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Transcription factor GATA-4 is expressed in a sexually dimorphic pattern during mouse gonadal development and is a potent activator of the Müllerian inhibiting substance promoter

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SUMMARY

Mammalian gonadal development and sexual differentiation are complex processes that require the coordinated expression of a specific set of genes in a strict spatiotemporal manner. Although some of these genes have been identified, the molecular pathways, including transcription factors, that are critical for the early events of lineage commitment and sexual dimorphism, remain poorly understood. GATA-4, a member of the GATA family of transcription factors, is present in the gonads and may be a regulator of gonadal gene expression. We have analyzed the ontogeny of gonadal GATA-4 expression by immunohistochemistry. GATA-4 protein was detected as early as embryonic day 11.5 in the primitive gonads of both XX and XY mouse embryos. In both sexes, GATA-4 specifically marked the developing somatic cell lineages (Sertoli in testis and granulosa in ovary) but not primordial germ cells. Interestingly, abundant GATA-4 expression was maintained in Sertoli cells throughout embryonic development but was markedly down-regulated shortly after the histological differentiation of the ovary on embryonic day 13.5. This pattern of expression suggested that GATA-4 might be involved in early gonadal development and possibly sexual dimorphism. Consistent with this hypothesis, we found that the Müllerian inhibiting substance promoter which harbors a conserved GATA element is a downstream target for GATA-4. Thus, transcription factor GATA-4 may be a new factor in the cascade of regulators that control gonadal development and sex differentiation in mammals.

Key words: Testis, Ovary, Gene expression, Sexual differentiation, Mouse

INTRODUCTION

In mammals, sexual differentiation is characterized by three sequential events: the establishment of genetic sex at fertilization, gonadal development and differentiation, and finally the development of the proper sexual phenotype. In both sexes, early gonadal development is characterized by the migration of extraembryonically derived primordial germ cells into the surface epithelium and underlying mesenchyme of the mesonephros and the appearance of the sexually indifferent gonad or genital ridge. Several genes are now known to have definitive roles in gonadal development and sex differentiation; they include steroidogenic factor1 (*SF1*; Luo et al., 1994), the testis-determining gene (*SRY*; Koopman et al., 1991), Wilms' tumor antigen (*WT1*; Kreidberg et al., 1993), and Müllerian inhibiting substance (*MIS*; Matzuk et al., 1995; Mishina et al., 1996). To date, the earliest known marker of gonadal development is the orphan nuclear receptor *SF-1* whose transcripts first appear in the urogenital ridge of day 9.0 mouse embryos (Ikeda et al., 1994); targeted disruption of the *SF-1* gene results in complete gonadal and adrenal agenesis indicating the essential role of this factor for gonadal development in both sexes (Luo et al., 1994). In genotypic XY males, the indifferent gonad is directed away from ovarian development and towards testicular differentiation through the action of the testis-determining gene, *SRY*, that is present on the Y chromosome (Gubbay et al., 1990; Sinclair et al., 1990). In the mouse, fetal *Sry* expression is limited to the period of sex differentiation (E10.5-E12.5) and is thought to act solely in the supporting cell lineage (Palmer and Burgoyne, 1991), triggering them to differentiate into Sertoli cells and organize into testicular cords (Koopman et al., 1990; Palmer and Burgoyne, 1991). Testicular cord formation is then believed to induce the remaining gonadal cell types to follow the male differentiation pathway (Byskov and Hoyer, 1994). Thus, while it is clear that SF-1 and SRY are vital for testicular

development, many other transcription factors are likely required at different stages of gonadal development and/or differentiation either as regulators or downstream targets of these two factors. Indeed, two transcription factors, SOX9 and DAX-1, have been proposed as candidate targets for SRY (Ikeda et al., 1996; Morais da Silva et al., 1996) while the only known gonadal targets of SF-1 are *MIS* and the P-450 steroid hydroxylases (Haqq et al., 1994; Shen et al., 1994; Giuili et al., 1997). The actions of SF-1 and/or SRY on these putative targets may be direct, or indirectly mediated through other intermediary factors. For example, SF-1 has been shown to directly regulate the *MIS* gene both in vitro and in vivo through a conserved upstream regulatory element (Shen et al., 1994; Giuili et al., 1997). In contrast, the binding of SRY to the *MIS* promoter (Haqq et al., 1993) is not sufficient for *MIS* gene activation despite the fact that SRY can induce substantial *MIS* expression when transiently transfected in a genital ridge-derived cell line, raising the possibility that *MIS* transcription may be regulated by SRY-induced factors (Haqq et al., 1994).

Another class of transcription factors that are expressed in the gonads are members of the GATA family of zinc finger proteins. These proteins, which bind the consensus sequence WGATAR in the 5′-flanking region of target genes, have received considerable attention recently due to their importance in cell differentiation and organ development (Simon, 1995; Weiss and Orkin, 1995). GATA regulatory motifs were first identified in the promoters of globin and other erythroid-specific genes which led to the cloning of the prototypic GATA-1 transcription factor about ten years ago (Evans et al., 1988; Tsai et al., 1989). Six vertebrate GATA factors (GATA-1 to GATA-6) have been identified so far and they can be divided into two subgroups based on sequence homology and tissue distribution: the hematopoietic (GATA 1-3) and the cardiac (GATA 4-6) groups. Although GATA factors have similar DNA-binding properties, they exhibit within each group, distinct spatial and developmental expression patterns and play essential, non-redundant functions (Simon, 1995; Weiss and Orkin, 1995). Three GATA factors have been reported to be expressed in the gonads: GATA-1, GATA-4, and GATA-6 (Arceci et al., 1993; Ito et al., 1993; Tamura et al., 1993; Grépin et al., 1994; Yomogida et al., 1994; Heikinheimo et al., 1997). Targeted mutagenesis and antisense RNA approaches have shown that GATA-1 and GATA-4 play essential roles in erythroid cell differentiation (Pevny et al., 1991; Simon et al., 1992) and cardiac morphogenesis (Grépin et al., 1995; Kuo et al., 1997; Molkentin et al., 1997), respectively. Unfortunately, the gonadal roles of these factors were not addressed and at present, no downstream gonadal target genes have been identified.

In the testis, GATA-1 is specifically expressed in prepubertal and some adult Sertoli cells where its expression may be negatively regulated by one or more paracrine factors produced by germ cells (Yomogida et al., 1994). The *GATA-4* gene was also shown to be transcribed in adult testis where its likely site of expression was suggested to be germ cells (Arceci et al., 1993). As a first step towards elucidating the role of GATA-4 in gonadal development and function, we have analyzed the pattern of GATA4 expression during embryonic and postnatal gonadal development in the mouse. The data show that GATA-4 is abundantly expressed in somatic cells of the mouse gonad as early as the genital ridge stage of E11.5 embryos and thus, marks the beginning of gonadal formation in both sexes. Thereafter, GATA-4 exhibits a marked sexual dimorphism since its expression becomes highly restricted to Sertoli cells of the developing testis. Consistent with a role in sexual differentiation, GATA-4 was found to potently activate the *MIS* promoter through a proximal, species-conserved GATA element. Taken together, these data suggest that GATA-4 may be a key factor in the molecular cascade controlling mammalian gonadal development and sex differentiation.

