding of the molecular and cellular mechanisms underlying pigment cell specification in sea urchin embryos will first require isolation of the genes responsible for this developmental process. In sea urchin embryos, cell fate specification occurs very early in development. By blastula stage, different embryonic territories can be identified: the small micromeres, the large micromeres or primary mesenchyme cell (PMC) precursors, the vegetal plate territory that will give rise to both the secondary mesenchyme cells (SMCs) and endoderm, the oral and aboral ectoderm (reviewed by Davidson et al., 1998; Angerer and Angerer, 2000; Ettensohn and Sweet, 2000). The central region of the vegetal plate is specified as SMCs, while the region around the SMCs is specified as endoderm (Ruffins and Ettensohn, 1993; Ruffins and Ettensohn, 1996).

SMCs give rise to four cell types: pigment cells, blastocoelar cells, circumesophageal muscle and coelomic pouch cells (Cameron et al., 1991; Ruffins and Ettensohn, 1996). Pigment cells are the first SMC type to migrate into the blastocoel at the early gastrula stage and by pluteus stage they are embedded in the ectoderm (Gustafson and Wolpert, 1967; Gibson and Burke, 1985; Kominami et al., 2001). The pigment produced by pigment cells is a naphthoquinone called echinochrome (McLendon, 1912; Kuhn and Wallenfells, 1940; Griffiths, 1965). The other SMC types generally delaminate from the archenteron tip during gastrula stage. Blastocoelar cells are fusiform cells wandering in the blastocoel (Burke, 1978; Tamboline and Burke, 1992). The muscle cells originating from the SMCs form the circumesophageal muscles (Burke and Alvarez, 1988). Coelomic pouches derive from SMC and small micromere descendents and they evert from the side of the archenteron tip at late gastrula (Gustafson and Wolpert, 1963).

SMCs are specified at the blastula stage during the eighth to tenth cleavage stages (Horstadius, 1973; Cameron et al., 1991; Ruffins and Ettensohn, 1993; Ruffins and Ettensohn, 1996; Sherwood and McClay, 1999; McClay et al., 2000). Several studies have proven that micromeres have the ability to induce the specification of the endo-mesodermal territory (Horstadius, 1939; Ransick and Davidson, 1993; Minokawa and Amemiya, 1999; Sweet et al., 1999; McClay et al., 2000). Notch (N) signaling from the micromeres to the SMC precursors has been proven to be necessary for the differential specification of presumptive SMC and endodermal territories (Sherwood and McClay, 1999; Sweet et al., 1999; McClay et al., 2000). It has also been shown that LiCl treatment of embryos, which expands both presumptive endoderm and mesoderm territories, shifts the boundary of the N receptor localization towards the animal half of the embryo (Sherwood and McClay, 1997). Moreover, the N ligand, Delta (Dl), has been recently shown to be expressed in PMC precursors during blastula stage and to activate the N signal to the SMC precursors (Sweet et al., 2002; Oliveri et al., 2002).

Only a few genes expressed in pigment cells have previously been isolated: the transcription factors *hmx* (Martinez and

Davidson, 1997) and *not* (Peterson et al., 1999), the actin-binding *profilin* (Smith et al., 1992; Smith et al., 1994) and an uncharacterized gene, *S9* (Miller et al., 1996). The expression of the known pigment cell genes begins at late blastula at the earliest, and their expression is not always restricted exclusively to the SMC lineage. In a recent study, a gene expressed in pigment cells and in other SMCs was identified from a cDNA library from late gastrula embryos (Shoguchi et al., 2002). It is not known yet if this gene is expressed earlier than late gastrula. In other recent studies, some pigment cell specific genes were identified through macroarray screening: the transcription factor *glial cells missing* (*gcm*) (Ransick et al., 2002), a cAMP-dependent protein kinase (*capk*), a dopachrome tautomerase-like (*dopt*) and *PI103*, a functionally uncharacterized gene (Rast et al., 2002).

In this work, a large-scale screen to isolate genes involved in the pigment cell specification was undertaken. To maximize the efficiency of the gene discovery, we screened a hatched blastula cDNA macroarray library of sufficient coverage to contain most expressed genes (Cameron et al., 2000) and we employed a subtractive hybridization procedure that allows the identification of low-prevalence transcripts (Rast et al., 2000). About 400 cDNA clones were isolated and sequenced. A group of these genes was characterized for temporal and spatial expression through real time quantitative PCR (QPCR) and whole-mount in situ hybridization. Six pigment cell specific genes were identified. Two of these genes were functionally characterized and the results suggest that they are required for the biosynthesis of the echinochrome pigment.

Materials and methods

Embryo culture

Gametes were collected from adult sea urchins (*S. purpuratus*) and after fertilization embryos were cultured at 15°C in filtered sea water containing penicillin 20 U/ml and streptomycin 50 μg/ml.

DnN mRNA injection and LiCl treatment

DnN mRNA was synthesized in vitro from the construct LvN^{neg} (Sherwood and McClay, 1999) using the mMessage mMachine kit (Ambion). Free nucleotides were removed through Sephadex-G50 Quick Spin Columns (Boehringer Mannheim). Injection was carried out as described previously (Mao et al., 1996) with 3-6 pg/zygote of mRNA (Sherwood and McClay, 1999). To check for the efficiency of the N signaling block, embryos were observed at mid-gastrula stage (36 hours), when SMCs delaminate from the archenteron tip in normal embryos. Pigment cell number was scored on pluteus stage embryos (72 hours). LiCl treatment was performed as described (Ransick et al., 2002). Embryos were collected at 19 hours (hatched blastula stage).

