SOX9 Regulates Prostaglandin D Synthase Gene Transcription in Vivo to Ensure Testis Development*

Received for publication, October 11, 2006; revised form, January 22, 2007*; published, JBC Papers in Press, February 2, 2007; DOI 10.1074/jbc.M609578200

Dagmar Wilhelm‡, Ryuji Hiramatsu+, Hirofumi Mizusaki‡, Laura Widjaja‡, Alexander N. Combes‡, Yoshiakira Kanai†, and Peter Koopman*‡

From the ‡Division of Molecular Genetics and Development, Institute for Molecular Bioscience, The University of Queensland, Brisbane, Qld 4072, Australia, the +Department of Veterinary Anatomy, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan, and the ¶ARC Centre of Excellence in Biotechnology and Development, Institute for Molecular Bioscience, The University of Queensland, Brisbane, Qld 4072, Australia

In mammals, male sex is determined by the Y-chromosomal gene Sry (sex-determining region of Y chromosome). The expression of Sry and subsequently Sox9 (SRY box containing gene 9) in precursors of the supporting cell lineage results in the differentiation of these cells into Sertoli cells. Sertoli cells in turn orchestrate the development of all other male-specific cell types. To ensure that Sertoli cells differentiate in sufficient numbers to induce normal testis development, the early testis produces prostaglandin D$_2$ (PGD$_2$), which recruits cells of the supporting cell lineage to a Sertoli cell fate. Here we show that the gene encoding prostaglandin D synthase (Pgds), the enzyme that produces PGD$_2$, is expressed in Sertoli cells immediately after the onset of Sox9 expression. Promoter analysis in silico identified a paired SOX/SRY binding site. Interestingly, only SOX9, and not SRY, was able to bind as a dimer to this site and transactivate the Pgds promoter. In line with this, a transgenic mouse model showed that Pgds expression is not affected by ectopic Sry expression. Finally, chromatin immunoprecipitation proved that SOX9 but not SRY binds to the Pgds promoter in vivo.

Sexual differentiation in mammals is unique in that an indifferent organ system, including gonads, genital tracts, and external genitalia, has the potential to develop in two completely different directions, either down the male pathway or down the female pathway. In mice, the decision as to which pathway to follow takes place between 10.5 and 12.5 days post coitum (dpc), when the gene Sry (sex-determining gene on the Y-chromosome) is expressed in the genital ridge of an XY animal or not expressed, as in the case of an XX animal. The expression of Sry drives the differentiation of the genital ridge into a testis rather than an ovary, which in turn directs all secondary sexual differentiation via secretion of male-specific hormones (for review see Ref. 1).

SRY belongs to the family of SOX transcription factors that are characterized by the high mobility group DNA binding domain. It is expressed specifically in the supporting cell lineage that gives rise to immature Sertoli cells in the developing testis. The expression of Sry resembles a wave, starting in the center of the genital ridge, expanding to the poles and turning off first at the anterior pole with the last SRY-positive cells being detectable at the posterior pole at around 12.5 dpc (2–6). Almost immediately after Sry, another gene of the Sox family, Sox9, is expressed in the same cells following a similar wave of up-regulation, but unlike Sry, its expression continues throughout testis development (7–9). Subsequently, SOX9-positive cells differentiate into Sertoli cells, which form the testis cords by enclosing clusters of germ cells. Sertoli cells then coordinate the differentiation of all other testis-specific cell types such as the steroidogenic Leydig cells within the interstitium and peritubular myoid cells, which surround Sertoli cells and thereby take part in testis cord formation. It has been shown that both Sry and Sox9 are necessary and sufficient for male development in mice and in humans (10–17).

Both SRY and SOX9 bind sequence-specifically to DNA via their highly conserved high mobility group domains. Consistently, in vitro experiments have shown that both factors recognize essentially the same DNA motifs with very similar affinities and induce sharp bends at similar angles (18, 19). This, together with the fact that Sry and Sox9 are expressed in the same cells in overlapping time windows during gonad development, presents a major challenge in identifying specific targets for each factor. Although SOX9 contains two well defined transactivation domains and has been implicated in the up-regulation of several genes such as anti-Müllerian hormone (Amh) (7, 20, 21) and Vanin-1 (Vnn1) (22), no in vivo target gene for SRY has been identified to date, and it is even unclear whether SRY functions as a transcriptional activator and/or repressor. The best candidate so far for being an authentic SRY target is Sox9 itself. However, experimental proof has not been forthcoming.

The direct or indirect up-regulation of Sox9 expression by SRY is crucial for testis development. Studies have shown that in XX ↔ XY chimeric mouse embryos, the ratio of XX to XY

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a grant from the National Institutes of Health.

‡ An Australian Professorial Research Fellow of the Australian Research Council.

