Sox17 and β-catenin cooperate to regulate the transcription of endodermal genes

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Summary

Recent studies have led to a model of the molecular pathway that specifies the endoderm during vertebrate gastrulation. The HMG box transcription factor Sox17 is a key component of this pathway and is essential for endoderm formation; however, the molecular events controlled by Sox17 are largely unknown. We have identified several direct transcriptional targets of Sox17, including *Foxa1* and *Foxa2*. We show that β-catenin, a component of Wnt signaling pathway, physically interacts with Sox17 and potentiates its transcriptional activation of target genes. We identify a motif in the C terminus of Sox17, which is conserved in all the SoxF subfamily of Sox proteins, and this motif is required for the ability of Sox17 to both transactivate target genes and bind β-catenin. Nuclear β-catenin is present in endoderm cells of the gastrula, and depletion of β-catenin from embryos results in a repression of Sox17 target genes. These data suggest that in a mechanism analogous to Tcf/Lef interacting with β-catenin, Sox17 and β-catenin interact to transcribe endodermal target genes.

Supplemental data available online

Key words: Endoderm, Transcription, Sox17, β-catenin, *Foxa*, Gata, *Xenopus*

Introduction

In the vertebrate embryo the endoderm gives rise to the epithelial lining of the respiratory and gastrointestinal tract, as well as to the liver, lungs, pancreas, thyroid and thymus (reviewed by Wells and Melton, 1999). Recent work in *Xenopus*, zebrafish and mouse have resulted in a model of the molecular pathway that controls vertebrate endoderm development (reviewed by Stainier, 2002).

In *Xenopus*, endoderm development is initiated by the maternal T-box transcription factor VegT, which is localized to vegetal region of the egg and early embryo. VegT starts the cascade of endoderm specification in the vegetal cells by activating the transcription of zygotic endodermal genes (Clements et al., 1999; Xanthos et al., 2001; Zhang et al., 1998), which encode nodal-related proteins (Xnr1,2,4,5,6) and Derriere – members of the TGFβ growth factor family (Jones et al., 1995; Joseph and Melton, 1997; Sun et al., 1999; Takahashi et al., 2000), homeodomain proteins of the Mixer/Mix/Bix family (Casey et al., 1999; Henry and Melton, 1998; Rosa, 1989; Tada et al., 1998; Vize, 1996), the zinc-finger factors Gata4, Gata5 and Gata6 (Jiang and Evans, 1996; Weber et al., 2000; Xanthos et al., 2001), and two closely related HMG domain transcription factors, Sox17α and Sox17β (Hudson et al., 1997).

Several of these zygotic factors, including Xnr1, Xnr5 and Sox17α are known to be direct targets of VegT (Hyde and Old, 2000; Hilton et al., 2003; Engleka et al., 2001). In the case of Sox17 it is thought that VegT initially activates Sox17 transcription but then nodal signaling is required to maintain its full expression (Engleka et al., 2001; Yasuo and Lemaire, 1999; Clements et al., 1999). Indeed, the available data indicates that nodals are the primary target of VegT and that nodal signaling acts up stream of and is required to maintain the expression of the *Mixer/Mix/Bix*, *Gata4*, *Gata5* and *Gata6*, and *Sox17* transcription factors (Alexander and Stainier, 1999; Clements et al., 1999; Kofron et al., 1999; Xanthos et al., 2001; Yasuo and Lemaire, 1999).

The epistatic relationships between Mixer, Mix1, Mix2, Bix1, Bix2, Bix3, Bix4, Gata4, Gata5, Gata6 and Sox17α/β are unresolved, but the limited data indicate that Mixer and Gata function upstream of Sox17. In both frog and fish, overexpression of Mixer and Gata4/5 can induce Sox17 transcription but Sox17 cannot induce the expression of Mixer (Alexander and Stainier, 1999; Henry and Melton, 1998; Weber et al., 2000; Xanthos et al., 2001). Furthermore mutations in the zebrafish *Mixer/Mix* family member (*bon*) and *Gata5* (*faust*) indicate that they are both required for *Sox17* expression (Kikuchi et al., 2000; Reiter et al., 2001). In *Xenopus*, a dominant-negative version of Sox17 can inhibit Mixer function, but dominant-negative Mixer cannot inhibit Sox17 function (Henry and Melton, 1998), suggesting that Mixer acts via Sox17. Thus, although *Sox17* is initially transactivated by the maternal VegT, these data suggests that Sox17 functions as one of the most downstream component of the pathway leading to endoderm differentiation. However, this model of the endoderm specification pathway is likely to be an

over simplification and the exact relationships between Sox17, Gata4, Gata5, Gata6 and the Mixer/Mix/Bix family still need to be carefully resolved.

In *Xenopus Sox17α/β* are specifically expressed in the presumptive endoderm at late blastula and gastrula stages and they can induce endoderm differentiation when ectopically expressed in naïve ectoderm (Clements and Woodland, 2000; Hudson et al., 1997). Blocking endogenous Sox17 function with a dominant-negative Sox17 engrailed transcriptional repressor (Sox17:EnR) construct (Hudson et al., 1997) or by antisense oligos (Clements et al., 2003) disrupts endogenous endoderm development. Similarly, targeted deletion of Sox17 in mouse causes severe definitive endoderm defects and embryonic lethality (Kanai-Azuma et al., 2002). Although Sox17 is clearly crucial for endoderm formation, the downstream transcriptional targets of Sox17, which subsequently direct endodermal differentiation, are largely unknown.

How Sox17 regulates the transcription of its targets is also an important unresolved issue. Although all Sox proteins, ~30 in the vertebrate genome, have remarkably similar DNA-binding properties (Bowles et al., 2000; Kamachi et al., 2000), distinct Sox proteins none-the-less regulate unique target genes. The prevailing idea is that interacting protein partners are key determinants of Sox protein specificity and activity (Kamachi et al., 2000; Wilson and Koopman, 2002), but in the case of Sox17, its interacting transcriptional co-factors were previously unknown.

We have sought to extend our understanding of endoderm development by focusing on the targets of Sox17 and the mechanism by which Sox17 regulates their transcription. We have identified a number of transcriptional targets of Sox17 and provide evidence that β-catenin is an essential transcriptional co-factor of Sox17. β-catenin is best known as a mediator of Wnt responsive transcription (Wodarz and Nusse, 1998), and in the *Xenopus* blastula β-catenin interacts with Tcf/Lef HMG transcription factors to activate dorsal organizer gene expression (Heasman, 1997; Moon and Kimelman, 1998). We had previously shown that β-catenin can also physically interact with Sox17 (Zorn et al., 1999a) but the biological relevance of this interaction was unclear. We now show that the transactivation domain of Sox17 mediates this interaction, suggesting that β-catenin binding is important for Sox17 activity. In animal cap experiments, Sox17 and β-catenin cooperate to activate Sox17 target genes, while depletion of β-catenin from embryos results in a repression of Sox17 target gene expression. These results extend our understanding of early endoderm development and suggest that Sox17 and β-catenin cooperate to regulate endodermal gene expression. Our findings also suggest that, like the Tcf/Lef family of HMG box transcription factors, Sox proteins may act as Wnt/β-catenin effectors.

Materials and methods

Embryo culture and manipulations

Embryo manipulations and microinjections were preformed as previously described (Zorn et al., 1999b) and staged according to the normal table of development for *Xenopus laevis* (Nieuwkoop and Faber, 1994). Typically, eight to ten animal cap explants, or three to four whole embryos were used for each condition and assayed at stage 11. For GR:Sox17β experiments, animal caps were incubated in 1×MBS with 0.1% BSA with 10 μg/ml of cycloheximide for 1 hour, starting 30 minutes prior to addition of dexamethasone (10^{-6} M), to ensure that translation was inhibited before GR:Sox17 was activated. Recombinant human activin A (R&D Systems) was used at 5 ng/ml. The antisense β-catenin morpholino oligo, previously described by Heasman et al. (Heasman et al., 2000) was used at a dose of 10-20 ng/embryo. Antisense morpholino oligos and RNAs were sequentially injected rather than mixed together.

RT-PCR analysis

Each experiment was repeated at least three times and a representative example is shown. Total RNA was extracted from embryonic tissue and RT-PCR analysis was preformed as previously described (Wilson and Melton, 1994). The primers in this study are shown in the supplementary data (see Table S1 at http://dev.biologists.org/supplemental). For RT-PCR analysis by gel electrophoresis the number of cycles required for each primer set was empirically determined and a dilution series of whole embryo cDNA was included in every assay to ensure that the PCR reaction was in the log-linear range. Controls without reverse transcriptase (–RT) were always included. Owing to space constraints these linearity and –RT controls are not shown.