MATERIALS AND METHODS

Animals

All animals were purchased from Charles River Canada (St. Constant, QC).

Isolation of immature Sertoli cells

Highly enriched populations of immature Sertoli cells were prepared from 6- and 12-day-old Sprague-Dawley rat testes using standard procedures as outlined by Tung and Fritz (1994). Final Sertoli cell aggregates were resuspended in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS); approximately 10×10^6 cells were seeded in 90 mm plastic dishes and cultured at 32°C under 5% CO_2. Cell medium was changed every 24 hours to remove contaminating germ cells and Sertoli cells were finally harvested after a 3-day culture period.

Plasmids

The −180 base pair *MIS* promoter (−180 to +51) was amplified by polymerase chain reaction (PCR) from mouse genomic DNA (forward primer: 5′-TAGGATCCGTTATGGGCCCAGCTCTGA-3′; reverse primer: 5′-AGCAGTACCAGTGGAGAGAGGT-3′) and cloned into the *Bam*HI/*Sma*I sites of the luciferase reporter vector pXP1 (Argentin et al., 1994). The identity of the *MIS* promoter was confirmed by sequencing. Deletion and mutation constructs were generated by PCR and cloned into pXP1 as described above. The −83 bp GATA mutant construct was obtained by substituting a single nucleotide (G**A**TA→G**G**TA) in the forward oligonucleotide primer that was used to generate the wild type −83 bp construct. Expression vectors for mouse GATA-1, human GATA-2, and human GATA-3 were kindly provided by B. Emerson, J. Adams, and P.-H. Roméo, respectively. Expression vectors for rat GATA-4, GATA-5, and GATA-6 were prepared by subcloning their respective coding regions into the cytomegalovirus-driven pCG expression vector as previously described (Grépin et al., 1994; Durocher et al., 1997).

Cell culture and transfections

Monkey kidney fibroblast (CV-1) and murine fibroblast L cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Transfections were done 24 hours after plating using the calcium phosphate precipitation method (Chen and Okayama, 1987). Cells were harvested 36 hours after transfection and luciferase activity was assayed using an EG&G berthold Autolumat LB 953 luminometer as previously described (Argentin et al., 1994). In all experiments, 3 μg of reporter construct was used in addition to 1 μg pRSV-hGH as an internal control to monitor transfection efficiency, and the total amount of DNA was kept constant at 10 μg per dish.

RNA isolation and northern blot analysis

Male Sprague Dawley rats at 1, 3, 7, 14, 21, 35, 42, 49, 63, and 91 postnatal days of age were obtained from Charles River Canada. Testes were removed and total cellular RNA was prepared by the single-step acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). For Northern blot analyses, 20 μg

of total cellular RNA were separated by agarose-formaldehyde gel electrophoresis and transferred to Hybond-N nylon membranes (Amersham Canada, Oakville, ON) as previously described (Viger and Robaire, 1991). Blot hybridization and washing conditions were as previously described (Viger and Robaire, 1991). The probes used were full-length cDNAs for mouse GATA-1 (Tsai et al., 1989) and rat GATA-4 (Grépin et al., 1994). RNA loading was verified by reprobing all Northern blots with an 18S ribosomal RNA-specific ^{32}P-labeled oligonucleotide (ACGGTATCTGATCGTCTTCGAACC) using standard protocols (Viger and Robaire, 1991).

Immunohistochemistry

Timed pregnant CD-1 mice were obtained from Charles River Canada. Noon of the day on which a vaginal plug was found was considered as E0.5 and the day of delivery as day 1. Pregnant mothers were killed by cervical dislocation and the embryos were dissected free of the uterine horns. Gonads from E11.5-E13.5 embryos were removed and directly immersed into Ste. Marie's fixative (95% ethanol/glacial acetic acid, 99:1) for 1 hour. At E11.5, male versus female indifferent gonads were distinguished by determining the genetic sex of the embryos using reverse transcriptase PCR and primers specific for the *Zfy1* and *Zfy2* genes which are present on the Y chromosome (Nagamine et al., 1989). Gonads from E14.5-E18.5 embryos and postnatal animals were perfused through the left ventricle, first with saline and then Ste. Marie's fixative. Gonads were removed and then placed into the same fixative for 1 hour. Fixed tissues were embedded in paraffin and 5 μm serial sections were cut and mounted on 0.5% gelatin coated glass slides. GATA-1 and GATA-4 were immunolocalized using the horseradish peroxidase method or by immunofluorescence. In brief, tissue sections were blocked for 30 minutes with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.2. Sections were subsequently incubated with αGATA-1 (1:100 dilution of N6 antibody, Santa Cruz Biotechnology, Santa Cruz, CA) or αGATA-4 (1:500 dilution, Santa Cruz) overnight at 4ºC. After washing in PBS containing 0.2% Tween-20, sections were blocked with 5% BSA, incubated with either biotinylated αgoat (GATA-4) or αrat (GATA-1) IgGs (Vector Laboratories, Burlingham, CA) for 1 hour at room temperature (RT), and finally reacted with an avidin D-biotinylated horseradish peroxidase complex or avidin D-FITC (Vector) for 1 hour at RT. Diaminobenzidine (DAB) was used as substrate for the peroxidase reaction. Sections were counterstained with 0.1% methylene blue for immunoperoxidase and 0.5 μg/ml propidium iodide (Molecular Probes, Eugene, OR) for immunofluorescence. Either preimmune serum or the absence of primary antibody was used in negative control experiments. For immunofluorescence experiments, confocal microscopy was performed as described by Laird et al. (1995) and images were printed on a Kodak XLS 8300 high resolution printer.

Table 1. Oligonucleotides used in this study

Oligonucleotide	Sequence*	Reference
MIS	GATCCTGGTGT**TGATAG**GGGCGTA	This study
MIS_m	GATCCTGGTGTTGGTAGGGGCGTA	This study
BNP	GATCCGACCCC**AGATAA**AAGGCAG	(Grépin et al., 1994)

*GATA motifs are in boldface; the mutation is underlined.

Western blot analysis and DNA-binding assays

Recombinant GATA proteins were obtained by transfecting L cells (which are devoid of GATA activity) with 20 μg of mouse GATA-1, rat GATA-4 and rat GATA-6 expression plasmids as described above. Nuclear extracts were prepared 48 hours following transfection by the procedure outlined by Schreiber et al. (1989). In western analyses, 10 μg aliquots of nuclear extract were separated by SDS-polyacrylamide electrophoresis and transferred to Hybond PVDF membranes (Amersham). Immunodetection of GATA-1 and GATA-4 was achieved using the DAB-peroxidase method as previously outlined. DNA-binding assays were performed using ^{32}P-labeled double-stranded oligonucleotides. The sequences of the oligonucleotides used as probes or unlabeled competitors are given in Table 1. Binding reactions using 3-5 μg of nuclear extract were done in 20 μl buffer (4 mM Tris-HCl, pH 7.9; 24 mM KCl; 0.4 mM EDTA, pH 8.0; 0.4 mM dithiothreitol, 5 mM $MgCl_2$, 10% glycerol, and 1 μg poly(dI-dC) for 1 hour at 4°C in the presence of normal goat or rat sera or antisera to mouse GATA-1 and GATA-4, respectively (Santa Cruz). GATA-containing complexes in the reaction mixtures were analyzed by electrophoresis through a 4% non-denaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 200 V for 3 hours at 4°C followed by autoradiography.

