

# Distinct Transcriptional Mechanisms Direct Expression of the Rat *Dmrt1* Promoter in Sertoli Cells and Germ Cells of Transgenic Mice<sup>1</sup>

Ning Lei,<sup>3</sup> Tatiana Karpova, Kaori I. Hornbaker, Daren A. Rice, and Leslie L. Heckert<sup>2</sup>

Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas

## ABSTRACT

DMRT1 is a transcription factor expressed only in Sertoli cells and undifferentiated spermatogonia of the postnatal testis, where it is required for proper cellular differentiation and fertility. To elucidate the transcriptional regulatory regions that provide DMRT1's cell-specific expression, transgenic mice containing a *LacZ* reporter gene driven by variable amounts of rat *Dmrt1* 5' flanking sequence, 9 kb and smaller, were evaluated. Examination of transgene expression by RT-PCR indicated that multiple promoter regions direct *Dmrt1* to the testis and that sequences upstream of 2.8 kb are needed for both Sertoli cell expression and limiting transcriptional influence imposed by surrounding chromatin. Thus, whereas many of the transgenes were expressed in the testis, the ones with smaller promoters were significantly more prone to expression at ectopic sites or to complete silencing. Transgene expression in Sertoli cells and germ cells was assessed by immunohistochemistry and RT-PCR following busulfan treatment to remove germ cells. Both evaluations indicated expression of the 9- and 3.2-kb promoters in Sertoli cells and germ cells, whereas activity of smaller promoters was largely restricted to germ cells. In all, the present study provides *in vivo* evidence that distinct promoter sequences participate in *Dmrt1* regulation in somatic cells and germ cells, with the -3.2 kb/-2.8 kb region directing expression in Sertoli cells and downstream sequences ( $\leq 1.3$  kb) directing it in germ cells. Further exploration of the mechanisms restricting *Dmrt1* expression to the testis revealed that FOXL2, a transcription factor required for differentiation of the ovary, repressed *Dmrt1* promoter through the -3.2 kb/-2.8 kb regulatory region, offering a potential mechanism for *Dmrt1* transcriptional silencing in granulosa cells.

*Dmrt1*, FOXL2, testis, testis development, transcription, transcription regulation, transgenic mice, transgenic mice spermatogenesis

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<sup>2</sup>Correspondence: Leslie L. Heckert, Department of Molecular and Integrative Physiology, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160. FAX: 913 588 7430; e-mail: lheckert@kumc.edu

<sup>3</sup>Current address: Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey, 195 Little Albany St., New Brunswick, NJ 08903.

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## INTRODUCTION

DMRT1 (doublesex and mab-3 related transcription factor-1) is an evolutionarily conserved transcription factor that contains a unique, cysteine-rich, DNA-binding motif, known as the DM domain, that was first identified in DOUBLESEX and MAB-3, which are transcription factors that regulate sexual differentiation in flies and worms, respectively [1]. As the putative mammalian ortholog of DOUBLESEX and MAB-3, DMRT1 has been implicated in mammalian sex determination and differentiation. This link to sexual differentiation is further supported by DMRT1's expression profile, which is limited to the developing gonads and postnatal testis [2–10]. Studies in mice have best exposed DMRT1's expression pattern and showed that during embryonic development, it is localized to somatic cells and germ cells of XX and XY gonads and, subsequently, is restricted to the testis after sex determination [2, 6, 10–13]. Male-specific expression continued through embryonic and postnatal development, with DMRT1 evident in Sertoli cells at all ages, in gonocytes up to Embryonic Day 15.5, and in undifferentiated spermatogonia after birth [13].

In mammals, evidence from both humans and mice clearly support DMRT1's importance in testis differentiation but contrast with respect to the time that its activity impinges on testis development [14–17]. Thus, DMRT1 is implicated in embryonic testis development and sex determination in humans, whereas in mice, it is needed for postnatal testis differentiation. Evidence in humans for the participation of DMRT1 in sex determination and gonad development stems largely from its chromosomal location (9p24.3), which links it to known breakpoints responsible for human 46,XY sex reversal and testis dysgenesis [14]. In mice, functional deletion of *Dmrt1* resulted in male- and testis-specific defects that were discernible only after birth [15]. Characterization of the mice showed that Sertoli cells were poorly differentiated and that germ cells failed to survive beyond Postnatal Day 14 [16]. Subsequently, conditional deletion of *Dmrt1* indicated autonomous requirements in both Sertoli cells and germ cells and nonautonomous activity in Sertoli cells for support of the germ line [17]. Thus, studies in mice showed that DMRT1 is required within Sertoli cells for self-directed postnatal differentiation and germ line maintenance, whereas in germ cells, it acts independently to orchestrate migration to the tubule periphery, mitotic reactivation, and cell survival [17].

The expression pattern and function of DMRT1 suggest that its regulatory position is at a key point in the genetic network leading testis differentiation; thus, unraveling transcriptional components controlling *Dmrt1* expression likely will contribute substantially to our understanding of this pathway. The rat *Dmrt1* promoter has been characterized in primary cultures of Sertoli cells, which identified several important regulatory elements within the first 5 kb of 5' flanking sequence [18, 19]. Deletion analysis of the promoter identified two major regions that contributed to its transcriptional activity. These regions were located between -3.2 kb and -2.8 kb (the distal

regulatory region) and downstream of  $-150$  bp (the proximal promoter), relative to the major transcriptional start site. Mutagenesis and DNA/protein-binding studies subsequently uncovered important elements and proteins required for activity of the proximal and distal regions. In the proximal promoter, several elements were identified in maintaining basal promoter activity that bound members of the ubiquitous specificity protein (SP) and early growth response (EGR) transcription factor families. Unlike the proximal promoter, the distal region was active only in Sertoli cells through direction of several critical regulatory elements, three of which bound the transcription factor GATA4, a protein previously identified as important for mammalian sex determination and testis differentiation [18, 20]. Furthermore, in mice, loss of GATA4's requisite partner, ZFPM2 (previously known as FOG2), reduced *Dmrt1* mRNA levels in gonads from XY embryos but not from XX embryos, providing additional support for GATA4 in *Dmrt1* regulation [18].

