Regulatory sequences driving expression of the sea urchin Otp homeobox gene in oral ectoderm cells

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Abstract

PlOtp (Orthopedia), a homeodomain-containing transcription factor, has been recently characterized as a key regulator of the morphogenesis of the skeletal system in the embryo of the sea urchin Paracentrotus lividus. Otp acts as a positive regulator in a subset of oral ectodermal cells which transmit short-range signals to the underlying primary mesenchyme cells where skeletal synthesis is initiated. To shed some light on the molecular mechanisms involved in such a process, we begun a functional analysis of the cis-regulatory sequences of the Otp gene. Congruent with the spatial expression profile of the endogenous Otp gene, we found that while a DNA region from −494 to +358 is shown to drive in vivo GFP reporter expression in the oral ectoderm, but also in the foregut, a larger region spanning from −2044 to +358 is needed to give firmly established tissue specificity. Microinjection of PCR-amplified DNA constructs, truncated in the 5′ regulatory region, and determination of GFP mRNA level in injected embryos allowed the identification of a 5′-flanking fragment of 184 bp in length, essential for expression of the transgene in the oral ectoderm of pluteus stage embryos. Finally, we conducted DNAse I-footprinting assays in nuclear extracts for the 184 bp region and detected two protected sequences. Data bank search indicates that these sites contain consensus binding sites for transcription factors.

Keywords: Sea urchin development; Skeletogenesis; Orthopedia homeobox gene; Oral ectoderm

1. Results and discussion

In the sea urchin embryo skeletogenesis is a complex morphogenetic event guided by the positioning of the migrating primary mesenchyme cells (PMCs) at ventral sites of the blastocoel (Gustafson and Wolpert, 1967). Although the spicule formation process is carried out by the PMCs, the patterning of the embryonic skeleton strictly depend on local short-range cues emanating from the ectodermal wall with which PMCs make intimate contacts (Armstrong et al., 1993; Di Bernardo et al., 1999; Guss and Ettensohn, 1997; Gustafson and Wolpert, 1963; Hardin et al., 1992).

Recent findings indicate that genes differentially expressed in the oral ectoderm territory are part of a gene regulatory network that governs the morphogenesis of the larval endoskeletal system. Among them are the homeodomain-containing regulators encoded by the PlOtp and SpDri genes. PlOtp is a Orthopedia related gene (Simeone et al., 1994) that in Paracentrotus lividus, displays a highly restricted expression pattern confined to a small subset of oral ectoderm cells, symmetrically located in a ventrolateral position and overlying PMC-clusters (Di Bernardo et al., 1999). As we have previously shown, ectopic expression of the Otp regulator, causes abnormal skeletal development to occur at multiple sites (Cavalieri et al., 2003; Di Bernardo et al., 2000; Di Bernardo et al., 1999). Conversely, inhibition of the Otp function either by the microinjection of a specific morpholino antisense oligonucleotide or through the expression of a dominant-negative En-Otp fusion, impairs skeletogenesis and interferes with the regular migration of the PMCs (Cavalieri et al., 2003). Based on these line of evidence, we previously suggested that Otp triggers, in the oral
ectoderm, a signal that is transmitted to the PMC-clusters to initiate skeletogenesis (Cavalieri et al., 2003; Di Bernardo et al., 2000). The deandringer homeodomain protein encoded by SpDri must be a component of this complex signalling process and should acts upstream of the Plootp gene. In fact, the inhibition of its function blocks the expression of Otp and the whole skeletogenic program (Amore et al., 2003). However, it is not known whether the Dri protein binds to the promoter region of the Otp gene, thus directly regulating its expression.

The elucidation of the cis-regulatory DNA elements that control the Otp expression should give clues to the hierarchy of the mechanism(s) responsible for ectoderm-to-PMC signalling transmission and skeletal patterning itself. Here we report the initial functional analysis of the 5'-flanking region of the Otp gene in transgenic embryos of the sea urchin P. lividus.