RNA isolation

Hatched blastula embryos were dissociated in RNA STAT-60 and total RNA was isolated following the manufacturer's procedure (Leedo Medical Laboratories). Polyadenylated RNA was isolated from total RNA with Poly-$T_{(25)}$ magnetic beads (Dynal, Lake Success, NY).

Selectate preparation

Selectate was prepared from LiCl-treated embryos. Polyadenylated RNA was isolated from 85 μg of total RNA with Poly-$T_{(25)}$ magnetic beads (Dynal, Lake Success, NY). The mRNA isolated was used for the synthesis of double-stranded cDNA as described by Rast et al. (Rast et al., 2000).

A linker was ligated to the cDNA to allow PCR amplification (Rast et al., 2000). A 600 to 800 bp cDNA was size selected by agarose gel electrophoresis. Two μl of the size-selectate cDNA was then amplified by 20 cycles of PCR using the biotinylated BT-LT7PRIMER and the linker primer. The (+)-strand cDNA selectate was prepared from the amplified cDNA as described elsewhere (Rast et al., 2000).

Driver preparation

5862 zygotes were injected with DnN mRNA to provide mRNA for the driver preparation and for the dnN unsubtracted probe preparation (see below). Polyadenylated RNA was isolated from total RNA with Poly-$T_{(25)}$ magnetic beads (Dynal, Lake Success, NY). Double-strand cDNA was synthesized from the isolated mRNA as described for the selectate. To allow PCR amplification, a linker, different from the one used in the selectate preparation, was ligated to the cDNA according to Rast et al. (Rast et al., 2000) but with the following exception: the short oligonucleotide used for the linker had a 3′ amino modifier C3 CPG instead of a dideoxycytidine. Half of the cDNA was amplified by six cycles of PCR under the following conditions: 94°C for 5 minutes followed by six cycles of 94°C for 30 seconds, 57°C for 1 minute and 72°C for 5 minutes, and a final step of 72°C for 10 minutes. The (–)-strand RNA driver was prepared from the amplified cDNA by transcription using a Megascript T7 RNA polymerase kit (Ambion, Austin, TX).

One quarter of the cDNA ligated to the linker was amplified by eight cycles of PCR using the same conditions described above. The amplified cDNA was used as template to synthesize the radiolabeled RNA driver probe for the macroarray filter hybridization (see below).

Subtractive hybridization

A pseudo-first order subtractive hybridization was performed with 100 ng of (+)-strand cDNA selectate and 5 μg of (–)-strand RNA driver in a 10 μl volume of 0.34 M phosphate buffer (PB) pH 6.8, 0.05% SDS at 65°C for 40 hours (driver $C_0t=2.40\times10^2$). Two bacteriophage λ single-stranded DNA sequences of 500 nt (λ_1 and λ_2) were each added to the selectate at a concentration equivalent to 5 copies/average embryo cell, as a control for the subtractive hybridization efficiency. The same linker and T7 sequences present in the selectate were added to the λ sequences.

After subtractive hybridization, hydroxylapatite (HAP) chromatography was performed to separate single strand sequences, unique to selectate, from double-strand sequences (common to selectate and driver). This was performed using a small-scale adaptation of the HAP chromatography described by Britten et al. (Britten et al., 1974). The chromatography bed volume was reduced to 200 μl in a water-jacketed column with internal diameter of 0.6 cm and a reservoir volume of 2 ml. The HAP chromatography was performed at 0.12 M PB, pH 6.8, 0.05% SDS at 60°C. Single-stranded nucleic acid was eluted with 6 ml of 0.12 M PB, pH 6.8, 0.05% SDS and double stranded with the same volume of 0.5 M PB, pH 6.8, 0.05% SDS. From tests done earlier it was observed that about 90% of the single stranded and double stranded nucleic acids were eluted in the first 2 ml. Therefore, 2 ml of the 0.12 M PB and 0.5 M PB elutions were desalted and concentrated with Centricon YM-30 centrifugal filter devices (Millipore, Bedford, MA) followed by drop dialysis on a 0.025 μm filter (Millipore, Bedford, MA). The single-stranded fraction was amplified by nine cycles of PCR and was used to synthesize a radiolabeled RNA probe (see below).

Probe preparation and macroarray cDNA library filter hybridization

A 20 hour cDNA macroarray library was spotted onto Hybond N+ nylon filters (Amersham, Pharmacia Biotech, Piscataway, NJ) by a QBot robot (Genetix, New Milton, UK).

The subtracted radiolabeled RNA probe was synthesized by transcription from the minimally amplified single stranded fraction using a MAXIscript T7 kit (Ambion, Austin, TX) in the presence of [α^{32}P]UTP. The unsubtracted RNA radiolabeled probes were prepared in the same way from 19 hour LiCl-treated and dnN-injected embryo

amplified cDNA. Between 2 and 2.5 μg of radiolabeled RNA probes with a specific activity of approximately 1×10^8 cpm/μg were obtained. Filter hybridization was carried out as described in Rast et al. (Rast et al., 2000). The same set of filters was used for the three hybridizations to eliminate the effect of colony growth differences between filters. Hybridized filters were exposed to phosphor screens for about 48 hours and then scanned at 100 μm resolution (Phosphorimager Storm 820; Molecular Dynamics, Sunnyvale, CA).

Macroarray filter analysis

Digital images of the hybridized filters were analyzed with the program BioArray (Brown et al., 2002). Normalization over background was calculated as the ratio between the spot intensity quantile 80 and quantile 20 values of each block.

Sequencing

The 5′-ends of the selected cDNA clones were sequenced using ABI prism BigDye Terminator Cycle Sequencing with an ABI 377 sequencer (Applied Biosystems, Foster City, CA). Clone 5′-end sequences were compared to the nonredundant GenBank using BLASTX (Altschul et al., 1997).

