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GATA Factors and Androgen Receptor Collaborate To Transcriptionally Activate the *Rhox5* Homeobox Gene in Sertoli Cells▽†

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How Sertoli-specific expression is initiated is poorly understood. Here, we address this issue using the proximal promoter (*Pp*) from the *Rhox5* homeobox gene. Its Sertoli cell-specific expression is achieved, in part, through a negative regulatory element that inhibits *Pp* transcription in non-Sertoli cell lines. Complementing this negative regulation is positive regulation conferred by four androgen-response elements (AREs) that interact with the androgen receptor (AR), a nuclear hormone receptor expressed at high levels in Sertoli cells. A third control mechanism is provided by a consensus GATA-binding site that is crucial for *Pp* transcription both in vitro and in vivo. Several lines of evidence suggested that GATA factors and AR act cooperatively to activate *Pp* transcription: (i) the GATA-binding site crucial for *Pp* transcription is in close proximity to two of the AREs, (ii) GATA and AR form a complex with the *Pp* in vitro, (iii) overexpression of GATA factors rescued expression from mutant *Pp* constructs harboring defective AREs, and (iv) incubation of a Sertoli cell line with testosterone triggered corecruitment of AR and GATA4 to the *Pp*. Collectively, our results suggest that the *Rhox5* gene achieves Sertoli cell-specific transcription using a combinatorial strategy involving negative and cooperative positive regulation.

The mechanisms by which cell-type-specific expression is achieved in higher organisms remains poorly understood despite many years of intensive investigation. In this study, we report the identification of a set of transcription factors and regulatory elements that together drive cell-type-specific transcription in the adult Sertoli cell. This postmitotic somatic cell, which is in intimate contact with developing germ cells in seminiferous tubules in the testis, is crucial for all phases of male gametogenesis, including germ cell proliferation, meiosis, and differentiation (15). Genes necessary for the Sertoli cell to support male gametogenesis have been identified, and some progress has been made in identifying regulatory mechanisms that control the transcription of some of these genes, including follicle-stimulating hormone receptor (*Fshr*), cathepsin L, the inhibin-β B subunit, transferrin, Mullerian inhibitory substance, androgen-binding protein, Dnmrt 1, GATA1, GATA4, and tissue plasminogen activator (12, 13, 15, 17, 18, 21, 35, 41, 43, 53). Regulatory regions and specific *cis* elements that participate in the transcriptional regulation of these genes have been identified in primary Sertoli cells and Sertoli cell lines. Analysis of these *cis* elements has led to the identification of transcription factors that act through them to regulate transcription in Sertoli cells, including TATA box-binding factor, SP1, EGR1, CREB, SRY, E2F, SF-1, the basic helix-loop-helix factor E12, GATA1, and GATA4 (21, 22). While these studies have identified transcription factors contributing to expression in Sertoli cells, it remains to be determined what sets of transcription factors together confer Sertoli cell-specific expression.

Many studies have been conducted to attempt to identify regulatory regions responsible for adult Sertoli cell transcription in vivo. Using various lengths of 5′ upstream regions ranging from 0.5 kb to more than 10 kb, investigators have assessed whether these sequences are sufficient to confer Sertoli cell-specific transcription in transgenic mice. Most of the studies have failed to identify regulatory regions sufficient to drive expression specifically in adult Sertoli cells in the testis in vivo. Some studies have reported that the transgene is not expressed in Sertoli cells, presumably due to missing regulatory element(s) (23). Other studies have reported misexpression of the transgene in cell types that do not express the endogenous gene (26). In other cases, the transgene did not exhibit a normal developmental expression pattern in embryonic and postnatal testes (63) or adult testes (43). In the rare cases in which a region was identified that conferred normal Sertoli cell expression, the regions defined were typically quite long (3 to 6 kb), complicating the identification of the specific *cis* elements that contribute to regulation (5).

Here, we report specific *cis* elements in the *Pem* (*Rhox5*) gene that confer Sertoli cell-specific expression in vitro and in vivo. Rhox5 is the founding member of the *r*eproductive *ho*meobox on the *X* chromosome (*Rhox*) gene cluster that we recently identified (24, 39, 58). The *Rhox* gene cluster resides on the X chromosome and contains more than 30 genes in mice, making it the largest homeobox gene cluster known in any species (29, 40, 44, 65). The *Rhox* homeobox genes are

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selectively expressed in male and female reproductive tissues, and thus they are likely to encode a set of transcription factors devoted to promoting and regulating gametogenesis (44). In support of this, we recently showed that mutational inactivation of *Rhox5* causes aberrant germ cell apoptosis in stages V to XI of the seminiferous epithelial cycle, increased germ-cell apoptosis in stages I to IV and XII of the cycle, decreased sperm cell count, impaired sperm motility, and subfertility in mice (39). These aberrations are not the result of a germ cell defect but, rather, a Sertoli cell defect, as the *Rhox5* gene and RHOX5 protein are expressed specifically in Sertoli cells within the postnatal and adult testis (38, 51, 59). This non-cell-autonomous function probably results from the ability of RHOX5 to regulate the transcription of genes in Sertoli cells that encode cell surface and/or secreted molecules dictating the behavior of the adjacent germ cells.

RHOX5 is expressed from both a distal promoter (*Pd*) and a proximal promoter (*Pp*). The *Pd* is transcribed in the embryo, where it is imprinted and regulated by DNA methylation (11, 33, 47). The *Pd* is also transcribed in trophoblast cells in the placenta, granulosa cells in the ovary, and a wide variety of tumors from diverse cell lineages and tissues (36, 55). The subject of this paper is the *Pp*, a cell-type-specific promoter that we previously demonstrated is expressed exclusively in Sertoli cells and caput epididymal cells in both mice and rats (38, 51, 59). Its expression in both of these cell types depends on testosterone and the nuclear hormone receptor androgen receptor (AR) (10, 25, 37, 38, 51, 59). In Sertoli cells, the *Pp* is dramatically induced between postnatal day 8 (P8) and P9, the time point when adjacent germ cells enter the preleptotene stage of meiosis (37). In adult mice, *Rhox5* is expressed specifically in Sertoli cells in stages IV through VIII of the seminiferous epithelial cycle (38, 51, 59). Using transgenic mice, we identified a relatively small (~0.6 kb) region upstream of the mouse *Rhox5 Pp* transcription start site sufficient to confer its normal temporal, androgen-dependent, and stage- and cell-type-specific expression pattern in vivo (51). Deletion analysis revealed that a transgene retaining only ~0.3 kb of the *Pp* 5′ flanking region retained both its cell-type-specific and androgen-dependent expression pattern, implying that all the elements necessary for these two properties are housed within this region (51).

In this paper, we report the identification of both positive and negative *cis* elements within this ~0.3-kb *Pp* region that drive Sertoli cell-specific transcription. We identify specific factors that bind to the positive elements and stimulate *Pp* transcription. AR is one of these factors; its ability to directly activate *Pp* transcription in Sertoli cells is significant for several reasons. First, very few genes have been identified that are directly regulated by AR in Sertoli cells (41). In fact, to our knowledge, RHOX5 is the only transcription factor known to be transcriptionally induced by AR in Sertoli cells in vitro and in vivo. Second, recent studies in conditional knockout mice have pinpointed the Sertoli cell as a crucial cellular target of AR action. AR signaling in Sertoli cells was shown in these studies to be required for two distinct steps in spermatogenesis: the meiotic progression of germ cells and their maturation into elongated spermatids (41). Since *Rhox5* is an androgen-induced gene expressed specifically in Sertoli cells, this makes it a good candidate to have a role in such AR-dependent steps of spermatogenesis. Third, because *Rhox5* encodes a homeodomain-containing protein, we suggest that it participates in such AR-dependent events by regulating the transcription of secondary androgen response genes. Such genes are not directly regulated by AR or testosterone but instead are controlled by androgen-regulated transcription factors (41). Because very few genes directly regulated by AR have been identified in Sertoli cells, it is possible that most androgen-dependent events in spermatogenesis are mediated by secondary androgen response genes.

We also demonstrate that GATA transcription factors activate the *Pp*. This well-studied class of zinc-transcription factors has a conserved and important role in embryonic development and activates the transcription of several key transcription factor genes during the formation of gonads (62). GATA factors are also expressed in Sertoli cells in male gonads after birth, leading to widely held notion that they serve to drive spermatogenesis (34, 43, 62, 63). Our finding that GATA factors transcriptionally activate *Rhox5* suggests that upregulation of this germ cell survival factor is one means by which GATA factors could promote spermatogenesis.

MATERIALS AND METHODS

Plasmids. We generated the *Rhox5* plasmids for this study from either the Pem-214 (51) or Pem-250 plasmid, as described in Table 1. Pem-250 was made by a two-step cloning procedure. In the first step, *Rhox5 Pp* 5′ flanking sequences (−306 to −1 with respect to the translation start site) were amplified by PCR from the plasmid Pem-121 (51) using the primers MDA-1598 and MDA-1599 (Table 1) and cloned into the pGEM-T Easy vector (Promega Corporation, Madison, WI). The *Rhox5 Pp* 5′ flanking sequences were excised using HindIII and SalI and cloned into the pRL-null vector (Promega Corporation, Madison, WI). The three *Gata* riboprobe template plasmids shown in Table 1 were made by inserting PCR products into the pGEM-T Easy vector (Promega Corporation, Madison, WI). The six GATA expression plasmids used in this study were kind gifts from the following laboratories: mouse GATA1 (plasmid G-609) from Robert Viger (CHUL Research Centre, Canada), GATA2 (G-610) and GATA3 (G-611) from James Douglas Engel (University of Michigan Medical School, MI), GATA4 (G-586) from Robert J. Schwartz (Baylor College of Medicine, TX), and GATA5 (G-612) and GATA6 (G-608) from Michael Parmacek (Hospital of the University of Pennsylvania, PA) and Roger K. Patient (King's College London, United Kingdom). The human AR pcDNA 3.1 plasmid (G541) was kindly provided by Zhengxin Wang (The University of Texas M. D. Anderson Cancer Center, Houston, TX).

