The Cerebellin 4 Precursor Gene Is a Direct Target of SRY and SOX9 in Mice

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ABSTRACT

In most mammals, the expression of SRY (sex-determining region on the Y chromosome) initiates the development of testes, and thus determines the sex of the individual. However, despite the pivotal role of SRY, its mechanism of action remains elusive. One important missing piece of the puzzle is the identification of genes regulated by SRY. In this study we used chromatin immunoprecipitation to identify direct SRY target genes. Anti-mouse SRY antibody precipitated a region 7.5 kb upstream of the transcriptional start site of cerebellin 4 precursor (Cbln4), which encodes a secreted protein. Cbln4 is expressed in Sertoli cells in the developing gonad, with a profile mimicking that of the testis-determining gene SRY-box containing gene 9 (Sox9). In transgenic XY mouse embryos with reduced Sox9 expression, Cbln4 expression also was reduced, whereas overexpression of Sox9 in XX mice caused an upregulation of Cbln4 expression. Finally, ectopic upregulation of SRY in vivo resulted in ectopic expression of Cbln4. Our findings suggest that both SRY and SOX9 contribute to the male-specific upregulation of Cbln4 in the developing testis, and they identified a direct in vivo target gene of SRY.

Cbln4, developmental biology, gene regulation, mouse, Sertoli cells, sex determination, SOX9, SRY, testis, transcriptional regulation

INTRODUCTION

A pivotal point in the growth of all sexually dimorphic organisms is the initiation of either the male or the female pathway of development. The gene that initiates the male pathway in almost all mammals, including humans, is SRY. The requirement for SRY in testis development was indicated by the discovery of mutations in this gene in two sex-reversed XY women but not their fathers [1, 2]. Male development of transgenic XX mice expressing Sry confirmed the significance of this gene [3]. SRY protein has been characterized as a DNA-binding transcription factor, but the molecular mechanisms of its action remain a mystery (reviewed in Polanco and Koopman [4]).

SRY belongs to the SOX family of transcription factors that are distinguished by the presence of a high mobility group domain. SRY has been shown to bind and bend DNA in vitro [5, 6]. However, whether SRY acts as a transcriptional activator, repressor, or architectural protein remains unclear. In vitro biochemical assays showed that SRY activated transcription of a reporter gene driven by multiple copies of the SRY-binding motif [7]. In addition, it has been shown recently that mouse SRY binds to a SRY-box containing gene 9 (Sox9) enhancer region in vivo and activates Sox9 expression [8]. Conversely, analysis of more than 100 human XX males supports a model in which SRY acts by repressing a negative regulator of male sex determination [9]. Other in vitro experiments have demonstrated that SRY can act as a repressor, depending on its phosphorylation status [10].

SRY is expressed in the supporting cell lineage within the developing XY genital ridge. Its expression in mice resembles a wave starting in the central portions of the genital ridge, expanding to the anterior part, and finally encompassing the posterior region. Expression levels then subside in a similar center-anterior-posterior wave, suggesting that Sry is active for only a few hours in each single cell [11–13]. Closely following the onset of Sry expression, another gene of the Sox family, Sox9, is expressed in the developing testis. Sox9 expression follows a wave similar to that of Sry, but rather than being rapidly extinguished, its expression continues in the supporting cell lineage of the testis for the remainder of embryonic development [14]. SOX9-positive cells become Sertoli cells that not only form cords surrounding germ cells but also are thought to orchestrate the differentiation of the other cell types in the testis. SOX9 protein has been shown to bind to the same DNA motifs as SRY [15, 16], and a number of direct target genes expressed during testis differentiation have been identified, including anti-Müllerian hormone (Amh) [17, 18], prostaglandin-d synthetase (Ptgds) [19], and vanin 1 (Vnn1) [20].

