INTRODUCTION

COUP-TFs are transcription factors that constitute a subclass (Qiu et al., 1994a) of the steroid-thyroid-retinoic acid superfamily of hormone receptors (Evans, 1988; Green and Chambon, 1988). Although much is known about their target genes in different animals, as well as their ability to form heterodimers with other receptors and suppress transcriptional activation (Qiu et al., 1994a), there is no cognate ligand found to date and, thus, these receptors are classified as orphans. Activation of COUP-TFs is possible in tissue culture cells, in a non ligand-dependent pathway probably through phosphorylation (Power et al., 1991). COUP-TF members are highly conserved in phyla belonging both to protostome and deuterostome animals (Qi et al., 1994a; Mlodzik et al., 1990; Wang et al., 1989; Chan et al., 1992; Matharu and Sweeney, 1992; Fjose et al., 1993; Lutz et al., 1994; Qi et al., 1994b). The extensive sequence similarity of these nuclear receptors has prompted a search for functional correlation in various species. The Drosophila melanogaster COUP-TF homologue, seven up (svp), is expressed in the larva nervous system and is implicated in cell fate determination of photoreceptor neurons (Mlodzik et al., 1990). The zebrafish COUP-TF is specifically expressed in the central nervous system (Fjose et al., 1993) and, in birds and mammals, the COUP-TFs are also expressed predominantly in the developing central nervous system and the embryonic motor neurons (Lutz et al., 1994; Qi et al., 1994b).

The SpCOUP-TF gene, isolated from Strongylocentrotus purpuratus, is a sea urchin homologue of this subfamily of orphan steroid receptors which shows an extensive sequence similarity to both vertebrate and invertebrate COUP-TFs. For example, the human COUP-TF I and SpCOUP-TF genes share 96% and 92% of the amino acids within the DNA- and hormone-binding domains, respectively (Chan et al., 1992). SpCOUP-TF was identified in sea urchin embryonic nuclear extracts (Chan et al., 1992) and shown to form specific complexes with a hormone response element (Niemeyer and Flytzanis, 1993), which is found in the upstream regulatory region of the ectoderm-specific CyIIIb actin gene (Flytzanis et al., 1989). Site-specific mutation of this SpCOUP-TF response element leads to aberrant expression of fusion gene constructs in developing transgenic sea urchin embryos. Thus, the mutant gene construct is expressed in cells belonging to all embryonic cell lineages, whereas the wild type is specifically expressed in the aboral ectoderm (unpublished results from this laboratory). It is evident that, in the developing sea urchin embryo, the SpCOUP-TF response element leads to aberrant expression of fusion gene constructs in developing transgenic sea urchin embryos. Thus, the mutant gene construct is expressed in cells belonging to all embryonic cell lineages, whereas the wild type is specifically expressed in the aboral ectoderm (unpublished results from this laboratory).

In order to elucidate the role of the maternal SpCOUP-TF mRNA in embryonic development, we began a study of its spatial distribution in sea urchin eggs and embryos. The results
presented herein reveal that the SpCOUP-TF mRNA is localized in the oocyte, egg and early embryo in a fixed position, which is lateral to the animal/vegetal (A/V) axis and 45° clockwise to the oral/aboral (O/A) axis of the embryos. The early zygotic SpCOUP-TF transcripts are present exclusively in the oral ectoderm (blastula and gastrula stage) and later in the neurogenic ciliated band of the pluteus larva. This study presents the first report of a localized maternal mRNA encoding a transcription factor in sea urchins with possible implications concerning the mechanisms of specification in this embryo.

MATERIALS AND METHODS

Sea urchin culture

Strongylocentrotus purpuratus and Lytechinus pictus adults were purchased from Pacific Biomarine (Long Beach, CA) and Marinus Inc. (Long Beach, CA). Lytechinus variegatus were purchased from Susan Decker (Florida). Gametes were collected by intracoelomic injection of 1 ml 0.5 M KCl. Eggs were washed by repeated settling in Millipore filtered sea water, fertilized and the embryos cultured in stirring vessels containing Millipore filtered sea water with penicillin (10,000 units/liter) and streptomycin (50 mg/liter).