The current study used transgenic analysis to examine *in vivo* activity of the *Dmrt1* promoter to validate the importance of the identified regulatory regions in testis and Sertoli cell-specific expression. Various amounts of rat *Dmrt1* 5' flanking sequence, ranging from 9 kb to 150 bp, were used to direct expression of a *LacZ* reporter gene in transgenic mice. Multiple tissues from the mice were then examined for transgene expression, which supported a role for the distal regulatory region ( $-3.2$  kb to  $-2.8$  kb) in Sertoli cell-specific expression of *Dmrt1* and implicated sequences between  $-1.3$  kb and  $-400$  bp in germ cell expression of *Dmrt1*. In addition, transfection studies showed that the transcription factor FOXL2 specifically repressed *Dmrt1* promoter activity, in part, through the distal regulatory region, suggesting that FOXL2 participates in silencing *Dmrt1* in granulosa cells. In all, the data suggest distinct mechanisms for direct transcription of *Dmrt1* in somatic and germ cells of the gonads and that the upstream regulatory region is required for gene activation in Sertoli cells and gene silencing in granulosa cells.

## MATERIALS AND METHODS

### Plasmid Generation

Cloning of rat *Dmrt1* promoter-reporter constructs *Dmrt1(-3280/+75)Luc* and *Dmrt1(-2800/+75)Luc* has been described elsewhere [18, 19]. The pGL3-control, pGL3-basic, and pRL-TK (internal control containing *Renilla* luciferase driven by the thymidine kinase promoter) vectors were purchased from Promega Corp.

The *Foxl2* cDNA (~1155 bp) was synthesized using Superscript III reverse transcriptase (Invitrogen), 0.5  $\mu$ g of oligo-dT15, and 2  $\mu$ g of total ovarian RNA from adult Swiss Webster mice (isolated with TRIZOL reagent; Life Technologies, Inc.) and then amplified by PCR using *Foxl2*-specific primers containing recognition sequences for *HindIII* and *XbaI* (5' primer *Foxl2 HindIII*, 5'-ATATAAGCTTGCGGAGAGCCGGCTTTGTC-3'; 3' primer *Foxl2 XbaI*, 5'-GAAGTCTAGATTGGCACTCAGAGATCCAGACGC-3'). The amplified *Foxl2* cDNA was digested with *HindIII* and *XbaI* and cloned into the corresponding sites of pcDNA3 to generate pcDNA3-*Foxl2*.

### Cell Preparation and Transfection Analysis

Preparation and transient transfection of Day 15 primary rat Sertoli cells were as described elsewhere [19]. For transfection, cells were incubated with 0.1  $\mu$ g of the reporter vector [either *Dmrt1(-3280/+75)Luc*, *Dmrt1(-2800/+75)Luc*, or pGL3-control] and increasing concentrations of pcDNA3-*Foxl2* combined with an empty expression vector (pcDNA3) to make a total of 0.2  $\mu$ g of cotransfected DNA together with 1.5  $\mu$ l of lipofectamine and 2  $\mu$ l of plus reagents (Life Technologies, Inc.). In addition, 20 ng of pRL-TK were included to control for transfection efficiency. At 48 h after transfection, cells were collected, lysed, and assayed for both firefly and *Renilla* luciferase activities using the Dual-Luciferase Reporter Assay System (Promega Corp.) as described elsewhere [19, 21]. Data are represented as the firefly/*Renilla* luciferase activity of *Dmrt1*-luciferase constructs relative to the firefly/*Renilla*

luciferase activity of SV40-promoter control vector pGL3-control. All plasmid DNAs were prepared from overnight bacterial cultures using QIAwell 8 plasmid kit (Qiagen) according to the supplier's recommendations. All buffers used for plasmid DNA preparation were endotoxin-free.

### Generation of *Dmrt1*-*LacZ* Transgenes

Approximately 5200 bp of *LacZ/Hprt1* DNA (generously provided by Dr. Mario Capecchi), containing most of the *LacZ* coding region and *Hprt1* exons 8 and 9, intervening sequences, and polyadenylation (see Fig. 1), was cloned into the *BamHI* and *XbaI* sites of pBKS (Stratagene). A 150-bp fragment with the 5' end of *LacZ* with a nuclear localization signal was digested with *BamHI* and inserted upstream of the 5200 bp at the *BamHI* site to generate pBKS-*LacZ/Hprt1*. The rat *Dmrt1* gene from  $-12$  bp to  $+75$  bp relative to the main transcriptional start site was inserted 5' to *LacZ* to generate pBKS-*LacZ(+)*. In addition, 9 kb of rat *Dmrt1* 5' flanking sequence was digested from lambda clone 56.1611 [19] using *KpnI* and *XhoI* and cloned between *KpnI* and *XhoI* sites in pBKS-*LacZ(+)*. The resulting plasmid is referred to as pBKS-*Dmrt1 9kb-LacZ*. The pBKS-*Dmrt1 2.8kb-LacZ* and pBKS-*Dmrt1 150bp-LacZ* were generated by isolation of the 2.8-kb and 150-bp *Dmrt1* promoters, respectively, after digestion of *Dmrt1(-2800/+75)Luc* and *Dmrt1(-150/+75)Luc* with *KpnI* and *XhoI* and ligation of them with pBKS-*LacZ(+)* [18, 19].

### Generation and Genotype of Transgenic Mice

For production of transgenic mice, transgene fragments of *Dmrt1 9kb-LacZ*, *Dmrt1 3.2kb-LacZ*, *Dmrt1 1.3kb-LacZ*, and *Dmrt1 400bp-LacZ* were isolated from vector sequences following digestion of pBKS-*Dmrt1 9kb-LacZ* with the appropriate restriction enzymes (e.g., *Dmrt1 9kb-LacZ*: *KpnI* and *NotI*; *Dmrt1 3.2kb-LacZ*: *SpeI* and *NotI*; *Dmrt1 1.3kb-LacZ*: *XbaI*; and *Dmrt1 400bp-LacZ*: *SalI* and *XbaI*). Transgene fragments of *Dmrt1 2.8kb-LacZ* and *Dmrt1 150bp-LacZ* were isolated by digestion of pBKS-*Dmrt1 2.8kb-LacZ* and pBKS-*Dmrt1 150bp-LacZ*, respectively, with *KpnI* and *NotI*. The DNA was purified using agarose gel DNA purification kit (UltraClean GelSpin; Mo Bio Laboratories, Inc.) according to the manufacturer's instructions and eluted in TE buffer (10 mM Tris and 0.25 mM EDTA, pH 7.5). Transgenic founders were produced by pronuclear injection into C57BL/6  $\times$  SJL F<sub>1</sub> zygotes through the Transgenic and Gene-Targeting Institutional Facility at the University of Kansas Medical Center. Founders were crossed into a C57BL/6 background for production of transgenic offspring. All animal studies were conducted in accordance with the principles and procedures outlined in *Guideline for Care and Use of Experimental Animals*.

