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Developmental stage- and spermatogenic cycle-specific expression of transcription factor GATA-1 in mouse Sertoli cells

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SUMMARY

GATA-1 is an essential factor for the transcriptional activation of erythroid-specific genes, and is also abundantly expressed in a discrete subset of cells bordering the seminiferous epithelium in tubules of the murine testis. In examining normal and germ-line defective mutant mice, we show here that GATA-1 is expressed only in the Sertoli cell lineage in mouse testis. GATA-1 expression in Sertoli cells is induced concomitantly with the first wave of spermatogenesis, and GATA-1-positive cells are uniformly distributed among all tubules during prepubertal testis development. However, the number of GATA-1-positive cells declines thereafter and were found only in the peripheral zone of seminiferous tubules in stages VII, VIII and IX of spermatogenesis in the adult mouse testis. In contrast, virtually every Sertoli cell in mutant *W/W^v*, *jsd/jsd* or cryptorchid mice (all of which lack significant numbers of germ cells) expresses GATA-1, thus showing that the expression of this transcription factor is negatively controlled by the maturing germ cells. These observations suggest that transcription factor GATA-1 is a developmental stage- and spermatogenic cycle-specific regulator of gene expression in Sertoli cells.

Key words: GATA-1, Sertoli cell, spermatogenic cycle, transcription factor, *W/W^v* mouse

INTRODUCTION

GATA-1 binds to the consensus WGATAR sequence through a zinc finger DNA-binding motif, which is highly conserved among the multiple members of this transcription factor family (deBoer et al., 1988; Evans and Felsenfeld, 1989; Yamamoto et al., 1990; Engel et al., 1992; Tsai et al., 1991; Orkin, 1992). The carboxyl finger is responsible for site-specific DNA association (Martin and Orkin, 1990; Yang and Evans, 1992; Yang et al., 1994), while the amino finger is required for nuclear localization of the factor (Yang et al., 1994). GATA-1 mRNA is found in erythroid, megakaryocytic and mast cells (Evans and Felsenfeld, 1989; Tsai et al., 1989; Martin et al., 1990; Romeo et al., 1990) and is required for functional erythropoiesis (Pevny et al., 1991; Simon et al., 1992), thereby suggesting that it also contributes to the transcriptional regulation of genes specifically expressed in all these hematopoietic cell lineages.

We recently found that GATA-1 is also expressed in the seminiferous tubules of mouse testes (Ito et al., 1993). This novel transcript utilizes a testis-specific promoter and first exon. The testis first exon is located 8 kbp 5′ to the hematopoietic cell first exon (both are non-coding), but the remaining five exons which code for the GATA-1 protein are common in both testis and erythroid mRNAs; thus identical GATA-1 proteins are expressed in hematopoietic and testis cells (Ito et al., 1993).

One of the most important remaining questions in male reproductive physiology regards the elucidation of the mechanism(s) by which the spermatogenic cycle is regulated. Growing evidence supports the hypothesis that the complex functional interdependence of germ and Sertoli cells in seminiferous tubules may play a pivotal role in regulation of the cycle (reviewed in Russell et al., 1993).

GATA-1-positive cells in the mouse testis were originally identified in the seminiferous tubules using a monoclonal antibody (mAb) prepared against the erythroid GATA-1 protein. While GATA-1-positive cells were abundant in the 2-week-old testis, the number declined thereafter, with only few cells found in the periphery of seminiferous tubules as testis development progressed. Since this developmentally and spatially restricted pattern of any early testis marker had not been previously described, we could not unambiguously determine which cell lineage expressed GATA-1 (Ito et al., 1993). Fortunately for simplification in resolving this problem, there are only two cell types expressed inside seminiferous tubules: germ cells and Sertoli cells (reviewed in Russell et al., 1990; Nishimune and

Okabe, 1993). Germ cells constitute the male meiotic contribution to the reproductive cycle, while Sertoli cells support the growth and differentiation of germ cells.