Cell culture, transfection, and luciferase assays. The MSC1, 15P1, TM4, NIH 3T3, LNCap, TEPI, and MME cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 50 mg/ml of both penicillin and streptomycin. All cell culture reagents except Fugene 6 were obtained from Invitrogen Technologies (Carlsbad, CA). NIH 3T3, LNCap, TEPI, and MME cells were transfected using Lipofectamine following the manufacturer's instructions. MSC1 cells were transfected using Fugene 6 (Roche Diagnostics, GmbH, Mannheim, Germany) following the manufacturer's instructions. TM4 and 15P1 cells were transfected using Lipofectamine 2000 (Invitrogen Technologies) following the manufacturer's instructions. Cells were plated on 24-well culture dishes and then cotransfected with 100 ng of the *Rhox5*-containing plasmid and 100 ng of either the AR expression plasmid or empty vector. After a 6-h incubation in serum-free DMEM, the medium was replaced with DMEM supplemented with 10% charcoal-stripped bovine serum (HyClone Laboratories, Inc., Logan, UT) containing 10 nM R1881 or vehicle alone (ethanol at a concentration of 0.02%). Total cellular extracts were prepared 48 h after transfection. Luciferase activity was measured using a Promega Dual-Luciferase reporter system (Promega Corporation, Madison, WI). We generated Sertoli cells stably expressing AR by transfecting MSC1 cells with 1 μg of AR expression plasmid using Lipofectamine, isolating cell clones resistant to 700 μg/ml G418, and screening them for expression of *Ar* mRNA. The MR531c cell clone was used for this study; it expresses four times as much *Ar* mRNA as adult mouse testes.

TABLE 1. Primer Sequences used for the study

Construct	Primer direction	Primer sequence	Primer name	Strategy	Parent vector
Pem-250	F	CGACAAGCTTGTAACTGGGCACCCTAAG	MDA-1598	Deletion PCR, subcloning	Pem-124
	R	ACGCGTCGACACCCTGAATAGGATCAATG	MDA-1599		pRL-null
Pem-251	F	CATCACAGATCTCATTCTGTTCCCG	MDA-1614	Deletion PCR	Pem-250
	R	GATTTGCTCACAGGACGTTCCTG	MDA-1615		
Pem-252	F	CCACAGGAACGTCCTGTGAGCAATC	MDA-1651	Deletion PCR	Pem-250
	R	GATGTAATGAGACGATGTGCTTGCAAG	MDA-1652		
Pem-262	F	CAGAACTTAGGGTGCCCAGTTAC	MDA-1820	Deletion PCR	Pem-250
	R	GCAAGCACATCGTGCTCATTACATC	MDA-1821		
Pem-267	F	CATCCCCAAACTGCTCACACTTGTGTACCCCAAAG	MDA-1858	Site-directed mutagenesis	Pem-250
	R	CTTTGGGGTACACAAGTGTGAGCAGTTTGGG GATG	MDA-1859		
Pem-278	F	CCCATGAACTGTGTCCACTTTGCAAGCACATC	MDA-1998	Site-directed mutagenesis	Pem-250
	R	CGATGTGCTTGCAAAGTGGACACAGTTCATGGG	MDA-1999		
Pem-279	F	CATCTTGCAAGCACACTGTGCTCATTACATCCCC	MDA-2000	Site-directed mutagenesis	Pem-250
	R	GGGGATGTAATGAGCACAGTGTGCTTGCAAGATG	MDA-2001		
Pem-281	F	CATCTTGCAAGCACACTGTGCTCATTACATCCCC	MDA-2000	Site-directed mutagenesis	Pem-278
	R	GGGGATGTAATGAGCACAGTGTGCTTGCAAGATG	MDA-2001		
Pem-282	F	CTTGCAAGCACATCGTTCTAATTACATCCCCAAAC	MDA-2014	Site-directed mutagenesis	Pem-250
	R	GTTTGGGGATGTAATTAGAACGATGTGCTTG CAAG	MDA-2015		
Pem-290	F	CATCCCCAAACTGCTCACACTTGTGTACCCCAAAG	MDA-1858	Site-directed mutagenesis	Pem-281
	R	CTTTGGGGTACACAAGTGTGAGCAGTTTGGG GATG	MDA-1859		
Pem-270	F	CATCCCCAAACTGCTCACACTTGTGTACCCCAAAG	MDA-1858	Site-directed mutagenesis	Pem-214
	R	CTTTGGGGTACACAAGTGTGAGCAGTTTGGG GATG	MDA-1859		
G-740a	F	AAGGCCGGGAGTGTGTCAAC	MDA-2084	PCR amplified	GATA2
	R	CCATTCATCTTGTGGTAGAGCCC	MDA-2378		
G-770	F	CACAAGATGAACGGCATCAACC	MDA-2088	PCR amplified	GATA4
	R	ATGTAGAGGCCGCAGGCATT	MDA-2089		

Transgenic mice. A 4.6-kb fragment containing the mutated *Rhox5* gene in Pem-270 (Table 1) was excised using the restriction enzymes EcoRV and Not1. This fragment was gel purified and injected into the male pronuclei of C57BL/6 mouse embryos by the M. D. Anderson Cancer Center transgenic mouse core laboratory. Positive transgenic mice were detected by PCR using tail DNA as a template and primers specific for the bovine growth hormone (bGH) 3′ untranslated region (UTR; between nucleotide [nt] 3840 and nt 4182 with respect to the *Rhox5* translation start site in Pem-270). Six founder lines containing the Pem-270 transgene DNA were obtained (Pem-270.1 to Pem-270.6).

RNA isolation and analysis. Total tissue RNA was isolated by lysis in guanidinium thiocyanate buffer and centrifugation over a 5.7 M CsCl cushion as described previously (9). RNase protection analysis was performed as described previously (37) using the following probes. The *Rhox5* transgene-specific riboprobe is 311 nt long and contains 61 nt of *Rhox5* exon 6 and 250 nt of the bGH 3′ UTR; it was transcribed from Pem-121 digested with NdeI (66). The *Gata2* riboprobe (containing the 5′ end of exon 4, nt 1 to nt 109) was prepared by linearizing plasmid G-740a with NdeI and transcribing with T7 RNA polymerase. The *Gata4* riboprobe (164 nt long; starting at nt 119 of exon 3 and ending at nt 112 of exon 4) was prepared by linearizing plasmid G-770 with NdeI and transcribing with T7 RNA polymerase. The *Gata6* riboprobe (197 nt long; starting at nt 39 of exon 3 and ending at nt 45 of exon 4) was prepared by linearizing plasmid G-771 with NcoI and transcribing with RNA polymerase SP6. The β-actin riboprobe (34 nt of β-actin exon 3) was prepared by linearizing the plasmid G-98 with BanI and transcribing with SP6 RNA polymerase, as previously described (45). For real-time reverse transcriptase (RT)-PCR analysis, cDNAs were generated using an iScript RT kit (Bio-Rad Laboratories Inc., Hercules, CA). Real-time RT-PCR was done using Sybr Green fluorescence and was analyzed using the ΔC_T method (where C_T is the cycle number at which the PCR signal crosses the threshold) that takes primer set efficiencies into consideration (49). Standard curves were derived in order to determine the efficiencies of primer sets corresponding to the different *Gata* transripts. All primer sets had efficiencies of 98% to 102%. Expression data were presented as relative expression above the background signal and normalized to the L19 housekeeping transcript. We were unable to distinguish *Gata2* transcripts from other *Gata* transcripts by Sybr Green quantitative PCR, so we used TaqMan quantitative PCR primers purchased from Applied Biosystems (Foster City, CA) (assay Mm00492300_m1).

EMSA. Ten micrograms of adult testes nuclear extract, prepared as described elsewhere (6), and ^{32}P-labeled double-stranded oligonucleotide (2×10^5 cpm) were incubated for 15 min at 37°C in binding buffer (100 mM HEPES, pH 7.9, 50 mM EDTA, 100 mM dithiothreitol, 10% glycerol, 2 μg of poly(dI-dC) [Amersham Biosciences, Piscataway, NJ]) in a total volume of 20 μl. The reaction was terminated by the addition of 4 μl of 6× DNA loading dye, and the tubes were placed on ice. The samples were loaded on a prerun 5.5% polyacrylamide gel (29:1, acrylamide/bisacrylamide ratio) and electrophoresed at 150 V for 3 h under nondenaturing conditions. The gel was dried and placed next to film for autoradiography. For supershift analysis, nuclear extracts were incubated with 2 μg of polyclonal antiserum specific for AR (Upstate Cell Signaling Solutions, Charlottesville, VA) or GATA transcription factors (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 15 min at room temperature before the addition of binding buffer. Cold competition experiments were performed by incubating unlabeled oligonucleotides (50 to 250 ng) with the nuclear extract and binding buffer for 10 min at room temperature prior to the addition of ^{32}P-labeled oligonucleotide probes. The oligonucleotide sequences of probes used for electrophoretic mobility shift assay (EMSA) are provided in Table S3 in the supplemental material. All oligonucleotides were obtained from Sigma-Genosys, Inc. (Houston, TX).