An Opticon machine (MJ Research) was used for semi-quantitative analysis. The only change to our PCR reaction conditions was the inclusion of SYBR green dye in the PCR mix, for convenience we used Qiagen SYBR green PCR mix. For each experiment and primer pair a serial dilution of whole embryo cDNA was used to generate a standard curve from which the amount of product in the experimental samples was determined at the log-linear amplification phase. The data for each sample is normalized to the expression level of the ubiquitously expressed gene *ornithine decarboxylase* (*ODC)* and presented as a ratio of *ODC* expression as previously described (Xanthos et al., 2001).

DNA constructs and synthetic mRNA

The following DNA constructs and details of RNA synthesis have been previously described: pT7TS-HA-Sox17β, pT7TS-HA-Sox17-deletions constructs, pGEX-β-catenin, UAS:luciferase reporter, pcDNA6 Sox17β-V5, pcDNA6 Sox17α-V5 and pcDNA6 d1-315-V5 (Zorn et al., 1999a); and pCS2+MT-pt-β-catenin, pCS2+MT-ΔN-β-catenin, pCS2+GSK3β and pCS2+kdGSK3β (Yost et al., 1996). pT7TS-GR:Sox17β (*Pst1* and T7) was constructed by inserting the hormone-binding domain of the human glucocorticoid receptor (a gift from Paul Krieg) in frame into pT7TS-HA-Sox17β. The Gal4:Sox17 deletion constructs were generated by PCR amplifying indicated fragments of Sox17β (Fig. 3A) with *Pfu* polymerase and these were cloned in frame with the Gal4 DNA-binding domain in pCMVGT (Zorn et al., 1999a). The 3G and ΔTA mutations were made from pT7TS-HA-Sox17β, pT7TS-HA-Sox17α, pcDNA6 Sox17β-V5 and pcDNA6 Sox17α-V5 parent plasmids using a GeneTailor mutagenesis kit (Invitrogen).

Antisense probes were synthesized using Ambion MEGA script kits and digoxygenin-11-UTP as follows *Sox17α* (pSK-Sox17α *Kpn*I, T3), *Sox17β* (pSK-Sox17β, *Eco*RI, T7), *FoxA1* (pSK-XFKH2; *Hin*dIII, T3) (Bolce et al., 1993), *Foxa2* (pCS2-XFD3; *Eco*R1, T7, a gift from Dr Knoechel), *endodermin* (pSK-edd, *Eco*R1, T7) (Sasai et al., 1996), *Xnr4* (pSK-Xnr4; BamH1, T7), *Gata4* (pSK-Gata4, *Sac*I, T7, a gift from Tom Drysdale). Whole-mount in situ hybridization was performed using the standard protocol (Sive et al., 2000).

Luciferase assays

COS-1 cells in 24 well plates were co-transfected with 100 ng of 5×Gal4 luciferase reporter, 50 ng of pTK:Renilla, and 300 ng of Gal4DBD:Sox17β fusion constructs using Fugene (Roche). Cells were harvested, after 36 hours, extracts prepared, and luciferase activity was measured and normalized for transfection efficiency with

Renilla luciferase activity using a Promega Luciferase/Renilla Assay System. Experiments were carried out in triplicate and the average result is shown.

Western blots and protein binding assays

Standard western immunoblotting procedures were used with the following antibodies: anti-V5-HRP (1:4000, Invitrogen), rabbit anti-β-catenin (1:1000, Santa Cruz Biotechnology, #sc-7199), goat anti-rabbit:HRP (1:20,000, Jackson ImmunoResearch), mouse anti-tubulin (1:1000, Sigma), mouse anti-histone H1 (1:500, AE-4, Santa Cruz) and goat anti-mouse:HRP (1:10,000, Jackson ImmunoResearch). Affinity-purified rabbit anti-XSox17β antibodies or rabbit anti-humanSox17 antibodies were raised to 'IYTIDQDSGAY-STNLLPSLI' and 'CKPEMGLPYQGHDCGVNLSDS' peptides respectively (1:1000, produced by Bethyl Laboratories).

For embryos extracts tissue was homogenized on ice in 10-20 μl per animal cap or embryo in 250 mM sucrose, 10 mM HEPES (pH 6.8), 1 mM EDTA, 0.5 mM EGTA, 2 mM Na_3VO_4, 0.2 mM NaF with protease inhibitors. If the sample was also to be assayed by RT-PCR, half of the extract was removed and processed to isolate total RNA. The remaining extracts were cleared by centrifugation (14,000 ***g***, 45 minutes at 4°C), which pellets most of the cytoskeleton and membrane bound β-catenin, allowing preferential analysis of the cytosolic signaling pool of β-catenin (Heasman et al., 2000).

For β-catenin binding experiments, COS-1 cells were transfected with the indicated constructs in pcDNA6-V5. Thirty-six hours after transfection, cells were lysed on ice with 1 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.5% NP-40 and protease inhibitors) and centrifuged at 14,000 ***g*** for 10 minutes at 4°C. Equal amounts of lysate were incubated for 3 hours at 4°C, with 5 μg of either purified GST or GST-β-catenin bound to agarose beads in lysis buffer adjusted to 20% glycerol and 1 mM DTT. Agarose beads were washed five times with lysis buffer containing 500 mM NaCl and bound proteins were eluted in SDS sample buffer.

SW480 cell fractionation and co-immunoprecipitation

Approximately 10^9 SW480 cells were homogenized in 3.5 ml of cell lysis buffer (250 mM sucrose, 30 mM KCl, 6 mM $MgCl_2$, 20 mM HEPES pH 7.9, 0.5 mM EDTA, 0.2 mM NaF, 2 mM Na_3VO_4 and 0.1% Triton X-100 with protease inhibitors). Nuclei were liberated from the cells by 15 gentle strokes of a tight fitting pestle in a dounce homogenizer. The extract was centrifuged at 500 ***g*** for 10 minutes at 4°C. The supernatant was used as the cytosolic fraction. The pellet was resuspended in nuclear lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1% NP-40 and 0.5% Triton X 100 with protease inhibitors) and sonicated to lyse the nuclei and thoroughly sheer the genomic DNA. The resulting extract was centrifuged at 12,000 ***g*** for 15 minutes at 4°C and the supernatant was used as the nuclear fraction.

For co-immunoprecipitation, equal amounts of nuclear extract were precleared with 1 μg of rabbit preimmune serum and Protein-A agarose for 1 hour at 4°C. The extracts were then incubated for 4 hours at 4°C with Protein-A agarose and either 1 μg of anti-Sox17 antibody or 1 μg of anti-HA as a negative control. In some samples, 2 μg of either a competing Sox17 peptide recognized by the Sox17 antibody or a negative control peptide, not recognized by the Sox17 antibody (from a different part of the Sox17 protein) were included in the incubations. Immunoprecipitates were washed four times in nuclear lysis buffer, resolved by SDS-page and subjected to anti-β-catenin immunoblotting.

Immunocytochemistry and confocal microscopy

Embryos were fixed in 80 mM PIPES (pH 6.8), 5 mM EGTA (pH 8.0), 1 mM $MgCl_2$, 0.2% TritonX-100, 3.7% formaldehyde for 30 minutes; bisected with a razor blade; re-fixed for 1 hour; rinsed in PBS; and then stored in 80% methanol/20% DMSO at –20°C. Rehydrated embryos were blocked in 10% lamb serum, 4% BSA, 2% DMSO, 0.2% Tween-20 in PBS overnight at 4°C, incubated in rabbit anti-β-catenin antibodies (1:200, H-102, Santa Cruz Biotechnology, #sc-7199 or rabbit-anti-β-catenin from Dr P McCrea) in PBST (PBS + 0.2% Tween-20) for 30-36 hours at 4°C, washed in PBST three times for 1 hour per wash and then overnight at 4°C, followed by incubation with goat anti-rabbit Cy5, (1:250, Jackson ImmunoResearch, #111-175-144) in PBST for 30-36 hours at 4°C. Embryos were again washed, as above, dehydrated in methanol, and cleared in 2 volumes benzyl benzoate: 1 volume benzyl alcohol. Images were captured with LSM 510 software using a Zeiss 510 Laser Scanning Confocal Microscope (10× objective; HeNe laser scanning at 633 nm). A *z*-series projection of 16 serial scan 10 μM sections is shown as an optical stack.