To identify unambiguously the lineage of cells in the testis that express GATA-1, we examined its expression in *W/W^v* (dominant white spotted or c-*kit*) mutant mice, which harbor greatly reduced numbers of germ cells (Sawada et al., 1991; Kurohmaru et al., 1993). The migration of primordial germ cells to germinal ridges is impaired in *W/W^v* mice due to a well-characterized mutation in the c-*kit* proto-oncogene (Chabot et al., 1988; Yoshinaga et al., 1991). We also examined the expression of GATA-1 in adult *jsd/jsd* and cryptorchid mouse testes, both of which are also known to lack germ cells. The results showed that GATA-1 is abundantly expressed in all of these animals, thus demonstrating that expression of GATA-1 is restricted to the Sertoli cell lineage. Furthermore, examination of the developmental stage- and spermatogenic cycle-specificity of GATA-1 expression in Sertoli cells clearly demonstrated that GATA-1 is induced concomitantly with the first wave of spermatogenesis in prepubertal mouse testis, and is then synthesized synchronously during the spermatogenic cycle in the mature adult mouse testis.

MATERIALS AND METHODS

RNA blot hybridization analysis

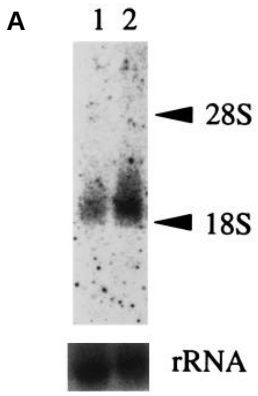
W/W^v mice and Balb/c mice were supplied from Shizuoka Experimental Animal Farm and the Mouse Center at Tohoku University, respectively. Total RNAs were prepared from the testes of 2-week-old Balb/c, *W/W^v* and wild-type litter mates using the acid guanidine/phenol/chloroform method (Chomczynski and Sacchi, 1987). 20 μg of total RNAs were electrophoresed on an 1% agarose/formaldehyde gel. After transfer to a nitrocellulose filter (Hybond-C extra, Amersham), the RNA was hybridized to ^{32}P-labeled mGATA-1 cDNA probe (Ito et al., 1993).

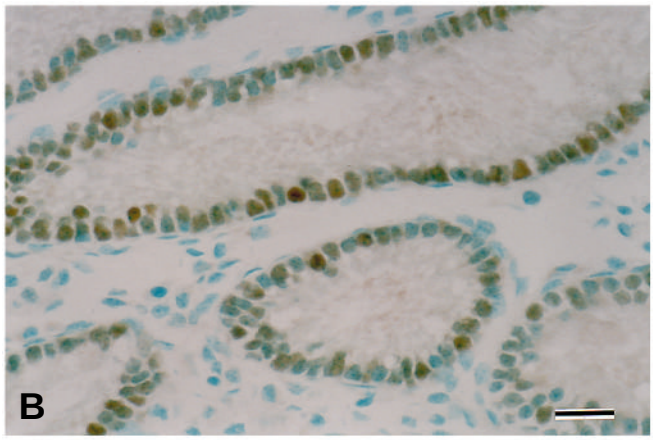
Immunohistochemical analyses of mouse testes

Immunohistochemistry was performed using the indirect immunoperoxidase method (Ohtani et al., 1993). Briefly, sliced testes of *W/W^v*, Balb/c, *jsd/jsd* and cryptorchid mice were fixed in periodate-lysine-4% paraformaldhyde for 2 hours at room temperature. Testes were washed in phosphate-buffered saline (PBS) with 20% sucrose and rapidly frozen after embedding in OCT compound (Miles). After rinsing in sheep serum, 5 mm-thick frozen sections were reacted with the N6 antibody (1: 50 dilution of ascites) for 24 hours at 4°C. After washing, the specimens were incubated with horseradish peroxidase-conjugated F(ab′)$_2$ fragments of anti-rat IgG (Amersham; 1: 200 dilution) overnight at 4°C; diaminobenzidine was used as chromogen. Normal mouse serum was added to the second antibody solution to block nonspecific reaction, and the sections were counterstained with methyl green. In control experiments, either no primary antibody was added or indifferent antibody was used (Ito et al., 1993). The *jsd/jsd* mice were maintained in the Experimental Animal Facility in the Research Institute for Microbial Diseases at Osaka University (Mizunuma et al., 1992). Cryptorchid mice were prepared as described (Nishimune et al., 1978). Immunoelectron microscopy was carried out using frozen sections of 8-week-old Balb/c mouse testis prepared as above. After diaminobenzidine reaction, the specimens were fixed in 1% osmium tetroxide and embedded in EPON (Ohtani et al., 1993).