ChIP analysis. Testis tissue from C57BL/6 mice were isolated and homogenized, and samples were cross-linked and processed for chromatin immunoprecipitation (ChIP) analyses as described elsewhere (67). Antiserum was added to the precleared chromatin and incubated overnight at 4°C. The extracts were incubated with a slurry of protein A or G (for polyclonal or monoclonal antiserum, respectively) bound to agarose and containing salmon sperm (Upstate, Charlottesville, VA) for 3 to 4 h at 4°C and washed following the manufacturer's instructions. Immune complexes were disrupted with 1% sodium dodecyl sulfate and 0.1 M $NaHCO_3$, and the DNA was reverse cross-linked by incubation with 200 mM NaCl at 65°C for 4 h, deproteinated with proteinase K for 1 h, extracted with phenol-chloroform, ethanol precipitated, and resuspended in 30 μl of H_2O. PCR amplification was performed with 3 μl of DNA and a 6-carboxyfluorescein-labeled *Rhox5* TaqMan probe specific for *Rhox5 Pp* transcripts (Applied Biosys-

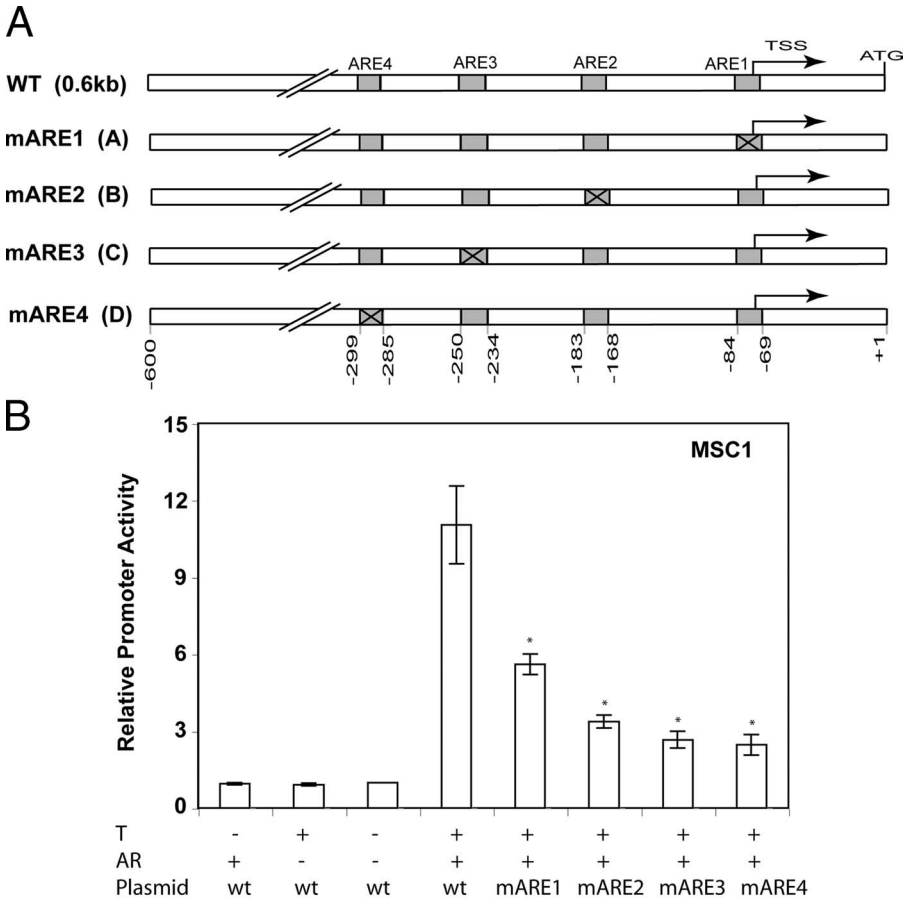


FIG. 1. Identification of four AREs responsible for *Rhox5 Pp* transcription in Sertoli cells. (A) Schematic diagram of a wild-type (WT) *Rhox5 Pp* construct and ARE-mutant derivatives. All constructs harbor 0.6 kb of 5′ flanking sequence and have the *Renilla* luciferase gene downstream (not shown). (B) MSC1 cells were transiently transfected with the constructs shown in panel A (100 ng), an AR expression vector (100 ng), and a simian virus 40 promoter-driven firefly luciferase plasmid PGL3-E-V (50 ng) used as an internal control. The cells were incubated with the synthetic androgen R1881 (T). The level of reporter was measured by normalizing against the internal control. Average values ± standard deviation from three experiments done in triplicate are shown. The asterisk indicates values significantly different from those for the wild-type constructs ($P < 0.05$). TSS, transcription start site; m, mutant.

tems, Foster City, CA) and oligonucleotides MDA-2117 and MDA-2118 (corresponding to nt −76 to −92 and nt −257 to −270, respectively) to specifically amplify the *Rhox5 Pp* region. The magnitude of recruitment was calculated as the percentage enrichment for the factor studied (using a specific antibody) subtracted from percentage of background enrichment (nonspecific antibody negative control). For calculating enrichment, we used the following formula: percent enrichment = $\{[1 \div 2^{(C_T \text{ of specific antibody} - C_T \text{ of input})}] - [1 \div 2^{(C_T \text{ of nonspecific antibody} - C_T \text{ of input})}]\} \times 100$.

Protein analysis. Histological and immunohistochemical analyses were performed as described previously (50). Sections were incubated with rabbit anti-AR polyclonal antiserum (Upstate, Charlottesville, VA) and rabbit anti-GATA2 antibody polyclonal antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:500 and 1:2,000 (vol/vol) dilutions, respectively. Following incubation with the secondary antibody, biotinylated anti-rabbit immunoglobulin G (IgG), the sections were incubated with streptavidin-horseradish peroxidase (Vectastatin ABC kit; Vector Laboratories, Inc., Burlingame, CA) and the substrate 3,3′-diaminobenzidine peroxidase.

Statistical analysis. Statistical analysis was performed using a Student's unpaired *t* test. Probability (*P*) values equal to or less than 0.05 were considered statistically significant.

RESULTS

Identification of AREs essential for *Rhox5 Pp* transcription in Sertoli cells. We previously reported that 0.6 kb of *Rhox5 Pp* 5′ flanking sequence confers expression restricted to the Sertoli cells in the testis and somatic cells in the caput epididymis in transgenic mice in vivo (51, 52). Here, we sought to identify within this 0.6-kb region the *cis* elements that are responsible for engendering Sertoli cell-specific expression. As a first step, we generated a luciferase reporter construct containing this 0.6-kb region and the *Pp* transcription start site [Fig. 1A, WT (0.6b)]. When cotransfected into the MSC1 Sertoli cell line with an AR expression plasmid and incubated with the testosterone analog R1881, this construct expressed ~10 times more luciferase activity than the promoterless control vector pRL-null (Fig. 1B, WT). Cells incubated without R1881 and the AR expression plasmid expressed only low levels of luciferase from the *Pp* reporter vector (only slightly above that of pRL-null), consistent with earlier studies showing that the *Pp* depends on AR and androgen for expression (3, 39, 51). To assess whether AR might directly regulate the *Pp*, we scanned the 0.6-kb *Pp* 5′ flanking region for sequences conforming to consensus AR-binding sites (androgen-response elements, or AREs) (57). We identified four such sites, each of which we mutated (Fig. 1A).

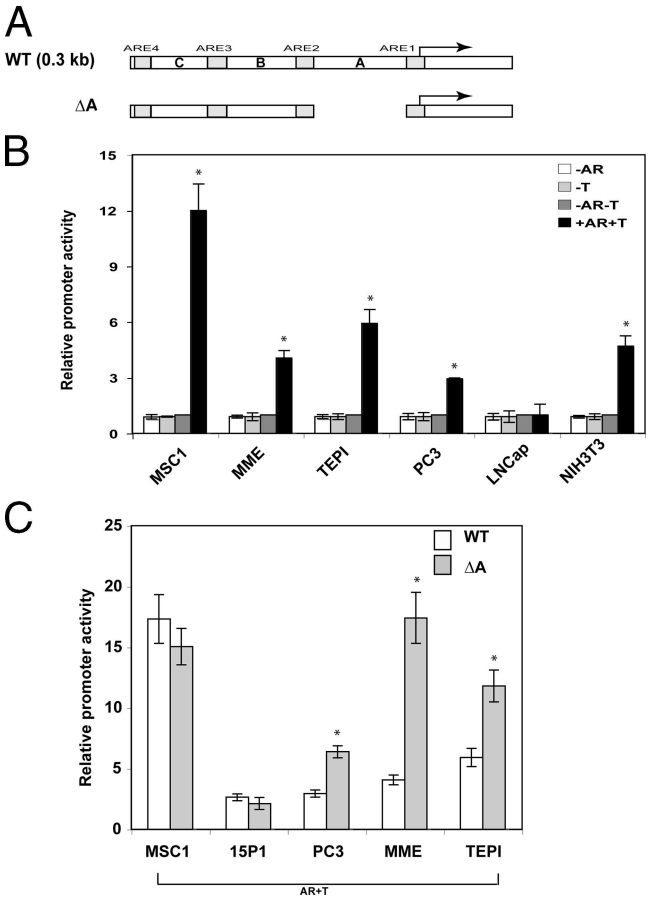


FIG. 2. A silencer region in the *Pp* inhibits expression in non-Sertoli cells. (A) Schematic of a wild-type (WT) construct identical to the wild-type construct described in the legend of Fig. 1A, except that it contains only 0.3 kb of 5′ flanking sequence. The ΔA construct lacks a 72-nt region between ARE1 and ARE2. (B and C) Transient transfection analysis performed as described in the legend of Fig. 1B, with the cell lines and constructs indicated in the presence or absence of the AR expression vector (AR) and R1881 (T). Average values ± standard deviations from three experiments done in triplicate are shown.

Mutation of any one of these sites (ARE1 to ARE4) reduced promoter activity relative to the control construct, indicating that all four sites are required for maximal *Pp* transcription (Fig. 1B). Oligonucleotides corresponding to ARE2 and ARE4 bound to AR, as assessed by EMSA using extracts from adult mouse testes or MSC1 cells transfected with an AR expression plasmid (our unpublished observations); Barbulescue and Handler reported the same for ARE1 and ARE3 (3). We conclude that the *Pp* is directly regulated by AR through at least four AREs. These AREs are likely to contribute to the Sertoli cell-specific expression pattern of the *Pp*, as AR is highly expressed in Sertoli cells (1).

Negative regulation: a transcription start site-proximal region inhibits *Pp* transcription in non-Sertoli cells. Because all four AREs are clustered together in a 0.3-kb promoter-proximal region that we previously showed was sufficient for Sertoli cell-specific expression in vivo (51), we examined a luciferase reporter construct containing only this 0.3-kb region (Fig. 2A, WT). Similar to the 0.6-kb 5′ flanking construct (Fig. 1), the 0.3-kb 5′ flanking construct was expressed at high levels in the MSC1 Sertoli cell line in response to AR and R1881 stimulation (Fig. 2B). In contrast, this construct was relatively poorly inducible in MME mammary epithelial cells, TEPI thymic epithelial cells, PC3 prostate epithelial cells, LNCap prostate epithelial cells, and NIH 3T3 fibroblast cells (Fig. 2B). This suggested the possibility that these other cell types lack positive-acting factors that are required in addition to AR for *Pp* transcription and/or that they express repressor proteins that inhibit *Pp* transcription. In fact, as described below and in the following sections, we obtained evidence that both repressor and activator factors collaborate to confer Sertoli cell-specific transcription to the *Pp*.