Real-time quantitative PCR

Real-time quantitative PCR (QPCR) was performed using the SYBR Green system (PE Biosystems, Foster City, CA) on an ABI 5700 Real-Time PCR machine (Rast et al., 2000). To measure the efficiency of the subtractive hybridization, normalized measurements of the prevalence of the λ sequences in the before and after HAP fractions were carried out as described previously (Rast et al., 2000).

After the macroarray screen, QPCR analysis was performed to verify that the positive clones were in fact differentially expressed. First strand cDNA was synthesized from LiCl-treated, dnN-injected and normal 19 hour embryo total RNA by random primed reverse transcription (TaqMan RT, Roche ABI). Primer sets were designed from the clone sequences. Reactions were prepared in triplicate using three to five embryo equivalents of cDNA per reaction. QPCR amplification conditions were: 95°C for 30 seconds, 57°C for 30 seconds, 60°C for 1 minutes, for 40 cycles. QPCR analysis was replicated using at least two different cDNA batches. Cycle threshold values (Ct) for each primer set were normalized to the ubiquitin Ct for each reaction. Ubiquitin expression is known to be approximately constant throughout gastrula stage (Nemer et al., 1991; Ransick et al., 2002).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described previously (Ransick et al., 2002; Arenas-Mena et al., 2000). When following the method of Arenas-Mena et al., a modified embryo fixation solution was used consisting of 4% paraformaldehyde, 32.5% filtered sea water, 32.5 mM MOPS pH 7 and 162.5 mM NaCl (T. Minokawa and E.H.D., unpublished).

Digoxigenin (DIG)-labeled RNA probes were synthesized by transcription. The *SpPks*, *SpFmo1*, *SpFmo2*, *SpFmo3* and *SpSult* DIG-labeled RNA probes were synthesized using as template the linearized plasmid of clone E62, D88, E64, E36 and B35, respectively. The resulting probe length was about 500 nucleotides for *SpPks* and *SpSult*, 1000 nucleotides for *SpFmo1* and 1500 nucleotides for *SpFmo2* and *SpFmo3*.

Antisense morpholino injection into zygotes

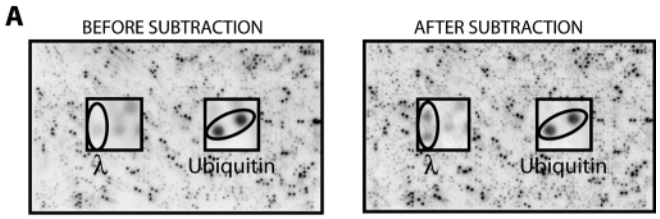
The antisense morpholino oligonucleotides for *SpPks* and *SpFmo1* were purchased from Gene Tools, Philomath, OR. The *SpPks* morpholino oligonucleotide sequence was 5′-AGCTGGTTTTATTG-CTTCCCATGTT-3′ and the *SpFmo1* was 5′-CATGCACACGTT-GCAGGAAAACTGG-3′. A random sequence morpholino oligonucleotide was injected in parallel as control. A 200 μM solution (2-4 pl) of morpholino oligonucleotide was injected into zygotes.

Results

Differential screening of blastula macroarray library to isolate pigment cell specific genes

In order to isolate the genes activated during pigment cell specification, a large-scale differential screening was undertaken using an *S. purpuratus* cDNA macroarray library made from hatched blastula stage embryos. The differential screening involved the comparison of the transcript population of LiCl-treated embryos, which have an expansion of the endo-mesodermal territory (Horstadius, 1973; Ransick et al., 1993; Sherwood and McClay, 1997), and dnN-expressing embryos, which are blocked in SMC specification (Sherwood and McClay, 1999). Overexpression of a dominant-negative form of the N receptor (dnN) practically eliminates the formation of pigment cells and severely reduces the formation of blastocoelar cells, circumesophageal muscles and coelomic pouch cells (Sherwood and McClay, 1997; Sherwood and McClay, 1999). Transcripts need to be present at 40-50 copies/average cell to be detected by complex probe hybridization on macroarray filters. Most transcripts though are present at lower prevalence in the sea urchin embryo, especially those encoding transcription factors (Davidson, 1986). By using subtractive hybridization technology, the complex probe was enriched for sequences that are differentially expressed, allowing the identification of transcripts expressed at about five copies per average cell (Rast et al., 2000). The subtractive hybridization reaction was carried out using a selectate prepared from LiCl-treated embryos, which contain the sequences of interest (the SMC specific transcripts) and a driver prepared from dnN-injected embryos, which lack those sequences. The subtractive hybridization procedure requires that the selectate specific sequences are practically absent in the driver. With the procedure employed, we expected to isolate genes involved in pigment cell specification more efficiently than those in the other three SMC types, because the latter are not completely absent in dnN-injected embryos that were used for the driver (Sherwood and McClay, 1999). The subtractive hybridization was performed with single stranded components and excess driver RNA. To measure the efficiency of the subtractive hybridization procedure, two different single strand bacteriophage λ sequences of 500 nucleotides were added at low concentration only to the selectate before subtraction. The subtractive hybridization reaction was followed by hydroxylapatite (HAP) chromatography to separate single strand from double strand sequences. The λ sequence enrichment achieved in the subtractive hybridization was about eightfold (Fig. 1B).

A set of 20 hour macroarrayed cDNA library filters was hybridized sequentially with the LiCl-treated unsubtracted probe, the subtracted probe and the dnN unsubtracted probe. Fig. 1A illustrates the digital image of a hybridized macroarray filter. As expected, the spot intensity value of the λ clones in the ‘before subtraction’ filter hybridization are at background level, but they became clearly visible in the ‘after subtraction’ hybridization (Fig. 1A). On the contrary, the spot intensity of clones corresponding to sequences common to both selectate and driver, such as ubiquitin, are not enhanced (Fig. 1A). After normalization to background, the ratio between each spot intensity value after and before subtraction was calculated to identify the clones with higher spot intensity after subtraction.