Riboprobe preparation

A 510 nt fragment spanning the putative ligand-binding domain of SpCOUP-TF and a 600 nt fragment of the 3' untranslated region of the SpSHR2 cDNA were amplified and subcloned into the PCR™ II vector (Invitrogen). Clones for Spec1, early H2b histone and LpN1.2 were kindly provided by Drs William Klein, Rob Maxson and Gary Wessel, respectively. All clones were in vitro transcribed in the presence of digoxigenin-labeled UTP (Boehringer, Mannheim). The concentration of the SpCOUP-TF probe used for hybridization varied between 0.006 μg/ml and 0.2 μg/ml for different probe preparations, as estimated by incorporation of radioactive [32P]ribonucleotides in the in vitro transcription reaction. The SpSHR2 probe was used at a final concentration of 0.1 μg/ml. For hybridization to S. purpuratus embryos, the SpCOUP-TF probe was heated at 70°C for 5 minutes in hybridization solution before addition to the embryos.

Whole-mount in situ hybridization

In situ hybridization was carried out according to Harkey et al. (1992), with the following modifications: Eggs and early embryos fixed in glutaraldehyde solution (2.5% glutaraldehyde [Sigma], 0.14% NaCl, 0.2 M PB pH 7.3) were incubated for 5 minutes with 1 μg/ml (S. purpuratus) or 2.5 μg/ml (L. variegatus and L. pictus) Proteinase K in PBST (0.2 M PB, 0.15 M NaCl, 0.1% Tween-20, pH: 7.3). Following postfixation in 4% paraformaldehyde (Sigma) in PBST, and prehybridization at 50°C for at least one hour, embryos were mixed with the appropriate probes in hybridization buffer (50% Formamide, 10% PEG, 0.6 M NaCl, 5 mM EDTA, 20 mM Tris pH: 7.5, 500 μg/ml yeast tRNA, 2× Denhards, 0.1% Tween-20) and transferred to 100 μl glass capillaries. Hybridization was carried out at 50°C for 16 hours in a rotational hybridization oven. Removal of the unbound probe was achieved by extended washes with PBST (once at room temperature and several times at 50°C) and finally with 0.5× SSC (3 times for 30 minutes each at 60°C). Following a 30 minute blockage with 4% sheep serum in PBST, embryos were incubated for at least one hour with a dilution of 1:750 anti-digoxigenin Ab (Boehringer, Mannheim) in PBST containing 2% sheep serum. After several washes with PBST, embryos were conditioned with several washes in alkaline phosphatase buffer pH 8.0 (0.1 M Tris pH: 8.0, 0.1 M NaCl) containing gradually increasing concentrations of MgCl2 (10-50 mM). Staining was carried out in alkaline phosphatase buffer pH 9.5 (0.1 M Tris pH: 9.5, 0.1 M NaCl, 50 mM MgCl2) containing 1 mM Levamisol (ICN) and 6 μl/ml of each of the alkaline phosphatase color substrates A and B (Biord). Following color development for an empirically set length of time, the reaction was terminated with two washes in PBST plus 1 mM EDTA and the cells were dehydrated and processed for microscopic observation as described by Harkey et al (1992).