RESULTS

GATA-4 marks Sertoli cells during testicular development

In order to study the function of GATA-4 in the testes, we first

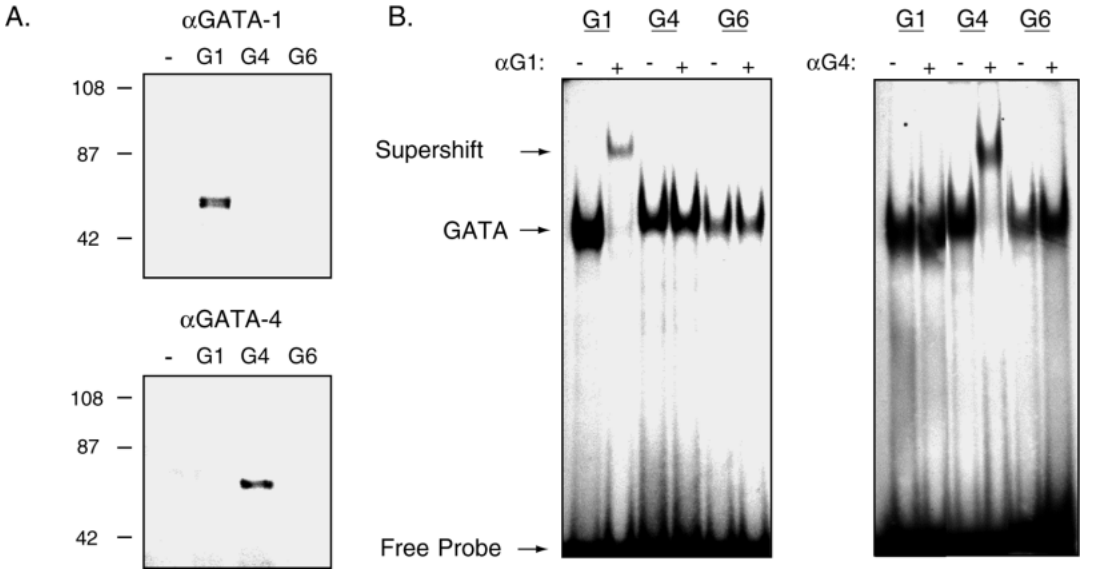

Fig. 1. Specificity of GATA-1 and GATA-4 antisera. (A) Western blot analysis. 10 μg aliquots of recombinant GATA-1, GATA-4 and GATA-6 were separated by SDS-PAGE and blotted to Hybond-PVDF membranes as described in Materials and Methods. The αGATA-1 (upper panel) and αGATA-4 (lower panel) sera reacted specifically with their proper recombinant proteins. Similarly in gel mobility shift assays (B), GATA-1 and GATA-4 DNA binding was supershifted solely by their respective antisera.

analyzed the spatial and temporal pattern of GATA-4 expression during testicular development and compared it to that of GATA-1 which is known to reside exclusively within Sertoli cells of prepubertal and some adult seminiferous tubules (Yomogida et al., 1994). Two antisera were used in our immunolocalization studies: a goat polyclonal antiserum directed against the carboxy terminus of mouse GATA-4 and a rat monoclonal antibody (N6) specific for the mouse GATA-1 protein. To confirm that the antibody preparations would not cross-react with other members of the GATA family, the antisera were first tested by western blot and supershift assays. As shown in Fig. 1, on immunoblots the GATA-1 and GATA-4 antisera specifically recognized their own recombinant proteins and not other GATA factors including GATA-2, GATA-3, GATA-5 and GATA-6 (Fig. 1A and data not shown). The specificity was also observed in supershift experiments where GATA-1 and GATA-4 binding activity was altered solely by their respective antisera (Fig. 1B).

GATA-4 was first immunolocalized in mouse testes between embryonic days 11.5-18.5; five representative ages (E11.5, E13.5, E15.5, E17.5 and E18.5) are shown in Fig. 2. Interestingly, GATA-4 protein was detected as early as E11.5 in the sexually indifferent gonad of both XY and XX embryos (Fig. 2A,B). At low power, the demarcation between the positively staining gonad (G) and the unreactive mesonephros (M) was particularly striking (Fig. 2A). Higher power magnification revealed that GATA-4 was expressed solely in the somatic cell lineage of the gonad (Fig. 2B) and not the primordial germ cells (Fig. 2B). Abundant GATA-4 protein was still observed on embryonic day 13.5 when the developing male gonad takes on its characteristic stripped appearance (Fig. 2C). At low magnification, it was clearly evident that GATA-4 immunoreactivity was specific to the seminiferous cords and interstitium of the testis and not the adjacent mesonephric structures (Fig. 2C). In the testicular cords, GATA-4 protein was found exclusively in nuclei of newly differentiated Sertoli cells (Fig. 2D). The larger germ cell nuclei present in the centre of the cords were completely unreactive (Fig. 2D). Weaker GATA-4 staining was also detected in the nuclei of E13.5 undifferentiated mesenchymal cells (Fig. 2D) which are the precursors for the interstitial cells of the testis.

Throughout mid to late embryogenesis (E15.5-E18.5), GATA-4 protein expression remained high in Sertoli cell nuclei localized along the periphery of the growing testicular cords (Fig. 2E-G). Interestingly, GATA-4 immunoreactivity was never apparent in the developing germ cell population (gonocytes) of the embryonic testis. Similar results were obtained even when using fluorescence as a more sensitive immunodetection method. Under these conditions, the absence of GATA-4 protein in gonocytes and its abundance in Sertoli cells was even more striking (Fig. 3). As expected for a transcription factor, GATA-4 immunostaining was clearly nuclear (Fig. 3D). Immunostaining was specific for GATA-4 since a similar reaction was not observed in negative control experiments using preimmune serum (Figs 2H, 3A, and data not shown). Moreover, and consistent with a previous study (Yomogida et al., 1994), GATA-1 immunoreactivity was undetectable in embryonic testis at all stages (data not shown). Thus it appears that, unlike GATA-1, GATA-4 marks immature Sertoli cells from their earliest differentiation stage.