B

Clone	Before HAP	After HAP	Enrichment
	ΔCt Ubiq	ΔCt Ubiq	
λ_1	7.15	3.84	8.36
λ_2	7.13	3.89	8.00

Fig. 1. Differential macroarray screen. (A) Digital image of macroarray colony filters hybridized with a radiolabeled RNA probe synthesized from LiCl-treated embryos (Before subtraction) and with a radiolabeled RNA probe synthesized after subtractive hybridization (After subtraction). Before the subtractive hybridization, two different 500 nucleotide bacteriophage λ sequences (λ_1 and λ_2) were added to the selectate at a concentration equivalent to five copies/average cell and were also spotted onto the library filters. A magnification of the spots corresponding to the λ and ubiquitin clones are shown. The λ clones became detectable only after subtraction while the ubiquitin sequence, common to both selectate and driver, was not enriched after subtraction. (B) Table showing the level of enrichment obtained by the subtractive hybridization, measured by QPCR as enrichment of the λ sequences relative to the ubiquitin sequence. QPCR amplification of the λ and ubiquitin sequences was carried out on an aliquot of the subtractive hybridization reaction before HAP chromatography (Before HAP) and on the single-strand fraction after HAP chromatography (After HAP). The differences in cycle threshold (Ct) between the λ sequences and ubiquitin were calculated (ΔCt Ubiq); Ct values were means of triplicates. The enrichment values are the cycle amplification efficiency (1.9) raised to the power of the difference [(before HAP ΔCtUbiq)-(after HAP ΔCtUbiq)].

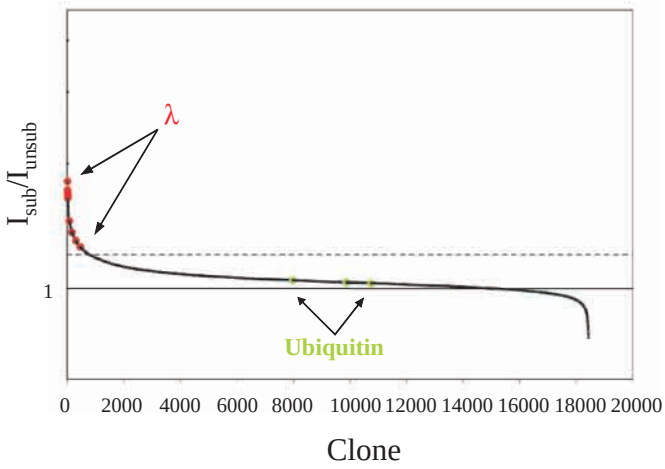


Fig. 2. Spot intensity enhancement of macroarray clones after subtractive hybridization. Data for one filter are shown as a representative example. On the *x*-axis are the clone numbers and on the *y*-axis are the ranked spot intensity ratios of the after (I_{sub}) and before subtraction (I_{unsub}) screen. To select the putative pigment cell specific clones, an enrichment threshold value (broken line) was determined based on the range of enrichment values of the 24 λ phage clones spotted onto each filter. Most clones with enrichment values below threshold are clones the spot intensity of which is very close to the background both before and after subtraction. The spot intensity values of the selected group of enriched clones were checked on the dnN (driver) screen. The clones for which spot intensity was above background were discarded as false positive.

These should be the putative pigment cell specific sequences. Fig. 2 contains a graph of the after/before subtraction spot intensity ratio of one filter (18,432 clones). In the end, 421 putative pigment cell specific genes were selected for further analysis.

Sequence analysis of selected clones

The 421 clones selected after macroarray filter analysis were sequenced from the 5′-end. About 83% yielded good quality sequence with a read length ranging from 500 to 700 bp (Table 1). The remainder of the clones (17%) either had sequences with GC-rich repeats that blocked the sequencing reaction or they contained inserts less than 200 bp. A BLASTX comparison of each clone sequence with the nonredundant GenBank database was carried out and about 30% of the clones showed a significant similarity with genes already identified (see Table S1 at http://dev.biologists.org/supplemental/). These genes encode proteins belonging to a wide range of categories: metabolic enzymes, transcription factors and co-factors; signal transduction proteins; nuclear non-transcriptional proteins; extracellular matrix proteins; protein belonging to the protein translation and protein degradation pathways; and cell movement proteins (see Table S1 at http://dev.biologists.org/supplemental/). The clones encoding genes similar to the polyketide synthase gene cluster (*pks-gc*), the flavin-containing monoxygenase (*fmo*) and the sulfotransferase (*sult*) were isolated multiple times (see Table S1 at http://dev.biologists.org/supplemental/). The sequence comparison of the 31 clones similar to the *pks* formed three contigs, which all perfectly matched to the sequence of a *S. purpuratus* BAC clone containing a *pks*-like gene (C.C. and E.H.D., unpublished). The five *fmo*-like clones fall into three classes according to gene sequence and they show orthology to separate vertebrate FMO family members. The nucleotide sequence identity among the three sea urchin genes is low (about 45%), indicating that they represent completely independent non cross-hybridizing isolates of the differential screen. The three *sulfotransferase*-like clones had the same sequence.

Table 1. Sequence analysis of putative pigment cell specific clones isolated from hatched blastula cDNA macroarray library

	Clone number (%)
Total sequences	421
Good quality sequence*	349 (83%)
Significant BLAST matches†	95 (27%)
Non significant BLAST matches	254 (73%)

*Readable sequences longer than 200 bp.
†BLASTX sequence comparison to the nonredundant GenBank database (E-value≤1×10^{-4}) at NCBI (http://www.ncbi.nlm.nih.gov).