RESULTS

SpCOUP-TF mRNA is localized in the oocyte and egg cytoplasm

Detection of the maternal SpCOUP-TF transcripts in the eggs of Strongylocentrotus purpuratus was achieved by whole-mount in situ hybridization, using a 510 nucleotide long antisense RNA probe, which spans part of the putative ligand-binding domain of the SpCOUP-TF-coding sequence. The detected hybridization signal is distributed unevenly. A region of the egg close to the cortex is intensively stained with the signal diminishing towards the opposite side (Fig. 1B). The specificity and fidelity of the whole-mount in situ hybridization technique for sea urchin eggs was demonstrated with the use of different probes, complementary to three different maternal mRNAs of S. purpuratus. In contrast to the localized SpCOUP-TF transcripts (Fig. 1B), the transcripts of another sea urchin orphan steroid hormone receptor, SpSHR2 (Kontogianni and Flytzanis, unpublished data), are evenly distributed throughout the egg (Fig. 1C). The transcripts of the early histone H2b gene are localized in the pronucleus of the mature egg (Fig. 1D) as previously shown (Maxson et al., 1983; DeLeon et al., 1983), whereas the sense SpCOUP-TF RNA hybridization probe does not detect any transcripts (Fig. 1E). A few oocytes were often included in the egg samples that were used for in situ hybridization. As Fig. 1A shows, the SpCOUP-TF transcripts which are synthesized during oogenesis are also localized in one side of the oocyte towards the cortex region.

SpCOUP-TF mRNA is localized in a fixed position in regard to the A/V and O/A embryonic axes

The position of the highest mRNA concentration relative to the
animal-vegetal (A/V) axis of the egg has been indirectly determined. There are no molecular markers to date that could be used to answer this in a direct way i.e. by in situ hybridization or by immunocytochemical detection. Assuming that there is no major redistribution of the mRNA after fertilization, the SpCOUP-TF mRNA localization in the cleavage stage embryos (2- to 16-cell stage), where the A/V axis is morphologically evident, should reveal its relative position within the egg. This assumption is validated by our observation that drastic changes in the concentration profile of the localized transcripts do not occur during early cleavage, i.e. it seems that the anchored SpCOUP-TF mRNA is not released from its position in the egg shortly after fertilization.

In situ hybridization experiments carried out with 2-, 4-, 8- and 16-cell-stage embryos of three sea urchin species, Strongylocentrotus purpuratus, Lytechinus pictus and Lytechinus variegatus emphasize the unequal distribution of the maternal SpCOUP-TF mRNA in the embryonic blastomeres, and also the species-specific positioning in regard to the cleavage planes. The Lytechinus COUP-TF homologous genes have not been characterized yet, but because of the extensive sequence homology within the COUP-TF subclass, their transcripts are

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**Fig. 2.** Segregation of maternal COUP-TF mRNA during cleavage. Whole-mount in situ hybridization of digoxygenin-labeled antisense SpCOUP-TF probe to eggs and cleavage stage embryos of *L. variegatus* (A-E) and *S. purpuratus* (F-J). (A,F) eggs; (B,G) 2-cell-stage embryos; (C,H) 4-cell-stage embryos; (D,I) 8-cell-stage embryos; (E,J) 16-cell-stage embryos. The eggs, 2-cell and 4-cell-stage embryos are depicted with the A/V axis perpendicular to the picture plane, whereas the 8-cell and 16-cell-stage embryos are arranged with the embryonic animal pole towards the top of the picture.

**Fig. 3.** Spatial restriction of COUP-TF mRNA in early and late embryos. *S. purpuratus* blastula (A), gastrula (B) and pluteus (C) were hybridized with the SpCOUP-TF antisense probe. As control, *S. purpuratus* plutei were hybridized with the Spec1 antisense probe (D) and the SpCOUP-TF sense probe (E). *L. pictus* plutei were hybridized with the SpCOUP-TF antisense probe (F), the LpN1.2 antisense probe (G) and the SpCOUP-TF sense probe (H). oe, oral ectoderm; cb, ciliated band; a, anus; g, gut; ae, aboral ectoderm.
expected to hybridize to the \textit{S. purpuratus} probe. It is evident from our results (Fig. 2A–E) that COUP-TF maternal transcripts are asymmetrically distributed in the eggs and early embryos of \textit{L. variegatus}. Following the first cleavage, the highest COUP-TF mRNA concentration is found in one of the two blastomeres at a 45° angle to the cleavage plane (Fig. 2B). After the second cleavage, the majority of the transcripts are found in one of the four cells with lesser amounts present in the two neighboring cells, whereas the fourth cell is depleted of transcripts (Fig. 2C). These results suggest that the location within the egg that shows the highest concentration of COUP-TF mRNA is not along the first cleavage plane (i.e. the A/V axis), nor is it along the second cleavage plane which forms at a 90° angle to the first one. The \textit{L. variegatus} COUP-TF transcripts are localized at a 45° angle relative to the two dissecting cleavage planes, at a position between the animal and vegetal poles, at one side of the embryo, i.e. laterally to the A/V axis of the egg. This position is at a 45° angle to the first cleavage plane and consequently to the oral-aboral (O/A or ventral-dorsal) axis, which in the genus \textit{Lytechinus} coincides with the first cleavage plane (Henry et al., 1992). The same maternal COUP-TF mRNA localization results were also obtained with eggs and embryos of the pacific sea urchin \textit{L. pictus} (data not shown).