The genotypes of all founders and their offspring were determined by PCR amplification of mouse tail DNA. Tail snips were digested in 500  $\mu$ l of lysis buffer (100 mM EDTA, 50 mM Tris [pH 8.0], and 1% SDS) in the presence of 400  $\mu$ l/ml of proteinase K (Invitrogen) at 55°C overnight. Next, 250  $\mu$ l of saturated NaCl (concentration,  $>6$  mol/l) were added, and the digestion was mixed by inversion (400 $\times$ ), followed by incubation on ice for 10 min. The sample was then centrifuged at 6000 rpm for 10 min, and 500  $\mu$ l of supernatant were precipitated by adding 1 ml of 100% ethanol and mixing the sample by inversion (10–20 $\times$ ). The precipitated DNA was spooled and rinsed by dipping in a solution of 70% ethanol. The DNA was resuspended in 150  $\mu$ l of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). Transgene amplification (PCR; 28 cycles for 30 sec at 94°C, 30 sec at 59°C, and 30 sec at 72°C) was performed using 5' primers, *Dmrt1*-DM2b (5'-AGCCTGCCTCAGAGAAGCG-3') for *Dmrt1 9kb-LacZ*, *3.2kb-LacZ*, *2.8kb-LacZ*, *1.3kb-LacZ*, and *400bp-LacZ* transgenic lines and *Dmrt1*-BS#15-sense (5'-AGGCAGAAAAAAGG TACA-3') for *Dmrt1 150bp-LacZ* transgenic mouse lines, and 3' primer, *LacZ4* (5'-GGAGGAGTAGAATGTTGAGAGTC-3'). Amplified products were analyzed by agarose gel electrophoresis, and positive animals were identified by the presence of a 376-bp band (for *Dmrt1 9kb-LacZ*, *3.2kb-LacZ*, *2.8kb-LacZ*, *1.3kb-LacZ*, and *400bp-LacZ* transgenic lines) or a 233-bp band (for *Dmrt1 150bp-LacZ* transgenic line).

### RT-PCR Analysis of Transgene Expression in Transgenic Mice

Tissue-specific expression of all transgenes was evaluated using RT-PCR as described elsewhere [22]. Prepubertal transgenic mice (15 days old) were killed and cDNA prepared from various tissues as described above. The cDNA synthesis was confirmed by testing 1  $\mu$ l of each reaction as template in a PCR reaction with primers for the ribosomal protein L7 (primer *Rp17.1*, 5'-GGGGGAAGCTTCGAAAGGCAAGGAGGAAGCT-3'; primer *Rp17.2*, 5'-GGGGGGTGCCTCCTCCATGCAGATGATGCC-3'). For analysis of *LacZ* expression, PCR parameters were kept constant across all transgene constructs, and if required, the amount of added cDNA template was adjusted to equalize

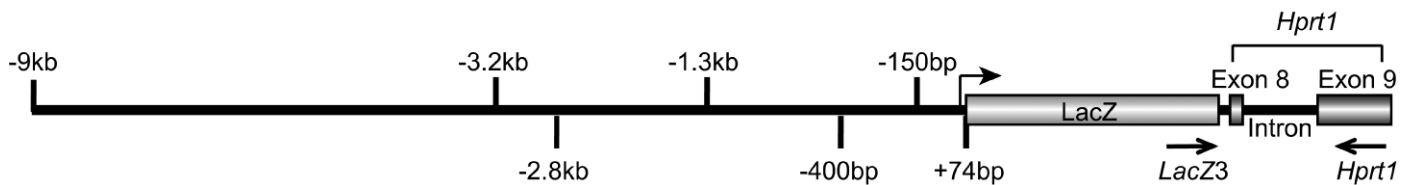


FIG. 1. Schematic representation of *Dmrt1-LacZ* transgenes. *Dmrt1-LacZ* transgenes contain the indicated *LacZ/Hprt1* reporter cassette driven by various lengths of rat *Dmrt1* 5' flanking sequence, denoted on the figure as -150bp, -400bp, -1.3kb, -2.8kb, -3.2kb, and -9kb, that ends 74 bp (+74) 3' to the transcriptional start site (bent arrow). Hybridization positions and amplification directions for the RT-PCR primers *LacZ3* and *Hprt1* are indicated by arrows below the depicted transgene.

the template based on the amplified cDNA signal from the internal *Rpl7* control. Typically, 1 ng of cDNA template was employed together with 5' primer *LacZ3* (5'-GCCGCTACAGTCAACAG-3') and 3' primer *Hprt1* (5'-GGCCTATAGGCTCATAGTGCAA-3'). These primers hybridized to sequences located in the *LacZ* gene and exon 9 of *Hprt1* and span the intron within the *Hprt1* sequence (Fig. 1). This allowed differentiation between contaminating genomic DNA and processed mRNA. Amplified products were examined by agarose gel electrophoresis, which revealed a 757-bp product for properly processed transcripts and a 1412-bp product for that arising from genomic DNA contamination. Expression analysis of all transgenes was performed using a minimum of three different animals for each line associated with a specific transgene, and the prepared RNA was assayed at least twice for each of these. For example, data representing the 9-kb transgene (four independent lines) was generated from a minimum of 24 independent RT-PCR reactions, which employed RNA prepared from 12 animals (three animals from each of the four lines). Expression of endogenous mouse *Dmrt1* mRNA was determined by RT-PCR using 5' primer *Dmrt1.10* (5'-GGCGAAGCTTGCTCGGCAATCAGGCTGC-3') and 3' primer *Dmrt1.36* (5'-CGGCAAGCTTACAGGTCCAGCTGCGGTG-3'), with an expected amplified product of 418 bp.