Results

Sox17 target genes

Prior to this study, *Hnf1β* and *endodermin* (*Edd*) were the only known direct transcriptional targets of Sox17 (Hudson et al., 1997; Clements et al., 2003). In order to better understand the events downstream of Sox17, we used a candidate gene approach to identify additional Sox17 target genes. We determined which of the known endodermal genes were ectopically transcribed in naïve animal cap ectoderm expressing exogenous Sox17β. By RT-PCR analysis we found that in addition to *Hnf1β* and *Edd*; *Foxa1* (*Hnf3α*) (Bolce et al., 1993), *Foxa2* (*Hnf3β*) (Ruiz i Altaba et al., 1993), *Xnr4* (Joseph and Melton, 1997), *Gata4/5/6* (Jiang and Evans, 1996), *hydroxy-acid oxidase* (*26D10*) (Gawantka et al., 1998), *goosecoid* (*gsc*) (Cho et al., 1991) and *Sox17α* were transcribed in animal cap cells expressing Sox17β, but not in uninjected animal cap tissue (Fig. 1). By contrast, endoderm genes thought to act upstream of *Sox17*, such as *Mixer*, *Mix1*,*2*, *Bix1*, *Bix2*, *Bix3*, *Bix4*, *Xnr1* and *Xnr2*, were not induced by Sox17β. Relative to whole embryo expression levels, *Gata4/5/6*, *Xnr4*, *gsc* and Sox17α were only weakly induced by Sox17β in comparison with the other target genes. In similar experiments, Sox17α activated the transcription of the same target genes as Sox17β (data not shown). By contrast, other HMG box transcription factors, including Sox3, Sox5, Sox11, Lef1, Tcf1, Tcf3 and Tcf4 did not significantly induce the transcription of the same genes as Sox17 in animal cap assays, with the exception that low levels of *Gsc* and *Gata6* expression were consistently induced by Sox3 and Lef1 (see Figs S1-S3 at http://dev.biologists.org/supplemental).

This suggests that we have identified specific endodermal targets of Sox17 and that Sox17β and Sox17α have very similar activities. We have also identified a group of endodermal genes that are not regulated by Sox17, indicating these genes as either act upstream of Sox17 or their expression is controlled by other endodermal factors.

It was important to determine if these genes are direct Sox17 transcriptional targets or not. Therefore we repeated the animal cap experiments with a hormone inducible form of Sox17β consisting of the hormone-binding domain of the human glucocorticoid receptor fused to the N-terminus of Sox17β (GR:Sox17β; Fig. 2A). The resulting GR:Sox17β protein is translated immediately but remains inactive in the cytoplasm until dexamethasone is added to the medium, at which time GR:fusion enters the nucleus and becomes active (Hollenberg et al., 1993). This allowed us to control the time of Sox17 activity and repeat the induction of Sox17 target genes in the presence of 10 μg/ml cycloheximide, which blocks translation

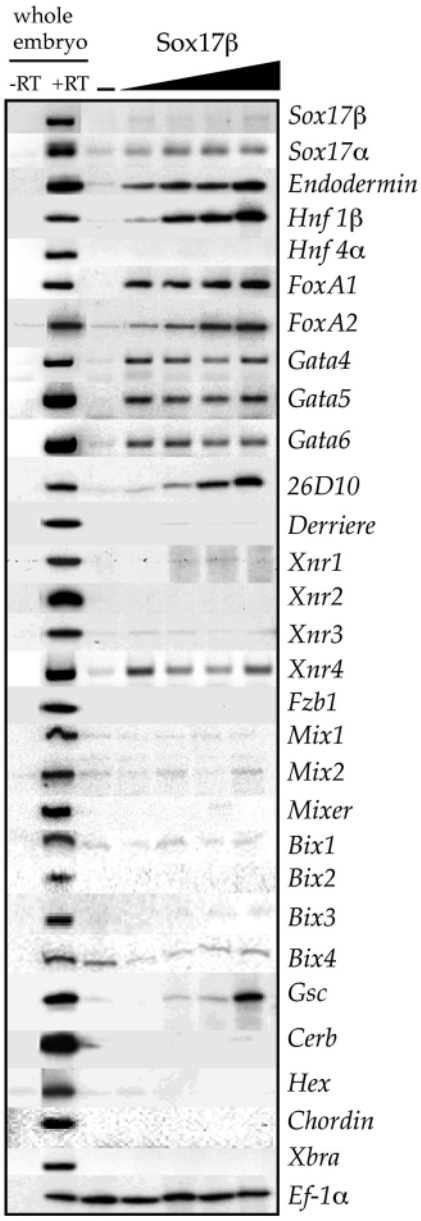

Fig. 1. Transcriptional targets of Sox17β. Embryos were injected at the two-cell stage with increasing doses mRNA of encoding Sox17β (62 pg, 125 pg, 250 pg, 500 pg), animal cap explants were isolated at blastula (stage 9), cultured until gastrula (stage 11) and assayed for endodermal gene expression by RT-PCR. None of the endodermal genes was expressed in uninjected animal cap tissue (–), but all were expressed in gastrula whole embryos. *Ef1α*, is a loading control; –RT, without reverse transcriptase; +RT, with reverse transcriptase.

(Cascio and Gurdon, 1987). Genes transcribed in response to GR:Sox17β when translation was blocked must be direct targets. As a control, we treated animal caps with activin protein, a Nodal-like TFGβ ligand, which is known to induce ectopic endoderm and mesoderm in animal cap tissue (Hudson et al., 1997). *Chordin* is an indirect mesodermal target of activin (Crease et al., 1998) and as expected it was not strongly induced by activin in the presence of cycloheximide, indicating that translation was effectively inhibited.

Fig. 2B shows that *Hnf1β*, *Foxa1*, *Foxa2* and *Sox17α* are direct Sox17β targets because their transcription was still induced by GR:Sox17β in the presence of cycloheximide. Occasionally, we found that *Edd* was induced by cycloheximide (Fig. 2B) while other times it was not (see Figs S1-S3 at http://dev.biologists.org/supplemental) – the source of variation was unclear. In cases when *Edd* was not induced by CHX alone, we found that it also was a direct Sox17 target, consistent with the findings of Clements et al. (Clements et al., 2003). The endodermal marker *26D10* was clearly an indirect target as it was not transcribed by GR:Sox17β when protein synthesis was blocked. Cycloheximide, either with or without dexamethasone, always induced the expression of *Gata4*, *Xnr4* and *Gsc* in animal caps (Fig. 2B; see Figs S1-S3 at http://dev.biologists.org/supplemental) and therefore we were unable to determine if these are direct Sox17 targets or not. In addition, *Gata5* (not shown) and *Gata6* were only weakly induced above background levels by GR:Sox17 and thus we could also not determine if these were direct Sox17 targets or not. Activin (which mimics nodal proteins) induced expression of all the endodermal genes. However, in the presence of cycloheximide, the activin induced transcription of *Hnf1β*, *Edd* and *Foxa2* was inhibited, indicating that activin/nodal activates their expression indirectly, probably via Sox17 and possibly other factors.

Whole-mount in situ hybridization to bisected gastrulae confirmed that the target genes are co-expressed with Sox17 in the deep endoderm (Fig. 2C). In summary, the data indicate that *Hnf1β*, *Foxa1*, *Foxa2*, *Sox17α* and *Edd* (this study) (Clements et al., 2003) are direct Sox17 targets, whereas *26D10* is an indirect target, and we could not determine if *Gata4*, *Gata5*, *Gata6*, Gsc and *Xnr4* are direct Sox17 targets or not.

The transactivation domain of Sox17β

To better understand how Sox17β regulates the transcription of its targets, we performed a structure-function analysis to localize its transactivation domain. Similar studies have been useful for identifying potential co-factor interaction sites in other Sox proteins (Kamachi et al., 1999; Kamachi et al., 2000; Nowling et al., 2000). Different parts of Sox17β were fused to the GAL4 DNA-binding domain and assayed for transactivation capacity on a UAS:Luciferase reporter in mammalian COS-1 cells (Fig. 3A). GAL4 fused to the entire Sox17β had little activity, but a latent transactivation domain in the C terminus was revealed when regions of the N terminus and HMG box were removed. This suggests that transactivation domain may be regulated somehow by inhibition from other regions of the protein. Testing further deletion construct allowed us to map the minimal transactivation domain to 25 amino acids between residues 315-340.

To determine if this transactivation domain was functionally important in embryos, we tested a similar set of Sox17β deletion constructs for the ability to induce the transcription of endogenous Sox17 target genes in animal cap assays. Consistent with the tissue culture experiments, C-terminal amino acids 315-373 were required to activate target gene transcription and deletion of the N terminus (d56-373) resulted in a more potent transcriptional activator (Fig. 3B). We noticed that deletion d1-340 (which based on the tissue culture