Determination of the relationship of the spermatogenic stages and GATA-1-positive seminiferous tubule

To determine the stages of seminiferous tubules, the immunohistochemistry was performed as described above, except that the nuclei were counterstained with hematoxylin. The stage of each seminiferous tubule was determined following the criteria described (Russell et al., 1990).





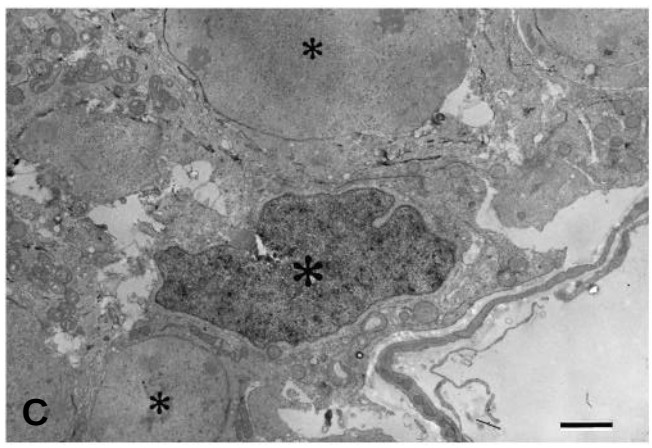


Fig. 1. GATA-1-positive cells in mouse testis are in the Sertoli cell lineage. (A) RNA blot hybridization analysis was performed using total RNAs isolated from the testes of 2-week-old Balb/c mice (lane 1) and *W/W^v* mice (lane 2). RNAs were transferred to a nitrocellulose filter and the filter was hybridized to the mouse testis type GATA-1 cDNA probe (Ito et al., 1993). The filter was then washed and rehybridized to ribosomal RNA probe to establish the amount of RNA loaded. Ribosomal RNA (18S and 28S) used as size mobility markers are indicated. (B) Immunohistochemical analysis of GATA-1 expression in the testis of 2-week-old *W/W^v* mouse. Thin sections of 2-week-old *W/W^v* mouse testis were stained using the N6 anti-mGATA-1 monoclonal antibody. Scale bar indicates 20 μm. (C) Immunoelectron microscopic analysis of testis from 8-week-old Balb/c mouse. Scale bar indicates 2 μm. Large asterisk indicates the GATA-1-positive nucleus of a Sertoli cell, whereas the small asterisks indicate GATA-1-negative nuclei of germ cells.

RESULTS

GATA-1-positive cells in mouse testis are in the Sertoli cell lineage

We first examined the expression of GATA-1 mRNA by blot hybridization of RNA samples isolated from the testes of 2-week-old W/W^v mice and 2-week-old wild-type Balb/c mice. The level of GATA-1 RNA in the W/W^v mutant mouse testes is much higher than that in the Balb/c mouse testes (Fig. 1A). A similar result was obtained when we examined RNA samples from W/W^v mouse testes, using a wild-type litter mate of the W/W^v mouse testes as a control (data not shown). Thus,