1.1. The 5'-region from the Otp gene drives expression of the GFP reporter gene in oral ectoderm cells

A genomic clone, termed λOtp, was isolated in the course of a screening of P. lividus genomic library aimed at the identification of homeobox-containing genes. An insert of 2.4 kb containing the 5'-coding region was excised from the λOtp clone by restriction enzyme digestion, sub-cloned into the Bluescript plasmid, and sequenced. The comparison of the DNA sequence of the genomic fragment with that of the full-length Otp cDNA (Di Bernardo et al., 1999) allowed us to define the 5'-flanking sequences from the putative transcription start site. To identify the promoter DNA elements required for proper regulation, DNA fragments of 2.4 kb and 0.85 kb in length, abutting at the 3' end the ATG start codon of the Otp gene (pOtp) were fused in frame with the Green Fluorescent Protein (GFP) reporter gene (Fig. 1). In order to distinguish injected from uninjected embryos, the pOtp–GFP constructs were microinjected in sea urchin zygotes together with Texas Red-conjugated dextran and embryos allowed to develop. Due to the mosaic distribution of the transgene coupled to the highly restricted expression of the Otp gene, we scored thousands of embryos for each microinjected construct. As expected from the timing and localization of the endogenous transcripts, the expression of the GFP transgene driven by the 2.4 kb and 0.85 kb Otp promoter fragments was undetectable during the first 20 h development, that in P. lividus corresponds to the early gastrula stage (not shown). Embryos at the pluteus stage (48–70 h of development) were collected for microscopic observation. The expression profile of the pOtp–GFP-2.4 construct in plutei at 70 h of development is shown in Fig. 2. Green spots of GFP can be observed in the oral ectoderm territory at or close to the tips of the antero-lateral (A’, B’) and at the tips of the post-oral (C’, D’) arms. This pattern exactly corresponds to the sites of expression of the endogenous Otp gene, previously shown to be strictly associated to active sites of skeletal growth (Di Bernardo et al., 1999; Cavalieri et al., 2003). In other embryos, like those shown in E’–F’, expression of the pOtp–GFP-2.4 construct occurs in cells of the oral epithelium, just flanking the mouth. Expression is always observed in the cuboidal oral epithelium, along the trajectory of the skeleton (G’, H’, I’). Altogether, the transgene expression pattern observed in different embryos recapitulate the spatial expression profile of the Otp endogenous gene (Di Bernardo et al., 1999). Based on our in situ pattern, we would roughly estimate that only 0.1–0.2 % of the cells of the embryo contain the Otp mRNA. As mosaic expression of transgene has irrefutably demonstrated also in other sea urchins, if one takes into account the size territory and normalizes to the same size as the Otp territory, a level of expression of about 5% is obtained (Yuh and Davidson, 1996). So, it is not surprising to find only 4–5% of the injected embryos positive for GFP expression. Strikingly, more than 95% of transgene-expressing embryos showed GFP localization in the oral tissue, while only less than 5% showed incorrect expression. This low level of ectopic expression is commonly observed, irrespective of the transgene used (Yuh and Davidson, 1996). Table 1 shows a summary of the results of three different microinjection experiments. In conclusion, these results strongly suggest that the 2.4 kb fragment contains the cis-regulatory DNA elements required for driving the proper temporal and spatial regulation of the Otp gene.

Fig. 1. Schematic drawn of the GFP transgene driven by the 2.4 kb Otp regulatory region and its 5’ deletion fragments. Two CCAAT boxes and a TATA box are indicated. Bent arrow denote the transcription start site. The ATG start codon of the sea urchin Otp sequence is indicated.
1.2. Characterization of the regulatory promoter sequences by deletion analysis

On the basis of the results described above and to identify the cis-regulatory elements of the Otp gene responsible for the restricted expression in the oral ectoderm, we tested the effect of the microinjection of a 0.85 kb Otp fragment spanning from −494 to +358 with respect to the putative transcription start site (Fig. 1). As shown in Fig. 3A, green spots of GFP-expressing cells were observed in the oral ectoderm of plutei at 48 h of development. In these embryos the fluorescence was confined to few cells localized near the tip of the post-oral arms (red arrow in a) and in the oral ectoderm at the place where the oral arms will bud off (white arrow in b). Again there is a striking correspondence between sites of active skeletal growth, Otp gene expression...
in the ventral region and the distribution of GFP-expressing cells. Fluorescent cells were also found flanking the foregut (b). However, the deletion of upstream sequences from the 2.4 kb *Otp* upstream region, lacking in the 0.85 kb GFP construct, caused the ectopic appearance of GFP-expressing cell patches also in the contiguous mouth and foregut territories in embryos at 70 h of development (Fig. 3A, c). Moreover, we observed a significant increase, up to 30%, of GFP-expressing embryos at this stage of development. This pattern of expression suggests that the 0.85 kb promoter fragment does contain the necessary cis-regulatory DNA elements for driving proper localization of the *Otp* gene in a restricted number of oral ectoderm cells, but probably lacks negative regulatory sequence elements that maintain this highly restricted pattern in the late pluteus stage (70 h of development).

Next we tested the effect of 5′ deletions in the 0.85 kb promoter fragment. Two constructs lacking, respectively, 184 and 380 bp, termed *pOtp–GFP 0.67* and *pOtp–GFP 0.47* (Fig. 1), and the *pOtp–GFP 0.85* as control, were microinjected into fertilized eggs. To exclude any possible interference of plasmid sequences, the functional analysis of this *Otp* promoter region was carried out by injecting PCR-amplified DNA fragments. We observed that the shortening of the 0.85 kb *Otp* DNA fragment caused a precipitous drop in the level of reporter expression and the loss of any oral tissue-specific expression (not shown).