Analysis of gene expression by real-time QPCR

Sixty-six of the 95 clones indicated in Table S1 (see

Table 2. QPCR analysis of putative pigment cell specific clones isolated from hatched blastula cDNA macroarray library

Gene	Abundance relative to Ub* (ΔCt)				
	LiCl	Norm	DnN	Norm-LiCl†	Norm-DnN‡
Pks (31)	7.4	7.7/4.7	10.3/7.2	0.3	**–2.6/–2.5**
	5.9/6.3/5.2	6.9/6.7/4.4	9.6/9.8/7.7	1/0.4/–0.8	**–2.7/–3.1/–3.3**
Sult (3)	7.7/7.0	7.4/7.2/4.7	10.1/9.8/7.6	–0.3/0.2	**–2.7/–2.6/–2.9**
Gcm (1)	9.1/5.2	7.6/4.6	10.7/8.1	–1.5/–0.6	**–3.1/–3.5**
Fmo1 (3)	nt	6/6.8	8.2/10	na	**–2.2/–3.2**
Fmo2 (1)	nt	7.5/6.7	10.0/9.1	na	**–2.5/–2.4**
Fmo3 (1)	10.4/8.4	10.0/9.5/7.2	12.6/12.0/10.8	–0.4/1.1	**–2.6/–2.5/–3.6**

Gene expression level of the putative pigment cell specific clones was analyzed on hatched blastula cDNA of LiCl treated, normal and dnN-injected embryos using real-time QPCR. In parenthesis are indicated the number of cDNA clones identified for each gene, within the group of sequences that showed significant BLASTX matches (E-value≤1×10^{-4}) in the nonredundant GenBank database.

*Values are the differences between Ct for each clone and Ct for ubiquitin in different cDNA batches; Ct values were means of triplicate measurements.

†Difference between column 3 and 2.

‡Difference between column 3 and 4. Values in bold are the differences between normalized Ct for normal and dnN-injected embryos that are significant (|Ct difference|≥1.6).

na, not applicable.

http://dev.biologists.org/supplemental/) were characterized further. Temporal and spatial analyses of gene expression of these putative positive clones were carried out, first using real-time QPCR and then whole-mount in situ hybridization. QPCR analysis using primer sets designed from the sequence available for each clone was a quick and efficient method to eliminate false-positive clones before carrying out whole-mount in situ hybridization on the embryos. The QPCR analysis data are shown in Table 2. QPCR amplification was performed on first-strand cDNA of LiCl-treated, dnN-injected and normal embryos at the hatched blastula stage. The genes positively regulated by N were *SpPks*, *SpSult*, *SpFmo1*, *SpFmo2*, *SpDimethylaniline monooxygenase* (similar to *fmo3*) and *SpGcm* (Table 2). Given that several clones were isolated for the *SpPks*, *SpSult* and *SpFmo* genes (see Table S1 at http://dev.biologists.org/supplemental/; Table 2) and taking into account that 29 clones were not tested, the percentage of differentially expressed clones was at least 42% on a total of 95 clones.

Pattern of gene expression analysed by whole mount in situ hybridization

Temporal and spatial expression analysis of *SpPks*, *SpFmo1*, *SpFmo2*, *SpFmo3* and *SpSult* genes was carried out using whole-mount in situ hybridization (Fig. 3). The *SpGcm* gene was also recently isolated in another differential macroarray screen for early endo-mesodermal regulators and its expression is described elsewhere (Ransick et al., 2002). At hatched blastula, all these genes are expressed in SMC precursors in a ring of about 20 cells around the PMCs (Fig. 3). At this stage, it is not possible to distinguish between the SMC precursor types by morphological observation of the embryo. At gastrula stage (38 hours) the expression of the *SpPks*, *SpFmo* and *SpSult* genes was detected in cells just beneath or embedded in the ectoderm. This is coincident with the distribution of pigment cells, which have been observed previously in *S. purpuratus* by immunofluorescence (Gibson and Burke, 1985). The expression of *SpPks*, *SpFmo* and *SpSult* genes was maintained throughout the pluteus stage in cells embedded in the aboral ectoderm, as is typical of pigment cells. According to a fate map of the SMC precursors constructed at the mesenchyme blastula stage, pigment cell precursors are excluded from the oral side, reflecting their ultimate distribution (Ruffins and Ettensohn, 1996). At mesenchyme blastula stage *SpPks* is expressed in a subset of SMC precursors forming an incomplete ring of cells (data not shown). *SpGcm* is expressed by 12 hours (early blastula) in a ring of cells immediately surrounding the PMCs (Ransick et al., 2002). At blastula and later at gastrula stages, *SpGcm* is expressed in the same pattern as *SpPks*, *SpSult* and *SpFmo* (Ransick et al., 2002).