§ Supported by grants from the Australian Research Council and the National Health and Medical Research Council (Australia). To whom correspondence should be addressed. Tel: 61-7-3346-2059; Fax: 61-7-3346-2101; E-mail: p.koopman@imb.uq.edu.au.

¶ The abbreviations used are: dpc, days post coitum; PGD$_2$, prostaglandin D$_2$; PGDS, prostaglandin D synthase; SRY, sex-determining region of Y chromosome; SOX, SRY box containing gene 9; ChiP, chromatin immunoprecipitation; ts, tail somite; HEK, human embryonic kidney; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; GST, glutathione S-transferase; ISH, in situ hybridization.
Regulation of Pgds Transcription by SOX9

cells is ∼50:50 in all tissues, the only exception being Sertoli cells. These were found to be more than 90% XY, indicating that the differentiation of Sertoli cells needs the presence of the Y chromosome (23), and therefore, SRY acts cell-autonomously. However, these experiments also imply that Sry is not essential for the differentiation of all Sertoli cells. XX cells were recruited to differentiate into Sertoli cells contributing almost one-tenth of the total number of Sertoli cells. Thus, it is not surprising that in addition to the cell-autonomous mechanism, at least one non-cell-autonomous mechanism exists to ensure the differentiation of sufficient Sertoli cells to a level above the estimated threshold of 20% (24, 25) to guarantee testis differentiation. We and others have shown that this non-cell-autonomous mechanism involves prostaglandin D2 (PGD2 (6, 26, 27)), produced by the enzyme prostaglandin D synthase (PGDS), which catalyzes the isomerization of PGH2, a common precursor of several prostanooids. PGD2 has been shown to play a role in various physiological and pathological functions as an endogenous somnogen, anti-coagulant, vasodilator, broncho-constrictor and as an allergic and inflammatory mediator released from mast cells (for review see Ref. 28).

Here we show that Pgds is expressed in Sertoli cells shortly after the expression of Sry and Sox9 in a similar dynamic wave. Biochemical, cell culture, and genetic experiments indicate that this up-regulation is mediated by SOX9, but not SRY, via a paired SOX recognition site within the Pgds 5’-flanking region. Furthermore, chromatin immunoprecipitation (ChIP) assays confirm that SOX9 binds in vivo to the identified regulatory region.

EXPERIMENTAL PROCEDURES

Animals and Cell Lines—Embryos were collected from timed matings of the Swiss Quackenbush outbred strain, with noon of the day on which the mating plug was observed designated as 0.5 dpc. For more accurate staging, the tail somite (ts) stage of the embryo was determined by counting the number of somites posterior to the hind limb (2). Using this method, 10.5 dpc corresponds to ~8 ts, 11.5 dpc corresponds to 18 ts, and 12.5 dpc corresponds to 30 ts. Human embryonic kidney (HEK) 293 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Cambridge). Cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Cambridge), 1X penicillin-streptomycin (Invitrogen), and 2 mM glutamine (Invitrogen) at 37 °C in 5% CO2.

In Situ Hybridization—The probes for Sox9 (29) and Pgds (27) were made as described previously. Section in situ hybridization was performed on 7-μm sections of paraformaldehyde-fixed, paraffin-embedded embryos. Sections were dewaxed, rehydrated, and incubated in 5 μg/ml proteinase K for 20 min at room temperature. After washing in PBS, sections were refixed with 4% paraformaldehde for 10 min at room temperature, acetylated, and prehybridized with hybridization solution (50% formamide, 5X SSC, 5X Denhardt’s, 250 μg/ml yeast RNA, 500 μg/ml herring sperm DNA) for 2 h at room temperature. Hybridization (hybridization solution +0.5 μg/ml probe) was performed overnight at 60 °C. Slides were washed in 5X SSC for 5 min, 0.2X SSC for 1 h at 60 °C, 0.2X SSC for 5 min at room temperature and NT buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) for 5 min at room temperature before incubating for 2 h with blocking solution (10% heat-inactivated sheep serum in NT buffer) in a humidified chamber. Antibodies for SOX9 (Roche Applied Science) at 1:2000 dilution in blocking solution was added to the slides and incubated overnight at 4 °C. Unbound antibodies were removed by washing three times in NT buffer. Sections were equilibrated in NTM buffer (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl2) and incubated in color solution (3.5 μl of 5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science), 3.5 μl of nitro blue tetrazolium (Roche Applied Science) per ml of NTM buffer) until purple staining was satisfactory. Whole-mount in situ hybridization was carried out essentially as described by Ref. 30.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA analysis was performed as described previously (31) using recombinant, bacterially expressed GST fusion proteins of the full-length mouse SRY, SOX9, and SOX2. Recombinant plasmids were constructed by using the vector pGEX-KG (32). Oligonucleotides harboring SOX-A/B sites had the following sequence: 5’-CCA AAT GTT CAA GGC ACA AAT GGT GCT TTG TGT TCC CTG CTG G-3’; harboring SOX-C site, 5’-GGT AGG GCT TGT GAG AAG CAG G-3’; mut-A (mutated nucleotides are underlined), 5’-CCA AAT GTT CAA GGG ACG TGT GGT TTG TGT CTG CTG CTG G-3’; mut-B, 5’-CCA AAT GTT CAA GGC ACA AAT GGT TTG TGG TCT CCCT CTG CTG G-3’; mut-AB, 5’-CCA AAT GTT CAA GGG ACG TGT GGT TTG TGT CTG CTG CTG G-3’. Oligonucleotides were annealed and subsequently labeled using [γ-32P]ATP and T4 polynucleotide kinase (Roche Applied Science) for 1 h at 37 °C and purified using Nick columns (Amersham Biosciences). Binding reactions were performed by using 20 ng of purified protein and 3000 cpm of end-labeled probe.