In \textit{S. purpuratus}, the first cleavage plane which also runs along the A/V axis forms two cells; one that contains the majority of the SpCOUP-TF mRNA, but with the highest concentration at a 90° angle to the cleavage plane and one that has only a fraction thereof (Fig. 2G). The second cleavage, which occurs at a 90° angle to the first one and passes also through the A/V axis, separates the four blastomeres into two that have seemingly equal amounts of the SpCOUP-TF transcripts and two which are devoid of them (Fig. 2H). Two cell divisions later, at the 16-cell stage, the SpCOUP-TF mRNA is detected in four of the eight animal cap mesomeres and in two of the four vegetal macromeres (Fig. 2E,J). The extreme vegetal micromeres are not stained suggesting that the localization of the mRNA does not extend to the vegetal pole of the egg and embryo. Therefore, as shown for the \textit{Lytechinus} embryos, the maternal SpCOUP-TF mRNA is also localized in \textit{S. purpuratus} at a lateral position relative to the A/V embryonic axis.

The second embryonic axis, O/A, is set in \textit{S. purpuratus} after the first cleavage at a 45° angle clockwise to the cleavage plane (Cameron et al., 1989). As observed for \textit{Lytechinus}, in \textit{S. purpuratus} the position of the SpCOUP-TF mRNA’s highest concentration is also found at a 45° angle relative to the O/A axis (90° clockwise to the first cleavage plane). It is thus evident that, in all three sea urchin species studied, the localization of the COUP-TF maternal mRNA within the egg and cleavage stage embryo is not random. Furthermore, although not aligned with the embryonic axes, it is found at a fixed position relative to them, i.e. always lateral to the A/V axis and at 45° angle to the O/A axis.

The localization pattern of the maternal COUP-TF mRNA was analyzed in about 3000 eggs, 2-cell and 4-cell stage embryos of all three sea urchin species. These experiments have been repeated many times with similar results. The percentage of eggs that stained asymmetrically was 77%, 91% and 93% for \textit{S. purpuratus}, \textit{L. pictus} and \textit{L. variegatus}, respectively. Since the embryonic axes are not morphologically evident in sea urchin eggs, in order to orient the position of the maternal COUP-TF transcripts in regards to these axes, we present graphically the 2-cell-stage embryo. As Table 1 shows, in the large majority of embryos that stained asymmetrically (67–88%), the localization of the maternal SpCOUP-TF mRNA is fixed at a 45° angle to the O/A axis. In 2-cell \textit{S. purpuratus} embryos, there are two possible positions, marked with numbered arrowheads in Table 1, where the maternal SpCOUP-TF transcripts could be found. Either position is 45° clockwise to the O/A axis. In \textit{Lytechinus} embryos, our results can not determine at present whether the COUP-TF transcripts are localized clockwise or counterclockwise to the O/A axis because the two reference points - first cleavage plain and O/A axis - coincide. Thus, in the latter embryos, the COUP-TF transcripts could be localized at any of four possible positions (Table 1). In each experiment, there is a minority of embryos that, although they are also asymmetrically stained, the position of the SpCOUP-TF transcripts does not follow the principal localization pattern. These embryos, which show a deviation of 45° compared to the majority pattern, may express a genetic variability in the positioning of the SpCOUP-TF mRNA.