Functional analysis of *SpPks* and *SpFmo1*

Embryos depleted of SpPKS and SpFMO1 were produced by antisense technology. The SpPKS and SpFMO1 knockdown embryos looked morphologically normal but did not show

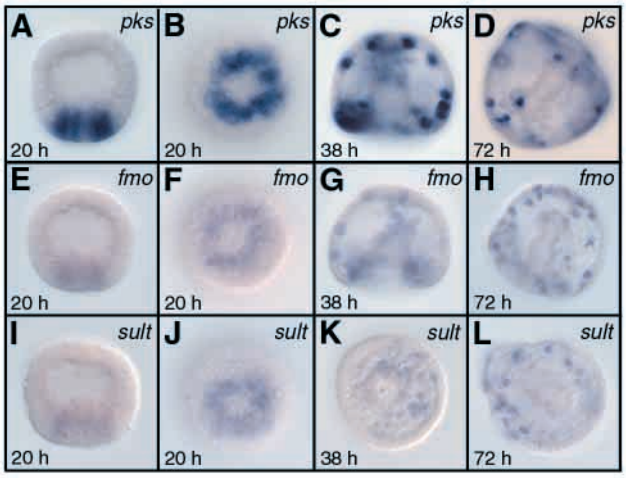


Fig. 3. Whole mount in situ hybridization of pigment cell specific genes. In situ hybridization with antisense RNA DIG-labeled probe for the *SpPks* gene (A-D), *SpFmo1* gene (E-H) and *SpSult* gene (I-L) on embryos at different developmental stages: hatched blastula side view (A,E,I) and vegetal view (B,F,J); gastrula side view (C,G) and apical view (K); pluteus oral view (D,H,L). In situ hybridization for *SpFmo2* and *SpFmo3* were also carried out (data not shown). All the genes examined are expressed in pigment cell precursors at the blastula stage and their expression is maintained in pigment cells throughout the gastrula and pluteus stages.

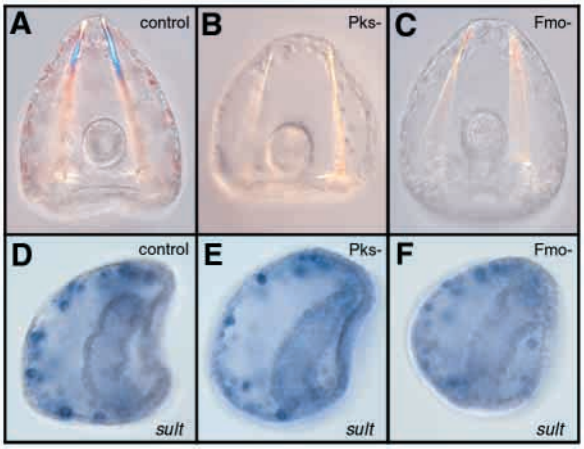


Fig. 4. SpPks and SpFmo1 antisense morpholino oligonucleotide injected embryos. (A-C) Aboral view of prism stage embryos injected with 200 μM antisense morpholino oligonucleotide against the *SpPks* (B) and the *SpFmo1* (C) genes and with a 200 μM random sequence morpholino oligonucleotide (A). The embryos injected with the *SpPks* and *SpFmo1* morpholinos did not show accumulation of the echinochrome pigment. (D-F) Whole mount in situ hybridization with antisense RNA DIG-labeled probe for *SpSult* on prism stage embryos injected with a random sequence morpholino oligonucleotide (D), or with the *SpPks* (E) or the *SpFmo* (F) morpholino oligonucleotides. (E,F) Pigment cells are present in embryos lacking SpPKS or SpFMO.

echinochrome accumulation in pigment cells up to 72 hours of development (Fig. 4A-C; Table 3). Later stages were not observed.

A whole-mount in situ hybridization for the pigment cell gene *SpSult* was carried out on pluteus stage embryos in order to determine whether the lack of pigment was due to a block of pigment biosynthesis itself or to the absence of pigment cells (Fig. 4D-F). The whole-mount in situ hybridization showed that pigment cells were indeed present in the embryos injected with the antisense morpholino oligonucleotide against *SpPks* or *SpFmo1* (Fig. 4E,F; Table 3). These results indicate that SpPKS and SpFMO1 are required for echinochrome biosynthesis.

Discussion

Isolation of SMC-specific genes

In this study we isolate sea urchin SMC specific genes, in particular pigment cell genes. A large-scale differential screening was performed using embryos with a block in N signaling and consequently in the SMC specification process (Sherwood and McClay, 1999). The SMC specific genes identified are highly similar to the transcription factor *glial cells missing* (*gcm*), to the *polyketide synthase* gene cluster (*pks-gc*), three members of the *flavin-containing monooxygenases* multigene family (*fmo*) and to a *sulfotransferase* (*sult*). All of the isolated genes were expressed exclusively in pigment cell precursors at blastula stage and their expression was maintained throughout the pluteus stage in pigment cells.

Through an antisense approach, we show that *SpPks* and *SpFmo1* are required for the biosynthesis of the echinochrome pigment (Fig. 4; Table 3).

Table 3. Effect of SpPKS and SpFMO1 downregulation on echinochrome accumulation in pigment cells

Percentage of embryos with pigment* (*n*)			
Uninjected	Control MASO	SpPks MASO	SpFmo1 MASO
100% (98/98)	100% (94/94)	1% (1/97)	0.8% (1/120)

*The *SpPks* and *SpFmo1* MASO-injected embryos showed a complete depletion of pigment in the whole embryo, while the control MASO embryos did not differ from the uninjected embryos

MASO, morpholino antisense oligonucleotide; *n*, number of embryos observed.

Data are percentages of embryos in two different plate cultures combined. The same results were obtained in separate injection sessions with different batches of embryos.

Glial cells missing (GCM)

gcm is a transcription factor that was first isolated from *Drosophila* (Akiyama et al., 1996). A second *gcm* gene, *gcm*/*glide2*, has been recently identified in *Drosophila* (Kammerer and Giangrande, 2001). Two Gcm genes have also been isolated in mammals, *gcm*1/*gcm*A and *gcm*2/*gcm*B (Akiyama et al., 1996; Kim et al., 1998). This appears to be the result of separate gene duplication events in arthropods and vertebrates (Kammerer and Giangrande, 2001). The *SpGcm* identified in this work is identical to the one isolated recently (Ransick et al., 2002) and so far no other *gcm* genes have been identified in the sea urchin. The highest region of similarity of *SpGcm* to the *Drosophila* and mammals *gcm* corresponds to the DNA-binding domain (Ransick et al., 2002), which recently has been shown to be a new type of zinc-coordinating DNA-binding domain (Cohen et al., 2002).