Luciferase Assays—HEK293 cells were plated at 3 × 105/well in 12-well plate 24 h before transfection. The cells were transfected in triplicates with the relevant combination of plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Each well received 800 ng of luciferase reporter plasmid (pGL2-Basic without or with the wild type or mutated Pgds promoter fragment), increasing amounts (32, 80, 200 ng) of expression plasmid (pSgSox9 (9) or pCHASry (33)), and 600 ng of β-galactosidase expression plasmid pCH110 as an internal control. The empty pHA vector was added to equalize amounts of transfected plasmid DNA concentrations. Cells were harvested 24 h after transfection and assayed for luciferase and β-galactosidase activity as described previously (7). To verify expression of SOX9 and SRY, Western blot analysis with antibodies specific for SOX9 (6) and the hemagglutinin tag (Sigma) was performed as described previously (34). Values were normalized for transfection efficiency according to β-galactosidase activity and with respect to the effects of the expression constructs on the empty pGL2-Basic reporter plasmid. Three independent experiments were performed.

ChIP—Male and female genital ridges (gonad + mesonephros) were dissected from ~500 staged mouse embryos (one genital ridge at 11.5 dpc yields ~1 μg of protein) in cold PBS, and a single cell suspension was prepared by passaging several
times through a 21-gauge needle. Cells were collected by centrifugation, and proteins and DNA were cross-linked by incubating the cells in 1% formaldehyde/PBS for 10 min at room temperature. Reaction was stopped by the addition of glycine in a final concentration of 0.125 M. Cells were washed three times with PBS, collected by centrifugation, resuspended in hypotonic lysis buffer (150 mM NaCl, 1% Nonidet P40, 0.1% SDS, 50 mM Tris-HCl, pH 8), and sonicated to shear DNA into fragments <=2 kb. Insoluble material was removed by centrifugation, and the extract was precleared with 30 μl of preswollen protein A-Sepharose (Amersham Biosciences) for 1 h at 4 °C to reduce nonspecific background. The precleared extract was split into two equal aliquots and incubated with either anti-mouse SRY or anti-mouse SOX9 antibodies (6) at 4 °C overnight. After incubation with 30 μl of preswollen protein A-Sepharose for 2 h at 4 °C, the immunoprecipitates were washed three times with sonication buffer and once with TE (10 mM Tris-HCl, pH 8, 1 mM EDTA), and protein-DNA complexes eluted by incubation twice with 100 μl of elution buffer (1% SDS in TE) at 37 °C for 20 min with vigorous shaking. Co-immunoprecipitated DNA was purified by 0.5 mg/ml proteinase K digest (overnight at 55 °C), phenol/chloroform extraction, and washing with Microcon YM-100 filter tubes (Millipore). Identity of the amplified fragments was verified by sequencing. Primers used to investigate the enrichment of *Pgds* promoter fragments by PCR were as follows: PCR1.F, 5’-GTC GAC CAG AAA TAA CTT TAG AGA AGA AA-3’; PCR1.R, 5’-TTA AGC TTC CTG TGT GTG GGG TCC TG TGT A-3’; PCR2.F, 5’-TTG TCG ACC AGG GTA GAG TGC GTG AGC TTC TT-3’; PCR2.R, 5’-GGA AGC TTG CGC TTC CCT GCT GCT GGA AA-3’.

**RESULTS**

*Pgds Expression in Developing Mouse Testes*—To address the question of how the expression of *Pgds* is regulated in the fetal gonad, we first compared *Pgds* and *Sox9* expression in detail by whole-mount *in situ* hybridization (ISH) analysis of ts-staged mouse genital ridges from 13 to 21 ts. At each developmental stage, left and right genital ridges from the same embryo were assessed in parallel for *Pgds* and *Sox9* expression. *Pgds* transcripts were first detected in the center of the testis at 17 ts (Fig. 1A), shortly after the onset of *Sox9* expression at 15 ts. Subsequently, the expression domain of *Pgds* expanded to include the anterior and posterior poles. This dynamic, wave-like expression pattern closely resembles those of *Sry* and *Sox9* in the embryonic testis (Figs. 1A and 5, A and B) (3, 6, and 7). *Pgds* was not detected in the ovary at any investigated stages (data not shown).