#### Generation and Morphological Analyses of Germ Cell-Free Transgenic Mice by Fetal Busulfan Treatment

The C57BL/6 female mice were mated to transgenic males and subsequently treated with busulfan (25–40 mg/kg body wt; dosage was determined experimentally for different transgenic lines) by intraperitoneal injection at 12.5 days postcoitus (dpc), as described previously [23]. On Day 15 after birth, one testis from transgenic animals treated in utero with busulfan was isolated for RNA extraction and RT-PCR and the other testis for histological examination to confirm germ cell depletion [13]. For immunohistochemical evaluation, at least two animals for a same genotype were assayed. Isolated gonads were fixed at 4°C overnight in 4% paraformaldehyde in PBS buffer (0.137 M NaCl, 2.7 mM KCl, 8.0 mM Na<sub>2</sub>HPO<sub>4</sub>, and 2.0 mM KH<sub>2</sub>PO<sub>4</sub>; pH 7.4) and embedded in paraffin. Samples were cut into sections (thickness, 5 μm) and analyzed for either DMRT1 or β-galactosidase. The DMRT1 immunohistochemical analyses were conducted as described elsewhere [13]. For β-galactosidase staining, sections were cleared in xylene, washed in absolute ethanol, and then incubated in methanol with 0.3% H<sub>2</sub>O<sub>2</sub> for 1 min. After being rehydrated through graded alcohol solutions, sections were incubated in 10 mM sodium citrate buffer (pH 6.0) at 100°C for 10 min for antigen retrieval. Slides were cooled, 10% normal goat serum blocking solution (Zymed) plus 1% BSA was added, and samples were incubated at room temperature for 60 min. Primary antibody incubations were carried out overnight at 4°C in blocking solution using a 1:750 dilution of rabbit polyclonal antibody generated to β-galactosidase (catalog no. ab616; Abcam, Inc.). Samples were next washed twice in PBST (PBS buffer with 0.1% Triton X-100) for 10 min, followed by another 20 min in blocking solution. Samples

were then incubated for 60 min with biotinylated goat anti-rabbit secondary antibody (Zymed) and washed twice (10 min each wash) with PBST. Next, histostain plus streptavidin peroxidase (Zymed) solution was added for 10 min, and the slides were again washed twice (10 min each wash) in PBST. Aminoethyl carbazole substrate solution (Zymed) was applied to the sections for 5–10 min, and the reaction was stopped by rinsing in PBS. With two or three drops of GVA mounting solution (Zymed), sections were mounted on glass slides. All the steps after primary antibody incubations were performed at room temperature.

## RESULTS

### Generation and Analysis of *Dmrt1-LacZ* Transgenic Mice

To determine the transcriptional requirements for correct temporal and spatial expression of *Dmrt1*, the activity of its promoter was evaluated in vivo by analyzing mice carrying transgenes with varying amounts of *Dmrt1* 5' flanking sequence that direct β-galactosidase (*LacZ*) expression (Fig. 1). Transgenes were generated by placing sequences residing upstream of *Dmrt1*'s transcriptional start site, ranging in size from 150 bp to 9.0 kb, 5' to a *LacZ* reporter cassette, which also included *Hprt1* genomic sequence corresponding to part of exon 8, exon 9, and the intervening sequence (Fig. 1). Transgenic mice were produced from six different *LacZ* reporter constructs, distinguished by the amount of promoter sequence, and for each construct, tissue expression was evaluated in a minimum of three independent lines by RT-PCR of *LacZ* mRNA (Table 1 and Fig. 1, primers *LacZ3* and *Hprt1* denoted by directional arrows).

### The 3.2-kb *Dmrt1* Promoter Directs Testis-Specific Expression

Tissue expression profiles from the different mouse lines revealed transgene expression patterns that varied between mice carrying different constructs and, in some instances, between mice harboring the same construct but representing distinct founder animals (i.e., different integration sites) (Fig. 2, representative expression profiles for each transgene, including multiple profiles for those with integration site differences). The latter is exemplified by lines 77 and 29, both of which carried the 2.8-kb transgene but showed two distinct expression patterns: testis restricted (line 77) and unrestricted (line 29). All

TABLE 1. Tissue expression of *Dmrt1-LacZ* transgenes.

Construct	Male (n) <sup>a</sup>	Testis	Brain	Stomach	Heart	Kidney	Spleen	Lung	Liver	No expression	Testis specific	Female (n) <sup>a</sup>	Ovary
9.0 kb	4	4	1	0	0	0	0	0	0	0	3	3	0
3.2 kb	3	3	0	1	0	0	0	0	0	0	2	2	0
2.8 kb	5	5	5	4	2	2	1	0	0	0	0	6	1
1.3 kb	7	6	2	0	2	1	1	1	0	1	3	5	1
400 bp	9	4	3	0	1	2	1	2	1	5	1	9	2
150 bp	4	3	3	2	2	2	1	1	1	1	0	2	0

<sup>a</sup> n = Number of individual lines evaluated for the indicated transgene construct.



transgenic data were summarized graphically by plotting, for each transgenic reporter, the percentage of mouse lines that express in a given tissue (Table 1). Notably, of the seven lines representing both 9- and 3.2-kb constructs, only two lines exhibited ectopic expression, and each in only a single tissue other than testis. In addition, 9- and 3.2-kb transgene expression levels were significantly lower in the ectopic tissues than in the testis (data not shown). However, deleting the promoter to 2.8 kb or below greatly expanded the number of lines expressing the transgene in nontesticular tissues. Thus, whereas many of the promoters directed expression to the testis, the smaller ones were significantly more prone to expression at ectopic sites or to complete silencing. The data suggest that different promoter regions participate in testis-specific expression of *Dmrt1*, with distinct sequences indicated for directing testis expression and for preventing incorrect expression induced via influences imposed by the site of integration. With the exception of a single nonexpressing line for the 1.3-kb transgene, the 1.3- and 2.8-kb transgenes expressed *LacZ* in the testis, but the percentage of mice demonstrating testis-specific expression was lower than that of the larger constructs (43% for 1.3 kb and 0% for 2.8 kb, compared to 75% and 67% for 9 and 3.2 kb, respectively). The present study also revealed that transgenes containing promoters smaller than 1.3 kb were even more prone to silencing and ectopic expression, with only one of the combined thirteen 400- and 150-bp lines having testis-specific expression (Table 1). Thus, with deletion below 1.3 kb, transgene expression showed no preference for the testis and was significantly more susceptible to silencing and ectopic expression, indicating transgene expression was directed predominantly by sequences near the site of integration, as opposed to those within the *Dmrt1* promoter.