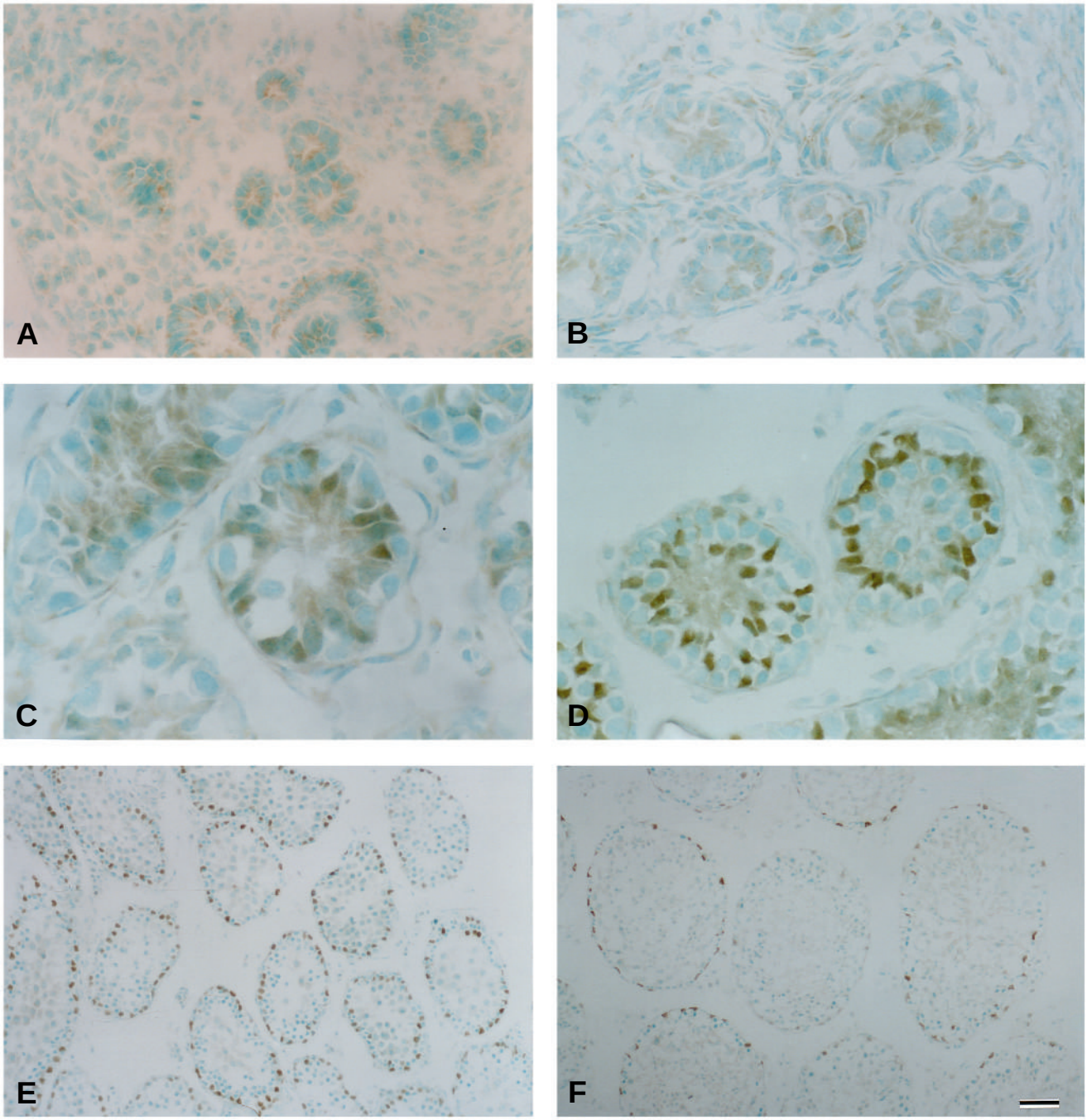


Fig. 2. Developmental stage-specific expression of GATA-1 in mouse Sertoli cells. Genital ridges and testes were isolated from various stages of Balb/c mice, and examined by immunohistochemical methods as described in the legend to Fig. 1. Male genital ridges were isolated from 13. 5 days p.c. (not shown) and 14.5 days p.c. embryo (A). GATA-1 is not detected in the nuclei of Sertoli cell precursors. Testes were isolated from newborn (B), 7-day (C), 9-day (D), 3-week (E) and 5-week (F)-old mice. Scale bar corresponds to 20 μm (A), 15 μm (B and D), 10 μm (C) and 50 μm (E and F), respectively. The expression of GATA-1 in the nuclei was first identified in 7-day-old mouse Sertoli cells. Note that 5-week-old mouse testis contains a segment of seminiferous tubules which lack GATA-1-positive cells. The nature of the weak signals in the cytoplasm of Sertoli cell precursors is currently unknown (see text).