To identify the cell type that express *Pgds*, section ISH was carried out on whole embryos from 11.5 to 14.5 dpc using an RNA probe specific for *Pgds* in comparison with the germ cell-specific marker gene *Oct4*. At all stages, *Pgds*, like *Sry* and *Sox9*, was expressed by Sertoli cells but not by germ or interstitial cells (Fig. 1B, left panels, and data not shown), whereas *Oct4* showed the expected expression in germ cells only (Fig. 1B, right panels).

**Identification of the Mouse Pgds Promoter**—Our expression analysis suggested that SRY and/or SOX9 might directly regulate *Pgds* transcription. To test this hypothesis, we compared the genomic region of mouse *Pgds* with that of human and dog by means of the ECR (evolutionary conserved region) browser. Approximately 600 bp 5’ of the transcription start site was found to be highly conserved between human and mouse and ~300 bp between human, mouse, and dog (Fig. 2A). Cloning and sequence analysis of the conserved 600-bp fragment revealed three putative SRY/SOX binding sites (Fig. 2B). Two of

**FIGURE 1.** Expression profiling of *Pgds* in comparison with *Sox9* and *Oct4*. A, whole-mount *in situ* hybridizations for *Pgds* (left gonads) in comparison with *Sox9* (right gonads) were performed on XY urogenital ridges collected from embryos between 13 and 21 ts. Expression of *Pgds* started in the center of the genital ridges between 15 and 17 ts, only a few hours after *Sox9* expression (between 13 and 15 ts), before extending to the poles (marked by arrowheads). B, in situ hybridization on sagittal paraffin sections showed *Pgds* expression specifically in Sertoli cells in the embryonic testis at 14.5 dpc, whereas *Oct4* was expressed by germ cells only. The boxed areas indicate the magnified area in the panel below. The dotted line demarcates a testis cord. Scale bars, 200 μm (upper panel), 100 μm (middle panel), and 50 μm (bottom panel).
Regulation of Pgds Transcription by SOX9

FIGURE 2. Sequence analysis of the Pgds promoter. A, using the mouse sequence as the base, −600 bp (depicted by dark gray bars) 5’ to the Pgds coding region was identified to be conserved between mouse and human, and −300 bp was identified to be conserved between mouse and dog using the ECR browser. Black bars indicate exons e1 to e6. The conserved region is magnified in a schematic representation below with the three identified putative SRY/SOX binding sites (A–C). TSS, transcription start site. B, sequences of and surrounding SOX-A, -B, and -C representing the EMSA probes used in Fig. 3. Gray shading highlights the SRY/SOX binding sites.

these binding sites (SOX-A and SOX-B) were separated by 4 bp and arranged in opposite orientation, representing a so-called paired SOX site similar to the sites identified in the Col2a1, matrilin-1, Col11a2, and Col27a1 promoters (7, 35–38). The third SOX site was an isolated, single site.

Characterization of the SOX Binding Sites within the Pgds Promoter—To determine whether SRY and/or SOX9 protein is able to bind to these putative binding sites, we performed EMSAs using labeled duplex oligonucleotides that encompassed the paired SOX sites and the single SOX site, respectively (Fig. 2B). After incubation of these oligonucleotides, bacterially expressed GST-SRY fusion protein was bound to the paired binding sites (SOX-A and SOX-B), forming one complex, even with increasing amounts of protein (Fig. 3A, lanes 2 and 3). In contrast, incubation of the labeled oligonucleotide encompassing SOX-A and SOX-B with SOX9 revealed the formation of two complexes (Fig. 3A, lane 4), presumably corresponding to the binding of SOX9 as a monomer to one site or as a dimer to both sites. Neither GST alone nor the dissimilar SOX protein SOX2 bound to the fragment under these conditions (Fig. 3A, lanes 1 and 5). In contrast, the single SOX site C was not bound by any of the proteins tested (Fig. 3A, lanes 6–10).

Having identified the paired SOX site within the Pgds promoter, we sought to characterize these binding sites further by mutation analysis. Introduction of four point mutations in SOX-A resulted only in a slight reduction of SRY binding when compared with the wild type sequence (Fig. 3B, compare lanes 2 and 5), whereas for SOX9, only monomeric but no longer dimeric complexes were detected (Fig. 3B, lanes 3 and 6). In contrast, mutation of SOX-B almost completely abolished binding of either SRY or SOX9 to the EMSA probe (Fig. 3B, lanes 4 and 7). Mutation of both sites completely abolished binding of SRY and SOX9.