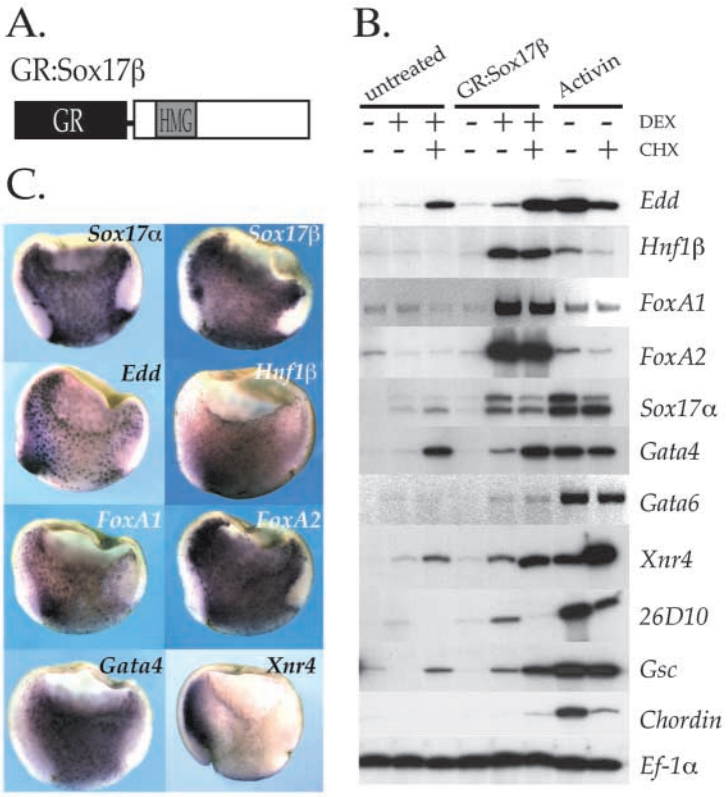

Fig. 2. Direct targets of Sox17β. (A) A schematic of the GR:Sox17β fusion protein consisting of the hormone-binding domain of the human glucocorticoid receptor fused to Sox17β. (B) Isolated blastula animal cap explants either injected with mRNA encoding GR:Sox17β (150 pg) or uninjected were each cultured in three conditions: 1×MBS, 1×MBS + 10^{-6} dexamethasone (DEX) or in 1×MBS with 10^{-6} dexamethasone + 10 μg/ml cycloheximide (CHX). Dexamethasone activates the GR:Sox17β fusion protein and cycloheximide blocks translation. Control animal caps were treated with 5 ng/ml human activin A either with or without 10 μg/ml cycloheximide. At gastrula (stage 11) the explants were assayed by RT-PCR. *GR:Sox17β* induced *Hnf1β*, *Foxa1*, *Foxa2* and *Sox17α* transcription when translation was blocked and are therefore direct Sox17 targets. A repeat of this experiment assayed by real-time RT-PCR is presented in Figs S1-S3 at http://dev.biologists.org/supplemental. (C) Whole-mount in situ hybridization to bisected gastrula (stage 11) embryos with the indicated probes confirms that Sox17 target genes are expressed in the endoderm. Dorsal/anterior towards the left.

experiments, still contains the putative activation domain) could not transactivate *Hnf1β*, *Edd* and *Foxa2*, whereas *Foxa1* and *Sox17α* were still induced, suggesting that in vivo the activation domain is compromised in the d1-340 mutant. The deletion mutant d1-315, which lacks the activation motif, was unable to stimulate transcription of any Sox17 targets, except *Foxa1*, at a reduced level. This indicates that the transactivation motif is essential in vivo and also suggests that different Sox17 targets have a differential requirement for the transactivation domain. Western blot analysis indicated that all of the truncated Sox proteins were expressed to similar levels in COS-1 and animal cap cells (data not shown).

Close examination of the transactivation domain sequence revealed a short motif conserved in all the Sox F subclass of Sox proteins (Fig. 3C), which includes Sox17, Sox18 and Sox7 (Bowles et al., 2000). Interestingly, zebrafish Sox17 does not have this motif, but it is present in Casanova, a zebrafish Sox17-like protein essential for endoderm formation (Dickmeis et al., 2001; Kikuchi et al., 2001). Outside of the highly conserved HMG domain, the sequences of Sox17, Sox18, Sox7 and Casanova are very divergent, only 5-10% identical amino acids, with the exception of this short conserved motif. To test if this conserved motif is essential for transactivation, we mutated the conserved the amino acids EQY or DQY to GGG (referred to as 3G mutant) or we deleted the amino acids EFDQY or EFEQY (referred to as ΔTA mutant) in Sox17α and Sox17β respectively (Fig. 3C). In GAL4:fusions-reporter assays (Fig. 3A; compare 200-340 with 200-340-3G and 200-340-ΔTA) and in *Xenopus* animal cap experiments (Fig. 3D), the mutant Sox17 proteins had significantly reduced transactivation capacity. Again, we observed that the transcription of *Hnf1β* and *Foxa2*, was more sensitive to an intact transactivation motif than *Foxa1* and *Edd*.

In summary we have identified a conserved motif in SoxF class proteins, which is essential for both Sox17α and Sox17β transcriptional activity and which may mediate interactions with important protein co-factors.

Sox17 associates with β-catenin

Sox proteins generally require interacting protein partners to function (Kamachi et al., 2000; Wilson and Koopman, 2002) and we had previously shown in overexpression experiments that Sox17α and Sox17β can directly bind the armadillo repeats of β-catenin (Zorn et al., 1999a), but the biological relevance of this Sox17/β-catenin interaction was unclear. We hypothesize that analogous to Tcf/Lef, β-catenin may be an important transcriptional co-factor of Sox17.

If Sox17 and β-catenin interact to regulate endodermal gene expression, then β-catenin must be present in the nuclei of gastrula endoderm cells. We therefore closely examined the subcellular distribution of β-catenin protein by immunostaining and confocal microscopy and observed obvious nuclear β-catenin throughout the Sox17 expressing deep endoderm cells of the gastrula (Fig. 4A), as previously reported (Schohl and Fagotto, 2002).

Another prediction of our hypothesis is that endogenous Sox17 and β-catenin should physically associate in the nucleus. With current Sox17 antibodies, we cannot robustly detect the low levels of endogenous Sox17 protein in *Xenopus* embryos and we therefore turned to a human colorectal cancer cell line, SW480, which expresses endogenous Sox17 (J. Kordich and J. Wells, personal communication). SW480 cells are $Apc^{-/-}$ mutant and as a result they have elevated levels of nuclear β-catenin, mimicking active Wnt signaling (Munemitsu et al., 1995). We fractionated SW480 cells to produce cytosolic and nuclear extracts. Immunoblotting with tubulin and histone H1 antibodies verified the efficient cell fractionation and as expected Sox17 and β-catenin were both present in the nuclear fraction (Fig. 4B). After immunoprecipitating the nuclear fraction with either anti-Sox17 antibodies or anti-HA antibodies as a negative control, immunoblotting of the precipitates with anti-β-catenin antibodies demonstrated that endogenous Sox17 and β-catenin