Like Sry, Sox9 is essential for male development, and its ectopic expression in mice leads to XX sex reversal [21, 22]. However, in these cases of sex reversal, it is unclear whether SOX9 is regulating its normal targets or, as a consequence of
its early expression, is recapitulating the function of SRY. Given that normal testis development requires SRY to be expressed within a narrow time window [23, 24] and that SRY and SOX9 recognize similar or identical DNA-binding sites, it may be that SOX9, expressed at the right time, can fulfill the early functions of SRY. This possibility raises the question of whether Sox9 is the single gene through which SRY influences male determination, or whether SRY regulates multiple targets, one of which is Sox9.

To understand the early events in sex determination, and particularly to disentangle the relationship between SRY and SOX9, we sought here to identify direct target genes of SRY. We performed chromatin immunoprecipitation (ChIP) with an anti-SRY antibody and identified a noncoding region 7.5 kb upstream of the transcriptional start site of the gene cerebellin 4 precursor (Cbln4). Cbln4 is expressed in Sertoli cells of the developing gonad, with a profile mimicking that of Sox9 upstream of the transcriptional start site of the gene cerebellin 4.

**Materials and Methods**

**Animal Strains**

Embryos were collected from timed matings of CD1 outbred and W' mutant mice [25], with noon of the day on which the mating plug was observed designated as 0.5 days postcoitum (dpc). For more accurate staging, the tail somite number of the embryo was determined by counting the number of somites posterior to the hind limb [26]. Using this method, 10.5 dpc corresponds to approximately 8 ts, 11.5 dpc to 18 ts, and 12.5 dpc to 30 ts.

Embryos at 11.5 dpc or younger were sexed by PCR using primers that correspond to approximately 8 ts, 11.5 dpc to 18 ts, and 12.5 dpc to 30 ts. Embryos at 11.5 dpc or younger were sexed by PCR using Zfy gene-specific primers Zfy-F, 5'-CTATTCATGAGCTGACAGTAT-3'; Zfy-R, 5'-GACAAGATGGATTTACATC-3'. Older embryos were sexed by morphological criteria. Protocols and use of animals in these experiments were approved by the Animal Welfare Unit of the University of Queensland, registered as an institution that uses animals for scientific purposes under the Queensland Animal Care and Protection Act (2001).

**ChIP Assay**

Male and female genital ridges (gonad and mesonephros) were dissected from approximately 550 staged mouse embryos, and ChIP was performed as described previously [19]. Antibodies used in the ChIP assays were anti-mouse SRY [13, 19]. Primers used were ChIPPCR1 F, 5'-TAGAACCACGTCTCTCGGTGATG-3'; ChIPPCR2 R, 5'-AAGTITACAAACCCCTTGGAGCGC-3'.

**Western Blot Analysis**

Western blot analysis was performed as described previously [29], with an anti-HA antibody (HA-7; Sigma) at a 1:1000 dilution.

**Cell Culture and Transfections**

MCF-7 cells (catalogue no. HTB-22; American Type Culture Collection) were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions and were analyzed 24–48 h after transfection. Plasmids used were isoprenylcysteine-O-carboxyl methylethertransferase-GFP (IGMT-GFP) based on the vector pEGFP-C1 (Clontech), Transgolgi network 38 (TGN38-YFP) [30] based on the vector pYFP-C1 (Clontech), and Cbln4-myc, containing the open reading frame of Cbln4 with a 3' myc tag cloned into pCDNA3 (Invitrogen).

**Immunofluorescence**

For immunofluorescence, cells were fixed in 4% paraformaldehyde (PFA) for 10 min on ice and then permeabilized with 0.25% Triton X-100 for 5 min at room temperature prior to labeling. Antibodies used were a rabbit polyclonal antibody to GFP (Molecular Probes) and a mouse monoclonal antibody to the MYC tag (9B11; Cell Signaling Technology). As a nuclear stain, 4',6-diamidino-2-phenylindole (DAPI; Roche) was used.

**Western Blot Analysis**

For immunofluorescence microscopy of fixed specimens was performed using an IX81 microscope with a 60x, 1.40 numerical aperture objective (Olympus), and imaging was performed with Orca-1 ER cameras (Hamamatsu) driven by Metamorph imaging software (Universal Imaging). Background correction and contrast adjustment of raw data images were performed with ImageJ (National Institutes of Health) or Adobe Photoshop (Adobe).