To determine what other elements besides AREs drive the Sertoli cell-specific transcription pattern of the *Pp*, we generated deletions in the regions between the four AREs. We first examined the functional role of the 72-nt stretch of DNA between ARE1 and ARE2, shown as region A in Fig. 2A. We found that while deletion of region A (Fig. 2A, ΔA) had little or no effect on *Pp*-driven reporter activity in MSC1 Sertoli cells, it increased reporter activity in PC3 prostate epithelial cells, MME mammary epithelial cells, and TEPI thymic epithelial cells (Fig. 2C, compare ΔA and WT). This suggested that region A is a silencer region that interacts with one or more repressor proteins that are expressed in non-Sertoli cells. This predicts that Sertoli cells lack the expression of the repressor(s), which was supported by the finding that the 15P1 Sertoli cell line, while not expressing high levels of *Pp*, acted like the MSC1 Sertoli cell line in that *Pp* expression was not affected by deletion of region A (Fig. 2C). While we did not study region A in more detail in this report, our data suggest that region A is a negative element that reduces misexpression of the *Pp* in non-Sertoli cells.

Positive regulation: a GATA-binding site critical for *Rhox5 Pp* transcription. We next extended our analysis to the regions between ARE2 and ARE3 (region B) and ARE3 and ARE4 (region C) (Fig. 3A). We found that in contrast to region A, regions B and C (39 and 25 nt, respectively) both had a positive effect on *Pp* transcription: reporter expression was reduced by 67 to 80% when either of these regions was deleted (Fig. 3B). Inspection of region B revealed a putative GATA-binding sequence that we named G1 (Fig. 3A). To determine whether G1 was responsible for the activity of region B, we made a TA to GT mutation in the GATA site to destroy its ability to bind GATA transcription factors. We found that this mutant (construct mG1) had strongly reduced reporter activity compared to the wild-type construct (Fig. 3B). Because reporter activity was reduced about as much by mG1 as by deletion of the entire region B, this GATA site is likely entirely responsible for region B's positive effect on *Pp* transcription.

We also identified a consensus GATA-binding site in region C (Fig. 3A, G3), and so we mutated it, too (construct mG3). mG3 reduced reporter expression, but not as much as mG1 did (Fig. 3B). We also identified a consensus GATA-binding site that overlapped with ARE3 (Fig. 3A, G2). To selectively disrupt its ability to interact with GATA factors, we mutated nucleotides in G2 that disrupt GATA binding but not the ARE consensus sequence (57). We found that this had no significant effect on reporter expression (Fig. 3B). Because transcription was not reduced by the GATA-selective mutation whereas

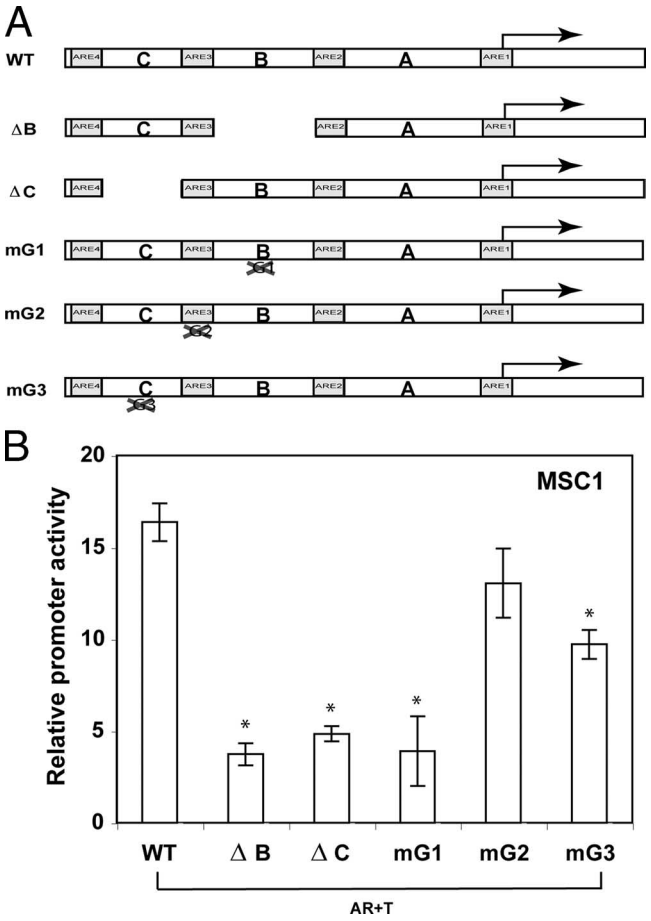


FIG. 3. Identification of GATA sites important for *Pp* transcription. (A) Schematic of the wild-type (WT) construct (also shown in Fig. 2A), deletion mutants lacking region B (39 nt) or region C (25 nt), and site-specific mutants that lacked GATA consensus site G1, G2, or G3. (B) Transient transfection analysis performed as described in the legend of Fig. 1B with the constructs indicated and deletions of regions B and C.

transcription was strongly reduced by a mutation that selectively disrupts the ARE (Fig. 1B, ARE3 mutant), we conclude that G2 is not a functional GATA site but instead a functional ARE.

The 15P1 and TM4 Sertoli cell lines have a defect in *Rhox5 Pp* transcription rescued by expression of GATA factors. In contrast to the MSC1 Sertoli cell line, the 15P1 and TM4 Sertoli cell lines only weakly expressed the *Pp*-driven reporter in response to AR and R1881 (Fig. 2C). We hypothesized that this defect in *Pp* inducibility is the result of lower levels of GATA transcription factors in 15P1 and TM4 cells. To test this prediction, we used real-time RT-PCR analysis to examine GATA transcription factor mRNA levels. Sertoli cells are known to express GATA4 and GATA6 (62), and thus we predicted that one or both of these factors would be lower in 15P1 and TM4 cells than in MSC1 cells. Indeed, we found that *Gata4* and *Gata6* transcripts were expressed at lower levels in 15P1 and TM4 cells than in MSC1 cells (Fig. 4A). The level of *Gata4* mRNA in 15P1 cells and TM4 cells was about 2% of that in MSC1 cells (also confirmed by RNase protection analysis [data not shown]). The level of *Gata6* mRNA in 15P1 was 33% of that in MSC1 cells, and the level in TM4 cells was only 10% of that in MSC1 cells (Fig. 4A). These data are consistent with the possibility that the low expression of one or both of these factors is responsible for limiting *Pp* expression in 15P1 and TM4 cells. We also examined the expression of *Gata1* mRNA, as GATA1 is expressed by Sertoli cells in vivo (62). None of the Sertoli cell lines, including MSC1, had appreciable levels of *Gata1* mRNA (less than 0.033% of the level in adult testis) (Fig. 4a).

We also analyzed the expression of *Gata2*, *Gata3*, and *Gata5* in Sertoli cells, which had not been reported before. We found that *Gata2* and *Gata3* transcripts were expressed in all three Sertoli cell lines at a level similar to that in adult testis (Fig. 4A). *Gata5* was also expressed in the Sertoli cell lines, albeit at very low levels in MSC1 cells.

To directly test whether the defect in *Pp* expression in 15P1 and TM4 Sertoli cells was the result of low GATA expression, we transiently transfected expression plasmids encoding each of the six GATA transcription factors into these cells. None of the GATA transcription factor expression vectors induced *Pp*-driven reporter expression on their own (see Fig. S2b in the supplemental material; also data not shown). In contrast, in the presence of AR and androgen, all six GATA transcription factors upregulated *Pp*-driven reporter expression in 15P1 and TM4 cells, indicating that all six GATA transcription factors were capable of activating *Pp* transcription if AR and testosterone were also present (Fig. 4B). In contrast, none of the GATA expression plasmids significantly upregulated *Pp*-driven reporter expression in MSC1 cells. Because MSC1 cells constitutively express high levels of some GATA transcription factors (Fig. 4A), we interpret this result as indicating that these cells constitutively express optimal or saturating levels of GATA factors; further increases would thus have no effect on *Pp* transcription (Fig. 4B).

To determine whether GATA-dependent *Pp* transcription is mediated through the crucial GATA site that we defined by mutagenesis (Fig. 3B, site G1), we examined whether the G1 mutant was impervious to the effect of forced GATA factor expression. For this transfection experiment, we used TM4 cells as they express only low endogenous levels of GATA factors. Unlike the wild-type construct, the G1 mutant did not respond to the GATA expression plasmids by significantly upregulating *Pp*-driven reporter expression (Fig. 4C). This result is strong evidence that GATA transcription factors directly regulate *Pp* transcription through the G1 GATA-binding site.