Analysis of SpCOUP-TF in situ hybridization pattern in 117 16-cell-stage \textit{S. purpuratus} embryos shows that, in the vast majority (106/117 embryos or 91%), the SpCOUP-TF mRNA

\begin{table}[h]
\centering
\caption{The position of maternal COUP-TF mRNA is fixed relative to the embryonic axes in 2-cell-stage sea urchin embryos}
\begin{tabular}{llll}
\hline
 & \textit{Sp} & \textit{Lp, Lv} \\
\hline
2-cell & & \\
\hline
45°: & 78% & 45°: & 69%, 63% \\
Other: & 22% & Other: & 31%, 37% \\
\hline
4-cell & & \\
\hline
45°: & 70% & 45°: & 61%, 75% \\
Other: & 30% & Other: & 39%, 25% \\
\hline
\end{tabular}
\end{table}

Analysis of the in situ hybridization pattern in eggs, 2- and 4-cell-stage embryos shows that, in all three species, the maternal COUP-TF mRNA is localized lateral to the A/V and at a 45° angle to the O/A axis. The percentiles refer to asymmetrically stained embryos, which varied from 67 to 88% of the total number of stained embryos. The remaining 12–33% of embryos were homogeneously stained. These percentiles were derived from the analysis of one experiment that included 128 2-cell and 119 4-cell-stage \textit{S. purpuratus}, 357 2-cell and 436 4-cell-stage \textit{L. pictus} and 40 2-cell and 19 4-cell \textit{L. variegatus} embryos. These experiments using the three sea urchin species were repeated many times with similar results. Other rare localization patterns include staining along the A/V or O/A axis, or localization of the SpCOUP-TF mRNA at other than 45° angle to the O/A axis. In each experiment, a small percentage of eggs and embryos remained unstained. The small black square denotes the A/V embryonic axis, which is assigned a perpendicular position to the drawing plane. The possible positions of the COUP-TF mRNA in the 2-cell-stage embryos relative to the cleavage planes and the embryonic axes, two for \textit{S. purpuratus} and four for \textit{L. pictus} or \textit{L. variegatus}, are designated by the arrowheads.
inherited by blastomeres located at one side of the embryo, lateral to the A/V axis. Half of the animal cap mesomeres (4 cells) and half of the vegetal macromeres (2 cells) contain SpCOUP-TF mRNA (Fig. 2E,J), whereas the most vegetal micromeres are devoid of transcripts (only in 9% of the 16-cell-stage embryos were some positive micromeres detected). The number and position of the positive blastomeres of the 16-cell-stage S. purpuratus embryo suggest that these cells belong primarily to ectodermal precursors (oral, aboral or both) and possibly endodermal, since the SpCOUP-TF mRNA is also found in two of the macromeres.

**SpCOUP-TF mRNA expression is confined to the oral ectoderm and the ciliated band of late embryos**

In blastula stage embryos, the SpCOUP-TF mRNA is exclusively present in the ectodermal cells on one side of the blastocoel wall, the presumptive oral side, whereas the cells of the vegetal plate are negative (Fig. 3A). It is safe to assume that, at this stage, the detected transcripts comprise, at least to some extent, newly synthesized embryonic mRNA. The assignment of the SpCOUP-TF-expressing cells to the blastula presumptive oral ectoderm is based on the localization of the transcripts in the definitive oral ectoderm at the following gastrula stage (Fig. 3B). At the gastrula and later stage embryos, we assume that the detected transcripts are exclusively embryonic and that the maternal mRNA has been turned over as previously shown for most maternal RNAs (Flytzanis et al., 1982). At the pluteus stage, the SpCOUP-TF mRNA is clearly present in the ectodermal cells that constitute the ciliated band and the supra-oral ectoderm in both *S. purpuratus* and *L. pictus* (Fig. 3C,F). The embryonic cell lineage of the ciliated band is the product of cell interactions between the progeny of blastomeres that contribute to both oral and aboral ectoderm (Cameron et al., 1993). As shown for the gastrula embryo, at pluteus stage too, the expression of SpCOUP-TF is exclusive to these cells and all other mesoderm, endoderm and aboral ectoderm derivatives are negative. Most intense is the signal over the region of the ciliated band that includes the anal arms in *S. purpuratus* (Fig. 3C) and the anal arms and the oral hood in *L. pictus* (Fig. 3F).