the level of GATA-1 mRNA in mouse testis is high regardless of the number of germ cells, suggesting that GATA-1 mRNA is expressed in Sertoli cells. The differences in the expression level of GATA-1 mRNA between W/W^v and wild-type testes probably reflect the fact that the ratio of Sertoli cells to the total testis cells is much higher in W/W^v mouse testes than in the wild-type testes.

The expression of GATA-1 was also investigated by immunohistochemical analysis of 2-week-old W/W^v mutant mouse testes using the N6 anti-mGATA-1 mAb (Ito et al., 1993). Since diaminobenzidine was used as chromogen, positive nuclei appear brown, whereas non-expressing nuclei (counterstained with methyl green) are green. In the seminiferous tubules of prepubertal (2-week-old) W/W^v mutant mice, almost all nuclei are GATA-1-positive (Fig. 1B).

We also performed an immunoelectron microscopic analysis of the GATA-1 expression in the adult Balb/c mouse testis. The GATA-1-positive nuclei in the adult mouse testis appeared to be very large in the light microscopic analysis in our previous study (Ito et al., 1993). In agreement with this observation, the GATA-1 protein localized in irregular-shaped nuclei (Fig. 1C, expressed as black color) in immunoelectron microscopic analysis, in contrast to the unstained round nuclei of germ cells (see Russell et al., 1990). These data comparing the coincidence of GATA-1 expression in both germ cell-defective and normal animals employing both light and immunoelectron microscopic analysis provide conclusive evidence that the cells expressing GATA-1 are in the Sertoli lineage, and not in germ cells.

GATA-1 expression in mouse Sertoli cells early in development

We next extended the immunohistological analysis of GATA-1 expression to examine testes at early developmental stages. GATA-1 was not detected in the nuclei of testis cell precursors in the male genital ridge of 13- or 14.5-day-old fetuses (Fig. 2A and data not shown) or in the nuclei of the testes of newborn animals (Fig. 2B); however, weak GATA-1 expression was detected in the cytoplasm of Sertoli cell precursors in the genital ridge (Fig. 2A) and newborn mouse testis (Fig. 2B) cells. Negative control experiments showed that these immunocytochemical signals were generated by the specific binding of the N6 antibody, and not by non-specific binding of the first or second antibodies. The specificity of the N6 mAb has been examined by immunoblot (Ito et al., 1993) and immunoprecipitation (unpublished) analyses, and, in both

