Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 310 (2007) 402–415
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Genomes & Developmental Control

Regulation of the *Xenopus Xsox17α₁* promoter by co-operating VegT and Sox17 sites

Laura Howard, Maria Rex, Debbie Clements[1], Hugh R. Woodland*

Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK

Received for publication 20 January 2007; revised 3 July 2007; accepted 23 July 2007
Available online 31 July 2007

Abstract

The gene encoding the Sox F-group transcription factor Xsox17α₁ is specifically expressed throughout the entire region of the *Xenopus* blastula fated to become endoderm, and is important in controlling endodermal development. *Xsox17α₁* is a direct target of the maternal endodermal determinant VegT and of Sox17 itself. We have analysed the promoter of the *Xenopus laevis Xsox17α₁* gene by transgenesis, and have identified two important control elements which reside about 9 kb upstream at the start of transcription. These elements individually drive transgenic endodermal expression in the blastula and gastrula. One contains functional, cooperating VegT and Sox-binding consensus sites. The Sox sites in this region are occupied in vivo. The other responds to TGF-β signals like Activin or Nodals that act through Smad2/3. We propose that these two regions co-operate in regulating the early endodermal expression of the *Xsox17α₁* gene.

Keywords: Sox17; VegT; Endoderm; *Xenopus*; Transgenic embryo; Promoter; TGF-β

Introduction

The endoderm of *Xenopus* embryo arises in two successive phases, involving firstly cell-autonomous gene action, followed by dependence on cell signalling. The cell-autonomous phase is directly initiated by the maternal T-box transcription factor VegT, but then some key genes associated with endodermal differentiation become dependent on signalling by the group of TGF-β family members that signal through Smad2/3 These include the Nodal-related proteins or Xnrs, Derrière, Vg1 and Activin. In this second phase, cells are sensitised to TGF-β signalling by the maternal VegT that they inherit (Clements et al., 2001; Clements and Woodland, 2003; Engleka et al., 2001; Hudson et al., 1997; Yasuo and Lemaire, 1999). Since the essential TGF-β signalling molecules are themselves induced by VegT, and VegT is indispensable for endoderm development (Xanthos et al., 2001), the overall effect is that only a critical mass of VegT-containing cells can generate sufficient signalling to sustain expression of these key endodermal genes. Scattered VegT containing cells will fail to become endoderm and conform to their surroundings. In contrast, some other genes (e.g. *Xnr4*), which are directly induced by VegT do not become signal-dependent. Finally, in the gastrula, endodermal gene expression becomes independent of cell signalling (Yasuo and Lemaire, 1999). This interpretation of endoderm initiation, establishment and maintenance is heavily based on studying the expression of the VegT target *Xsox17*, an HMG-box transcription factor, although *Mix.1* and *Mixer* behave in a similar fashion.

There are three *Sox17* genes in *Xenopus laevis*, *Xsox17α₁*, *α₂* and *β*, but since no differences in their activity have so far been detected, for most purposes we refer to them collectively as *Xsox17*. The transcription of these Sox F group genes is activated prior to the mid-blastula transition (MBT), when very low levels are found ubiquitously, but Xsox17 transcription in the vegetal pole is enormously upregulated at MBT, precisely marking out the territory of the future endoderm through late blastula, gastrula and neurula stages (Hudson et al., 1997; Zorn et al., 1999). The *Xsox17* genes have a key role in endoderm

* Corresponding author. Fax: +44 24 76523701.
E-mail address: h.r.woodland@warwick.ac.uk (H.R. Woodland).
[1] Present address: Division of Therapeutics, University of Nottingham, Queens Medical Centre, Nottingham, NG7 2UH, UK.

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.07.028

formation. Their ectopic expression induces endodermal gene expression, as well as changing the fate of cells. Interfering with their expression with a dominant negative Engrailed *Xsox17* fusion construct has the converse effect, inhibiting endodermal gene expression and shifting cells out of an endodermal fate in intact embryos (Clements and Woodland, 2000; Hudson et al., 1997). The use of antisense morpholino oligos shows that the individual genes have non-redundant late roles in the developing mid- and hindgut, but that together they are needed for the completion of gastrulation (Clements et al., 2003). This correlates well with the essential role of the single-murine *Sox17* gene for early development of endoderm fated to become mid- and hindgut, although in the absence of Sox17 there is also later loss of foregut cells (Kanai-Azuma et al., 2002). In zebrafish, two related Sox genes, *Casanova* and *Sox17*, are important in forming the endoderm; the former is more upstream and its mutants indicate that it has a vital role in endoderm development (Aoki et al., 2002). While mutations in the zebrafish *Sox17* gene have not been described, it is likely that in the zebrafish endodermal gene network the combined action of *Casanova* and *Xsox17* are roughly equivalent to the overall action of *Xsox17* in *Xenopus*, particularly allowing for the fact that *Casanova* expression in the yolk syncytial layer is Nodal-independent, allowing a parallel with the *Xenopus* cell autonomous phase to be drawn (Kikuchi et al., 2001; Woodland and Clements, 2003). In *Xenopus*, ablation of Xsox17 expression with morpholinos halts gastrulation at an early stage. However, the immediate effects on gene expression were initially reported to be modest and were restricted to the direct Xsox17 targets *Endodermin* and *Hnf-1β* (Clements et al., 2003). However, much wider effects have now been observed using microarrays (Sinner et al., 2006).