To further determine the effect of deletions of the *Otp* promoter region on the expression of the transgene, we examined the GFP mRNA level by semi-quantitative RT-PCR in microinjected pluteus stage embryos. To normalize the RT-PCR we co-amplified the transcripts of the constitutively expressed *Mbf-1* factor (enhancer binding protein of the α-H2A histone gene (Alessandro et al., 2002)). In agreement with the microscopic observations, both

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**Table 1**

Summary of the results of three independent microinjection experiments with the *pOtp–GFP 2.4* construct

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Injected embryos</th>
<th>GFP-expressing embryos</th>
<th>Oral ectoderm epithelium</th>
<th>Other tissues</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Antero-lateral arm tips</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Post-oral arm tips</td>
<td></td>
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<td></td>
<td>Near the mouth</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Other localizations a</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>782</td>
<td>36</td>
<td>7</td>
<td>10</td>
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<tr>
<td>2</td>
<td>749</td>
<td>35</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>766</td>
<td>35</td>
<td>8</td>
<td>10</td>
</tr>
</tbody>
</table>

The relative distribution of GFP-expressing cells in sub-districts of the oral epithelium and in other embryonic tissues is detailed.

a “Other localizations” oral territory is always along the trajectory of the skeleton, but not exactly correspond to the growing tips at this stage of development, as suggested by specimen G′, H′, I′, K′ in Fig. 2. These foci of GFP expression are likely to be earlier tips left behind after skeleton elongation.

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Fig. 3. (A) Expression of the *pOtp–GFP 0.85* transgene. Images were obtained under epifluorescent illumination (a–b) and merged with brightfield illumination (c). In (a-b), oral ectoderm GFP-expressing cells are observed in living specimen at 48 h of development. Embryos are oriented with the oral surface facing down. In (c), a lateral view of a 70 h-old embryo, shows GFP ectopic labelling in the contiguous “oral” territories, mouth and foregut. (B) Expression analysis by RT-PCR of *pOtp–GFP* transgenes in *P. lividus* pluteus stage embryos microinjected either with *pOtp–GFP 0.85* (1), *pOtp–GFP 0.67* (2) or *pOtp–GFP 0.47* (3) PCR-amplified DNA fragments. Total RNA was extracted from microinjected embryos, treated with DNAseI, retrotranscribed and co-amplified using GFP and endogenous *Mbf-1* specific primers (lanes 1–3). The expected amplified DNA products of 233 bp for GFP and 762 bp for *Mbf-1* are shown. The GFP transgene expression is severely down-regulated in embryos injected with the *pOtp–GFP 0.67* construct (2) and almost suppressed in embryos injected with the *pOtp–GFP 0.47* one (3), indicating that the upstream 184 bp *Otp* regulatory region is essential for to drive higher level of expression. As a negative control, an equal amount of total RNA from *pOtp–GFP 0.85*-injected embryos was directly amplified (lane 4).
pOtp–GFP 0.67 and pOtp–GFP 0.47 constructs similarly led to a significant reduction of expression of the GFP transgene (Fig. 3B). Altogether, these findings can only be explained with the presence in the region comprised between nucleotides −494 and −310 of one or more positive cis-regulatory elements necessary for proper temporal and spatial expression of the Otp gene.

1.3. Identification of protein-binding sites in the 184 bp Otp cis-regulatory region

To identify protein-binding sites in the functionally characterized 184 bp promoter region, we carried out DNase I footprinting experiments with both strands in nuclear extracts from gastrula stage embryos. The results

Fig. 4. (A) In vitro DNAsse I footprinting analysis. PCR fragments comprising the 184 bp Otp regulatory region (−494/−310) were 32P labeled at one 5’ end and incubated without (−) and either with nuclear extracts from gastrula stage embryos (+) or BSA, before being subjected to DNase I digestion. Protected sequences F1 and F2 on sense (left) and antisense (right) DNA strands are respectively indicated by light and dark grey boxes. Asterisks indicate DNase hypersensitive sites on sense strand. G + A are the Maxam and Gilbert purine sequencing tracks. (B) Nucleotide sequence of the 184 bp Otp regulatory region, in which the DNase I protected regions are boxed.
are presented in Fig. 4. Two distinct footprints, denoted F1 and F2 according to their position in the distal-to-proximal direction, were detected. We also observed some DNAse-hypersensitive sites on sense strand. The F1 site was mapped to the 5′-most region, from −494 to −465 nucleotides. The larger F2 footprint was located in the region spanning from −438 to −388 residues.