A GATA site crucial for *Rhox5 Pp* expression in vivo. To determine whether the G1 GATA site has a role in *Pp* transcription in vivo, we performed transgenic mouse studies. We previously showed that a construct containing 363 nt of *Rhox5 Pp* 5′ flanking region was sufficient to drive Sertoli cell-specific expression in transgenic mice (51). We mutated TA to GT in the G1 GATA site in this construct (Fig. 5A), rendering this site incapable of binding to GATA factors. Six transgenic mouse lines containing the mG1 construct were obtained. As shown in Fig. 5B, we found that the transgenic lines harboring the mutant construct expressed significantly lower levels of the transgene than the transgenic lines expressing the wild-type construct, as assayed by RNase protection analysis. Average

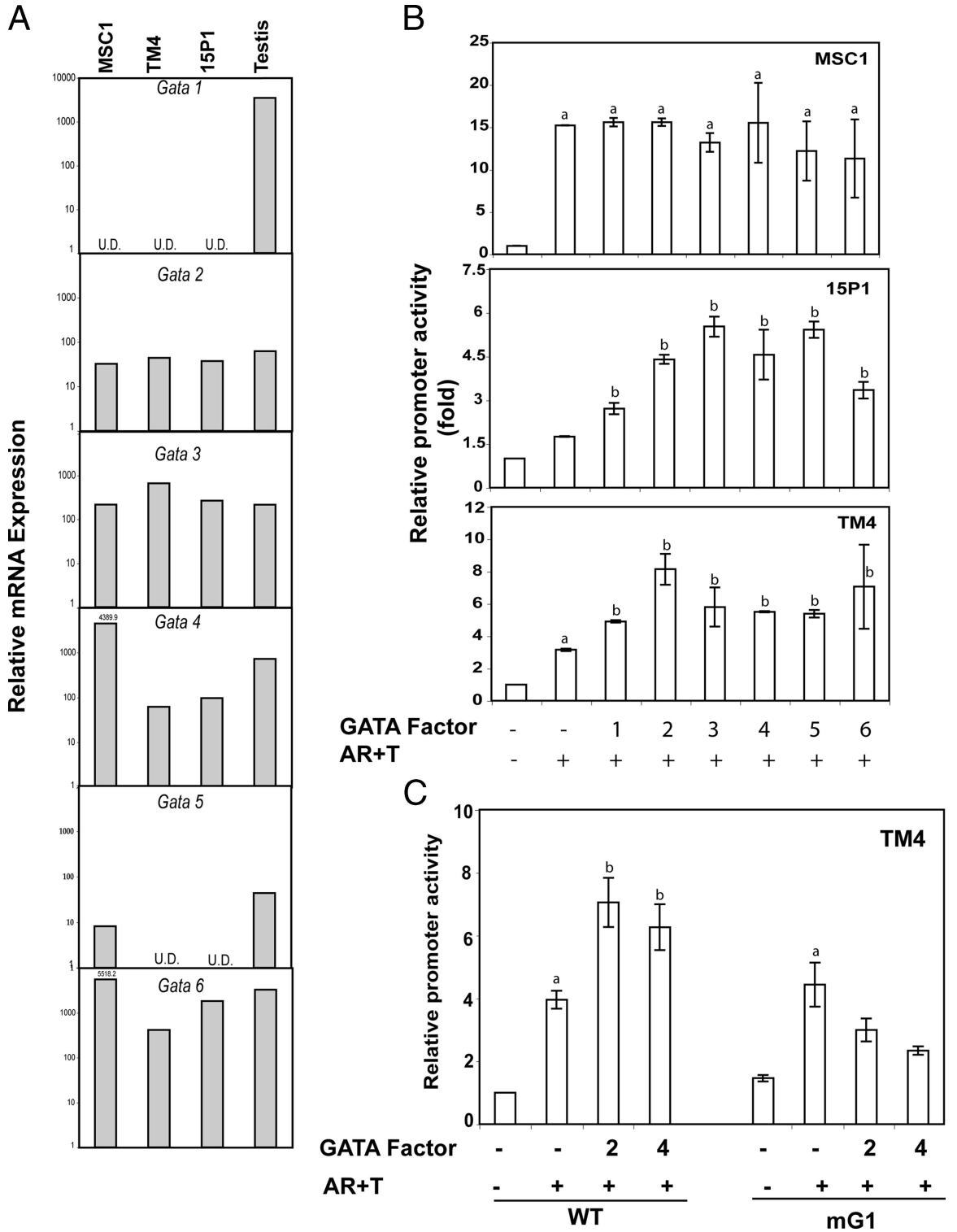


FIG. 4. Expression pattern of *Gata* transcripts and their functional activity in Sertoli cells. (A) Real-time RT-PCR analysis of *Gata* transcript levels. Values are from duplicate PCRs run on three independent samples that were normalized to the level of *L19* mRNA, which encodes a ribosomal protein. Primer efficiency was taken into account to calculate relative gene expression levels. The standard deviation was less than 10% in each case. (B and C) Transient transfection analysis performed as described in the legend of Fig. 1B with the WT construct shown in Fig. 2A in the presence or absence of the GATA expression plasmids indicated (cells not incubated with a GATA expression plasmid were transfected with the empty expression vector). The cells were incubated with or without the AR expression vector (AR) and R1881 (T) as indicated. Average values ± standard deviations from three experiments done in triplicate are shown. Statistical significance is shown as follows: a indicates cells with values significantly different from those of untreated cells; b indicates cells with values significantly different from those of cells that have both AR and T ($P < 0.05$). U.D., undetectable.

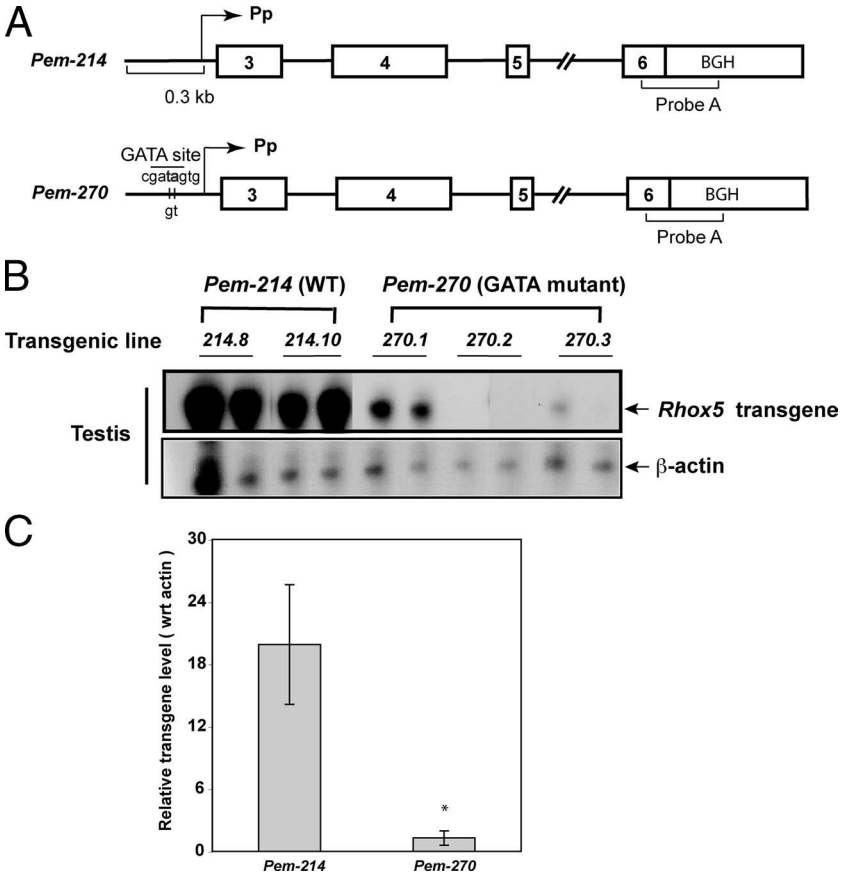
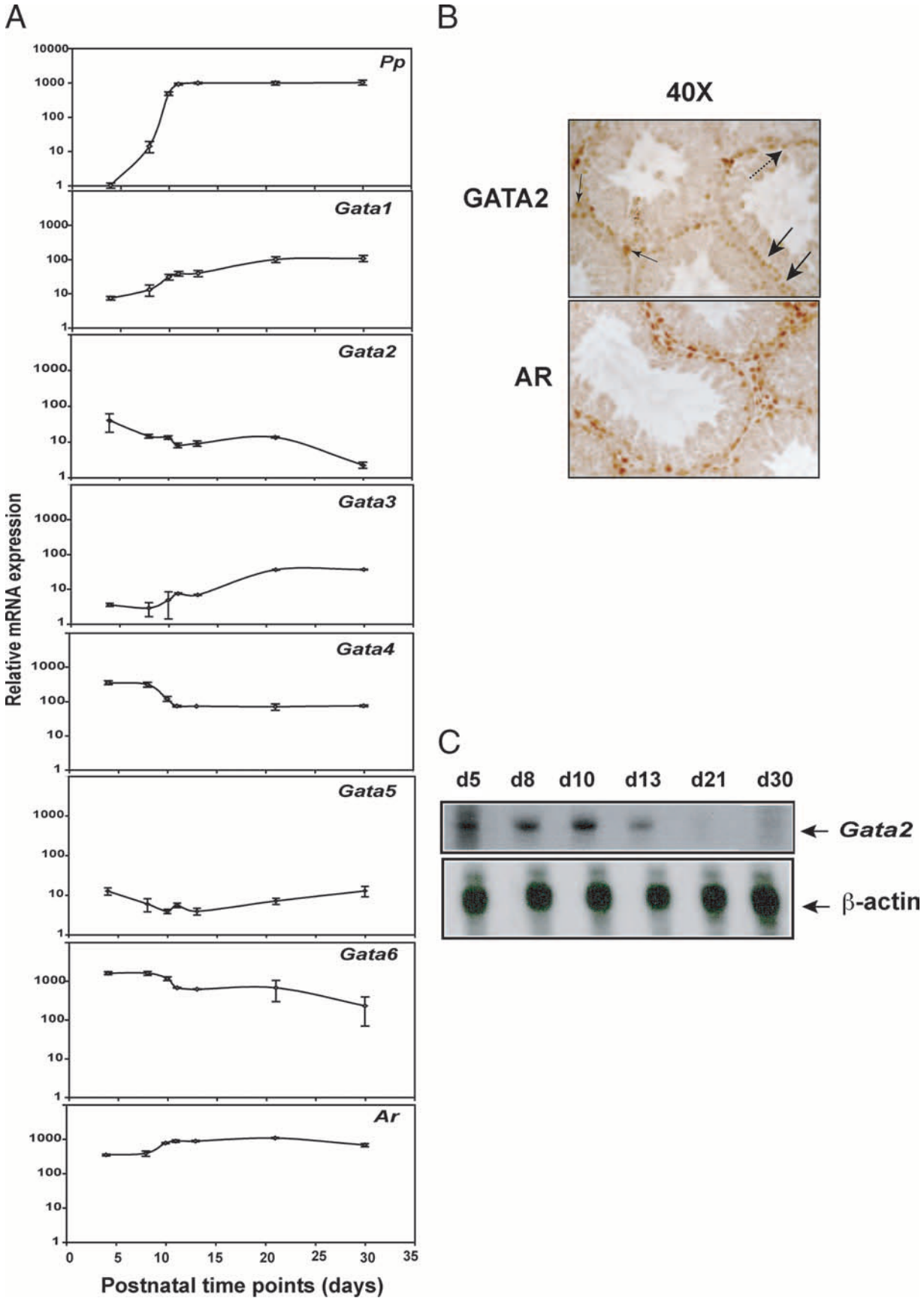


FIG. 5. The G1 GATA site is crucial for *Pp* transcription in vivo. (A) Schematic of the wild-type *Pp* transgene (Pem-214) previously described (51) and a derivative harboring the 2-nt mutation indicated (Pem-270). (B) RNase protection analysis of testis total cellular RNA (10 μg) from adult transgenic mice containing either the Pem-214 or Pem-270 transgene. The transgene-specific probe (probe A) contains bGH 3′ UTR sequences. A band of the expected size (~200 nt) was protected by testis RNA; this band was not protected by tRNA (data not shown). A β-actin probe was included in all assays as a loading control (the protected band was ~35 nt). (C) Transgene expression from six Pem-270 and two Pem-214 transgenic lines (average values ± standard deviation). WT, wild type.

transgene expression levels from mG1 and wild-type transgenic mice are shown in Fig. 5C.