**Secretion and Deglycosylation Assays**

MCF-7 cells were transfected with the Cbln4-myc construct for 6 h prior to PBS (wash and addition of fresh medium. Medium was removed following 24 h of incubation and was spun for 5 min at 5000 × g at 4°C to remove cell debris.

For deglycosylation, medium was heated to 100°C for 5 min in PBS containing 1% SDS and 1 M dithiothreitol (Sigma). Samples were then incubated with 1% Triton X-100 in PBS in the presence or absence of peptide-N-glycosidase (Roche) for 3 h at 37°C. Proteins in untreated and treated media, as well as cell lysates, were separated by SDS-PAGE. Cbln4-MYC was detected by Western blot analysis using a mouse monoclonal antibody to the MYC tag (9B11; Cell Signaling Technology), as described previously [29].

**In Situ Hybridization**

Probes for Amh and Oct4 were made as described previously [31, 32]. A 792-bp Chl0 fragment was cloned from cDNA from 13.5 dpc mouse testes. Primers used were Chl0-F, 5’-ATAGAACCCGACTTCTCCTCGTGATG-3’; Chl0-R, 5’-ACCAAGGAGGGTACCTTTGCAAAG-3’. Embryos and dissected gonads/mesonephros were fixed in 4% PFA in PBS for several hours at 4°C. Whole-mount in situ hybridization (ISH) with digoxigenin-labeled RNA probes was carried out as described by Hargrave et al. [33]. Section ISH was performed on 7-µm sagittal sections from paraffin-embedded embryos as described previously [19].

**RNA Isolation, cDNA Synthesis, and Quantitative Real-Time PCR**

Fetal gonads (pooled) or gonad and mesonephros (individual) were collected and frozen on dry ice. Total RNA was isolated using an RNasy Micro kit (Qiagen) according to the manufacturer’s instructions, including the optional DNAse step. cDNA was synthesized from 1 µg of RNA from pooled gonads or 400 ng of RNA from individual gonads and mesonephros by reverse transcriptase (Superscript III; Invitrogen) using random primers (Invitrogen) according to the manufacturer’s instructions. Relative cDNA levels were analyzed by quantitative real-time RT-PCR using an Applied Biosystems Inc. Prism-7000 Sequence Detector System or Light Cycler Instrument (Roche). Quantitative real-time RT-PCR was carried out on pooled samples in triplicate of three independent biological samples and is represented as mean ± SEM of the three individual experiments. Samples were analyzed in 25-µl reactions as described previously [34]. Reactions lacking reverse transcriptase were carried out in parallel with each cDNA sample to identify genomic DNA contamination. Primers used were rSRY-F, 5’-CAGCCCTGACATTGCTCAA-3’ and rSRY-R, 5’-GTTGTGACCTACCTCTGACT-3’; rSOX9-F, 5’-AGTACCCCGACTGCTCACAAC-3’ and rSOX9-R, 5’-TACGTGTAATCAGGGTGGTGT-3’; rAMH-F, 5’-GCCAAGCTTGGTGAAGTGCTA-3’ and rAMH-R, 5’-GAAGTCCACGGTTAGCACCAA-3’; rMVH-F, 5’-AAGTITACAAACCCCTTGGAGCGC-3’.
triplicate included Taqman PCR master mix (Applied Biosystems Inc.) and 1× Taqman gene expression sets (Applied Biosystems Inc.) with 200 nM each forward and reverse primers. Taqman gene expression sets (Applied Biosystems Inc.) were used for rt18S (4319413E), rtSRY (Mm00441712_s1), rtSOX9 (Mm00448840_m1), rtAMH (Mm00431795_g1), and rtCBLN4 (Mm0055863_m1).

Quantitative real-time RT-PCR was performed on Wt1::Sox9 and Sox9-KO mice as described previously [35]. Primers were Gapdh, Sry, Sox9 [35], and Cbln4 (5'-GCACCGAGAAAGGAATCTA-3' and 5'-TGCAGAGATGGACTGGTTTTCC-3' and universal probe library probe no. 21).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay analysis was performed as described previously [36] using recombinant, bacterially expressed GST fusion proteins of full-length SRY, SOX9, SF1, and SOX7. Recombinant plasmids were constructed using the vector pGEX-KG [37].