FIGURE 3. SOX9 and SRY binding to the Pgds promoter. A, EMSA of Pgds promoter fragments using oligonucleotides constituting SOX-A/-B (left) and SOX-C site (right), respectively, and GST only (lanes 1 and 6), GST-SRY (50 ng, lanes 2 and 7; 100 ng, lanes 3 and 8), -SOX9 (lanes 4 and 9), and -SOX2 (lanes 5 and 10) fusion proteins. The arrow indicates SRY binding as a monomer, one open arrowhead indicates SOX9 binding as a monomer, and a double open arrowhead indicates SOX9 binding as a dimer to SOX-A/-B. The third putative SOX/SRY site was bound neither by GST only, by GST-SRY or GST-SOX9, nor by GST-SOX2. B, EMSA of wild type (wt, lanes 1–3) or mutated (A-mut, SOX-A mutated, lanes 4–6; B-mut, SOX-B mutated, lanes 7–9; AB-mut, SOX-A and SOX-B mutated, lanes 10–12) Pgds promoter fragment harboring the paired SOX-A/-B site using GST only (lanes 1, 4, 7, and 10), GST-SRY (lanes 2, 5, 8, and 11), and GST-SOX9 (lanes 3, 6, 9, and 12) fusion proteins. The arrow indicates SRY binding as a monomer, one open arrowhead indicates SOX9 binding as a monomer, and a double open arrowhead indicates SOX9 binding as a dimer to SOX-A/-B. Mutation of SOX-A prevented only SOX9 binding as dimer, whereas mutation of SOX-B greatly reduced binding of both GST-SRY and GST-SOX9. Mutation of both sites completely abolished binding of SRY and SOX9.
SOX9 but Not SRY Activates the Pgds Promoter—Binding of SRY and SOX9 to these sequences suggested that these transcription factors are able to transactivate the Pgds promoter. To validate this possibility, we used HEK293 cells and co-transfected either Sry or Sox9 expression plasmids together with the Pgds promoter fragment driving the luciferase gene. Expression of SOX9 and SRY was confirmed by Western blot analysis (Fig. 4A). Surprisingly, increasing amounts of Sox9 expression plasmid appeared to repress rather than activate transcription (Fig. 4B, wt/Sry). However, this repression of the wild type promoter mediated by SRY was not changed by mutation of either or both SOX binding sites (Fig. 4B, A-mut, B-mut, AB-mut/Sry), suggesting that it is a non-specific, binding site-independent effect.

In contrast, increasing amounts of Sox9 expression construct resulted in activation of luciferase activity (Fig. 4B, wt/Sox9). Introduction of mutations in any or both of the SOX binding sites led to a complete loss of promoter activation by SOX9 (Fig. 4B, A-mut, B-mut, AB-mut/Sox9), indicating that SOX9 has to bind as a dimer to an intact paired SOX site to activate the Pgds promoter. In summary, only SOX9 dimers but not SOX9 monomers or monomeric SRY binding are sufficient for transactivation of the Pgds promoter.

Ectopic Sry Does Not Alter Endogenous Pgds Expression in Vivo—Our in vitro data implied that SOX9 but not SRY is able to activate Pgds transcription. To support the in vivo significance of this hypothesis, we performed two types of experiments. First, we made use of a transgenic mouse line in which Sry is expressed under the control of the Hsp70.3 promoter (39). These mice exhibit XX sex reversal with animals already at 10 ts along the whole length of the gonad, in contrast to endogenous Sry in wild type XY gonads, whose expression started at 13 ts. In both cases, Pgds expression was induced between 15 and 17 ts, suggesting that it is not directly regulated by SRY.

Regulation of Pgds Transcription by SOX9
Regulation of Pgds Transcription by SOX9

before extending to the poles (Fig. 5A) (3, 6). The onset of Sox9 expression is not altered in these transgenic mice, taking place between 13 and 15 ts (39). Therefore, these mice provide a unique model system with a time window of at least 8–10 h in which only Sry but not Sox9 is expressed. Using this mouse model, we investigated whether endogenous Pgds expression is altered by the ectopic expression of Sry. Whole-mount in situ hybridization of genital ridges from tail somite-staged mice revealed that Pgds expression is first detectable at 15 ts both in wild type XY as well as in transgenic XX genital ridges (Fig. 5B).

Therefore, ectopic expression of Sry is not sufficient to change the expression pattern of Pgds, supporting our hypothesis that Pgds is not directly regulated by SRY.

SOX9 Binds in Vivo to the Pgds Promoter—In a second series of experiments, we used in vivo ChIP assay with isolated 11.5 dpc XX and XY genital ridges, respectively. Any transcription factor that functions as a direct activator of Pgds in vivo would be expected to be associated with the identified promoter fragment in Sertoli cell nuclei. To test whether SRY and/or SOX9 bind to the Pgds promoter in vivo, immunoprecipitation was carried out with antibodies specific for mouse SRY and SOX9.