Developmental and cell-type-specific expression patterns of GATA and AR transcription factors in the testis. Our transgenic analysis described above indicated that the G1 GATA site was crucial for *Pp* transcription in vivo, but it did not reveal which particular GATA transcription factors were responsible for activating *Pp* transcription in vivo. Because we found that all GATA transcription factors were capable of activating *Pp* transcription (Fig. 4B), we reasoned that the particular GATA factors responsible for promoting *Pp* transcription depend, at least in part, on which GATA factors are available in the testis during the developmental stages when the *Pp* is expressed. To test this possibility, we determined the postnatal expression patterns of the *Pp* and all six GATA factors using real-time PCR analysis (Fig. 6A) and/or RNase protection analysis (Fig. 6C). This analysis demonstrated that the *Pp* was first induced on P8, that it was further upregulated between P9 and P12, and that it then remained expressed into adulthood (Fig. 6A). *Gata1*, *Gata2*, *Gata3*, *Gata4*, *Gata5*, and *Gata6* transcripts were all expressed on P8, indicating that all six of the encoded GATA transcription factors were potentially available in the testis to first activate *Pp* transcription in the postnatal testis. As postnatal development proceeded, the level of some *Gata* transcripts precipitously decreased, while the level of other *Gata* transcripts rose (Fig. 6A; note that this figure is plotted on a log scale). This suggests that some GATA factors contributed more in generating the first wave of *Pp* transcripts, while other GATA factors drive the expression of *Pp* transcripts at later developmental time points (see Discussion for more details). Because AR is required for *Pp* expression, we also analyzed its developmental expression pattern. We found that the *Ar* transcript level increased as testis development proceeded (Fig. 6A). The upregulation of *Ar* mRNA between P8 and P10 mirrored the upregulation of *Pp* between these times (albeit less markedly), suggesting that AR may have a role in this upregulation.

Because, to our knowledge, the expression of GATA2, GATA3, and GATA5 in testes had not been previously described, we examined whether they were expressed in Sertoli cells. Real-time RT-PCR analysis of enriched Sertoli cells (39) showed that only *Gata2* mRNA was significantly expressed (data not shown). *Gata2* transcripts were present in both the Sertoli and interstitial cell fractions, even when they were subjected to hypotonic shock to remove residual germ cells (see Fig. S2a in the supplemental material). Evidence that this


A
Pp
Gata1
Gata2
Gata3
Gata4
Gata5
Gata6
Ar
Relative mRNA expression
Postnatal time points (days)
B
40X
GATA2
AR
C
d5
d8
d10
d13
d21
d30
Gata2
β-actin

treatment successfully removed germ cells was the finding that the Sertoli cell-specific marker, *Gata1*, was increased in level by ~2.5-fold after hypotonic treatment of the Sertoli cell fraction, and the Leydig cell marker, leuteinizing hormone mRNA, was increased in level by ~4-fold after hypotonic treatment of the interstitial cell fraction (data not shown). Because the *Gata2* mRNA signal was not increased in hypotonic-shocked Sertoli cells, this implied that it is also present in germ cells. To verify these results we turned to immunohistochemical analysis with an anti-GATA2 antiserum. This detected a strong signal in Sertoli cell nuclei (Fig. 6B, thick arrows). In addition, GATA2 antibody staining was observed in Leydig cells (Fig. 6B, thin arrows) and spermatogonia (Fig. 6B, arrows with dotted line). No staining was seen in any of these cell types when slides were stained with only the secondary antibody (data not shown). Only some seminiferous tubules (including those in stages VII and VIII of the seminiferous epithelial cycle) had GATA2-positive cells, indicating that GATA2 is expressed in a stage-specific manner. Together, the results from both the mRNA and protein analyses indicate that GATA2 is present in Sertoli, Leydig, and germ cells.

Androgen-induced recruitment of AR and specific GATA factors to the *Pp*. To determine which GATA factors are recruited to the *Pp* in testes, we performed ChIP analysis. In particular, we examined the recruitment of GATA1, GATA2, GATA4, and GATA6 as these are the GATA factors expressed in Sertoli cells in the testis (2, 46, 68) (Fig. 6B). ChIP analysis showed that all but GATA6 were detectably bound to the *Pp* in the adult testis (Fig. 7A). The observation that GATA2 was recruited to the *Pp* confirms that this GATA factor is present in Sertoli cells in vivo (Fig. 6B). We also found that AR was recruited to the *Pp* in the testis (Fig. 7A), a finding consistent with the fact that *Pp* transcription is AR dependent (Fig. 1B).

To determine how GATA factors and AR are recruited to the *Pp*, we performed ChIP analysis on an MSC1 Sertoli cell clone stably expressing AR from an expression plasmid (MR531c; note that unlike parental MSC1 cells, these cells express *Gata1* mRNA) (our unpublished observation). We found that MR531c cells had GATA1, GATA2, and GATA4 but no GATA6 detectably bound to the *Pp* (Fig. 7B and data not shown). Upon treatment with the androgen analogue R1881, AR and an increased amount of GATA4 were recruited to the *Pp*. The corecruitment of GATA4 and AR in testes suggested the possibility that these two factors form a protein complex, an idea supported by the EMSAs described below.

Evidence for AR and GATA cooperativity. Our observation that GATA factors and AR factors are corecruited to the *Pp* (Fig. 7A), where they have adjacent binding sites (Fig. 3A), suggests the possibility that GATA factors and AR physically interact to activate *Pp* transcription. To test this possibility, we incubated a ^{32}P-labeled DNA probe containing the G1 GATA site and ARE2 with testes nuclear extracts (Fig. 8A). EMSA detected a band whose formation was specifically blocked by unlabeled competitor oligonucleotide corresponding to the

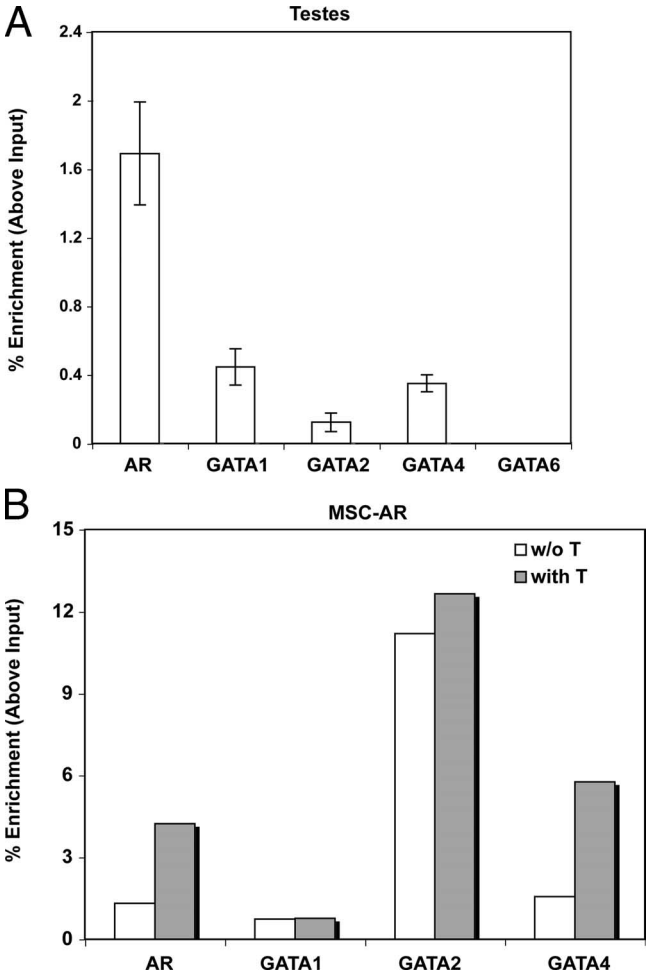


FIG. 7. GATA factor and AR recruitment to the *Pp* in adult testes and MSC1 Sertoli cells. (A) ChIP analysis of adult mouse testes using antiserum against the indicated factors and the corresponding negative control antiserum. The values shown are mean values obtained by real-time PCR (± standard error) from at least three pooled testis samples run in triplicate from three different experiments. (B) ChIP analysis of MR531c, an MSC1 cell clone stably expressing AR from an expression vector, in the presence or absence of R1881 (T). The analysis was performed as described in panel A. Representative data are shown from at least three experiments done with each antiserum.

FIG. 6. The postnatal expression pattern of *Pp*, *Gata*, and *Ar* transcripts and the expression of GATA2 protein in testes. (A) Real-time PCR analysis of total cellular RNA from postnatal testes at the time points indicated, performed as described in the legend of Fig. 4. Average values ± standard deviations are shown. (B) Immunohistochemical analysis of adult mouse testes. The upper panel shows a section incubated with a rabbit polyclonal anti-GATA2 antiserum followed by an anti-rabbit IgG antiserum conjugated to horseradish peroxidase (magnification, ×40). The large arrow points to a GATA2-positive Sertoli cell nuclei, the thin arrows point to GATA2-positive Leydig cells, and the dotted-line arrow points to a spermatogonia. The lower panel shows testis sections incubated with an anti-AR antiserum that specifically stains Sertoli, myoid cells, and Leydig cell nuclei (magnification, ×40). (C) RNase protection analysis performed as described in the legend of Fig. 5C, with a *Gata2* probe, a β-actin internal control probe, and the postnatal testis samples indicated. d, day.