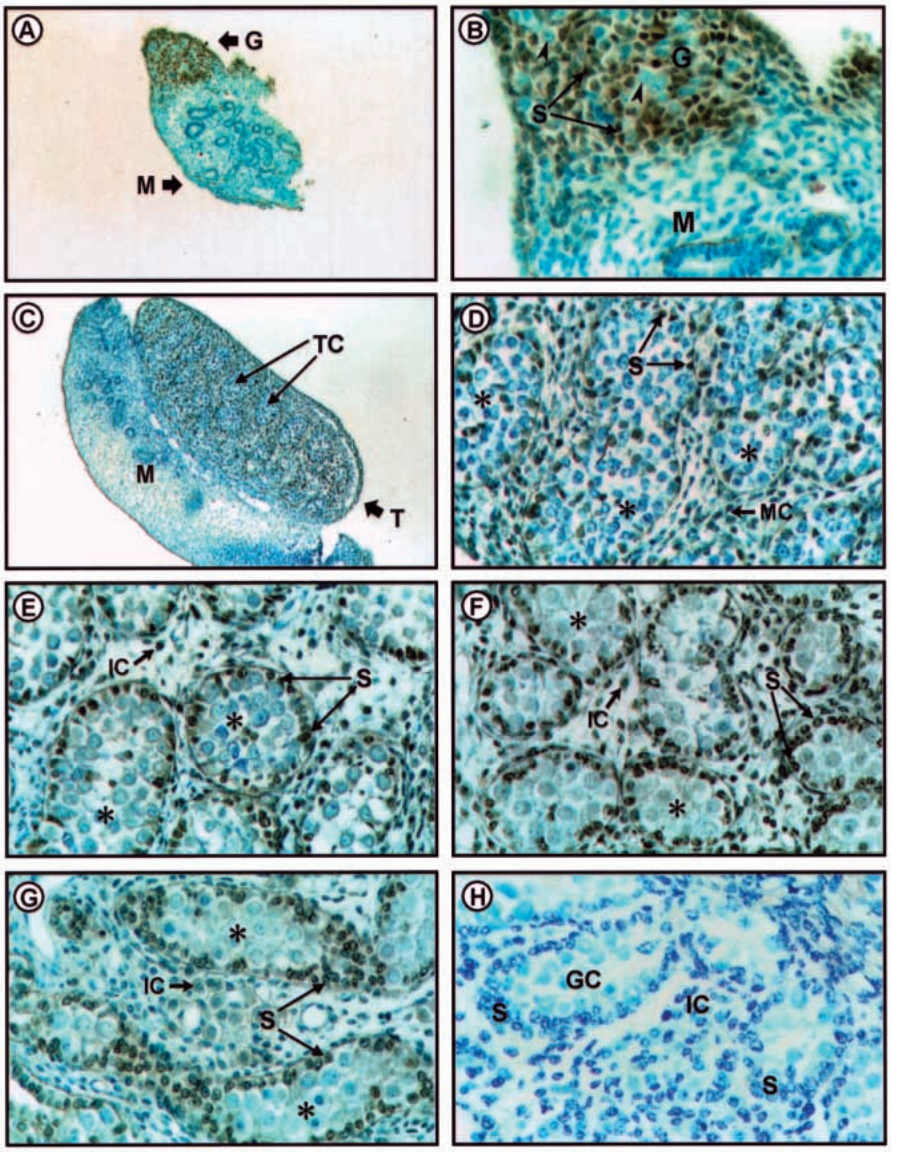

Fig. 2. Immunolocalization of GATA-4 in the embryonic testis. (A,B) E11.5; (C,D) E13.5; (E) E15.5; (F) E17.5; (G) E18.5; (H) E18.5 preimmune control. Intensely staining Sertoli cells (S) and unreactive germ cells (GC in H, asterisks in D-G and arrowheads in B) are indicated. G, gonadal component of genital ridge; IC, interstitial cells; M, mesonephric component of genital ridge; MC, mesenchymal cell; T, testis; TC, testicular cords. Magnification: (A,C), ×100; (B,D-H), ×400.

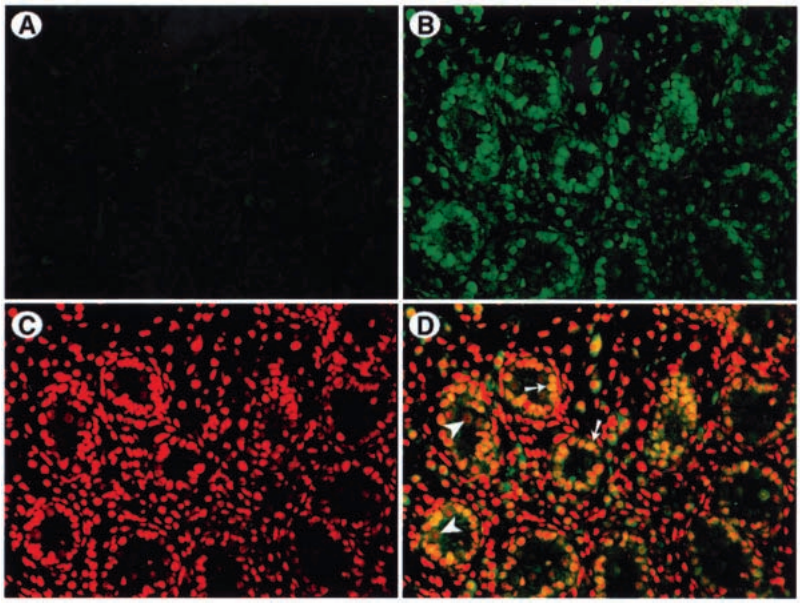

Fig. 3. GATA-4 protein expression in Sertoli cells is nuclear. Immunofluorescent staining of E17.5 testis with GATA-4 antiserum. (A) Preimmune. FITC was used as a green fluorescent tag for the GATA-4 antibody (B) and propidium iodide as red fluorescent counterstain for all nuclei (C). A nuclear localization is evident when both the FITC and propidium iodide overlap to produce an intense yellow color whereas negative cells appear red (D). Note the intense labeling of Sertoli cells present at the periphery of the cords (arrows) and the unreactive germ cells or gonocytes at the centre (arrowheads). Magnification, ×400.

Transcription factors GATA-1 and GATA-4 are expressed in Sertoli cells at different stages of testicular development

Next we examined the expression of GATA-4 during postnatal testicular development using northern blot analysis and immunohistochemistry. A full-length radiolabeled rat GATA-4 cDNA recognized a single mRNA species of approximately 3.1 kb in rat testis at all postnatal ages examined, from shortly after birth to the adult animal (Fig. 4, upper panel). GATA-4 transcripts were consistently highest in the immature testis with peak levels occurring between 1 and 14 days. The 3.1 kb RNA species proved to be specific for GATA-4 and not other closely related members of the GATA family, particularly GATA-5 and GATA-6, since a similar developmental pattern was observed when using radiolabeled probes corresponding to the 5′ untranslated or N-terminal regions of the GATA-4 cDNA which are least conserved among the GATA factors. In marked contrast to GATA-4, the GATA-1 gene in the rat testis only began to be expressed on day 7 and rapidly reached maximum levels prior to puberty on days 14-21 (Fig. 4, middle panel). GATA-1 mRNA levels decreased substantially thereafter but the 2.0 kb transcript remained detectable even in the mature testis (day 91). This expression pattern was consistent with what has been previously reported for the GATA-1 protein in Sertoli cells during postnatal testicular development (Yomogida et al., 1994).