In sea urchin, *SpGcm* positively regulates the expression of *SpPks*, *SpSult* and *SpFmo* in pigment cell precursors (Davidson et al., 2002). In *Drosophila*, *gcm* was shown to be required for the development of hemocytes/macrophages (Bernardoni et al., 1997; Lebestky et al., 2000; Alfonso and Jones, 2002) and for the regulation of the binary decision of glial versus neuronal cell fate (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). In mammals, Gcm genes do not seem to be involved in the specification of glial cells, but they are required for placental development (Schreiber et al., 2000) and parathyroid gland development (Gunther et al., 2000).

This study shows that, in the sea urchin embryo, N signaling positively regulates *SpGcm* expression in pigment cell precursors, either directly or indirectly. N signaling has been shown to regulate *gcm* expression in some other developmental contexts. In *Drosophila*, N signaling activates *gcm* in the central nervous system (Udolph et al., 2001) and in the dorsal bipolar dendritic sensory lineage in the embryonic peripheral nervous system (Umesono et al., 2002). N signaling has instead a negative regulatory effect on *gcm* in the bristle lineage of the peripheral nervous system of *Drosophila* (Van De Bor and Giangrande, 2001). Many studies show that N signaling is a very conserved mechanism in the development of many metazoa and it is used in several different types of cell fate decisions. It is not surprising then that N signaling can have either a positive or a negative regulatory effect, even on the same target gene, depending on the combinations of regulatory factors present in any particular developmental context.

Polyketide synthase (PKS)

Polyketide synthases are large multifunctional enzymes involved in the biosynthesis of a wide range of polyketide compounds (for reviews, see Hopwood, 1997; Hopwood and Sherman, 1990; Staunton and Weissman, 2001). The sea urchin echinochrome synthesized in pigment cells is a naphthoquinone that has the characteristics of a polyketide compound. In terms of precursors and enzymes used, the polyketide biosynthetic pathway is very similar to the fatty acid biosynthetic pathway. A difference is that in fatty acid biosynthesis there is a complete reduction of the keto groups with the production of completely saturated carbon chains, while these remain unreduced in polyketides.

Polyketide compounds have been isolated mainly from bacteria, fungi and plants. Several polyketides synthesized in bacteria and fungi are antibiotics, such as erythromycin, rifamycin and actinorhodin, and several mycotoxins in fungi. Higher plants synthesize polyketides, called flavonoids, which have various functions: flower pigmentation, defense against pathogens (phytoalexins), response to UV light and visible light exposure, and symbiotic plant-pathogen interactions (Schroder et al., 1998; Winkel-Shirley, 2002). Polyketide compounds have also been isolated from some marine organisms: dinoflagellate unicellular algae, macro-algae, sponges and molluscs (Garson, 1989). In marine organisms, polyketides are often toxic compounds used as defense mechanisms against predators, e.g. the brevetoxins synthesized by the dinoflagellate *Gymnodinium breve* (Garson, 1989).

The *S. purpuratus* sequences isolated in this work showed the highest similarity to bacterial and fungal PKSs (see Table S1 at http://dev.biologists.org/supplemental/). A lower sequence similarity of the *S. purpuratus* genes was observed to the human and *C. elegans* fatty acid synthases (FAS; NCBI Accession Numbers NP_004095 and NP_492417) and to *C. elegans* PKS (NCBI Accession Number NP_508923). The *S. purpuratus* PKS sequences also showed lower similarities to some uncharacterized *Drosophila* proteins (CG3524, CG17374, CG3523) that are similar to the vertebrate FASs and fungal PKSs (NCBI Accession Number AAD43562, AAB08104). A *pks* gene cluster has not been identified in human.

Generally the Pks genes are organized in clusters and they contain very large ORFs. The different ORFs either together encode a very large multifunctional protein (type I PKS), or they encode separate proteins (type II PKS). The cDNA sequence contigs of the isolated clones show that the sea urchin *SpPks* belongs to the type I class. In most PKSs, the series of ORFs each encode domains with a unique catalytic function. The *S. purpuratus* PKS sequences are highly similar to the ketosynthase (KS), the acyltransferase (AT) and the alcohol dehydrogenase-zinc-dependent domains (ADH-zinc). This specific combination of catalytic domains is present in several PKSs, e.g. in the bacteria *Streptomyces coelicolor*, *Stigmatella aurantica* and *Nostoc* (NCBI Accession Number NP_ 630898, CAD19090, NP_486720). The PKS domains cooperatively catalyze the condensation of simple carbon units (acetyl, propionyl or butyryl units) to produce the polyketone linear chain, which is then further modified by other PKS domains and cyclized. A biochemical study by Salaque et al. (Salaque et al., 1967) showed that acetic acid molecules are used as precursors in the biosynthesis of echinochrome A in sea urchin. Thus, the SpPKS sequence similarity data together with the chemical structure of the echinochrome and the simple carbon units used for its biosynthesis suggest that SpPKS is directly involved in the biosynthesis of this pigment.