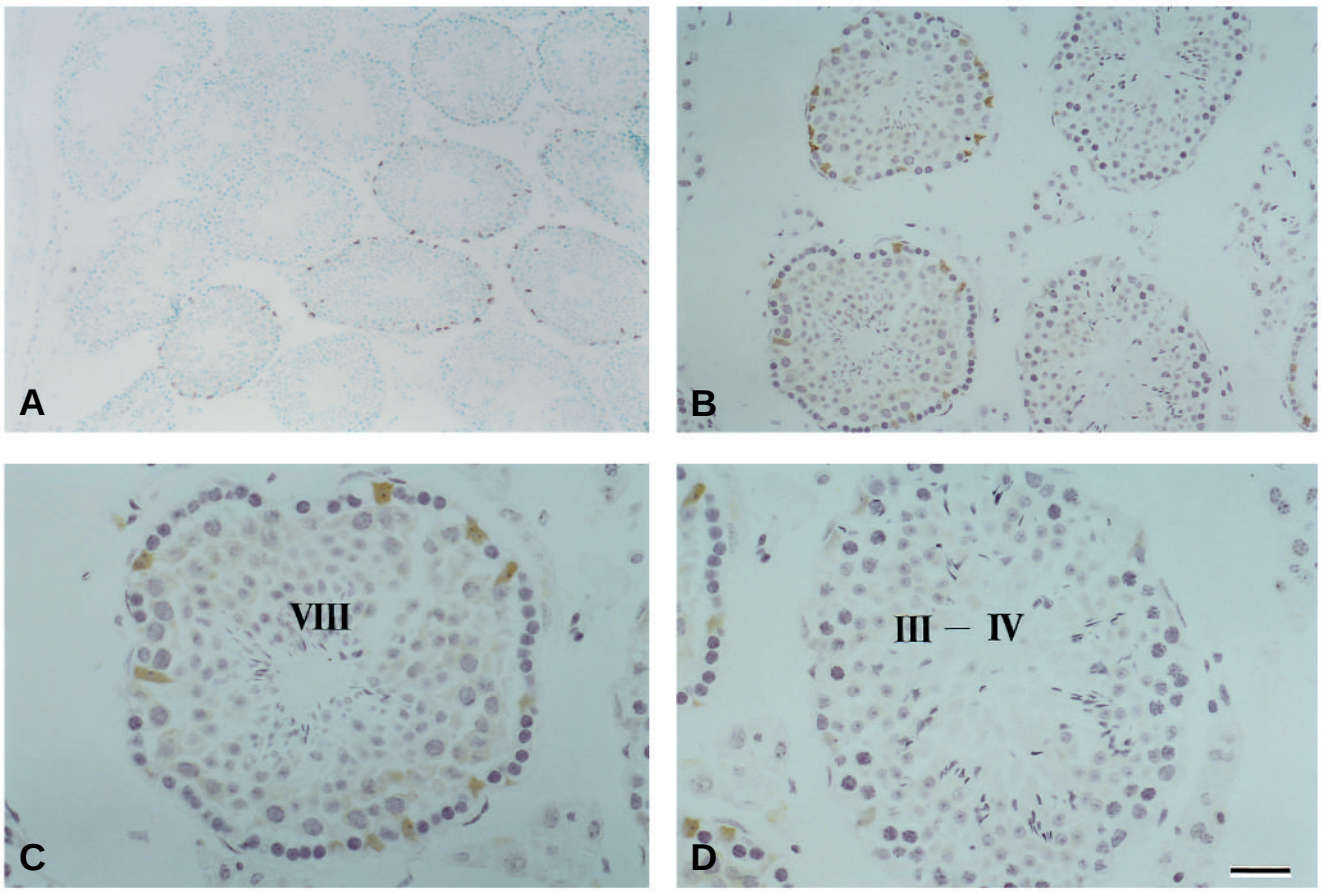


Fig. 3. Spermatogenic stage-specific expression of GATA-1 in mouse Sertoli cells. (A) Two types of seminiferous tubules exist in transverse sections of adult mouse testes: one containing, and one lacking, GATA-1-positive cells. Testis from a 10-week-old Balb/c mouse was analyzed as described in the legend to Fig. 1. (B-D) GATA-1-positive cells exist exclusively in cross-sections which correspond to stages VII, VIII and IX of spermatogenesis. Sertoli cell nuclei were counterstained with hemotoxylin to clearly identify the spermatogenic stages. C and D represent a tubule shown in B. Scale bar corresponds to 50 μm(A), 100 μm (B), and 200 μm (C and D), respectively.

cases, the antibody exclusively binds to the mGATA-1 protein. These data suggest that the signals may represent cytoplasmic GATA-1 expression in these immature Sertoli cells. Alternatively, it is possible that these signals possibly represent cross-reactive proteins detected by the mAb (not GATA-1) which reside specifically in the cytoplasm of Sertoli cell precursors. We think that the latter possibility is likely, since investigation of the subcellular localization of hGATA-1, hGATA-2 and hGATA-3 (Nagai et al., unpublished data; Yang et al., 1994) proteins have shown that these factors are all localized exclusively in nuclei.