RESULTS

Identification of Putative SRY Target Genes

To identify direct target genes of SRY, we performed in vivo ChIP using approximately 550 pairs of mouse genital ridges at 11.5 dpc as starting material. For the immunoprecipitation, we used a recently described antibody to mouse SRY [13, 27]. We showed that this antibody can precipitate mouse SRY protein by using extracts of cells transfected with an Sry expression construct (Supplemental Fig. S1, available online at www.biolreprod.org). Genomic fragments, presumably bound to endogenous SRY, were isolated and cloned into plasmid vector. In all, 162 cloned fragments were retrieved and sequenced. Of these, 53 mapped to single regions of the mouse genome with a low probability of false alignment and were larger than 200 bp (Supplemental Table S1, available online at www.biolreprod.org). Fragments with poor alignment, multiple alignments with similar probability, or those smaller than 200 bp were discarded. We examined these fragments bioinformatically to determine if they contained putative SRY consensus binding sites (Supplemental Table S1). Any genes close to these regions or containing these fragments in an intron were considered as candidate SRY target genes.

A bona fide SRY target gene ought to be either expressed at the same time and in the same cells as Sry—that is, in the supporting cell lineage of XY genital ridges at 11.5 dpc in mice—or downregulated in these cells at the onset of Sry expression. We first assessed candidate target genes by analyzing their expression in sorted gonadal cell populations at 11.5 dpc using Affymetrix microarray data (data not shown and Beverdam and Koopman [38]). Based on this analysis, we chose one candidate target gene, Cbln4, for further investigations. The immunoprecipitated genomic fragment mapped 7.5 kb upstream of the Cbln4 transcription start site within the qH3 region of mouse chromosome 2 (Fig. 1). Using the University of California, Santa Cruz genome browser [39], we confirmed that this genomic fragment is a noncoding region and is highly conserved between mouse and rat and, to a lesser extent, human, dog, cow, and chimpanzee genomes (Fig. 1), suggesting that it may be important in the regulation of Cbln4 gene expression.

Cbln4 Expression in Developing Mouse Testes

We next investigated in detail the expression of Cbln4 in relation to the known schedule of events in sex determination and testis differentiation. We used whole-mount ISH of mouse fetal gonad samples from 12 to 24 ts as a qualitative assay to assess the spatiotemporal expression pattern. This analysis showed that Cbln4 was expressed in XY and XX genital ridges until 16 ts. Approximately 4 h later, at 18 ts, the stage at which Sry expression reaches its maximum [11, 13], Cbln4 was downregulated in ovaries, whereas its expression seemed to increase in the developing testis (Fig. 2A). By 24 ts, Cbln4 was male-specifically expressed, with no detectable levels in ovaries (Fig. 2A).

To quantify and directly compare the mRNA expression of Cbln4 with that of Sry, we employed quantitative real-time RTPCR. Sox9 was included for comparison as an SRY target.
gene, and Amh as a SOX9 target gene [17, 18]. As expected, Sry expression increased over time and reached a maximum around 18 ts before declining in XY gonads, whereas it was absent in XX (Fig. 2B, top left). Sox9 and Amh, which showed similar expression profiles, were expressed in XX gonads at low levels at 10–14 ts, with a small, transient increase at 15 ts. In XY gonads, both genes were strongly upregulated after 18 ts (Fig. 2B, top right and bottom right). Although Cbln4 expression appeared the highest in XX gonads at 16 ts (Fig. 2A) in the ISH, quantitative RT-PCR showed that Cbln4
mRNA rapidly increased in XY gonads (Fig. 2D, bottom right), supporting the hypothesis that Cbln4 might be regulated by Sry.