The analysis of GATA-4 expression in the postnatal testis was extended at the protein and cellular level using immunocytochemistry. GATA-4 immunoreactivity in Sertoli cell nuclei was high in newborn (day 1) and 7-day-old testes but low on day 14 (Fig. 5). During this postnatal period (days 1-14), Sertoli cells remained the predominant cell type expressing GATA-4 and no staining was evident in germ cells or in the interstitium. However, Sertoli cells from pubertal (day 23) or adult testes no longer expressed the GATA-4 protein, which became detectable in germ cells (Fig. 5G and data not shown). Interestingly, the decrease in GATA-4 immunoreactivity on day 14 and its loss from Sertoli cells on day 23 coincided with the upregulation of GATA-1 in these cells (Fig. 5). In the postnatal testis, GATA-1 protein was first apparent in Sertoli cell nuclei on day 7 (Fig. 5D), reached its maximum between 14 and 23 days (Fig. 5F,H), and was undetectable in the adult (not shown). Consistent with a previous report (Yomogida et al., 1994), no other testicular cell type expressed significant GATA-1 protein. Again, immunostaining in postnatal testes was specific for GATA-4 or GATA-1 and no visible reaction was observed with preimmune serum (data not shown). Together with the northern blot analysis, the immunocytochemical data indicate that transcription factors GATA-1 and GATA-4 are expressed in Sertoli cells but essentially at different stages of testicular development.

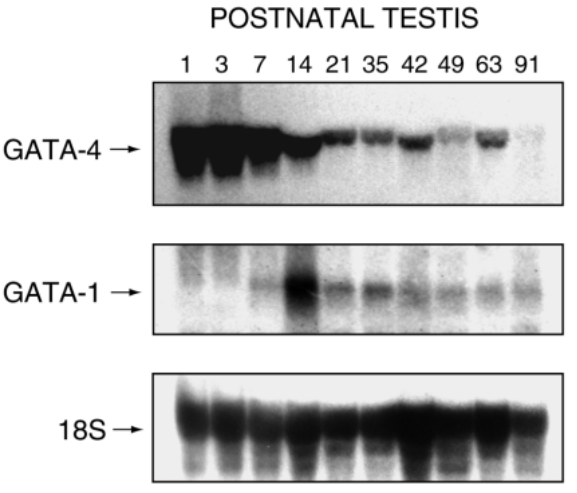

Fig. 4. GATA-4 and GATA-1 gene expression during postnatal testicular development as determined by northern blot hybridization. 15 μg of total cellular RNA were used in each lane. The blots were rehybridized with an oligonucleotide probe specific for the 18S ribosomal RNA in order to verify the quantity and integrity of the RNA used.

Sexual dimorphic expression of transcription factor GATA-4

Since GATA-4 was present in the sexually indifferent gonad of genotypically female mouse embryos, we analyzed GATA-4 expression during the ontogeny of the mouse ovary (Fig. 6). GATA-4 protein was detected in the differentiating ovaries of E13.5-14.5 mouse embryos where it specifically marked the somatic cell lineage and not germ cells (Fig. 6A-C). Remarkably, GATA-4 expression was dramatically down-regulated shortly after ovarian differentiation (Fig. 6D). Thus, in contrast to the testis, GATA-4 protein was not detected during late embryonic development and in neonate ovaries (Fig. 6D-G). Significant GATA-4 expression was apparent, however, in the adult ovary where it localized predominantly to granulosa cells of follicles at different stages of development and some interstitial cells (Fig. 6H). Thus, GATA-4 exhibits a striking sexually dimorphic pattern of expression during early mouse gonadal development, raising the possibility that this factor may also be involved in mammalian sex differentiation.

The Müllerian inhibiting substance promoter is a potential downstream target for GATA-4 in Sertoli cells

The expression pattern of GATA-4 in the sexually indifferent gonad and then in immature Sertoli cells suggested an important role for GATA-4 in early Sertoli cell function. Sertoli cells from fetal and newborn testis secrete Müllerian inhibiting substance (MIS) which is essential for the regression of the Müllerian ducts in the male and hence, normal male sex differentiation. As shown in Fig. 7, the expression profile of *MIS* closely follows that of GATA-4 suggesting that the *MIS* gene may be a downstream target for GATA-4. The first 180 base pairs (bp) of the *MIS* promoter are sufficient for cell specific expression in embryonic and neonatal Sertoli cells both in vivo and in primary Sertoli cell cultures and deletion to −65 bp reduces promoter activity by 90% (Shen et al., 1994; Giuili et al., 1997). Sequence alignment of this region reveals the presence around −75 bp of a GATA motif that is conserved across species (Fig. 8A). We tested whether this GATA element can interact with endogenous Sertoli cell GATA factors and with recombinant GATA-4 protein. Gel shift experiments showed that the *MIS* GATA element was bound specifically by GATA factors present in nuclear extracts prepared from neonate primary Sertoli cell cultures (Fig. 8B-D); the binding was supershifted by the GATA-4 antiserum confirming that GATA-4 is a major GATA factor of immature Sertoli cells (Fig. 8B). The *MIS* GATA element displayed a similarly high affinity for GATA-4 as the well characterized GATA element from the B type natriuretic peptide (BNP) promoter which is a downstream target for GATA-4 in the myocardium (Grépin et al., 1994).

We then tested whether the *MIS* promoter can be transactivated by GATA-4 in heterologous cells. Cotransfection of the −180 bp promoter with a GATA-4 expression vector resulted in a significant 10-fold activation of the *MIS* promoter