#### *The Distal Regulatory Region Is Required for Testis Somatic Cell Expression*

Because neither enzymatic or immunological detection of  $\beta$ -galactosidase provided reliable results that could distinguish testicular cells expressing the transgene (i.e., Sertoli versus germ cells), cell-specific promoter activity was evaluated by RT-PCR in testes isolated from mice born to mothers treated, during pregnancy, with the alkylating agent busulfan, which eliminates germ cells from male progeny [23]. Following treatment with sublethal doses of busulfan (25–40 mg/kg body wt; dosage was determined experimentally for different transgenic lines), pregnant females delivered litters of normal size compared to those of untreated controls, but male progeny were infertile (data not shown). Thus, similar to previous reports, busulfan treatment of 12.5-dpc pregnant females effectively prevented spermatogenesis of male progeny [23]. Immunohistological analyses for DMRT1 in testes isolated from 15-day-old mice born to either treated or untreated mothers revealed that more than 90% of the germ cells were depleted from the testis of males born to treated mothers, with only a few remaining spermatogonia observed to be attached to the basal membrane of the seminiferous epithelium (compare Fig. 3, A with B). Five different lines, representing three constructs (e.g., *Dmrt1 2.8kb-LacZ*, *Dmrt1 3.2kb-LacZ*, and *Dmrt1 9kb-LacZ*), were examined histologically to confirm germ cell depletion, followed by evaluation of transgene expression in the contralateral testis by RT-PCR. Notably, transgenes with promoter lengths of 3.2 or 9.0 kb were expressed in testes of mice born from either treated or untreated mothers, whereas those with the 2.8-kb promoter were expressed only in samples derived from untreated mothers (Fig. 3C and data not shown). The results indicated that the 2.8-

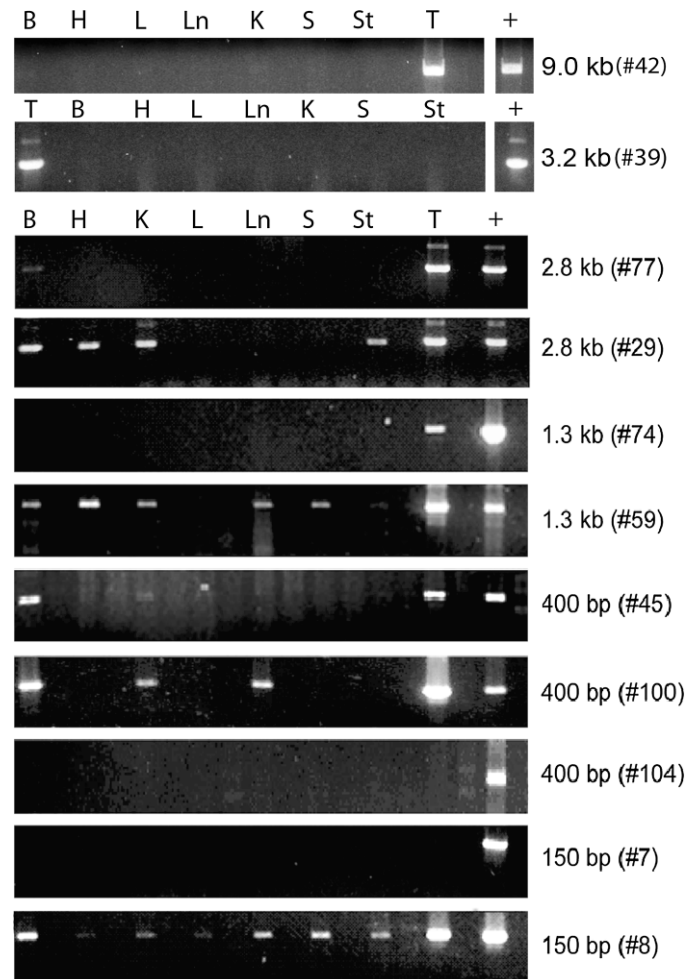


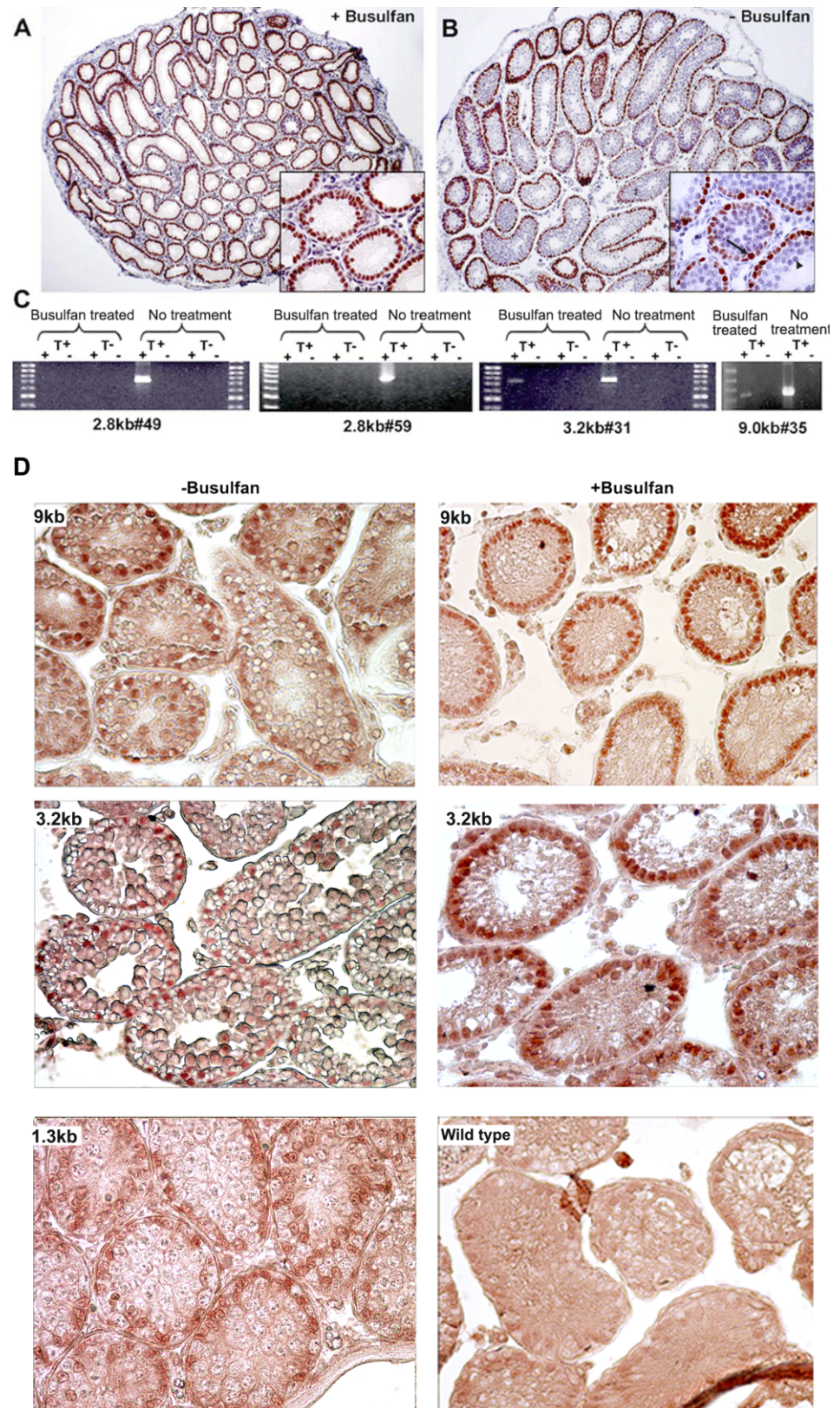
FIG. 2. Observed profiles for *Dmrt1-LacZ* transgenes. RNA extracted from tissues derived from 15-day-old transgenic mice was evaluated for transgene expression by RT-PCR. Complementary DNA synthesis was confirmed by evaluating cDNA amplification of the ribosomal protein L7, and if required, templates for *LacZ* amplification were adjusted to equalize template levels based on that of *Rpl7*. RT-PCR was performed in the presence (left lane) and absence (right lane) of added reverse transcriptase, and amplified products were examined by agarose gel electrophoresis (product of *LacZ* mRNA is 757 bp; genomic DNA is 1412 bp). Representative expression profiles are shown for each transgenic construct, with the promoter length denoted on the right together with the evaluated transgenic line number in parenthesis. For transgenic constructs that exhibited more than one expression pattern, representative profiles of each distinct pattern are shown. B, brain; L, liver; h, heart; Ln, lung; S, spleen; K, kidney; St, stomach; T, testis; G, genomic DNA from positive transgenic animal; +, positive control (cDNA generated from testis of *Dmrt1 9kb-LacZ* transgenic mice). Results for all transgenic lines are summarized in Table 1, which represents data derived from two or more independent PCRs from a minimum of three animals for each line of a specific transgene.