We next sought to determine whether Cbln4 is expressed in the same cell type as Sry. We first performed quantitative real-time RT-PCR analysis on W/W^* mice for Cbln4 in parallel with Sox9 as a Sertoli cell marker and mouse vasa homologue (Mvh) as a germ cell marker. W/W^* mice harbor a mutation in the Kit gene that prevents the migration of germ cells into the genital ridge, resulting in a lack of this cell type within the gonad [14]. As expected, Sox9 was expressed at approximately the same level in wild-type and W/W^* mice (Fig. 3A, left), whereas expression of the germ cell marker Mvh was lost in W/W^* mutants (Fig. 3A, middle). Cbln4 was expressed at similar levels in W/W^* compared with wild-type mice (Fig. 3A, right), demonstrating that Cbln4 expression is neither in germ cells nor dependent on the presence of germ cells. To further identify the cell type that expresses Cbln4, section ISH was carried out on whole embryos at 13.5 dpc. For comparison, we performed section ISH with the germ cell marker Pou5f1 (Oct4, octamer 4) and the Sertoli cell marker Amh on adjacent sections of the same embryo (Fig. 3B). Cbln4 expression resembled the expression of Amh within the testis cords, suggesting Cbln4 is expressed by Sertoli cells (Fig. 3B, left and right columns), whereas Pou5f1 showed the expected expression in germ cells only (Fig. 3B, middle column).

CBLN4 Is a Secreted Protein

Having established that Cbln4 is expressed by the same cell type and at the same time as Sry, we wanted to identify the type of protein that is encoded by Cbln4. Using the Web-based protein prediction tool TMHMM [40], CBLN4 has a predicted transmembrane helix domain from amino acids 7 to 24. SignalP 3.0 analysis [41] predicted a signal peptide with a cleavage site between amino acids 24 and 25, suggesting that it is a secreted factor or a membrane-bound protein (Fig. 4A). Using SMART [42], an online domain architecture tool, we found that CBLN4 also contains a C1Q domain (Fig. 4A), a protein-protein association domain present in a wide variety of secreted proteins [43, 44].

To confirm experimentally the intracellular location of CBLN4, we transfected MCF-7 cells with a construct expressing CBLN4 with an MYC-tag at the C-terminus, and we performed coimmunofluorescence using an antibody specific for the MYC-tag together with antibodies detecting marker proteins for the secretory pathway. CBLN4-MYC was trafficked through the secretory pathway. To test whether CBLN4, we transfected MCF-7 cells with a construct expressing CBLN4 with an MYC-tag at the C-terminus, and we performed coimmunofluorescence using an antibody specific for the MYC-tag together with antibodies detecting marker proteins for the secretory pathway. CBLN4-MYC was trafficked through the secretory pathway.
within the medium was approximately 20 kDa larger than that found in the cell lysate. Deglycosylation with peptide-N-glycosydase was able to reduce the secreted protein size to that of the lysate, indicating that secreted CBLN4 is posttranslationally glycosylated (Fig. 4C).

**SRY and SOX9 Bind to the ChIP Fragment In Vitro**

We next aimed to confirm that SRY regulates the expression of Cbln4 via the genomic fragment identified by ChIP analysis. To this end, we first examined bioinformatically the sequence of the ChIP fragment for the presence of SRY/SOX, steroidogenic factor 1 (SF1), and Wilms tumor suppressor 1 (WT1) binding motifs. We included SF1 and WT1 because both proteins have been shown to be expressed and to be important for the formation of the genital ridges [45, 46]. This approach identified three potential SRY/SOX sites (here referred to as SOX sites) and one SF1 and one WT1 binding site within the ChIP fragment (Fig. 5A). To determine whether SRY, SOX9, and SF1 can bind to the ChIP fragment, we performed electrophoretic mobility shift assays. All three factors—SRY, SOX9, and SF1—bound in vitro to the Cbln4 enhancer fragment. SRY bound to the fragment to either one (Fig. 5B, white arrowhead) or two (black arrowhead) of the three sites, whereas SOX9 (black arrowhead) binds at least two of the three sites simultaneously. In addition, SF1 binds to the ChIP fragment in contrast to GST-SOX7 and GST-only, which do not bind.