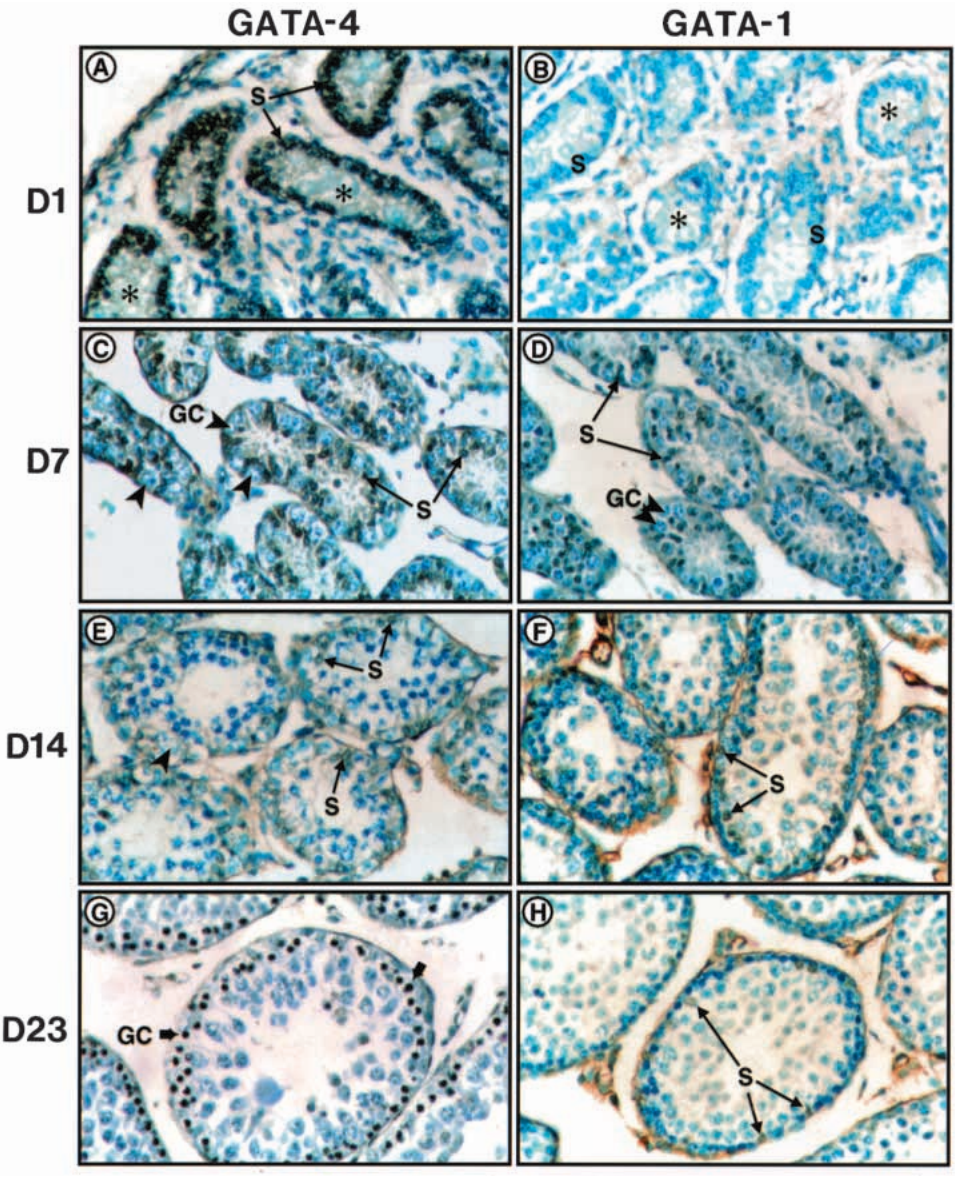

Fig. 5. Immunolocalization of GATA-4 (A,C,E,G) and GATA-1 (B,D,F,H) in the postnatal testis. (A,B) day 1; (C,D) day 7; (E,F) day 14; (G,H) day 23. Sertoli cells (S) strongly express GATA-4 on days 1 and 7 (A,C), weakly express it on day 14 (E), and not all by day 23 when germ cells (GC) begin to produce this factor (G). GATA-1 protein expression was first detected in Sertoli cells (S) on day 7 (D) and in contrast to GATA-4, was not turned off by day 23 (H). Moreover, germ cells (GC; asterisks in A,B, arrowheads in C-E, broad arrow in G) never expressed GATA-1. Magnification, ×400.

at very low GATA4 concentrations (Fig. 9A). Interestingly, the *MIS* promoter displayed specificity for GATA-4 relative to other GATA factors (Fig. 9A). This transactivation required an intact GATA element since a point mutation that abolishes GATA binding (Fig. 8B) abrogates GATA-4 responsiveness. The coexpression of GATA-4 and MIS in embryonic and neonatal Sertoli cells, together with the ability of GATA-4 to bind and transactivate the *MIS* promoter suggest that GATA-4 may be an important regulator of the *MIS* gene. Since the expression of *MIS* is essential for the acquisition of the normal male sexual phenotype, these data further support a role for GATA-4 in the regulation of sex differentiation in mammals.

DISCUSSION

The GATA transcription factors constitute a family of nuclear proteins that play crucial roles in cell differentiation and organ formation in many systems. We report here that one member of this family, GATA-4, is expressed at the onset of mouse gonadal development in both sexes and may be a potential regulator of early gonadal development in mammals. Moreover, the sexually dimorphic expression of GATA-4 at later stages and the identification of the Müllerian inhibiting substance gene as a potential downstream target for GATA-4 in embryonic Sertoli cells raise the possibility that this transcription factor may play a role in mammalian sex differentiation.

GATA-like factors are found in the gonads of both lower organisms and higher vertebrates (Drevet et al., 1994; Laverriere et al., 1994; Singh et al., 1994; Yomogida et al., 1994; Lossky and Wesink, 1995; Heikinheimo et al., 1997). The evolutionary conservation of this expression pattern suggests that these factors play important roles in gonadal function. In mammals, gonad formation is thought to be mediated by factors that act specifically within the somatic cell lineage of the gonad and not germ cells (Byskov and Hoyer, 1994). The fact that GATA-4 localizes to the somatic cell lineage (Sertoli in testis and granulosa in ovary) of the developing gonad is consistent with a role in gonadal development. As shown in Fig. 7, GATA-4 expression in Sertoli cells overlaps with several other genes, many of which are known to play important roles in male sex determination and testicular function. These include *SRY* (Koopman et al., 1990), *SF-1* (Ikeda et al., 1994, 1996), *WT-1* (Pritchard-Jones et al., 1990; Pelletier et al., 1991), *SOX9* (Morais da Silva et al., 1996), *DAX-1* (Ikeda et al., 1996; Swain et al., 1996; Tamai et al., 1996), *MIS* (Shen et al., 1994), kit ligand (Rossi et al., 1991; Manova et al., 1993), and the α and β subunits of inhibin (Shaha et al., 1989; Tone et al., 1990). It is noteworthy that several of these genes have one or more consensus GATA motifs in their 5′-flanking regions suggesting that GATA-4 may be an important regulator of Sertoli cell-specific gene expression. For example, conserved GATA elements are found in the proximal *SF-1* promoter of both mouse and human (Woodson et al., 1997) and deletion of these GATA elements has been shown to reduce *SF-1* promoter activity by more than 50% in a gonadal (GATA positive) but not adrenocortical (GATA negative) cell line (Woodson et al.,