As a control, primers were chosen that amplify a region ~2 kb upstream of the Pgds transcription start site without any putative SOX sites (Fig. 6, PCR1) in comparison with the region encompassing the characterized paired SOX site (Fig. 6, PCR2). No enrichment of either of the fragments was found in XX or XY genital ridges when immunoprecipitated with the SRY-specific antibody. Similarly, no SOX9 was bound to the upstream region in XX and XY genital ridges or to the paired SOX sites in XX genital ridges. However, SOX9 clearly occupied the paired SOX sites in XY genital ridges (Fig. 6). Altogether, these results demonstrated that SOX9 binds in vivo to the paired SOX binding sites in the Pgds promoter and is therefore likely to be responsible for its male-specific expression.

**DISCUSSION**

The involvement of PGD2 in male sex determination has been documented in several studies (6, 26, 27). PGD2, via the binding to its specific receptor DP (prostaglandin D receptor), is necessary and sufficient to recruit supporting cells that do not express Sry to express Sox9 and its downstream target Amh and subsequently differentiate into Sertoli cells (6). Previous studies involving XX ↔ XY chimeras have shown that the number of Sertoli cells has to reach a certain threshold to guarantee testis development (24, 25). The paracrine signaling via PGD2 could function as an important backup mechanism in case of impaired Sry function to ensure that the Sertoli cell number threshold is reached and male sexual differentiation will commence.

Several studies investigated the regulation of human PGDS transcription with relation to its expression in the brain and its involvement in sleep regulation, pain response, and inflammation (40–42). The molecules identified to be responsible for the activation or repression of its expression in these cells such as Notch, Hes-1, and AP-2 (40, 41) do not show any sex-specific expression within the developing gonad based on Affymetrix gene chip analysis (43–45), suggesting that SOX9 might be the critical factor for sex-specific expression in the testis, whereas other factors play a more general role.

In this study, we aimed to shed light on the molecular mechanisms of male-specific Pgds expression during testis development. By whole-mount and section ISH, we showed that Pgds, like Sry and Sox9, is expressed in a similar dynamic spatio-temporal expression pattern in the developing mouse gonads with the onset of expression in the center of the genital ridges and extending to the poles. Its expression starts soon after Sry and Sox9 in the same cells, namely the Sertoli cell lineage. Surprisingly, in contrast to published data (27), we could not detect any Pgds transcripts in germ cells by using paraffin section ISH. This might be due to the different techniques used: indirectly using whole-mount ISH followed by paraffin embedding and sectioning (27) versus direct section ISH on paraffin-embedded, sectioned whole embryos in this study.

Analysis of the Pgds promoter revealed a functional paired SRY/SOX recognition site. Interestingly, incubation with the SRY protein resulted in the formation of only one complex, in contrast to SOX9, which yielded two complexes, suggesting that SRY is capable of binding one of the two sites, and SOX9 binds either as a monomer to one site or as a dimer to both sites. Paired SOX sites that are recognized by SOX9 have been described for almost all Sox9 target genes identified so far (7, 35–38). Most of these genes, such as Col2a1, matrilin-1, Col11a2, and Col27a1, play a role in chondrogenesis. Their paired SOX regulatory sequences are invariably arranged in

---

4 A. Jackson and P. Koopman, unpublished data.
opposite orientation to each other and separated by 3 or 4 bp. The paired SOX site identified in the Pgds promoter exhibits the same features. However, mutation analysis uncovered an important functional difference. In contrast to the chondrogenic-specific enhancers in which mutation of either site completely prevented binding of SOX9, the paired site identified in the Pgds promoter contains one high affinity site (SOX-B), the occupancy of which facilitates binding of a second SOX9 molecule to the adjacent low affinity site (SOX-A). We found that mutation of SOX-A does not affect SOX9 binding to SOX-B. A similar scenario has been described for a SOX10 paired binding site in the promoter regulating expression of the gene encoding myelin Protein zero (P0) (46). It has been hypothesized earlier that SOX-A does not affect SOX9 binding to SOX-B. A recent study (46) showed that SOX9, is not sufficient to transactivate the promoter. However, we could detect relatively high basal luciferase activity driven by the Pgds promoter fragment (12.8-fold increase when compared with the empty luciferase construct). Mutation of the paired SOX site reduced this activity to 2.1-fold, demonstrating a high contribution of these sites to the full promoter activity in 293 cells and therefore the only moderate induction by exogenous Sox9.

Results from in vitro experiments such as transient transfection and EMSAs have to be treated with caution as they may not reflect the in vivo situation. To investigate whether Pgds is regulated by Sry in vivo, we made use of a transgenic mouse line in which ectopic Sry precedes Sox9 expression by at least 8 h. Endogenous Pgds expression was not altered in these mice, suggesting that SRY does not regulate its transcription directly. However, it could also be due to the lack of a specific SRY partner protein at these early time points whose expression is independent of Sry. The second type of in vivo data is based on ChIP. With this technique, we were able to convincingly demonstrate that SOX9 binds in vivo at the right time to the paired SOX binding site within the Pgds promoter. In contrast, with the SRY antibody, we could not detect an enrichment of the region including the paired SOX sites, suggesting that SRY does not bind to this element in vivo at 11.5 dpc.