**Cbln4 Expression Is Regulated by SRY and SOX9 In Vivo**

Both SRY and SOX9 bind to essentially the same binding motif [47, 48], and both Sry and Sox9 are expressed in the same cells in overlapping time windows during gonad development, making it difficult to determine which of these factors might be responsible for the male-specific expression of Cbln4 in vivo. To solve this problem, we made use of three mouse models in which the expression of Sry and Sox9 is uncoupled.
We first employed a mouse model in which SRY is expressed under the control of the Hspalα (Hsp70.3) promoter (Hspalα:Sry; Kidokoro et al. [49]). These mice express Sry in XX and XY genital ridges from a very early stage along the whole length of the gonad. However, although Sry is expressed at least 8 h earlier than normal, the onset of Sox9 expression is not altered [49]. Therefore, these mice provide a useful mouse model with a longer than normal time window in which Sry is expressed prior to the onset of Sox9 expression. Using this mouse model, we investigated whether Cbln4 expression is altered by the ectopic expression of Sry. Whole-mount ISH revealed that Cbln4 was expressed robustly along the whole length of the gonad by 9 ts, whereas in wild-type XX and XY gonads, Cbln4 expression was weak (Fig. 6A). These results demonstrate that ectopic SRY expression was able to elicit a corresponding ectopic upregulation of Cbln4 expression.

Next, we used quantitative real-time RT-PCR to measure the expression of Cbln4 in mice harboring a null mutation of Sox9 [35]. In these mice, Sox9 was conditionally deleted by CRE recombinase driven by the Sfl promoter (Sfl:Cre; Sox9lox/lox/Sox9lox), here referred to as Sox9-KO [35]. Expression of Sox9 mRNA in Sox9-KO embryos is variable from almost normal to a complete absence of transcription at 13.5 dpc, with the result that, unlike wild-type mice, Sry expression in the Sox9-KO gonads remains until at least 13.5 dpc [35]. This aberrant expression profile provides a time window of gonad development in which Sry is expressed but Sox9 is absent. We found that at 13.5 dpc Cbln4 expression was reduced in XY Sox9-KO mice compared with wild-type littermates (Fig. 6B), suggesting that, at least at this late developmental stage, SOX9 is necessary for Cbln4 expression in vivo.

Having established that SOX9 is necessary for Cbln4 expression in vivo, we next sought to determine whether SOX9 also was sufficient to initiate the expression of Cbln4. To answer this question, we used quantitative real-time RT-PCR analysis to examine the expression of Cbln4 in mice (Wt:Sox9) that express Sox9 driven by the Wt1 regulatory region of the XX gonads [22]. Ectopic expression of Sox9 was sufficient to upregulate Cbln4 in transgenic XX gonads at 13.5 dpc (Fig. 6C). Hence, Sox9 is not only necessary but also sufficient to activate Cbln4 expression in vivo in the absence of SRY. In summary, we showed that SRY and SOX9 are both able to upregulate Cbln4 expression and that SOX9 is essential for the maintenance of Cbln4 expression.

**SRY and SOX9 Bind to the ChIP Fragment In Vivo**

Induced ectopic expression in vivo does not necessarily mean that SRY and SOX9 bind directly and regulate the identified Cbln4 enhancer during normal development. To investigate whether endogenous SRY and SOX9 bind to the identified enhancer fragment in vivo, we performed PCR on ChIP extracts of isolated 11.5 dpc XX and XY genital ridges that have been used previously [19]. Any transcription factor that functions as a direct regulator of Cbln4 in vivo would be expected to associate with the promoter or enhancer fragment in Sertoli cell nuclei, which can be measured by an enrichment of these fragments in the ChIP extract. Two primer sets,
FIG. 7. SRY and SOX9 bind to putative Cbln4 enhancer region in vivo. Chromatin immunoprecipitation of XY (M) and XX (F) genital ridges at 11.5 dpc with antibodies specific to SOX9 (αSOX9) and SRY (αSRY), respectively. Polymerase chain reaction analysis with primer pair 1 (PCR1; black arrowhead 1) and primer pair 2 (PCR2; open arrowhead 2) shows an enrichment of fragment 2 in the SRY and SOX9 ChIP extracts of XY genital ridges. Genomic DNA before immunoprecipitation (input) was used as positive control, and PCR without DNA template (—) was used as negative control. The PCR fragment sizes are 361 bp (PCR1) and 367 bp (PCR2).