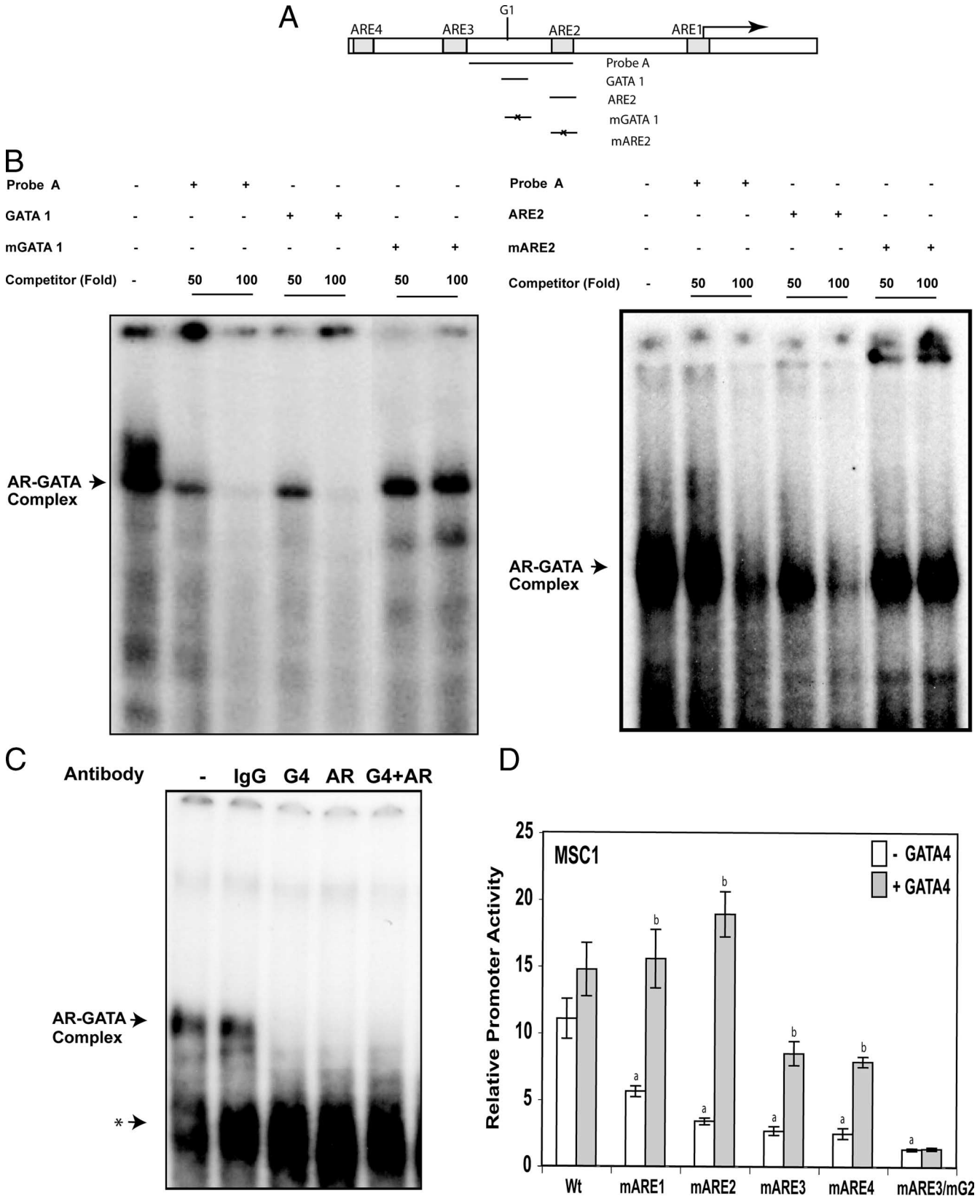


FIG. 8. Evidence for AR and GATA cooperativity. (A) Schematic of the *Rhox5 Pp*, showing the location of the EMSA probes and the G1 GATA site and AREs required for *Pp* transcription. (B and C) EMSA of testis nuclear protein extracts incubated with ^{32}P-labeled probe A, the indicated unlabeled competitors, and/or the indicated antiserum. The asterisk indicates a nonspecific band whose formation varied between different experiments and was not depleted upon addition of GATA or AR antiserum. While we do not know why this nonspecific band increased

probe itself or oligonucleotides that contain either the GATA or ARE2 sites alone (for sequences of these oligonucleotides, see Table S3 in the supplemental material). In contrast, a GATA mutant oligonucleotide or an ARE2 mutant oligonucleotide did not significantly affect complex formation (Fig. 8B and C). Together, these data suggested that this complex contained both GATA and AR factors. As further evidence for this, preincubation with antibodies against GATA4 or AR inhibited the formation of this complex (Fig. 8C).

The discovery that GATA4 and AR form a complex in vitro suggested the possibility that they cooperate to activate *Pp* transcription. This predicted that ablation of the binding site for one factor could be compensated for by overexpression of the other factor, as the remaining binding site would allow recruitment of both factors by virtue of the fact that they interact. To test this prediction, we determined whether overexpression of GATA4 would rescue the transcription of mutant *Pp* constructs harboring mutations that destroy each one of the four AREs (Fig. 1A). In agreement with the prediction, we found that transfection of a GATA4 expression plasmid increased *Pp*-driven reporter activity from each of the four ARE mutants (Fig. 8D). As a negative control, we tested a construct harboring mutations that destroy not only the ARE3 site but also the overlapping GATA G2 site (mARE3/mG2). GATA4 overexpression failed to increase *Pp*-driven reporter expression from this double-mutant construct (Fig. 8D).

GATA elements and AREs cooperate to regulate the *PSA* gene promoter. We determined whether the human prostate specific antigen (*PSA*) promoter was a candidate for coregulation by GATA and AR, as it is a well-studied androgen-induced gene promoter (27). Indeed, it has several GATA consensus sites in its enhancer (see Fig. S3 in the supplemental material), and, like the *Pp*, these GATA sites often closely flank the AREs. In agreement with past studies (48), we found that AR and R1881 dramatically upregulated reporter activity from the *PSA* gene promoter when in the context of its enhancer (see Fig. S3 in the supplemental material). Transfection of the GATA4 and GATA5 expression vectors further increased reporter activity, indicating that GATA4 and GATA5 work together with AR to activate *PSA* transcription (Fig. S3 in the supplemental material). In contrast, the other four GATA factors did not have this property, demonstrating specificity. These data indicate that, like the androgen-inducible *Pp*, the androgen-inducible *PSA* promoter depends on GATA factors for maximal transcription.

DISCUSSION

While, in theory, transcription restricted to Sertoli cells could be conferred by a single transcription factor expressed exclusively in Sertoli cells, to our knowledge no such transcription factor has been identified. An alternative means to achieve Sertoli cell-specific transcription is for several transcription factors expressed in Sertoli cells to collaborate. In this strategy, none of the transcription factors need to be exclusively expressed in Sertoli cells to achieve restricted expression. Another layer of specificity is achieved by having a negative regulator that reduces or prevents transcription in non-Sertoli cells. In this paper, we provide evidence that this combinatorial mechanism is responsible for the selective expression of the *Rhox5 Pp* in Sertoli cells.

One set of factors that participate in the *Pp*'s Sertoli cell-specific expression pattern are GATA transcription factors. These are highly conserved zinc finger-containing proteins that are found across the phylogenetic scale where they have conserved roles regulating cell differentiation, organ morphogenesis, and tissue-specific gene expression (32, 34, 61, 62, 68). In the reproductive system, GATA4 is essential for mouse gonad development and male sex determination (34, 62). While the precise mechanism by which GATA4 drives the indifferent gonad down the male pathway remains to be determined, various GATA4-regulated genes have been identified that are likely to have a role, including the male-determining gene *Sry* and the male-inhibitory gene *Mis* (60). GATA transcription factors are also expressed in testes after embryonic development, but their role there remains uncertain. Interestingly, most GATA transcription factors expressed in the testis appear to be present only in Sertoli cells, not germ cells, suggesting that their role in spermatogenesis is to regulate the transcription of genes in Sertoli cells that indirectly regulate germ cell events (30, 61, 68). Our discovery that the *Rhox5* homeobox gene is regulated by GATA transcription factors in Sertoli cells provides a plausible mechanism by which GATA transcription factors could promote spermatogenesis. By activating *Rhox5* transcription in Sertoli cells, we propose that GATA transcription factors promote germ cell survival. This follows from our previous studies in *Rhox5*-null mice showing that *Rhox5* expression in Sertoli cells is necessary for the survival of a subset of the adjacent germ cells (39).

Four GATA transcription factors are expressed in Sertoli cells and thus are candidates to activate *Pp* transcription (30, 31, 61, 68) (Fig. 6B). We provide several lines of evidence that GATA4 activates *Pp* transcription. First, we found high levels of *Gata4* mRNA in both postnatal and adult testes (Fig. 6A), and others have shown that GATA4 protein is present in Sertoli cell nuclei (4, 30). Second, we found a positive correlation between *Gata4* and *Pp* mRNA expression in different Sertoli cell lines (Fig. 4A). Third, transfection of a GATA4 expression vector increased *Pp*-driven reporter expression in Sertoli cell lines expressing low levels of GATA4 (Fig. 4B). Fourth, ChIP analysis showed that GATA4 is recruited to the *Pp* in adult testes and MSC1 Sertoli cells, both of which highly express *Gata4* mRNA (Fig. 7A and B). We speculate that GATA4 has a role in both the initial expression of the *Pp* in the

in intensity when GATA or AR antiserum was added, we suspect it is because more labeled probe was released from specific complexes, allowing more to bind to the nonspecific complexes. (D) Transient transfection analysis performed as described in the legend of Fig. 1B, with the constructs shown in Fig. 1A (an additional mutant, mARE3/mG2, contains both the ARE3 mutation and a G2 GATA mutation). The cells were cotransfected with a GATA4 expression plasmid or the corresponding empty expression vector. All cells were also cotransfected with the AR expression vector (Fig. 1) and R1881. Average values ± standard deviations from three experiments done in triplicate are shown.

testes during early postnatal development and its tissue-specific expression in the testis in adult mice. The former is supported by the finding that *Gata4* mRNA levels are at their highest during early postnatal testicular development (Fig. 6A), and the latter is supported by the fact that *Gata4* mRNA levels are much higher in adult testes than other adult tissues that we examined (see Fig. S1b in the supplemental material). In contrast to GATA4, the related GATA factor, GATA6, was not detected by ChIP at the *Pp* in either adult testes or MSC1 Sertoli cells (Fig. 7A and B), suggesting that GATA6 does not contribute to driving *Pp* transcription in Sertoli cells. *Gata6* mRNA and GATA6 protein are abundant in Sertoli cells (Fig. 4A and 6A) (30), suggesting that GATA6 is sequestered from the *Pp*, perhaps by a repressor protein. However, we cannot rule out the possibility that low levels of GATA6 insufficient to be detected by ChIP analysis have a role in regulating *Pp* transcription, as we found that forced GATA6 expression was capable of upregulating *Pp*-driven reporter expression (Fig. 4B).