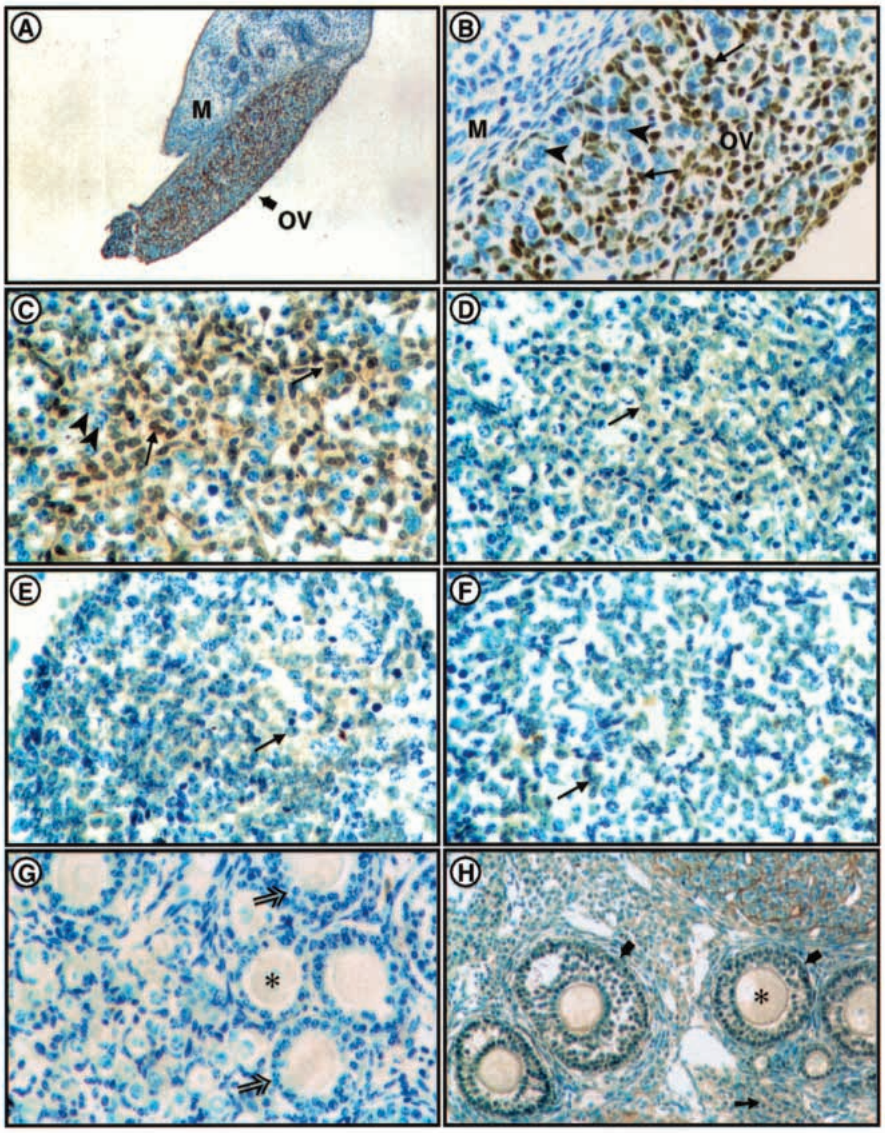

Fig. 6. Ontogeny of GATA-4 protein expression in the mouse ovary. (A,B) E13.5; (C) E14.5; (D) E16.5; (E) E17.5; (F) E18.5; (G) day 7; (H) adult. GATA-4 is strongly expressed in the somatic cell lineage (arrows) of the developing ovary shortly after gonadal differentiation (B,C), is markedly down-regulated during late embryonic development and in the neonate (D-G), and then reappears in granulosa cells (broad arrow) and some interstitial cells (curved arrow) of the adult (H). Note that embryonic germ cells (arrowheads) and oocytes within developing follicles (asterisks) never express GATA-4. OV, ovary; M, mesonephros. Magnification: (A) ×100; (B-G) ×400; (H) ×200.

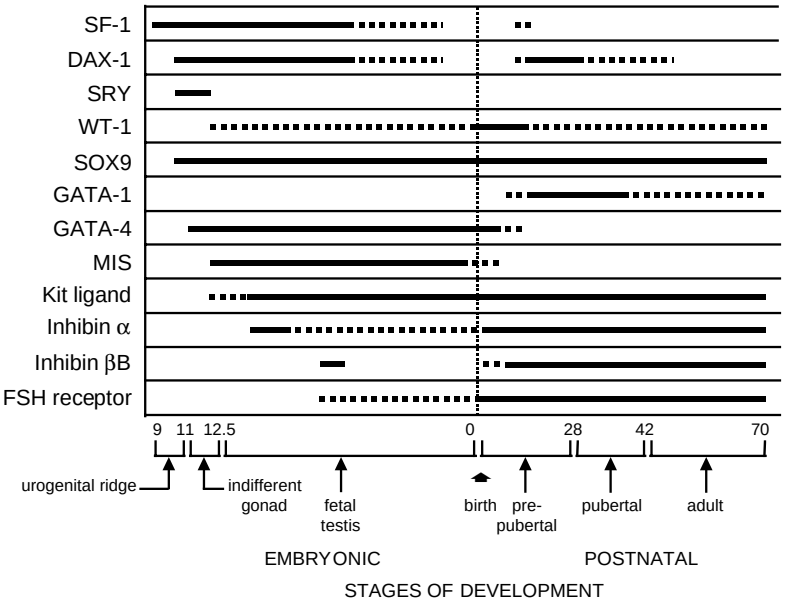

Fig. 7. Ontogeny of gene expression in Sertoli cells. Developmental expression patterns of genes known to be important for mammalian gonadal development, male sex determination and Sertoli cell function were compared to those obtained for GATA-1 and GATA-4. Expression patterns for *SF-1* (Ikeda et al., 1994; Shen et al., 1994), *DAX-1* (Ikeda et al., 1996; Tamai et al., 1996), *Sry* (Koopman et al., 1990), *WT-1* (Pritchard-Jones et al., 1990; Pelletier et al., 1991), *MIS* (Shen et al., 1994; Molkentin et al., 1997), c-*kit* ligand (Matsui et al., 1990; Keshet et al., 1991; Manova et al., 1993), and the FSH receptor (Rannikki et al., 1995; O'Shaughnessy et al., 1996) were drawn from in situ hybridization data obtained in the literature, the patterns of Sox9 (Morais da Silva et al., 1996), GATA-1 (this study; Yomogida et al., 1994), GATA-4 (this study), and the α and βB subunits of inhibin (Majdic et al., 1997) which were based on immunohistochemical data. While GATA-4 expression in early Sertoli cells overlaps with several genes that are important for gonadal development and male sex determination, the most striking correlation is found between GATA-4 and MIS.