GATA-1 was expressed at very low levels in some cell nuclei of 7-day-old newborn testis (Fig. 2C), and the expression becomes much more pronounced in 9-day-old mouse testis (Fig. 2D). While GATA-1 is expressed in all cross-sections of tubules in the 3-week-old mouse testis (Fig. 2E), some seminiferous tubules are GATA-1-negative in sections of testes in 5-week-old mice (Fig. 2F). A significant proportion of cells then became GATA-1-negative as the animals matured (see below).

Spermatogenic stage-specific expression of GATA-1 in mouse Sertoli cells

In examining immunohistological sections of the testes of mature mice, two easily distinguishable types of seminiferous tubule cross-sections were always observed: one contained GATA-1-positive cells in close juxtaposition to the basement membrane of the tubules, while other tubules did not contain any GATA-1-positive cells (Fig. 3A). We therefore sought to determine the reason for the difference between GATA-1-positive and -negative seminiferous tubules in adult mice and to determine the cause(s) of this difference. To this end, we correlated the emergence of GATA-1-positive seminiferous tubules with the various stages of spermatogenesis (Russell et al., 1990).

Adult testes were isolated from 8, 10, 14, 15 and 50-week-old mice, stained with mAb N6 and then counterstained by hematoxylin to clearly identify the distinct stages of spermatogenesis. GATA-1-positive cells were found exclusively in cross-sections of seminiferous tubules which corresponded to stages VII, VIII and IX, whereas seminiferous tubules in other stages were negative for GATA-1-staining (Fig. 3B-D). For example, when the spermatogenic stages in cross-sections of seminiferous tubules in 50-week-old mouse were characterized in detail following the criteria as described (Russell et al., 1990), 350 of 357 GATA-1-positive tubules scored were classified in stages VII to IX, 5 were in stage VI and 2 were in stage X. In contrast, no stage VII to IX tubules were found in 116 GATA-1-negative tubule cross-sections examined. The same expression pattern was observed in all of the adult mouse testes examined (not shown). Thus, the expression of GATA-1 in Sertoli cells is intimately linked to specific stages of spermatogenic development, which normally varies from segment to segment in seminiferous tubules. In stages VII-IX, meiosis begins, spermatocytes break into the adluminal compartment from the basal compartment through the blood-testis barrier and mature spermatids are released into the central lumen of the tubule (Russell et al., 1990).

Germ cells suppress GATA-1 expression in Sertoli cells

Based on the preceding observations, we hypothesized that GATA-1 may take part in the regulation of the onset of the spermatogenic cycle by regulating the expression of (a group of) stage-specific genes in Sertoli cells. As an initial test of this hypothesis, we examined the expression of GATA-1 in Sertoli cells in the adult W/W^v mutant mouse testis. The seminiferous