kb promoter and, presumably, smaller constructs express only in germ cells, whereas those containing sequences between  $-2.8$  kb and  $-3.2$  kb express in both germ cells and somatic cells, presumably Sertoli cells.

Immunohistochemistry also was employed to evaluate  $\beta$ -galactosidase expression in testes from treated and untreated animals with select transgene constructs (Fig. 3D).  $\beta$ -Galactosidase protein expression was similar to that of endogenous DMRT1 in both untreated (Fig. 3D, left) and treated (Fig. 3D, right) mice carrying either the 9- or 3.2-kb

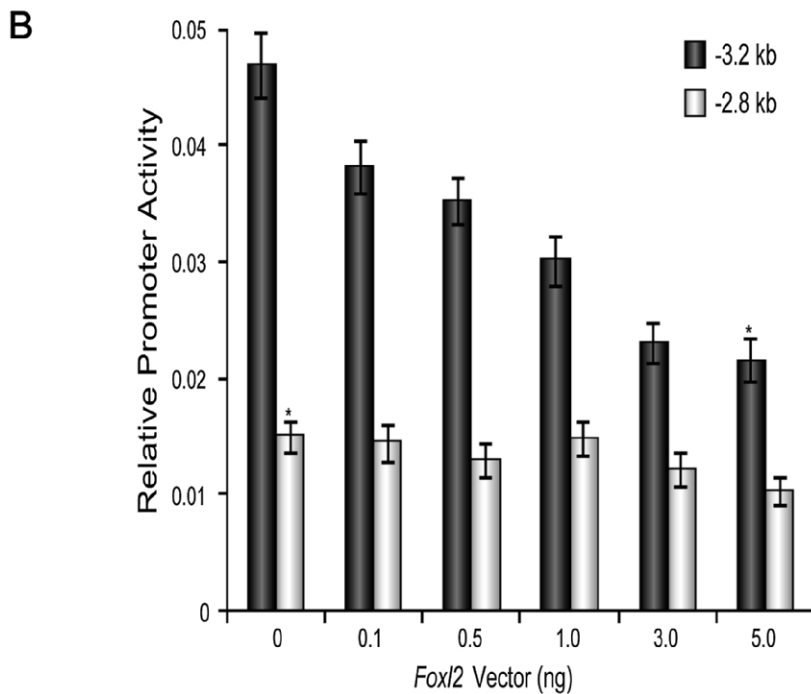
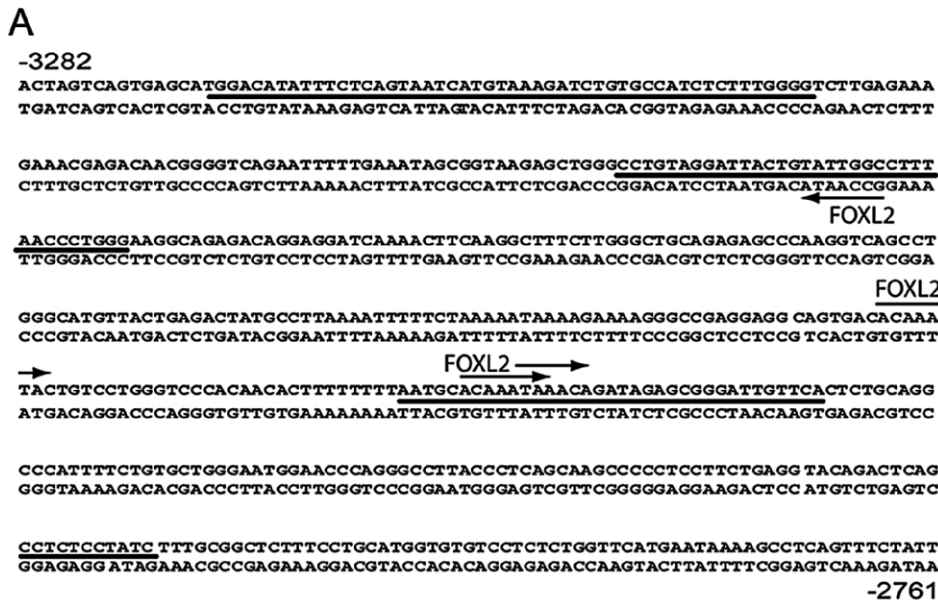