The Role of Cbln4

CBLN4 is a member of the C1q and tumor necrosis factor superfamily. Precerebellin 1, the first identified family member, was originally described as a precursor of the Purkinje cell-specific peptide cerebellin [54]. More recently, cerebellins 1 to 4 have been hypothesized to act as signal molecules themselves [55]. Our results here support this hypothesis by showing that Cbln4 is secreted as a glycoprotein.

One of the early roles of the supporting cell lineage in the testis is to initiate cell migration from the mesonephros into the differentiating gonad [56], cell proliferation within the gonad and the coelomic epithelium [57], and the formation of a male-specific vasculature [58]. For Sertoli cells to initiate these events, they must use some form of paracrine signaling. The expression of Cbln4 is upregulated and male specific at 18 ts, approximately the time when these processes are set in motion. It is therefore feasible that CBLN4 may be a paracrine factor produced and secreted by Sertoli cells to initiate one or several of these early testis-specific events.

In addition, Cbln4’s early expression in both male and female genital ridges suggests that it might also play a sex-independent role in the formation of the early bipotential gonad. It is known for other genes involved in gonad development to have multiple roles. For example, a dual role during gonad development has been described for WT1. Mice carrying homozygous null mutations in Wt1 lack gonads entirely in males and females [45], suggesting an early role for Wt1 in the formation of the primordial genital ridge. Other studies in which Wt1 was conditionally ablated in Sertoli cells by 14.5 dpc showed that Wt1 is essential also for the maintenance of Sox9 expression and tubular architecture in the developing testis [59]. Cbln4 null mice have not been generated to date. However, future functional analyses of Cbln4 could employ similar conditional knockout strategies to identify its role in the development of the bipotential gonad as well as during testis differentiation.

SRY as a Transcriptional Activator

A longstanding question in the biology of SRY is whether SRY acts as a transcriptional activator or a repressor. Our analyses showed an increase in Cbln4 expression in the Hspa1a:Sry transgenic mice. This upregulation could be due to a direct activation by SRY, or indirectly via repression of a repressor. In vitro luciferase assays may provide further information about the transcriptional control of Cbln4 by SRY, but extrapolation of in vitro results to the in vivo setting must be treated cautiously. However, the observed in vivo binding of SRY to the putative Cbln4 enhancer in the ChIP experiments confirmed a direct interaction, and it suggests that SRY acts as a transcriptional activator of Cbln4. This is in agreement with a
One of the complications in identifying a direct target of SRY has been the differentiation of a potential target of SRY from a potential target of SOX9. Based on the known, almost identical DNA binding and bending abilities of SRY and SOX9, any gene activated by one factor could theoretically also be activated by the other. Given that early ectopic expression of Sox9 is sufficient to induce XX sex reversal, it has been proposed that Sox9 is the only gene that is induced by SRY and that, once activated, Sox9 initiates all pathways necessary for male development. If this were the case, SRY would simply function as a switch that is present in most mammals but not in any other vertebrate class [4, 62]. However, published data do not exclude the possibility that the expression of multiple genes might be upregulated initially by SRY and subsequently maintained by SOX9. Indeed, it has been shown that Sox9 expression in the testis is initially SRY dependent. Thereafter, Sox9 transcription is maintained by SOX9 protein in a self-regulatory fashion [8]. We did not isolate the Sox9 enhancer region in our ChiP; however, the amount of starting material used is very small, and therefore it is not surprising to not have pulled down this regulatory region. Our results using the three different mouse models indicate a similar regulatory mechanism for Cbln4. The analysis of the Hspa1a:Sry mice showed that SRY might play a role in the early, male-specific upregulation of Cbln4 [8]. On the other hand, analysis of Sox9-KO mice demonstrated that SRY is not sufficient to maintain Cbln4 expression at later stages. This may be because of an absence of specific cofactors required for SRY to transactivate Cbln4 at 13.5 dpc or because of insufficient levels of SRY expression. However, we demonstrated that SOX9 is necessary and sufficient for the expression of Cbln4, which would explain why during normal development, Cbln4 is expressed in the Sertoli cells long after Sry expression has extinguished. Moreover, the second ChiP analysis proved that both SRY and SOX9 are bound to the identified Cbln4 enhancer in vivo at the right time of development, supporting the hypothesis of direct Cbln4 regulation by both SRY and SOX9. Further experiments are needed to establish the regulatory network that is needed to initiate Cbln4 expression in the bipotential genital ridges of both sexes.