The production of PGD2 by PGDS in response to SOX9 in turn activates Sox9 transcription (6), forming a positive feedback loop likely to play a role in maintaining Sox9 expression and therefore Sertoli cell character. In addition to PGD2, the transcription factor WT1 has been shown to be responsible for continued Sox9 expression during testis development (49). How far these two pathways interact has not been investigated to date. However, given the fact that the maintenance of Sox9 expression is essential for testis cord integrity and therefore proper testis development, it would not be surprising if it would be under the control of several independent regulatory mechanisms.

Acknowledgments—We thank Andrew Jackson for sharing unpublished results, Brett Hosking for technical advice, and Terje Svingen and Tara Davidson for helpful comments on the manuscript.

REFERENCES
1. Wilhelm, D., and Koopman, P. (2006) Nat. Rev. Genet. 7, 620–631
2. Hacker, A., Capel, B., Goodfellow, P., and Lovell-Badge, R. (1995) Development (Camb.) 121, 1603–1614
3. Bullejos, M., and Koopman, P. (2001) Dev. Dyn. 221, 201–205
4. Jeske, Y. W. A., Bowles, J., Greenfield, A., and Koopman, P. (1995) Nat. Genet. 10, 480–482
5. Koopman, P., Münsterberg, A., Capel, B., Vivian, N., and Lovell-Badge, R. (1990) Nature 348, 450–452
6. Wilhelm, D., Martinson, F., Bradford, S., Wilson, M. J., Combes, A. N., Bevendam, A., Bowles, J., Mizusaki, H., and Koopman, P. (2005) Dev. Biol. 287, 111–124
7. Schepers, G., Wilson, M., and Koopman, P. (2003) J. Biol. Chem. 278, 28101–28108
8. Morais da Silva, S., Hacker, A., Harley, V., Goodfellow, P., Swain, A., and Lovell-Badge, R. (1996) Nat. Genet. 14, 62–68
9. Kent, J., Wheatley, S. C., Andrews, J. E., Sinclair, A. H., and Koopman, P. (1996) Development (Camb.) 122, 2813–2822
10. Bishop, C. E., Whitworth, D. J., Qin, Y., Agoulnik, I. A., Agoulnik, I. U., Harrison, W. R., Behringer, R. R., and Overbeek, P. A. (2000) Nat. Genet. 26, 490–494
11. Huang, B., Wang, S., Ning, Y., Lamb, A., and Bartley, J. (1999) Am. J. Med. Genet. 87, 349–353
12. Vidal, V., Chaboissier, M., de Roij, J., and Schedl, A. (2001) Nat. Genet. 28, 216–217
13. Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., Pasantes, J., Bricarelli, F. D., Keutel, J., Hustert, E., Wolf, U., Tommerup, N., Schempff, W., and Scherer, G. (1994) Cell 79, 1111–1120
14. Foster, J. W., Dominguez-Steglich, M. A., Giulioli, S., Kwok, C., Wells, P. A., Weissenbach, J., Mansour, S., Young, I. D., Goodfellow, P. N., Brook, J. D., and Schafer, A. J. (1994) Nature 372, 525–530
15. Jäger, R. J., Anvret, M., Hall, K., and Scherer, G. (1990) Nature 348, 452–454
16. Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1991) Nature 351, 117–121
17. Berta, P., Hawkins, J. R., Sinclair, A. H., Taylor, A., Griffiths, B. L., Goodfellow, P. N., and Fellous, M. (1990) Nature 348, 448–450
18. Ferrari, S., Harley, V. R., Pontiggia, A., Goodfellow, P. N., Lovell-Badge, R., and Bianchi, M. E. (1992) EMBO J. 11, 4497–4506
19. McDowall, S., Argentaro, A., Ranganathan, S., Weller, P., Martin, S., Mansour, S., Tolmie, J., and Harley, V. (1999) J. Biol. Chem. 274, 24023–24030
20. de Santa Barbara, P., Bonneaud, N., Boizet, B., Desclozeaux, M., Moniot, B., Sudbeck, P., Scherer, G., Poulat, F., and Berta, P. (1998) Mol. Cell. Biol. 18, 6653–6665
21. Arango, N., Lovell-Badge, R., and Behringer, R. (1999) Cell 99, 409–419
22. Wilson, M. J., Jeyasuria, P., Parker, K. L., and Koopman, P. (2005) J. Biol. Chem. 280, 5917–5923
23. Palmer, S. J., and Burgoyne, P. S. (1991) Development (Camb.) 112, 265–268
24. Burgoyne, P. S., Buehr, M., and McLaren, A. (1988) Development (Camb.) 104, 683–688
25. Patek, C. E., Kerr, J. B., Gosden, R. G., Jones, K. W., Hardy, K., Muggleton-Harris, A. L., HANDyside, A. H., Whittingham, D. G., and Hooper, M. L.
Regulation of PgdS Transcription by SOX9