Since *Xsox17* is a crucial gene in the early endoderm, and its expression defines the endodermal territory, we have analysed the regulatory elements in the *Xsox17α$_1$* promoter approximately 9 kb upstream of transcriptional initiation. We have identified two small elements, which can confer endodermal expression on a reporter gene in the early embryo. We have analysed one of these in detail and show that its activity depends on co-operating VegT and Sox17-binding sites, whereas the other regulatory region responds to Activin.

Materials and methods

Biological materials

Eggs of *X. laevis* were obtained, fertilised, dejellied and cultured by standard methods, as described previously (Wilson et al., 1986). Oocytes, complete with their follicle, were manually removed using watchmakers forceps in modified Barths' saline (MBS; 110 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.8 mM MgSO4, 15 mM Tris–HCl, pH 7.5).

Transgenic methods

These followed the procedure of Kroll and Amaya (1996), except that a reduced amount of egg extract was used in the incubation of stored frozen sperm nuclei with DNA (2 μl in a 25 μl reaction). The reaction typically contained 150–200 ng of linearised plasmid DNA and 0.5 μl of a 1:200 dilution of restriction enzyme (2 units/ml). Injections of dejellied unfertilised eggs were performed in 6% Ficoll, 0.4× Marc's modified Ringer (MMR) or 0.4× MBS in polyheme-coated plastic dishes. Correctly cleaving eggs were sorted at the 2- to 4-cell stage and incubated in 6% Ficoll, 0.1× modified Barth's medium. GFP fluorescence was monitored using a Leica MZFLIII dissecting microscope.

Transient transgenesis in embryos and oocytes

For transient expression in embryos, DNA constructs were linearised and purified with the Qiagen gel extraction kit. 50 pg DNA together with 5 pg control *Renilla* luciferase reporter in 10 nl water were injected bilaterally, with or without mRNA, into the animal or vegetal poles of 2-cell embryos, cultured in 6% Ficoll, 0.4× MBS. Embryos were analysed at gastrula or neurula stages using the Promega Dual Luciferase Reporter Assay System. Triplicate batches of 10 embryos were homogenised in 600 μl passive lysis buffer and incubated on ice for 10 min. Samples were centrifuged at 13,000 rpm for 2 min at 4 °C, and supernatants removed. They were equilibrated to room temperature for 30 min, and 60 μl assayed for bioluminescence after addition of 50 μl luciferin stock using the Luminoskan RS apparatus (Labsystems). Normalisation of the experimental reporter was achieved by quenching of the firefly luciferase reaction and measurement of *Renilla* luciferase luminescence.

Oocyte nuclei were microinjected with 18 nl water containing 300–500 pg circular firefly test plasmid, plus one third this amount of control *Renilla* luciferase plasmid, with or without transcription factor mRNA. After culturing overnight in MBS, they were processed for luciferase activity as detailed above.

Cloning and characterisation of the Xsox17α$_1$ gene

The *Xsox17α$_1$* gene was isolated by screening a *X. laevis gilli* PAC library (RZPD) with an *Xsox17α$_1$* cDNA probe. One clone (BUMSP710J2012Q3) reacted strongly with this probe. A positive Not 1 fragment was subcloned into Bluescript and this sequence encompassed all 12 kb of 5′ upstream sequence present in the PAC, the transcribed region itself and 3 kb downstream of the 3′ UTR.

Transgenic constructs and mutagenesis

Mutant promoter constructs were created by hybrid overlap extension PCR, using a series of external and overlapping internal primers. Primer sequences were as follows: B1 ext up 5′CAACACTCACATTC 3′. B1 T-box ext down 5′CTTGAGAATGGGACTGTGTTAACAAACAATGATGATCAGAACTCTGG 3′, Sox A int up 5′ CTTGGGAACTAGTTGTGGATC 3′, B1 Sox A int down 5′GATCCACAACTAGTTCCCAAG 3′, B1 Sox B int down 5′ CTTGAGAATGGGACTGTGTTAACAACCATGGATGGTGTGAACTCTGG 3′, B1 Sox B+T-box ext down 5′CTTGAGAATGGGACTGTTTTAACAACCATGGATGATCAGAACTCTGG 3′. Underlined text denotes mutated sequence. External primers had 5′ *Sac1* and 3′ *Kpn1* extension sequences. Amplification was performed over 20 cycles with 55 °C annealing temperature. Products were Qiagen column purified, cut with restriction enzyme, and cloned into pGL3basic-act-luc (transient assay) or pGL3basic-act-mgfp5 (transgenic assay).

Electrophoretic band-shifts (EMSAs)

EMSAs were performed using VegT protein synthesised in vitro in the rabbit reticulocyte system. 10 pmol single-stranded DNA oligo was 5′ end-labelled with 20 μCi ^{32}P γ-ATP using T4 polynucleotide kinase. Forward and unlabelled reverse strands were annealed by heating to 90 °C for 5 min, followed by slow cooling overnight. DNA was recovered by ethanol precipitation and resuspended in TE buffer. 15 μl binding reactions contained 1 μl in vitro translated protein, 2 μg [poly dI.dC].[poly dI.dC], 3 μl MDB buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol w/v, 2 mM DDT). The probe was added following 10-min incubation at 30 °C, followed by a further 10-min incubation. Control binding reactions included a 50-fold excess of unlabelled specific competitor probe. Samples were analysed on a 15% polyacrylamide gel at 4 °C (200 V, 2 h). Gels were dried and autoradiographed or analysed with a phosphoimager. Probe sequences used were: B1 5′-TGTCCAGAGTTCACACCATCATTGTTTGTTA-3′, T-box mutant 5′-TCTCCAGAGTACGCACATTCATTGTTTGTTA-3′, T-box consensus 5′-