A Multiple-Target Model for SRY

Based on our data, we suggest a model for the male-specific expression of Cbln4 in which SRY initiates male-specific upregulation, and SOX9 further upregulates, and then maintains, Cbln4 expression in the developing gonad (Fig. 8). This model is similar to a feed-forward network, which has been described previously, where a given transcription factor controls the expression of another transcription factor, and together the two factors regulate the transcription of a shared target gene [50, 63]. It is possible that a cooperative interaction between SRY and SOX9 may operate to transactivate Cbln4, although no direct interaction between SRY and SOX9 has been shown to date. However, SOX9 has been shown previously to interact with other SOX proteins to cooperatively activate gene expression. For example, the L-SOX5/SOX6 protein complex and SOX9 cooperate in activating the expression of collagen type 2 alpha 1 (Col2a1) by binding to a 48-bp chondrocyte-specific enhancer [64, 65]. This coupling of transcription factors accommodates switchlike transcriptional responses that are important in development [63] and may help reinforce the molecular commitment down the male pathway.

Although our data support a model in which both SRY and SOX9 regulate Cbln4 expression, we suspect that this mode of regulation may be more broadly applicable in early sex determination. According to this hypothesis, SRY is responsible for the male-specific expression of a number of genes, including Cbln4 and Sox9 (Fig. 8A). However, because the expression of SRY is transient, it is not sufficient for the maintenance of gene expression throughout testis development. Consequently, SOX9 replaces SRY (Fig. 8B) to maintain the expression of genes required for testis differentiation, and it possibly represses genes of the female pathway (Fig. 8C).

If a multiple-target model for SRY is correct, then the male-specific increase in expression of Sox9, Cbln4, and Amh apparent at 15 ts might be due to transactivation by SRY. This early upregulation of Sox9, Cbln4, and Amh by SRY might then be counteracted by genes involved in the female pathway. Once SOX9 reaches a threshold level, it replaces SRY and upregulates testis-specific genes, and it represses ovary-specific genes. A detailed expression analysis, along with in vitro studies, is necessary to determine whether this mechanism is also the case for other genes that are regulated by SOX9. One SOX9 target gene, Ptgds, has been excluded already from being initially upregulated by SRY because its promoter was found to be regulated by SOX9 but not by SRY [19].

In summary, our data show that Cbln4 is upregulated in the same spatiotemporal pattern as Sox9, making it one of the first genes to be upregulated in early testis determination. The increased expression in the testis is mediated by both SRY and SOX9. CBLN4 is likely to work in a paracrine fashion and be part of the signaling mechanisms of early Sertoli cells to direct the differentiation of other cell types within the developing testis. Our data identify Cbln4 as a likely direct target gene of SRY and support a transcriptional model in which SRY has multiple targets and cooperates with SOX9 to regulate early gene expression in the developing testis.

ACKNOWLEDGMENTS

We thank the staff of the Institute for Molecular Bioscience Animal Facility for breeding mice used in this study; Marcel Dinger and Martin Frith for assistance with bioinformatics; Annemieke Beverdam, Fred Martinson, Jo Bowles, Cassy Spiller, Fiona Simpson, Rajith Aturaliya, Markus Kerr, and Andrew Jackson for technical help; and Annemieke Beverdam and Terje Svingen for comments on the manuscript.

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