FIG. 3. Cell-specificity of *Dmrt1-LacZ* transgenes. Transgene expression in specific testis cell populations was evaluated in testes from 15-day-old transgenic mice born to mothers treated with busulfan (+Busulfan) or without busulfan (-Busulfan) to eliminate germ cells. Efficacy of the busulfan treatment was evaluated by DMRT1 immunohistochemistry (red) to assess the DMRT1-expressing germ cell population (A and B). DMRT1-positive (arrow) and -negative (arrowhead) germ cells are indicated (B, inset). Note the paucity of germ cells in the busulfan-treated group (A). Transgene expression was evaluated in testes from both treatment groups by both RT-PCR (C) and immunohistochemistry for  $\beta$ -galactosidase (D). RT-PCR analysis (+RT, -RT) was used to evaluate testis expression of transgenic (T+) and wild type (T-) littermates that represent a total of five different transgenic lines (*Dmrt1-2.8kb-LacZ*, lines 49 and 59; *Dmrt1-3.2kb-LacZ*, lines 31 and 20 and data not shown; *Dmrt1-9kb-LacZ*, line 35).  $\beta$ -Galactosidase expression in testes from males with 9- and 3.2-kb transgenes (denoted to left) and born to mothers of both treatment groups (+Busulfan, right; -Busulfan, left) was evaluated by immunohistochemistry (D).  $\beta$ -Galactosidase expression in a wild-type testis from the treated group (+Busulfan) and in a 1.3-kb transgenic testis from the untreated group (-Busulfan) also are shown. Original magnification  $\times 4$  (A and B),  $\times 20$  (insets in A and B), and  $\times 40$  (D).



promoter constructs (compared Fig. 3D with Fig. 3, A and B). Thus, transgene expression was detected in Sertoli cells and spermatogonia of untreated animals and only in Sertoli cells of the treated animals, confirming that the 3.2-kb promoter contains sequences required for Sertoli cell expression. Staining in testes from untreated mice carrying the *1.3kb-LacZ* transgene showed expression predominantly within germ cells,

with less certain expression in Sertoli cells. For mice containing promoters of 2.8 kb and smaller, demonstrating Sertoli cell expression by staining testes from busulfan-treated mothers was ineffective, because a lack of staining could be attributed to either the transgene's inability to express in Sertoli cells or technical problems associated with the tissue preparation.



*FOXL2 Represses Dmrt1 Promoter Activity*

Expression studies indicate that *Dmrt1* undergoes a female-specific silencing in somatic cells of the ovary (pregranulosa) and a sexually independent germ cell silencing that accompanies their exit from the cell cycle during embryogenesis [13]. DMRT1 reappears only in male germ cells and coincides with reactivation of the cell cycle after birth. Interestingly, female transgenic mice carrying the same constructs evaluated in males showed little or no *LacZ* expression in the ovaries, with the exception of lines having ectopic expression (Table 1). This suggested that sequences needed for *Dmrt1* silencing are located within the evaluated promoter constructs, that sequences needed for ovarian activation are outside the tested promoter regions, or both. The existence of resident silencing elements initially was assessed by in silico sequence analysis to identify potential binding sites for transcriptional repressors within the

FIG. 4. FOXL2 represses *Dmrt1* promoter activity via the distal regulatory region. Depicted is the promoter sequence encompassing the distal regulatory region from -3.2 kb to -2.8 kb of rat *Dmrt1* (A). Arrows mark sequences matching the consensus binding site for FOXL2, and their direction indicates the putative element's orientation [28–30]. Transient transfection analysis of primary rat Sertoli cells was used to evaluate activities of the 3.2-kb [black bars, *Dmrt1*(-3280/+75)*Luc*] and 2.8-kb [gray bars, *Dmrt1*(-2800/+75)*Luc*] rat *Dmrt1* promoters in the presence of increasing amounts of FOXL2 expression vector (B). Graphed is the ratio of firefly luciferase to *Renilla* luciferase (RL-TK control) activity, for the indicated promoter and dose of transfected *Foxl2* vector, relative to the same ratio (firefly/*Renilla*) for pGL3-control transfected with the same amount of *Foxl2* vector. Transfections were done a minimum of three times, and error bars represent the SEM (\**P* = 0.0605, by Student *t*-test: two samples assuming unequal variances).

*Dmrt1* promoter. This revealed four candidate regulatory elements, between -3.2 kb and -2.8 kb, for FOXL2 [binding motif (G/A)(T/C)(C/A)AA(C/T)A], a member of the forkhead transcription factor family implicated in *Dmrt1* silencing by virtue of its granulosa cell expression profile, which opposes that of *Dmrt1*, and previous studies that showed *Foxl2* deletion led to induction of *Dmrt1* in the mouse ovary [24–30] (Fig. 4A). Based on these findings, we hypothesized that *Dmrt1* silencing in granulosa cells occurs via direct interactions between FOXL2 and the *Dmrt1* promoter. To test this possibility, primary cultures of Sertoli cells were transfected with a luciferase reporter containing 3.2 kb of rat *Dmrt1* promoter sequence [*Dmrt1*(-3.2kb/+75)-*Luc*] and different amounts of expression vector for *Foxl2*. The results showed that increasing the amount of cotransfected *Foxl2* caused a corresponding decrease in *Dmrt1* promoter activity (Fig. 4B, black bars). Importantly, the silencing effect of FOXL2 was



lost on removal of sequences containing the identified candidate binding sites (i.e., between  $-3.2$  kb and  $-2.8$  kb), as attested by the unmodified activity of the 2.8-kb promoter with increased FOXL2 expression (Fig. 4B, gray bars). Thus, promoter silencing depended on the amount of expressed FOXL2 and *Dmrt1* promoter sequences between  $-3.2$  kb and  $-2.8$  kb.

## DISCUSSION

Expression profiles for *Dmrt1* have documented its strict specificity for germ cells and somatic cells of embryonic gonads and its male-specific expression in postnatal testes [2, 3, 10, 13, 15–17]. Before gonad differentiation and sex determination, DMRT1 is expressed at similar levels in the somatic and germ cell populations of male and female gonads, but on differentiation, cell-specific, sexually distinct DMRT1 profiles emerge, each at a different stage of development [13]. In males, DMRT1 is maintained in the somatic (Sertoli) cells throughout development and postnatal life, whereas in females, it is lost from somatic (pregranulosa) cells at the time of sex determination [2, 3, 10, 13, 15–17, 31–33]. In addition, DMRT1 is expressed in germ cells of developing male and female gonads but subsequently extinguishes at times corresponding to either meiotic entry (XX germ cells) or mitotic arrest (XY germ cells) [13]. After birth, germ cell expression only returns in males and is coincident with their resumption of mitosis [13, 15–17]. Overall, the data suggest that differences in *Dmrt1* expression result from distinct transcriptional mechanisms in germ cells and Sertoli cells that depend on both specificity and developmental status of the expressing cells [13, 16, 17]. In somatic cells, differential expression results from male-specific maintenance of *Dmrt1* initiated just after sex determination, whereas in germ cells, differences result from male-specific activation of *Dmrt1* instituted at birth with resumption of mitosis [13, 16, 17].