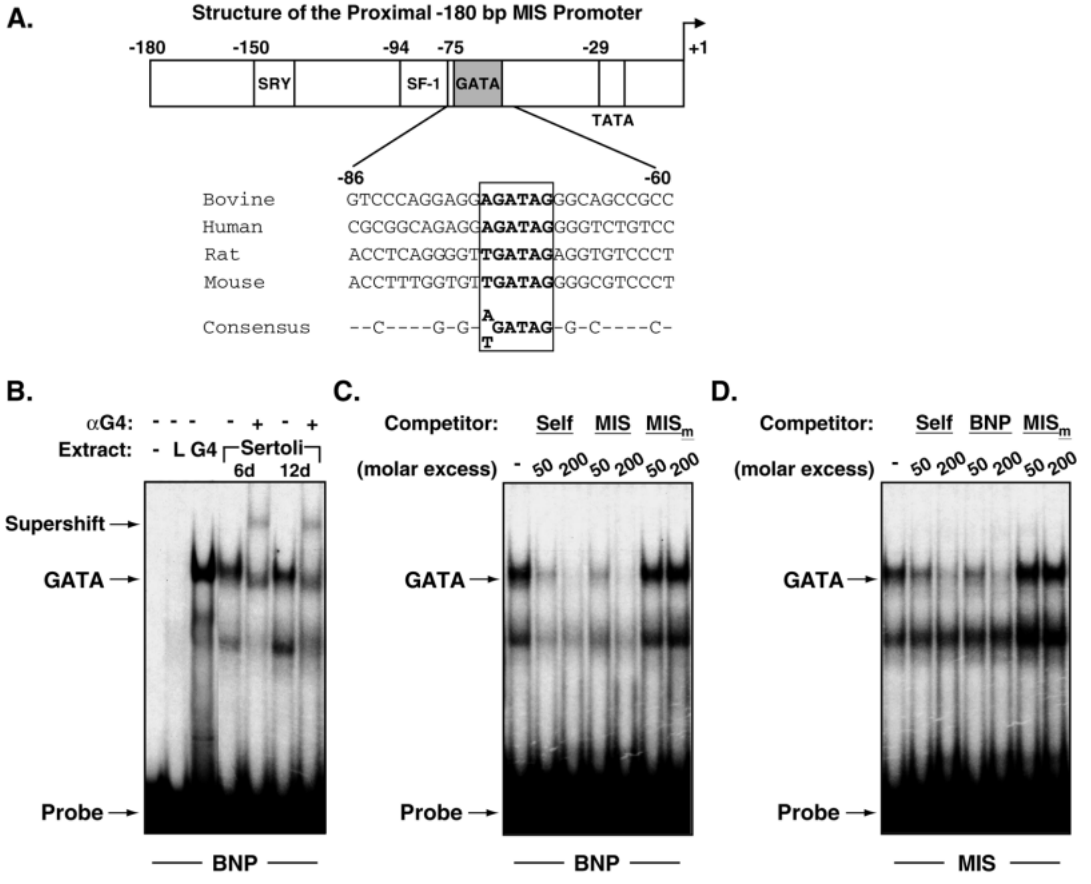

Fig. 8. A consensus GATA binding site is present in the proximal *MIS* promoter. (A) Structural organization of the proximal (−180 bp) *MIS* promoter. Putative regulatory elements of the *MIS* promoter are boxed and their locations relative to the transcriptional start site are shown. Alignment of the bovine (Cate et al., 1986), human (Guerrier et al., 1990), rat (Haqq et al., 1992) and mouse (Shen et al., 1994; Molkentin et al., 1997) proximal *MIS* promoter sequences reveals a consensus GATA motif at −75 bp that is conserved across species. (B) GATA-4 binding activity in neonate (6- and 12-day-old) primary Sertoli cell cultures. Endogenous GATA proteins present in nuclear extracts prepared from immature Sertoli cells bind to a consensus GATA element; this binding is supershifted by a specific GATA-4 antiserum indicating that GATA-4 is the major GATA factor of immature Sertoli cells. (C,D) Sertoli cell GATA proteins specifically interact with the *MIS* GATA element. GATA-binding to either the *BNP* (C) or *MIS* (D) GATA elements was specifically competed by excess unlabeled oligonucleotides but not by a mutant *MIS* GATA oligonucleotide (MIS_m) in which the GATA consensus motif had been changed to GGTA. The sequences of oligonucleotides used as probes or competitors are shown in Table 1. L, nuclear extract from L cell fibroblasts; G4, nuclear extract from L cells overexpressing recombinant GATA-4.

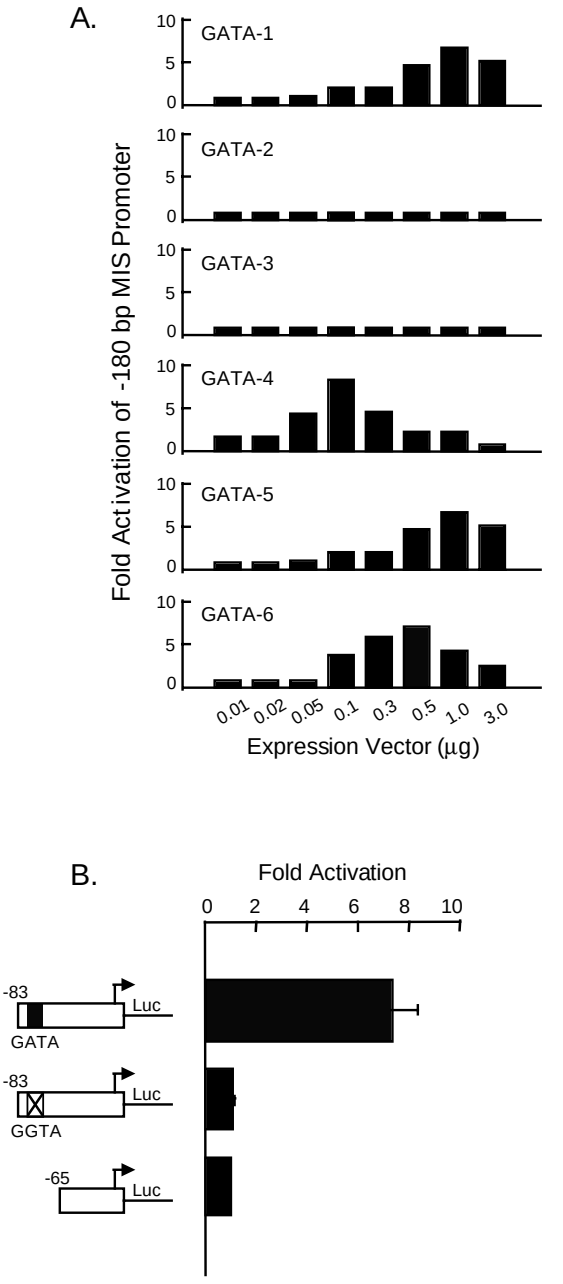

Fig. 9. (A) Transactivation properties of recombinant GATA proteins on the 180 bp *MIS* promoter. CV-1 cells were cotransfected with the mouse *MIS* −180 bp luciferase promoter construct and increasing amounts of GATA expression vectors. In all transfections, the total amount of DNA was kept constant by adding appropriate amounts of a control background vector. The data are expressed as fold activation over the control background vector and represent the means of two independent experiments done in duplicate.
(B) Mutation of the GATA element at −75 bp abolishes GATA responsiveness of the *MIS* promoter. CV-1 cells were cotransfected with 0.1 μg of recombinant GATA-4 and the above listed *MIS* luciferase promoter constructs. The 83 bp construct that retains the GATA motif at −75 responds to GATA-4 in a similar fashion to the −180 bp construct shown in A. In contrast, mutagenesis of the GATA element at −75 bp [−83 bp (mut)] that destroys GATA binding (Fig. 7), abolishes GATA responsiveness. The data are expressed as fold activation over the −65 bp construct and represent the means of two independent experiments done in duplicate.

1997). However, expression of *WT-1* has already been shown to be directly modulated by GATA factors in hematopoietic cells (Wu et al., 1995) and it is therefore possible that GATA-4 regulates *WT-1* expression in Sertoli cells. Of the different Sertoli cell gene expression patterns analyzed, the most striking correlation was found between GATA-4 and MIS (Fig. 7). Interestingly, the first 180 bp of the *MIS* promoter which are sufficient to confer Sertoli cell-specific expression, both in vitro and in vivo (Shen et al., 1994; Giuili et al., 1997) harbors putative binding sites for SRY and SF-1 but also a previously uncharacterized GATA consensus motif at −75 bp (Fig. 8A).

The present work provides evidence that this GATA element functionally interacts with GATA-4 and raises the possibility that the *MIS* gene may be a downstream target for GATA-4 in Sertoli cells. To date, the only other transcription factor shown to directly regulate *MIS* gene expression is SF-1 (Shen et al., 1994; Giuili et al., 1997). However, SF-1 cannot be the sole determinant for *MIS* gene expression in vivo since *SF-1* has many extra-gonadal sites of expression whereas *MIS* does not. Rather, Sertoli cell-specific expression of *MIS* may result from the combinatorial interaction of SF-1 and other transcriptional regulator(s), such as GATA-4, over the *MIS* promoter. Together with the sexually dimorphic expression of GATA-4 in gonadal development, the identification of the *MIS* gene as a putative downstream target for GATA-4 in Sertoli cells raises the intriguing possibility that GATA-4 may be an important regulator of sexual differentiation.

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