In order to identify candidate DNA-binding proteins we inspected the corresponding DNA regions for the presence of consensus binding sequences for known transcription factors using the MatInspector and TESS softwares (URL: http://www.cbil.upenn.edu/tess). By this approach we revealed the presence of some potential consensus sites for several transcription factors, with a core similitude of 1.00 and matrix similarity higher than 0.81. The output of this search are schematized in Fig. 5. In particular, a highly conserved Krüppel binding region lies in F1. In F2 we found two perfectly conserved consensus sequences for the binding of the TG-Interacting Factor (TGIF), belonging to the Three Aminoacid Loop Extension (TALE) class of atypical homeodomain factors, and for the Zn-finger factor Hunchback. In addition, a binding sequence for the SMAD family member Forkhead Activin Signal Transducer (FAST-1) is also present.

2. Experimental procedures

2.1. Embryo culture and microinjection of DNA constructs and PCR products

Gametes of P. lividus were harvested and eggs fertilized and cultured as previously described (Cavalieri et al., 2003). To be microinjected, eggs were de-jellied by treatment with acidified Millipore Filtered Sea Water (MFSW) and rapidly brought back to the normal pH value. Prior to injection, plasmids were linearized at unique restriction sites located upstream and in frame to the Otp transcription start site, was cloned into a pGL3 modified plasmid upstream and in frame to the GFP reporter gene, to give the pOtp–GFP 2.4 construct. Another fragment, spanning nucleotides −494 to +358, was generated by a Neol restriction enzyme digestion and fused in frame with the GFP reporter gene, to give the pOtp–GFP 0.85 construct. 5′ deletions, for microinjection, of the Otp DNA fragment were obtained from the pOtp–GFP 0.85 construct by PCR reactions. Primers were as follows:

E-Otp1 (5′-TGCCCTCCATCTTCTCTC-3′) and pGL-down (5′-CCTCTTCGCTATTACGCCAG-3′) for the pOtp–GFP 0.67 fragment; E-Otp2 (5′-GGCCTGACTCATTCAAAC-3′) and pGL-down for the pOtp–GFP 0.47 fragment. Amplification reactions were performed by Pfu DNA polymerase. DNA fragments were purified with the QIAquick PCR Purification Kit (Qiagen), and quantified by spectrophotometry.

2.3. RNA extraction and RT-PCR

Total RNA from 30 pluteus stage embryos microinjected with pOtp–GFP 0.85, pOtp–GFP 0.67 or pOtp–GFP 0.47 DNA fragments were purified by using the High Pure RNA Isolation kit (Roche). One-step RT-PCR, using the Titan One Tube RT-PCR kit (Roche), was employed to co-amplify GFP and MBF-1 mRNA sequences. An aliquot of RNA equivalent to that extracted from 10 embryos was reverse-transcribed and the resulting cDNA was amplified for 30 cycles (denaturation at 94 °C for 10 s, annealing at 55 °C for 30 s and extension at 68 °C for 55 s). One-fifth of the product from each reaction was resolved on agarose gel 1% in TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Primers used were as follows:

![Fig. 5. Summary of a combined data bank search made by MatInspector and Tess softwares in order to identify potential binding sequences for known transcription factors, within the F1 and F2 footprints of the −494/−310 Otp regulatory region. A collection of binding sites with core similarity of 1000 and a matrix similarity higher than 0.81 is shown in the upper diagram.](image-url)
GFP forward 5'-CATGAGCAAGGCGAGGAAC-3', GFP reverse 5'-TATGGTCTGGATCTGGAA-3'; MFB-1 forward 5'-GGAATGAAGACATGAGCAAGGGCGAGGAAC-3', MFB-1 reverse 5'-CTGGTAGACGACGAGGCCT-3', MFB-1 forward 5'-H11032-GGAATGAAAACACAGAGCAGCCT-3', MFB-1 reverse 5'-H11032-TATGGTCTGGGTATCTGGAA-3'.

2.4. DNaseI protection assays

Nuclear extracts proteins from *P. lividus* gastrula stage embryos and DNaseI footprint assays were as previously described (Palla et al., 1993). Two PCR-amplified fragments both covering the cis-regulatory region of 184 bp (from nucleotides −494 to −310) were 32P-end-labeled and incubated for 20 min on ice with 30 μg of gastrula stage nuclear extracts or 25 μg BSA in reaction mixtures consisting of 60 mM KCl, 1 mM DTT, 1 mM EDTA, 10 mM Hepes pH 7.9, 5 μg of poly(dA-dT)-(dA-dT), 4% Ficoll in a total volume of 50 μl. DNAseI (0.1 μg/μl) digestion was carried out at 4 °C for 2–5 min. Reaction products and Maxam–Gilbert sequencing reactions were analyzed on denaturing 6% polyacrylamide sequencing gels.

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References