By assessing in vivo the transcriptional role of *Dmrt1*'s 5' flanking sequence, the current study identified promoter regions that support different expression patterns, suggesting they are responsible for the differential transcriptional control implicated by DMRT1's expression profile and cell-specific functions. The results indicated distinct regulatory regions, located between  $-3.2$  kb and  $-2.8$  kb and between  $-1.3$  kb and  $-400$  bp, in directing *Dmrt1* transcription to Sertoli cells and germ cells, respectively. Thus, histochemical analysis confirmed expression of the 9- and 3.2-kb transgenes in Sertoli cells and germ cells, and RT-PCR analysis showed *Dmrt1* flanking sequences between  $-3.2$  kb and  $-2.8$  kb helped to restrict expression to the testis and supported somatic cell expression, because without this region, transgene expression was dependent on the presence of viable germ cells [2, 11, 13, 15–19, 34]. Interestingly, transient transfection analysis previously showed that sequences within this region were required for Sertoli cell-specific expression of *Dmrt1* and identified several critical response elements, as well as their associated transcription factors, that regulated *Dmrt1* promoter activity in primary Sertoli cells [18]. Two transcription regulatory proteins previously identified for their role in male sex determination, GATA4 and its coregulator, ZFPM2, also were implicated in *Dmrt1* regulation by studies that examined its expression in *Zfp2* mutant mice [18, 20].

FOXL2 is a winged-helix/forkhead transcription factor that exhibits female-specific gonad expression and reproductive function [27, 35]. Notably, its expression profile and in vivo function strongly support the predicted role in *Dmrt1* silencing. Thus, during development, FOXL2 is absent from indifferent

gonads and, on differentiation, is induced only in somatic cells of the ovary. Here, FOXL2 levels rise sharply between 11.5 and 13.5 dpc, while at the same time, DMRT1 levels decline, nearly reaching extinction by 13.5 dpc [13, 26, 27, 35, 36]. FOXL2 is maintained in the ovary and functions to establish and maintain the somatic cell lineage. With FOXL2 deficiency, female-to-male sex reversal is observed and marked by the continued presence of fetal sex cords, loss of female somatic cell markers, and induced expression of male-specific genes, including *Dmrt1* [24, 25, 37]. In *Foxl2*<sup>-/-</sup> mice containing a second mutation in *Wnt4*, which also functions to establish female identity in ovarian somatic cells, the sex reversal phenotype is more pronounced and shows earlier induction of the male-specific genes [25].

In addition to revealing a somatic regulatory region (i.e., distal regulatory region), the current study also implicated a region ( $-1.3$  kb and  $-400$  bp) in directing *Dmrt1* to testicular germ cells. Thus, except for one nonexpressing 1.3-kb line, all transgenes containing sequences between  $-2.8$  kb and  $-400$  bp (11 of the 12 lines) were expressed in the testis. Notably, a total of seven lines carrying either the 2.8 kb (three lines) or 1.3 kb (four lines) showed testis-specific (three lines) or nearly testis-specific expression (four lines; modest brain expression in three 2.8-kb and one 1.3-kb lines, data not shown), whereas the remaining lines showed expression in testes plus other tissues. In contrast, when the  $-1.3$  kb/ $-400$  bp region was removed by further deletion, transgene expression was determined largely by the site of integration, not by the *Dmrt1* promoter, because expression was either silent or without preference for the testis over other tissues. The data strongly implicate *Dmrt1* promoter elements located between  $-1.3$  kb and  $-400$  bp in directing expression to the testis. Furthermore, cellular expression revealed the region as one directing germ cell expression, because testis activity of both the 2.8- and 1.3-kb promoters was restricted largely to the germ cells (Fig. 3). Importantly, germ cell specificity likely results from the interplay between this region and those upstream (i.e.,  $>2.8$  kb) that help to prevent erroneous transcription induced by the surrounding chromatin.

Findings from the current study also are supported, in part, by a previous report on *Dmrt1* promoter activity in transgenic mice, which showed testis-specific expression directed by porcine *DMRT1* 5' flanking sequence [38]. In that study, a 2.6-kb promoter was used to direct transgenes with either green fluorescent protein or Cre recombinase and demonstrated expression in embryonic gonads of both sexes and adult testes. In the testis, transgene activity was found in both somatic cells and germ cells [38]. The study only characterized a single promoter, but it is consistent with results from the present study that delineated important, testis-specific regulatory sequences in the 5' proximal region of *Dmrt1*. However, the two studies conflict with respect to somatic cell regulation. The present study, which used the rat promoter, indicated that such sequences reside upstream of 2.8kb, whereas the earlier studies indicated that such sequences are located within 2.6 kb of the pig promoter. However, comparison of *Dmrt1* promoter sequences between various species revealed insertions and deletions that altered the relative location of several conserved sequences [38]. Likewise, sequence alignment of the rat and pig promoters identified similarities between the rat somatic regulatory region and sequences within the 2.6-kb pig promoter (data not shown). Thus, the apparent difference in promoter function may be explained by positional changes in elements required for somatic cell expression that placed them nearer the transcriptional start site in porcine *DMRT1*.

In summary, the current study identified two distinct regulatory regions in the *Dmrt1* promoter that help to direct

it to somatic cells and germ cells of the testis. The somatic regulatory region was localized to sequences between  $-3.2$  kb and  $-2.8$  kb and a germ cell regulatory region to sequences between  $-1.3$  kb and  $-400$  bp. It also revealed a role for FOXL2 in silencing *Dmrt1* transcription through the somatic regulatory region. From these studies, as well as a previous one identifying GATA4 and ZFP221 in *Dmrt1* activation via the somatic regulatory region, the following model emerges: Both GATA4 and ZFP221 activate *Dmrt1* in XX and XY somatic cells of indifferent gonads via GATA4 elements in the somatic regulatory region. On differentiation, FOXL2 is induced in the ovary, where it silences GATA4 activity (and, consequently, *Dmrt1* transcription) when bound to sequences near the GATA4 elements. In the testis, FOXL2 is absent, and DMRT1 persists. However, substantiation of the model requires demonstration that FOXL2 binds and silences GATA4 activity of the promoter, which forms the basis of future studies on *Dmrt1* transcription, together with those to unravel the elements and transcription factors that act through the germ cell regulatory region.

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