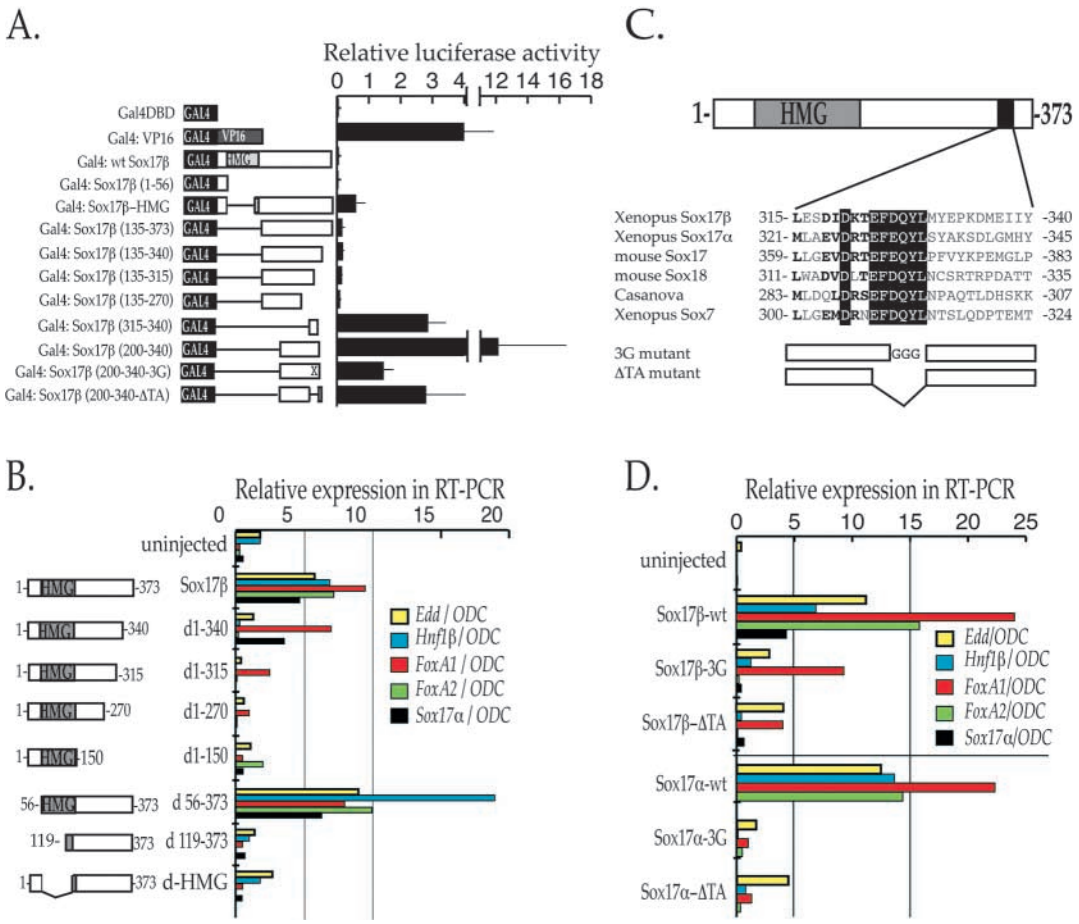

Fig. 3. Structure-function mapping of the transactivation domain of Sox17β. (A) The Gal4 DNA-binding domain (Gal4DBD) was fused to various parts of the Sox17β open reading frame. COS-1 cells were co-transfected with the indicated Gal4-fusion constructs (300 ng), UAS:luciferase reporter (100 ng) containing five Gal4 binding sites and a pTK-Renilla Luciferase plasmid (50 ng). The average relative luciferase activity normalized to renilla activity, from a triplicate experiment is shown. The Gal4 DNA-binding domain alone (Gal4DBD) is a negative control and Gal4:VP16 is a positive control containing the viral VP16 transactivation domain. (B) 200 pg of RNA encoding the indicated HA-tagged Sox17β deletion constructs was injected into two-cell stage embryos, animal cap tissue was isolated at blastula stage, cultured until gastrula stage and assayed by real-time RT-PCR for the expression of Sox17 target genes. The histogram shows the relative gene expression normalized to the loading control *ODC*. (C) The schematic shows the transactivation domain of Sox17β contains a sequence motif conserved in all members of the SoxF subfamily of Sox proteins. A sequence alignment of this conserved motif from representative proteins is shown. Identical amino acids are white on black, conserved resides are in bold. Below the schematic the '3G' and 'ΔTA' mutations generated in both Sox17α and Sox17β are shown. (D) 200 pg of RNA encoding either wild-type or mutant versions of Sox17β (top three constructs) or Sox17α (bottom three constructs) were injected into two-cell stage embryos, animal cap tissue was isolated at blastula stage, cultured until gastrula stage and assayed by real-time RT-PCR for the expression of Sox17 target genes. The histogram shows the relative gene expression normalized to the loading control *ODC*.

co-precipitate from the nuclear extract (Fig. 4C). The immunoprecipitation of Sox17/β-catenin complexes was abolished by the addition of excess Sox17 peptide epitope but not by a control peptide corresponding to another region of Sox17 not recognized by the Sox17 antibody. These data indicate that endogenous Sox17 and β-catenin interact in the nuclei of SW480 cells and suggests that they may also interact in the nuclei of *Xenopus* gastrulae.

If the interaction with β-catenin influences the ability of Sox17 to regulate the transcription of its target genes, we predicted that the conserved transactivation motif would be involved in β-catenin binding. To test this, we transfected COS-1 cells with V5-epitope tagged versions of either wild-type Sox17β, Sox17α or various versions with mutated transactivation domains: Sox17β-3G, Sox17β-ΔTA, Sox17β-d1-315, Sox17α-3G, Sox17α-ΔTA (details of these mutants are shown in Fig. 3). As a control, we also tested a Sox17β construct with a mutation in the HMG box where Gly93 was changed to arginine (Sox17β-G93R), which disrupts the HMG domain structure and DNA-binding activity (Love et al., 1995) (data not shown) but which should not effect β-catenin binding. Extracts were prepared from the resulting cells and incubated with either GST-agarose or GST-β-catenin-agarose beads. After washing, the bound proteins were visualized by anti-V5 western blotting.

We found that mutations or deletion of the conserved transactivation motif in either Sox17α or Sox17β impaired or abolished the ability to bind to β-catenin, while the mutation

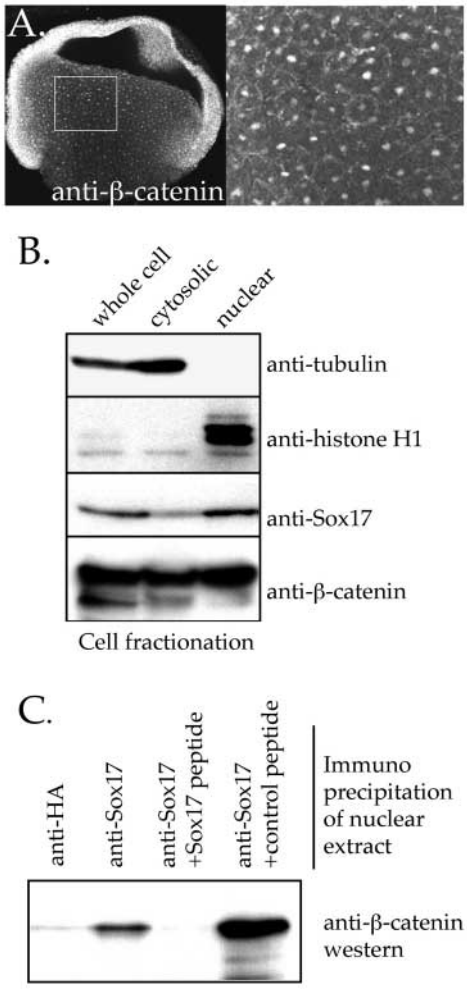

Fig. 4. Interaction of endogenous β-catenin and Sox17. (A) A 160 μM mid-sagittal optical *z*-series of anti-β-catenin immunofluorescence in a *Xenopus* midgastrula shows both membrane bound and nuclear β-catenin in the deep endodermal cells. The intense staining throughout the animal cap and mesoderm is the result of the cells in this region of the embryo being much smaller than in the endoderm (therefore the optical stack is several cells thick in these regions, resulting in an almost uniform staining). The right panel shows a higher magnification of the endoderm (white box) with the stained nuclei clearly visible. Dorsal/anterior towards the left. (B**)** Western blotting of whole cell, cytosolic and nuclear extracts (10 μg of protein each) from human SW480 colorectal cancer cells with anti-tubulin (cytosol antigen), anti-histone H1 (nuclear antigen), anti-Sox17 and anti-β-catenin antibodies. SW480 cells express both endogenous Sox17 and β-catenin in the nuclear fraction. (C) After immunoprecipitation of the nuclear extract with either anti-Sox17 or anti-HA (as a negative control) antibodies, associated β-catenin protein was detected by western blotting. Endogenous β-catenin co-immunoprecipitated with nuclear Sox17. The precipitation of Sox17/β-catenin complexes can be competed by the addition of Sox17 peptide recognized by the anti-Sox17 antibody but not by peptides to other regions of Sox17.

in the HMG box had no effect on β-catenin binding (Fig. 5). Thus, the ability of Sox17 to activate transcription of its target genes (Fig. 3) correlates with β-catenin binding, suggesting that β-catenin may facilitate the ability of Sox17 to activate transcription.

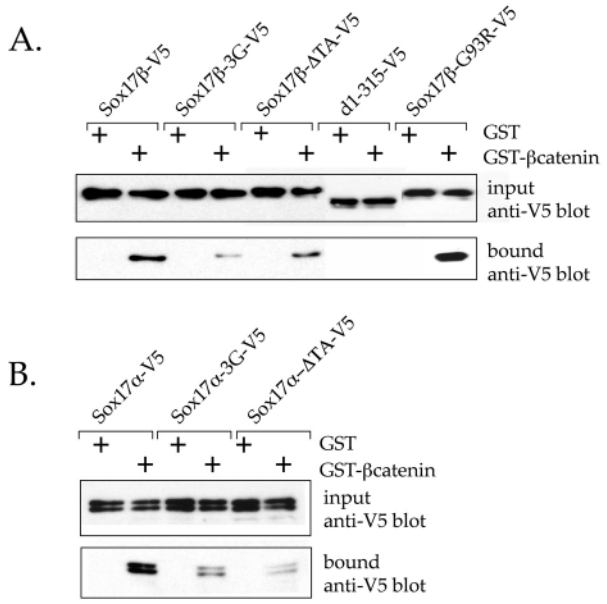

Fig. 5. The transactivation domain of Sox17β is required for β-catenin binding. COS-1 cells were transfected with (A) 3 μg of DNA encoding the indicated V5-epitope tagged Sox17β constructs or (B) 3 μg of DNA encoding the indicated V5-epitope tagged Sox17α constructs. The resulting cell extracts were either incubated with GST-agarose or GST-β-catenin-agarose. The input and bound V5-tagged proteins were visualized by anti-V5 immunoblotting. Mutations or deletion of the conserved transactivation motif in either Sox17β or Sox17α impairs or abolishes β-catenin binding.