Another GATA factor that may contribute to activating *Pp* transcription is GATA1. Like GATA4 and GATA6, GATA1 upregulated *Pp*-driven reporter expression in Sertoli cell lines (Fig. 4B). Like GATA4, GATA1 was detected by ChIP at the *Pp* in adult testes and the MSC1 Sertoli cell line (Fig. 7A and B). However, unlike *Gata4* and *Gata6* mRNA, *Gata1* mRNA was upregulated at the same postnatal day as *Pp* transcripts were induced (Fig. 6A). This suggests that GATA1 upregulation has a role in the induction of *Pp* transcription during early testes postnatal development. In agreement with this idea, Yomogida et al. showed that GATA1 protein is first detectable in Sertoli cell nuclei at P7 (68), approximately when *Rhox5* mRNA is first expressed (Fig. 6A). GATA1 may also be important in maintaining the tissue-specific expression pattern of *Pp* in testes at later postnatal times and in the adult, as *Gata1* mRNA levels increase during postnatal development (Fig. 6A); moreover, GATA1 protein and *Gata1* mRNA are expressed at high levels in adult Sertoli cells but not any other cell type in adult mice except for erythrocytes (see Fig. S1a in the supplemental material) (68).

The final GATA factor that we suggest may contribute to *Pp* transcription is GATA2. While GATA2 was not previously known to be expressed in Sertoli cells, we demonstrate here that GATA2 protein is present in Sertoli cell nuclei in adult testes (Fig. 6B) and that *Gata2* transcripts are expressed in three Sertoli cell lines (Fig. 4A) and purified Sertoli cells from the adult testis (see Fig. S2a in the supplemental material). Moreover, ChIP analysis showed that GATA2 protein is bound to the *Pp* in adult testes and the MSC1 Sertoli cell line (Fig. 7). Finally, transfection analysis showed that forced GATA2 expression increased *Pp* reporter expression (Fig. 4B). Like *Gata4* and *Gata6* mRNA, *Gata2* mRNA is expressed at the highest level early in postnatal development (Fig. 6A) (32, 61), suggesting that GATA2's main role is to promote *Pp* transcription early during development. In addition, our immunohistochemical analysis indicated that GATA2 is present in the nuclei of germ cells, mainly spermatogonia (Fig. 6B). Its expression in germ cells was confirmed by our analysis of highly enriched testicular germ cell fractions obtained using a recently developed fractionation procedure (69). Real-time RT-PCR analysis of these fractions indicated that *Gata2* transcripts were expressed in spermatogonia but not elongated spermatids (our unpublished observations). To our knowledge, this is the first evidence that any GATA factor is expressed in germ cells. This opens up the possibility that GATA2 may function directly in germ cells rather than only through its ability to regulate transcription in Sertoli cells.

We obtained several lines of evidence that GATA factors collaborate with AR to activate *Pp* transcription. First, the GATA site crucial for *Pp* transcription is in close proximity (<25 nt) to two AREs essential for maximal *Pp* transcription (Fig. 1A and 3A). Second, GATA factors and AR form a complex with the *Pp* in vitro (Fig. 8B). Third, depletion of either GATA site-binding factors or ARE-binding factors ablates formation of this complex (Fig. 8B and C). Fourth, increased expression of GATA factors rescued expression from mutant *Pp* constructs harboring defective AREs (Fig. 8D). Fifth, *Ar* and *Gata1* mRNA levels rise concomitantly with *Pp* transcripts during postnatal development (Fig. 6A), consistent with the idea that these factors rise above a threshold level to drive the expression of the *Pp*. Finally, testosterone treatment elicited corecruitment of AR and GATA4 to the *Pp* in a Sertoli cell clone stably transfected with AR (Fig. 7B). Together, these data suggest, but do not prove, that GATA factors and AR act cooperatively to activate *Pp* transcription.

A regulatory scenario analogous to that occurring at the *Rhox5 Pp* may occur at the mouse mammary tumor virus (MMTV) promoter. Archer and colleagues showed that glucocorticoid receptor (GR) cooperates with nuclear factor 1 (NF1) to activate MMTV transcription via close binding sites that act synergistically to activate MMTV transcription (19). Surprisingly, despite their cooperative behavior, Archer and colleagues could not detect an interaction between GR and NF1 by coimmunoprecipitation analysis. Thus, the affinity between these two transcription factors may be insufficient for them to significantly interact in the absence of a DNA target harboring binding sites for them. Alternatively, GR and/or NF1 may interact with a wide variety of other factors in the cell, leaving only a small proportion to interact with each other. By analogy, we have not been able to detect an interaction between AR and GATA4 in MCF-7 and LNCaP cells by coimmunoprecipitation analysis (data not shown).

AR and GATA factors probably collaborate to regulate the transcription of other genes besides *Rhox5*. One likely target is another androgen-inducible gene, *PSA*. The *PSA* enhancer harbors well-studied AREs responsible for its induction in response to testosterone (27), but it also has a large number of GATA-consensus sites (see Fig. S3 in the supplemental material). Evidence for the importance of these GATA sites was our finding that the *PSA* enhancer maximally activates a heterologous promoter only when both AR and GATA transcription factors are expressed (see Fig. S3 in the supplemental material). While we did not determine whether the GATA factors act directly on the *PSA* enhancer, it seems likely that they do, given the abundance of GATA sites in the *PSA* enhancer, their close juxtaposition with AREs (see Fig. S3 in the supplemental material), and a previous study demonstrating that the *PSA* enhancer binds to GATA factors in vitro (48). Further support for the generality of a collaboration between AR and GATA comes from a genome-wide study that found that the presence of consensus AREs near consensus GATA sites was a strong predictor that the AREs actually bound AR

(42). Yet further support comes from a recent genome-wide study demonstrating that GATA consensus sites are significantly enriched within AR-binding regions in the human genome (64). ChIP analysis of a prostate cell line demonstrated that GATA2 was bound to many of the AR-binding regions and that the amount bound increased in response to androgen. They ascribed this property to their finding that AR interacts with GATA2 in a hormone-dependent manner.

Given that AR and GATA transcription factors are likely to collaborate to drive transcription in cell types in addition to Sertoli cells, how could they contribute to Sertoli cell-specific expression? We suggest that the particular GATA factors that are recruited to the *Pp* may help restrict its expression to Sertoli cells. The *PSA* gene, which is expressed in the prostate, may attract a different combination of GATA factors that help drive its expression in this organ. In the case of the *Pp*, negative regulatory control conferred by DNA methylation (47; also our unpublished observations) and the negative regulatory region that we defined between ARE1 and ARE2 (Fig. 2C) are very likely to contribute to its Sertoli cell-specific expression.

It is axiomatic that genes exhibiting selective expression in Sertoli cells will use different strategies to achieve this goal. Using several approaches, including transient transfection experiments and protein-DNA binding assays, several groups have dissected a large number of Sertoli cell promoters to address this question (16, 21, 28, 43, 54). While various difficulties have been encountered, including misexpression in inappropriate tissues or developmental stages (see introduction) or regulatory elements so prohibitively far from the promoter that they are not even present in a large ~400-kb bacterial artificial chromosome (23), there has been some progress. For example, 5′ flanking regions from the *Gata4*, *inhibin*-α, and cathepsin L gene promoters that confer Sertoli cell expression in vivo have been defined (5, 26, 43). In the case of the cathepsin L promoter, a 3-kb region has been identified that provides Sertoli cell-specific transcription (5). In vitro studies have defined GC-box motifs and/or E boxes in minimal promoter regions from the cathepsin L, *Fshr*, and *Gata4* genes that are critical for promoter activity (14, 20–22). E-boxes are also found in combination with CRE (cyclic AMP response element)-like elements in many other Sertoli cell-expressed genes, including transferrin, *Abp*, *Mis*, *Fshr*, *inhibin*-α, and *Ar* (7, 8). Thus, GC-box-binding proteins, basic helix-loop-helix proteins, and CRE-binding proteins are all likely to collaborate to drive the transcription of many genes in Sertoli cells.

Rhox5 may employ a strategy involving AREs to drive Sertoli cell-specific expression because it encodes a transcription factor that serves to regulate androgen-dependent events occurring during spermatogenesis. While it has long been known that spermatogenesis depends on AR and androgen, the molecular basis for its dependence on these factors remains largely unknown (15, 41). *Rhox5* is one of very few genes known to be directly regulated by AR in Sertoli cells and, to our knowledge, is the only known androgen-induced transcription factor gene expressed in Sertoli cells (41). Interestingly, its *Pp* harbors "selective" AREs that respond to AR and not other nuclear hormone receptors, based on studies in cell lines (3) and a knock-in mouse that expresses a form of AR only able to activate the transcription of promoters harboring "nonselective" AREs (56). Recently, we discovered that a subset of other *Rhox* genes is induced by androgen and AR in the MSC1 cell line (39); most of these genes also depend on AR in Sertoli cells for their expression in testes in vivo (our unpublished observations). Together, these androgen-regulated *Rhox* genes are good candidates to encode a set of transcription factors that control the expression of secondary androgen-response genes (i.e., genes not directly regulated by AR) important for spermatogenesis. It remains for future studies to test this hypothesis and to examine precisely how AR collaborates with other factors to restrict the expression of *Rhox* genes to Sertoli cells.

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