The function of sea urchin embryonic pigment cells is not completely understood. Considering that pigment cells are embedded in the epithelium of the larva and that polyketides are also photoactive compounds, it is possible that pigment cells have a role in photoreception. In support of this hypothesis are observations of light-induced alterations of pigment cell shape and pigment granule displacement within the pseudopodia in the sea urchin *Centrostephanus longispinus* (Weber and Dambach, 1974; Gras and Weber, 1977; Weber and Gras, 1980). Pigment granule translocation accompanied by cell shape changes have also been observed in dermal photoreceptors (melanophores) of amphibians and fish (Wise, 1969; Schliwa and Bereiter-Hahn, 1973). By considering the echinochrome chemical structure, it might have antibiotic properties as many polyketide compounds. Some evidence supporting this hypothesis has been provided by Service and Wardlaw (Service and Wardlaw, 1984). In addition, the morphology and behavior of pigment cells are, to some extent, similar to those of macrophages. Pigment cells have a stellate shape with two or three pseudopodia, which can be rapidly extended and contracted, and they have the ability to migrate within the larval epithelium and the basal lamina (Gibson and Burke, 1987).

Flavin-containing monooxygenases (FMOs)

Generally FMOs are NADPH-dependent flavoproteins that catalyze the oxidation of a wide variety of compounds containing nucleophilic heteroatoms. FMOs are involved in the detoxification of several xenobiotics and in the molecular activation of different kinds of metabolites.

FMOs are found in bacteria, higher metazoa (Hines et al., 1994; Gasser, 1996; Schlenk, 1998; Cashman, 2000; Ziegler, 2002) and in plants (Zhao et al., 2001; Tobena-Santamaria et al., 2002). In this work three different sea urchin Fmo genes have been isolated. Five members of the Fmo multigene family have been identified so far in mammals (for reviews, see Hines et al., 1994; Gasser, 1996; Cashman, 2000).

The mammalian FMO enzymes exhibit different species-, developmental- and tissue-specific expression, and different substrate specificity (Dolphin et al., 1996; Dolphin et al., 1998; Koukouritaki et al., 2002). In this work, we show evidence that supports an involvement of *SpFmo1* in echinochrome biosynthesis. Taking into account that the FMOs show different substrate specificity in other organisms, it could also be that *SpFmo1*, *SpFmo2* and *SpFmo3* are involved in different catalytic steps of echinochrome biosynthesis.

The human *FMO1*, *FMO2*, *FMO3* and *FMO4* are all localized on the same chromosome arm (Dolphin et al., 1991; Shephard et al., 1993; McCombie et al., 1996). This indicates a possible co-regulation of the Fmo genes. The three Fmo genes isolated in this work are expressed in the same cell type, the pigment cells, and their expression starts at the same time (at about 15 hours; C.C. and E.H.D., unpublished). These results suggest that the three SpFmo genes might also be co-regulated.

Sulfotransferase (SULT)

Sulfotransferases catalyze the sulfate conjugation of a broad

range of substrates leading either to their detoxification or bioactivation. Sulfotransferases also show different developmental and tissue-specific gene expression (Her et al., 1997; Dunn and Klaassen, 1998).

Sulfotransferases are present from bacteria to higher eukaryotes. In rats and humans they catalyze the sulfate conjugation of hormones, neurotransmitters and various drugs and xenobiotics (for reviews, see Falany, 1997; Weinshilboum et al., 1997). The *sult* gene identified in this work is expressed exclusively in pigment cells, suggesting that it could be involved in a specific function of pigment cells.

New insight into the pigment cell specification process

The molecular basis of pigment cell specification is largely unknown. N signaling at blastula stage was shown to be necessary for pigment cell specification as well as for the other SMC types (Sherwood and McClay, 1999; Sweet et al., 1999; McClay et al., 2000). The large-scale screening for SMC-specific genes carried out in this work allowed the isolation of six different pigment cell specific genes downstream of N, one transcription factor *SpGcm* and five enzymes, *SpPks*, *SpFmo1*, *SpFmo2*, *SpFmo3* and *SpSult*.

Pigment cells might be the only SMC type exclusively regulated by the N pathway through the Dl-N signaling at seventh to ninth cleavage. A study on the role of the N ligand Dl in mesoderm specification showed that Dl depletion in micromeres completely eliminates pigment cells, while muscle cells were normal and blastocoelar cells decreased by about 50% (Sweet et al., 2002). Muscle cell specification does not require micromere descendant Dl expression, but it does require mesenchyme-blastula Dl expression in the SMC precursors (Sweet et al., 2002). Blastocoelar cell specification requires Dl expression in both micromere descendants and mesenchyme-blastula SMC precursors, and probably also additional genetic inputs (Sweet et al., 2002). DnN-injected embryos are completely depleted of pigment cells but they show only about 40% depletion of blastocoelar, muscle and of coelomic pouch cells (Sherwood and McClay, 1999). It is possible that the dnN receptor is not sufficient to eliminate N signaling completely through the endogenous N receptor and that the alternative SMC type specification processes are differentially sensitive to N signaling, probably having partially different N downstream effectors. Another possibility is that the non-pigment SMC types are specified by a combination of N signaling and other genetic pathway inputs.

Current gene expression profiles suggest a relatively shallow regulatory pathway for pigment cell specification. The N ligand Dl is expressed in 7th cleavage (8 hours in *S. purpuratus*) micromere descendants (Sweet et al., 2002; Oliveri et al., 2002), about 2 or 3 hours before *SpGcm* starts to be expressed. In addition, recent data show that *SpGcm* is a direct target of N (A. Ransick and E.H.D., unpublished). It is also known that *SpGcm* positively regulates the expression of *SpPks*, *SpSult* and *SpFmo1* (Davidson et al., 2002). *SpGcm* begins to be expressed between 10 and 12 hours (Ransick et al., 2002), while *SpPks*, *SpFmo* and *SpSult* gene expression begins at about 15 hours (C.C. and E.H.D., unpublished). Further studies could soon bring to light all the genetic components of the pigment cell specification pathway. The differentiation gene battery at the end of this pathway should include the genes discovered here, *SpPks*, *SpFmo1*, *SpFmo2*, *SpFmo3* and *SpSult*.

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