Sox17 and β-catenin regulate endodermal transcription

As the transactivation domain of Sox17 is essential for β-catenin binding, we determined if β-catenin could influence the ability of Sox17 to activate the transcription of its targets in vivo. Low doses of *Sox17β* RNA (20 pg, 60 pg and 180 pg of RNA) were injected into 2-cell stage embryos either with or without RNA encoding stabilized β-catenin (100 pg of pt-β-catenin) (Yost et al., 1996). Animal cap cells were isolated at blastula stage, cultured for 2-3 hours and assayed for the expression of direct Sox17 target genes by real-time RT-PCR. We found that Sox17β and β-catenin cooperated to induce the transcription of *Hnf1β*, *Edd*, *Foxa1* and *Foxa2* (Fig. 6). *Sox17α* was only weakly induced with these low doses of *Sox17β* RNA and there was little if any enhancement by β-catenin. As expected the mRNA levels of the ubiquitously expressed gene, *plakoglobin* (*Plako*), which is neither a Sox17 nor a β-catenin target, exhibited only minor variations that did not correlate with the experimental treatment. In four separate experiments, β-catenin always potentiated the induction of *Hnf1β*, *Foxa2*, and *Edd* by Sox17 more than it did for *Foxa1*, or *Sox17α*, suggesting that the interaction between Sox17 and β-catenin has a varying degree of importance for different target promoters.

β-Catenin alone did not induce the expression of the Sox17 target genes even though animal caps express endogenous Tcf1, Tcf3, Lef1 and Tcf4 (Molennar et al., 1998; Houston et al., 2002; Roel et al., 2003), further suggesting that Sox17 targets are not regulated by β-catenin/Tcf complexes. In

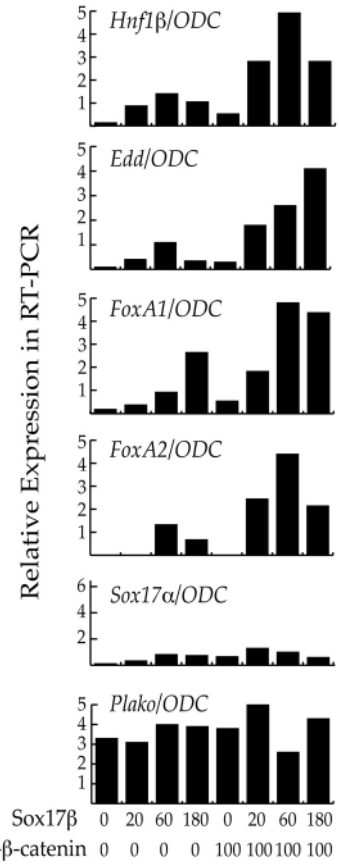

Fig. 6. Sox17 and β-catenin co-operate to transcribe endodermal genes. Embryos were injected at the 2-cell stage with Sox17β mRNA (20 pg, 60 pg, 180 pg) either with or without co-injection of RNA encoding a stabilized β-catenin (pt-β-catenin, 100 pg) (Yost et al., 1996). At blastula stage, animal cap tissue was explanted and cultured for 3-4 hours until gastrula stage when it was assayed by real-time RT-PCR for the expression of Sox17 target genes. The histograms show the relative expression levels normalized to the loading control *ODC*. *Plakoglobin* (*Plako*) is control gene that is neither a target of Sox17 nor β-catenin.

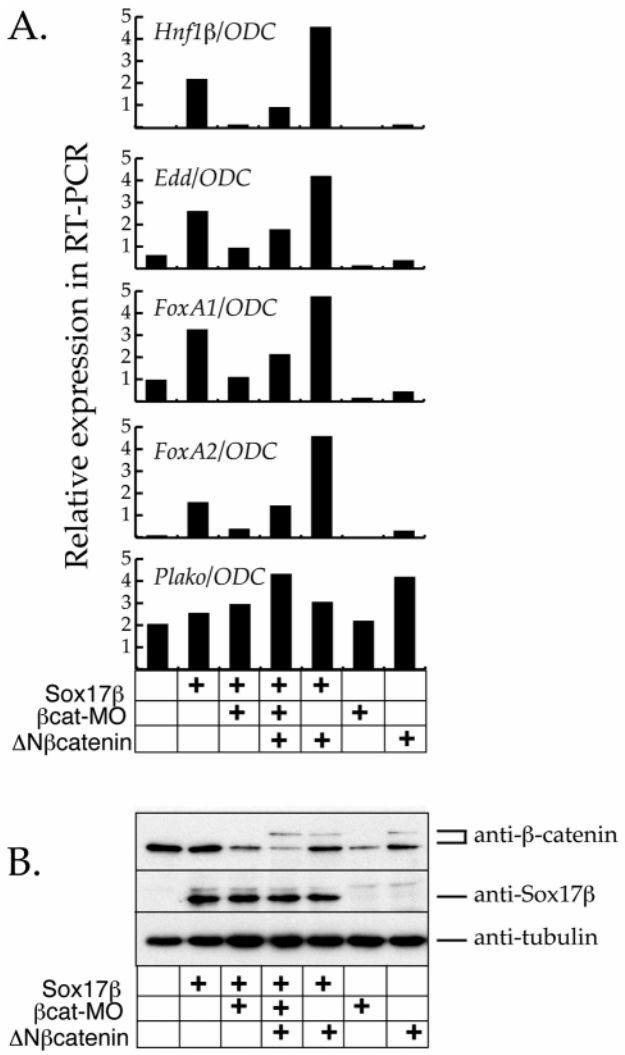

Fig. 7. Sox17β requires β-catenin to robustly activate target gene transcription in animal caps. (A) Embryos were injected at the two-cell stage with the indicated combinations of: Sox17β mRNA (200 pg), RNA encoding a N-terminal deleted form of stabilized β-catenin (ΔN-β-catenin, 100 pg) (Yost et al., 1996), or an antisense β-catenin morpholino oligos (βcat-MO; 20 ng). At blastula stage, animal cap tissue was explanted and cultured for 3-4 hours until gastrula stage, when it was assayed by real-time RT-PCR for the expression of Sox17 target genes. The histograms show the relative expression levels normalized to the loading control, *ODC*. *Plakoglobin* (*Plako*) is control gene that is neither a Sox17 nor β-catenin target. (B) A proportion of each sample from the same experiment was assayed by immunoblotting with anti-β-catenin, anti-Sox17β or anti-tubulin antibodies. Injected ΔN-β-catenin protein has a higher molecular weight than endogenous β-catenin because of the presence of an epitope tag. Tubulin is a loading control.

addition, neither β-catenin alone or β-catenin+Sox17 induced the expression of any other endodermal genes tested, including Mixer, Xnr1 or Xnr2 (data not shown), arguing that the enhanced expression of Sox17 target genes was not due to a secondary endoderm promoting factor induced by β-catenin.

We next asked if Sox17 required β-catenin to activate transcription of its targets. It is possible that the low level of nuclear β-catenin found in the animal cap cells (Schohl and Fagotto, 2002) (Fig. 4) facilitates the ability of Sox17 to activate its targets in this non-endodermal tissue. To test this, we expressed Sox17β in animal cap tissue where endogenous β-catenin had been depleted by antisense morpholino oligos (Heasman et al., 2000) and assayed for the expression of Sox17 target genes by real-time RT-PCR (Fig. 7A). Endogenous β-catenin protein levels and the expression of injected Sox17β were monitored by western blotting (Fig. 7B).

We found that Sox17β required β-catenin to robustly activate transcription of *Hnf1β*, *Edd*, *Foxa1* and *Foxa2*. This effect was specifically due to the loss of β-catenin, because injection of RNA encoding a stabilized β-catenin (ΔN-β-catenin) (Yost et al., 1996), which lacked the sequences recognized by the antisense oligo, rescued the inducing activity of Sox17β. These results suggest that β-catenin potentiates Sox17 transactivation activity and consistent with our previous experiments, the transcription of *Hnf1β* and *Foxa2* was more dependent on β-catenin levels than *Foxa1* and *Edd* (Fig.7).