It is possible that the *SpCOUP-TF* gene is not activated in the entire cell lineage of the ciliated band and there may be species-specific differences to the extent of embryonic expression within this tissue. The specificity of the in situ hybridization is demonstrated at the different stages by using the sense RNA probe which does not generate any hybridization signal (Fig. 3E,H) and by probes specific for other marker genes. Thus, the Spec1 (Hardin et al., 1985) antisense RNA has been used as a probe for aboral ectoderm-specific hybridization in *S. purpuratus* embryos (Fig. 3D) and the LpN1.2 (Wessel et al., 1989), antisense RNA as an endoderm-specific probe in *L. pictus* embryos (Fig. 3G). The embryonic expression pattern of SpCOUP-TF correlates with the postulated suppressor activity of this transcription factor, i.e. the target aboral ectoderm-specific *Cyllib* actin gene (whose spatial expression pattern is similar to the *Spec1* gene in Fig. 3D) is inactive in the ciliated band.

**DISCUSSION**

Preformed egg polarity, followed by asymmetric distribution during cleavage of developmentally important molecules such as growth and transcription factors, is considered to be the mechanism by which initial assignment of different fates is established in the early embryo (Wilson, 1925; Davidson, 1986; Melton, 1987; Nüsslein-Volhard et al., 1987). Hördstadus (1939) interpreted his results of manipulated sea urchin embryos by the theory of opposing gradients of morphogens within the sea urchin egg (Child, 1916; Runnström, 1975), which he considered responsible for the early cell lineage specification. Recently, however, Wilt (1987) and Davidson (1989) proposed (1) that such opposing gradients need not exist in order to explain the classical or more recent experiments of manipulated sea urchin embryos and (2) that the undisputed polarization along the A/V axis by specific determinants localized at the vegetal pole of the egg suffices to provide the embryo with the initial signals responsible for subsequent specification of the embryonic blastomeres. Based on the results that we present herein, we propose that the maternal SpCOUP-TF mRNA, which encodes a transcription factor, localized at a fixed position relative to the two embryonic axes, may play a role in early determinative events during or after the cleavage stages. Such a role may involve specific repression of genes within ectodermal cells as inductive processes determine future cell identities in this territory.

The embryonic role of the COUP-TFs in vertebrate (Matharu and Sweeney, 1992; Fjose et al., 1993; Lutz et al., 1994; Qiu et al., 1994b) and invertebrate embryos (Mlodzik et al., 1990; Chan et al., 1992) is unclear. The extensive sequence conservation of COUP-TFs and their preference of embryonic expression in the nervous system point to some specific function in neurogenesis. In the sea urchin embryo, based on the zygotic restrictive expression in the ectodermal derivative of the neurogenic ciliated band (Bisgrove and Burke, 1986, 1987) and the 16-cell embryonic pattern, which indicates that SpCOUP-TF transcripts are found primarily in precursor cells of the ectoderm, our hypothesis is that a possible function of this transcription factor maybe the determination of neurogenic progenitor cells which, by subsequent specification events, give rise to the larva nervous system.

We wish to thank Drs William Klein, Rob Maxson and Gary Wessel for supplying us with the clones for Spec1, early H2b histone and LpN1.2, respectively, and Drs. Eric Davidson, Rudolph Raff, Jeff Rosen and Ming-Jer Tsai for critical review of this manuscript. We are also grateful to our colleagues in the laboratory and the Department of Cell Biology for their advice and many helpful discussions. This work was supported by an NIH grant (HD22055) to CNF.

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(Accepted 10 November 1995)