(1991) Development (Camb.) 113, 311–325
26. Malki, S., Nef, S., Notarnicola, C., Thevenet, L., Gasca, S., Mejean, C., Berta, P., Poulat, F., and Boizet-Bonhoure, B. (2005) EMBO J. 24, 1798–1809
27. Adams, I., and McLaren, A. (2002) Development (Camb.) 129, 1155–1164
28. Urade, Y., and Hayaishi, O. (2000) Biochim. Biophys. Acta 1482, 259–271
29. Wright, E., Hargrave, M. R., Christiansen, J., Cooper, L., Kun, J., Evans, T., Gangadharan, U., Greenfield, A., and Koopman, P. (1995) Nat. Genet. 9, 15–20
30. Hiramatsu, R., Kanai, Y., Mizukami, T., Ishii, M., Matoba, S., Kanai-Azuma, M., Kurohmaru, M., Kawakami, H., and Hayashi, Y. (2003) Dev. Dyn. 228, 247–253
31. Wilhelm, D., and Englert, C. (2002) Genes Dev. 16, 1839–1851
32. Guan, K. L., and Dixon, J. E. (1991) Anal. Biochem. 192, 262–267
33. Beverdam, A., Wilhelm, D., and Koopman, P. (2003) Cytogenet. Genome Res. 101, 242–249
34. Wilhelm, D., Huang, E., Svingen, T., Stanfield, S., Dinnis, D., and Koopman, P. (2006) genesis 44, 168–176
35. Bell, D. M., Leung, K. K., Wheatley, S. C., Ng, L. J., Zhou, S., Ling, K. W., Sham, M. H., Koopman, P., Tam, P. P., and Cheah, K. S. (1997) Nat. Genet. 16, 174–178
36. Bridgewater, L. C., Walker, M. D., Miller, G. C., Ellison, T. A., Holsinger, L. D., Potter, J. L., Jackson, T. L., Chen, R. K., Winkel, V. L., Zhang, Z., McKinney, S., and de Crombrugghe, B. (2003) Nucleic Acids Res. 31, 1541–1553
37. Jenkins, E., Moss, J. B., Pace, J. M., and Bridgewater, L. C. (2005) Matrix Biol. 24, 177–184
38. Rentsendorj, O., Nagy, A., Sinko, I., Daraba, A., Barta, E., and Kiss, I. (2005) Biochem. J. 389, 705–716
39. Kidokoro, T., Matoba, S., Hiramatsu, R., Fujisawa, M., Kanai-Azuma, M., Taya, C., Kurohmaru, M., Kawakami, H., Hayashi, Y., Kanai, Y., and Yonekawa, H. (2005) Dev. Biol. 278, 511–525
40. Fujimori, K., Fujitani, Y., Kadoyama, K., Kumanogoh, H., Ishikawa, K., and Urade, Y. (2003) J. Biol. Chem. 278, 6018–6026
41. Fujimori, K., Kadoyama, K., and Urade, Y. (2005) J. Biol. Chem. 280, 18452–18461
42. White, D. M., Takeda, T., DeGroot, L. J., Stefansson, K., and Arnason, B. G. (1997) J. Biol. Chem. 272, 14387–14393
43. Beverdam, A., and Koopman, P. (2006) Hum. Mol. Genet. 15, 417–431
44. Nef, S., Schaad, O., Stallings, N. R., Cederroth, C. R., Pitetti, J. L., Schaer, G., Malki, S., Dubois-Dauphin, M., Boizet-Bonhoure, B., Descombes, P., Parker, K. L., and Vassalli, J. D. (2005) Dev. Biol. 287, 361–377
45. Small, C. L., Shima, J. E., Uzumcu, M., Skinner, M. K., and Griswold, M. D. (2005) Biol. Reprod. 72, 492–501
46. Peirano, R. I., and Wegner, M. (2000) Nucleic Acids Res. 28, 3047–3055
47. Bernard, P., Tang, P., Liu, S., Dewing, P., Harley, V. R., and Vilain, E. (2003) Hum. Mol. Genet. 12, 1755–1765
48. Sock, E., Pagon, R. A., Keymolen, K., Lissens, W., Wegner, M., and Scherer, G. (2003) Hum. Mol. Genet. 12, 1439–1447
49. Gao, F., Maiti, S., Alam, N., Zhang, Z., Deng, J. M., Behringer, R. R., Lecuireuil, C., Guiliou, F., and Huff, V. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 11987–11992