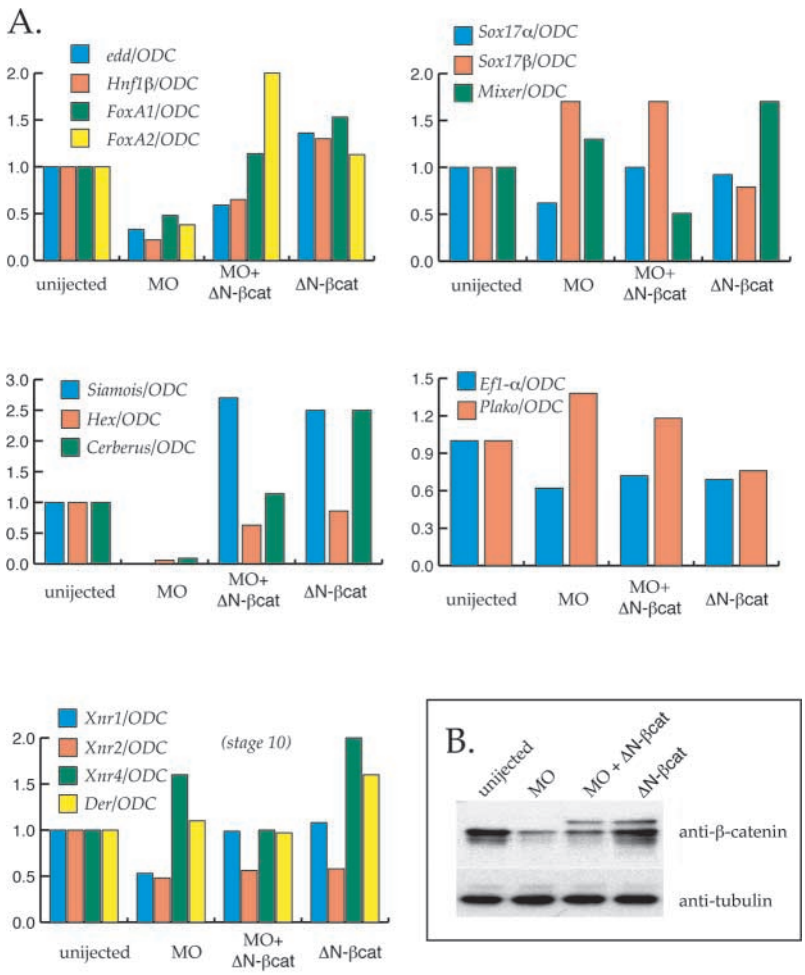

Fig. 8. β-catenin is required for the expression of Sox17 targets genes at gastrula stage. (A) Two-cell embryos were vegetally injected with either 20 ng of antisense β-catenin morpholino oligos (MO) and/or 100 pg of RNA encoding a N-terminal deleted form of stabilized β-catenin (ΔN-β-cat) (Yost et al., 1996), which does not contain the sequence targeted by the antisense oligo. At a series of stages throughout gastrulation (stages 10, 11 and 12) whole embryos were harvested and assayed by real-time RT-PCR for the expression of Sox17 target genes as well as several other control genes. The histograms show the relative expression levels normalized to the loading control, *ODC*. For simplicity only the stage 11 data (stage 10 for *Xnr1*, *Xnr2*, *Xnr4* and *Derriere*) is shown. *Edd*, *Hnf1β*, *Foxa1* and *Foxa2* are direct Sox17 target genes. *Siamois*, *Hex* and *Cerberus* are known β-catenin target genes. *Xnr1*, *Xnr2*, *Xnr4*, *Derreire* and *Mixer* are endodermal genes that are not Sox17 targets. *Plakoglobin* (*Plako*) and *Ef1α* are control genes that are neither Sox17 nor β-catenin targets. (B) A proportion of each sample from the same experiment was assayed by immunoblotting with either anti-β-catenin or anti-tubulin antibodies. Injected ΔN-β-catenin protein has a higher molecular weight than endogenous β-catenin because of the presence of an epitope tag.

β-Catenin is required for normal endoderm formation

If Sox17 and β-catenin interact to regulate expression of endodermal genes during normal development, then β-catenin should be required for the expression of Sox17 target genes. To test this prediction, we depleted endogenous β-catenin protein from embryos by microinjecting antisense β-catenin morpholino oligos into 2-cell stage embryos. The resulting embryos were assayed at gastrula stage by real-time RT-PCR for endodermal and organizer gene expression (Fig. 8A). A proportion of the same sample was assayed by western blotting to monitor the levels of β-catenin protein (Fig. 8B).

We found that *Edd*, *Hnf1β* and *Foxa2* were consistently downregulated in β-catenin-depleted embryos. Of all the Sox17 targets, *Hnf1β* transcripts were the most severely reduced, to ~10-20% of wild-type levels, whereas *Foxa1* mRNA levels were the least effected dropping at most by half and in some cases not at all (Fig. 8A; see Figs S1-S3 at http://dev.biologists.org/supplemental). This effect was specifically due to loss of β-catenin, because it was rescued by injection of RNA encoding a stabilized β-catenin (ΔN-β-cat) (Yost et al., 1996) that lacked the sequence complimentary to the antisense oligo. The anterior endoderm organizer genes *Hex*, *Siamois* and *Cerberus* (which are not Sox17 targets) were downregulated in the β-catenin-depleted embryos as previously shown (Xanthos et al., 2002).

We also assayed the expression of components of the endoderm specification pathway such as nodals and Mixer to determine if all endoderm development was compromised or just Sox17 targets. We observed that *Xnr1* and *Xnr2* were moderately downregulated while the expression of *Derriere* was unchanged and *Xnr4* was moderately increased in β-catenin-depleted embryos (Fig. 8A) (Xanthos et al., 2002). The reduction in *Xnr1* and *Xnr2* mRNA levels is unlikely to account for the reduced expression of Sox17 targets for several reasons. First, the levels of *Sox17* and *Mixer* RNA, both of which are nodal targets (Hudson et al., 1997; Henry and Melton, 1998) (Fig. 2), were changed only modestly by depletion of β-catenin (Fig. 8) (Xanthos et al., 2002). *Sox17α* transcripts were only reduced to ~60% of wild-type levels and *Sox17β* mRNA levels were actually increased to ~160% wild-type levels. Furthermore, nodal signaling regulates *Hnf1β*, *Edd* and *Foxa2* transcription indirectly, via Sox17. Blocking Sox17 function either with a dominant negative Sox17 or by depleting Sox17 with antisense oligos, inhibits activin (nodal) induction of *Hnf1β*, *Edd* and *Foxa2* in animal caps (Hudson et al., 1997; Clements et al., 2003). This is consistent with our results indicating *Hnf1β*, *Edd* and *Foxa2* transcription is indirectly regulated by activin but directly activated by Sox17. Therefore it is unlikely that the modest decrease in Xnr1, Xnr2 could account for the reduced expression of Sox17 targets.

In summary, these experiments show that β-catenin is essential for endoderm formation downstream of Sox17. Furthermore, our results suggest that β-catenin is an important co-factor of Sox17, assisting in the transcription of some of its downstream target genes.

Discussion

Sox17 targets

Although Sox17 is essential for endoderm formation, little was understood about its targets. We have identified a number of Sox17 target genes, at least five of which, *Edd*, *Hnf1β*, *Foxa1*, *Foxa2* and *Sox17α*, are direct transcriptional targets (this study) (Clements et al., 2003). In general, our findings fit with the gene hierarchy predicted by the current model of endoderm formation. Genes thought to be upstream of *Sox17* such as *Mix/Bix/Mixer*, *Xnr1*, *Xnr2* and *Derriere* were not activated by Sox17. However, *Gata4*, *Gata5*, *Gata6* and *Xnr4* were previously considered to be upstream of Sox17, but were induced by Sox17. As Gata, Gata5 and Xnr4 can also induce Sox17 expression (Clements et al., 1999; Weber et al., 2000), there appear to be feedback regulatory loops in operation, suggesting that the pathway of endoderm specification is more complex than predicted by the current model.

Endodermal genes reported to be downregulated in Sox17 loss-of-function studies are largely consistent with the Sox17 targets we have identified. In a recent study by Clements et al. (Clements et al., 2003) depletion of Sox17 in *Xenopus* embryos by antisense morpholino oligos resulted in a reduction of *Gata5* and *Edd* expression. In addition, analysis of Sox17 null mutant mice found that *Foxa1* and *Foxa2* expression was dramatically reduced (Kanai-Azuma et al., 2002). However, *Foxa2* was largely unaffected in Sox17-depleted *Xenopus* embryos (Clements et al., 2003), suggesting that other factors also regulate its expression.

The identification of *Foxa1* and *Foxa2* as direct Sox17 targets is particularly important as these hepatic nuclear factors are known to be involved in endodermal organ differentiation and tissue-specific gene expression (Duncan et al., 1998; Kaestner et al., 1999). Furthermore, *Foxa2* is essential for definitive endoderm development in mice (Ang et al., 1993; Hallonet et al., 2002), but its epistatic position in the endoderm specification pathway was unclear.

β-catenin is a Sox17 co-factor

Sox proteins generally require interacting protein partners in order to regulate the transcription of their target genes (Wilson and Koopman, 2002). We had previously shown that Sox17α and Sox17β could physically interact with the armadillo repeats of β-catenin (Zorn et al., 1999a), but at that time the biological significance was previously unclear. Our data now suggests that β-catenin is a transcriptional co-factor of Sox17.

It is interesting that the transactivation motif and β-catenin binding are not absolutely required for Sox17 to activate the transcription of *Foxa1*, whereas Sox17-induced transcription of *Hnf1β* and *Foxa2* is much more dependent on the transactivation motif and β-catenin. The basis of this difference and how β-catenin potentiates the ability of Sox17 to activate the transcription remains to be determined. One possibility is that β-catenin recruits the co-activator CBP/p300 (Hecht et al., 2000; Takemaru and Moon, 2000) to Sox17 target gene promoters.

The role of β-catenin in normal endoderm development

β-Catenin is best known in *Xenopus* development for activating the expression of organizer genes through Tcf/Lef complexes. We suggest that the same dorsoanterior β-catenin activity that regulates organizer gene expression, promotes high levels of Sox17 target gene transcription when complexed with Sox17 in the anterior endoderm. If fact, we have observed that most of the Sox17 target genes are first more strongly activated in the dorsoanterior endoderm relative to the ventroposterior (data not shown). It is unlikely that β-catenin/Tcf complexes directly regulate the transcription of the Sox17 targets, because even though Tcf1, Tcf3, Tcf4 and Lef1 are endogenously expressed in animal cap cells (Roel et al., 2003; Molenaar et al., 1998; Houston et al., 2002), overexpression of activated β-catenin or Tcf1, Tcf3, Tcf4 and Lef1 did not activate the transcription of Sox17 targets (see Figs S1-S3 at http://dev.biologists.org/supplemental). In addition, in *Xenopus* embryos depleted of Tcf3, the only Tcf/Lef protein shown to regulate organizer gene expression (Houston et al., 2002), we observed no changes in Sox17 target gene expression (see Figs S1-S3 at http://dev.biologists.org/supplemental). Together, these data suggest that β-catenin/Tcf complexes are unlikely to regulate Sox17 target genes in vivo.

Considering all of our data, the simplest interpretation is that Sox17 requires β-catenin to robustly activate the transcription of its target genes. Emerging evidence from other model systems also indicates that β-catenin is required for endoderm formation in *C. elegans*, sea urchin, ascidian and the mouse (Imai et al., 2000; Logan et al., 1999; Rocheleau et al., 1997; Stainier, 2002).

Sox proteins as effectors of β-catenin signaling

Our findings have broader implications for how Sox proteins may act as Wnt/β-catenin transcriptional effectors. Our data suggest that Sox17β and β-catenin interact to regulate endodermal gene transcription in a manner analogous to β-catenin/Tcf regulation of Wnt responsive transcription (Behrens et al., 1996; Molenaar et al., 1996; Wodarz and Nusse, 1998). The fact that the transactivation and β-catenin-binding motif in Sox17 is conserved in most SoxF subgroup members, suggests that other SoxF proteins may similarly interact with β-catenin. Indeed, human Sox7 was recently shown to interact with β-catenin in tissue culture reporter assays (Takash et al., 2001). Furthermore, in other contexts, Sox proteins (Sox17, Sox3 and Sox7) can antagonize β-catenin/Tcf-mediated transcription (Zorn et al., 1999a; Takash et al., 2001; Zhang et al., 2003). Thus, the interaction of β-catenin with different Tcf or Sox proteins may explain, in part, how the Wnt signaling pathway can elicit diverse transcriptional responses in different cellular contexts.

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Supplementary Table 1. Primers used in RT-PCR analysis.

mRNA	sequence	reference	acquisition temp.
Bix1	F: 5'- AGA GAC TCC CAG TTC ATC TGA -3' R: 5'- GGT AGG TGG GAA GTT GCT AAT -3'	Xanthos et al. 2001	78°C
Bix2	5'- TCT CGC ATT CAG GTT TGG TTC C -3' 5'- ATC TCC TTG TTA GGG ATC ATA C -3'	Xanthos et al. 2001	78°C
Bix3	F: 5'- TGC TCG AGT CAC GCA TAC AG -3' R: 5'- TGG ATG TCC TGG GAG TCT CTG GC -3'	Xanthos et al. 2001	78°C
Bix4	F: 5'- AGA TGC TAC AGG CTG GAG CAA -3' R: 5'- GTG TGT AAG GGG TGA GTC ATA -3'	Xanthos et al. 2001	78°C
Cerberus	F: 5'- GCT GAA CTA TTT GAT TCC ACC –3' R: 5' ATG GCT TGT ATT CTG TGG GGC -3'	Bouwmeester et al. 1996	74°C
Chordin	5'- AAC TGC CAG GAC TGG ATG GT -3' 5'- GGC AGG ATT TAG AGT TGC TTC -3'	Sasai et al. 1994	72°C
Derriere	F: 5'- CGC TCA TAT CGA GAT CAA GG -3' R: 5'- TCC TGC AAG TTC ACT GCT TG -3'	Yasuo and Lemaire, 1999	78°C
Ef1-α	F: 5'- CAG ATT GGT GCT GGA TAT GC -3' R: 5'- ACT GCC TTG ATG ACT CCT AG -3'	Wilson&Melton 1994	78°C
Edd	F: 5'- AGA ACG GAG ACT CAG ATC AAT -3' R: 5'- 5'- TTA CTC ATT GCT CCC ACC ATC -3'	this study	78°C
FoxA1	F: 5'- TCA CTC ATT GGC CCA TGA AAC -3' R: 5'- CCT AGG AGG TGT TTA GGA CAG -3'	this study	78°C
FoxA2	F: 5'- CCT ATC ATG AAC TCC TCA TAG -3' R: 5'- GGC CAG AAT ACA TAC AGC AGT C -3'	this study	74°C
Fzb1	F: 5'- AGT AAG CCT ACA CAT ACA GGT TGG -3' R: 5'- GCA GAC TCC TCT GTC ATA TAC GG -3'	Wang et al. 1997	78°C
Gata4	F: 5'- AGT GCT ACT GCT GCT ACC TC -3' R: 5'- AGT GCT ACT GCT GCT ACC TC -3'	Xanthos et al. 2001	78°C
Gata5	F: 5'- ACC TTC AGA GCT GCG ACA CT -3' R: 5'- CAG TGT ATT GCC ATA CTG GTC -3'	Xanthos et al. 2001	78°C
Gata6	F: 5'- CCA ACC GGG AGC CCC GAT A -3' R: 5'- GCT GCT GTA GCC TGT ATC C -3'	Xanthos et al. 2001	78°C
Gsc	F: 5'- ACA ACT GGA AGC ACT GGA -3' R: 5'- TCT TAT TCC AGA GGA ACC -3'	Wilson and Melton 1994	76°C
Hex	F: 5'- GGT TCC AGA ACA GAA GAG -3' R:5'- CCT TTG TCG CCT TCA ATG -3'	Zorn et al. 1999	78°C
Hnf1β	F: 5'- GCA TAT GGC ACA GCA GCC ATT -3' R: 5'- CAC CAT GCT TGC AAA GGA CAC -3'	this study	78°C
Mix1	F: 5'- ATG TCT CAA GGC AGA GGT -3' R: 5'- CAC TGA CAC CAG AAT CTG -3'	Wilson & Melton 1994	78°C
Mix2	F: 5'- TGC AAG CCA TCA TTA TTC TAG C -3' R: 5'- AGG AAC CTC TGC CTC GAG ACA T -3'	Xanthos et al. 2001	78°C
Mixer	F: 5'- CAC CAG CCC AGC ACT TAA CC -3' R: 5'- CAA TGT CAC ATC AAC TGA AG -3'	Henry and Melton 1998	78°C
ODC	F: 5'- GCC ATT GTG AAG ACT CTC TCC AAT C -3' R: 5'- TTC GGG TGA TTC CTT GCC AC -3'	Heasman et al. 2000	78°C
Plakoglobin	F: 5'- GCT CGC TGT ACA ACC AGC ATT C -3' R: 5'- GTA GTT CCT CAT GAT CTG AAC C -3'	Kofron et al., 1999	78°C
Siamois	F: 5'- AAA CCA CTG ATT CAG GCA GAG G -3' R: 5'- GTA GGG CTG TGT ATT TGA AGG G -3	Zorn et al. 1999a	78°C
Sox17α	F: 5'- GGA CGA GTG CCA GAT GAT G -3' R: 5'- CTG GCA AGT ACA TCT GTC C -3'	Hudson et al. 1997	78°C
Sox17β	F: 5'- TAT CAG TCC CAG AAG ACG GTC -3' R: 5'- CAT GTC ACA TCC ACA AGA GAG -3'	Zorn et al. 1999b	74°C
Xbra	F: 5'- GGA TCG TTA TCA CCT CTG -3' R: 5'- GTG TAG TCT GTA GCA GCA -3	Wilson & Melton 1994	74°C
Xnr1	F: 5'- CTG CCA ACC ATA TTG GCT TT –3 R: 5'- GTG GTG CCT CAA AAC AAC CT -3'	Yasuo and Lemaire, 1999	78°C
Xnr2	F: 5'- CAG ACC CTG ATT TTG GGA AA -3' R: 5'- CTG ACC TTC CTT GGT GTG GT -3'	Yasuo and Lemaire, 1999	78°C
Xnr3	F: 5'- AAA TCC ATG TGA GCA CCG TTC -3' R: 5'- GCA TTC TCT GTC TCA TTC TGT G -3'	Zorn et al. 1999a	74°C
Xnr4	F: 5'- ACT TGG CTG CTC TAC CTC -3' R: 5'- CAG CAA GTT GAT GTT CTT CC -3'	Sun et al. 1999	78°C
26D10	F: 5'- GGG GCA GAC GAT CAG CAA ACG C -3' R: 5'- AAT TGG CAT CGG ATT CTC TGG C -3'	de Souza et al. 1999	73°C