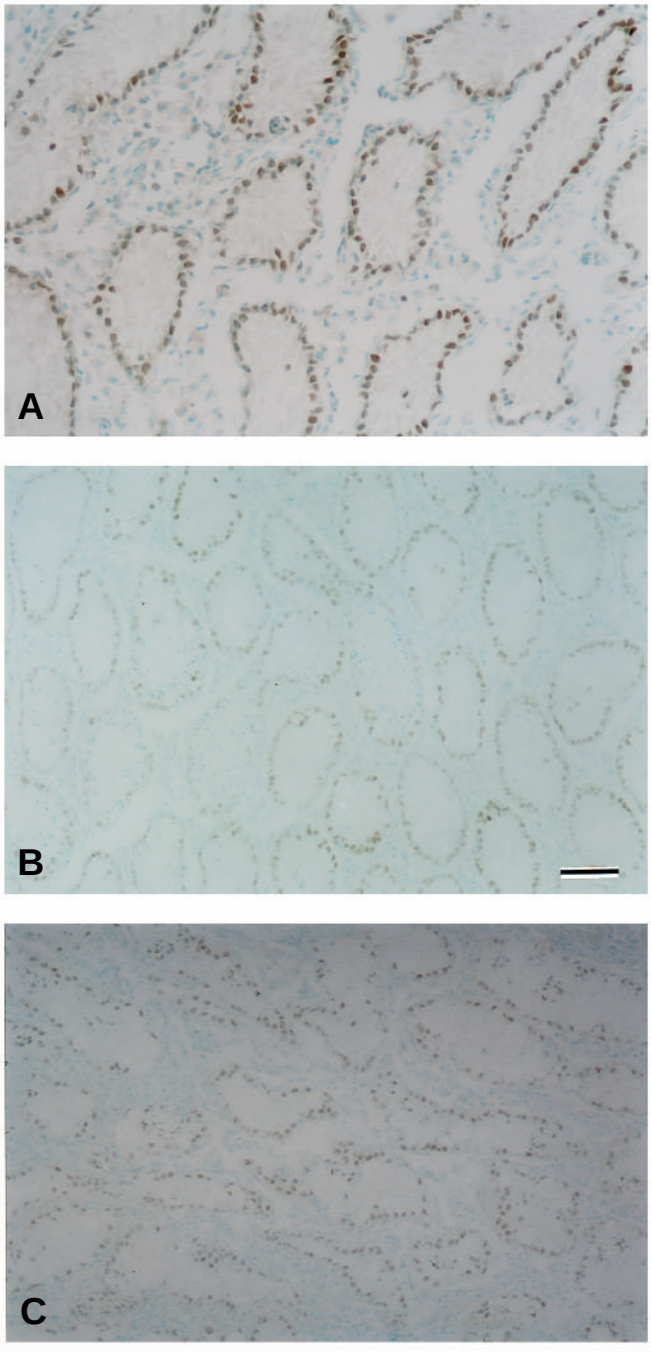


Fig. 4. Germ cells suppress GATA-1 expression in Sertoli cells. The expression of GATA-1 in 10-week-old W/W^v (A), *jsd/jsd* (B) and cryptorchid (C) mouse testes is shown, stained with mAb N-6 as described (legend to Fig. 1). Scale bar indicates 100 μm in (A) and 50 μm in (B,C). All Sertoli cells in the mutant mice stain positively for GATA-1 in all tubules, suggesting that GATA-1 expression in Sertoli cells is repressed by the presence of germ cells after the organ has fully matured (see Results and Discussion).

tubules in adult *W/W^v* mouse do not contain germ cells (Sawada et al., 1991; Kurohmaru et al., 1992), and virtually every Sertoli cell in every seminiferous tubule in these mutant animals express GATA-1 (Fig. 4A). This is in clear contrast to wild-type mice (see Fig. 3A), and demonstrates that the normally cyclic changes in GATA-1 expression in Sertoli cells are lost in the adult *W/W^v* mouse testis.

We also examined the expression of GATA-1 in the testes of *jsd/jsd* (juvenile spermatogonial depletion) mutant mice and cryptorchid mice. The initial wave of spermatogenesis is normal in *jsd/jsd* mice, but after the juvenile stage, type A spermatogonia in the testis fail to continue to differentiate and, as a consequence, only type A spermatogonia and Sertoli cells remain in the seminiferous tubules of adult *jsd/jsd* mice (Mizunuma et al., 1992; Beamer et al., 1988). In cryptorchid mice, germ cells fail to develop due to high body temperature (Nishimune et al., 1978). As in the c-*kit* mutant mice, all of the seminiferous tubules of both *jsd* and cryptorchid mice show uniform GATA-1-positive staining (Fig. 4B,C). These observations, taken together, strongly suggest that GATA-1 expression in Sertoli cells is repressed by the presence of germ cells after the organ has fully matured, since the only common feature shared by the two mutant mouse strains and cryptorchid mice is the virtual (or actual) absence of germ cells.

DISCUSSION

The data presented here clearly demonstrate that GATA-1 is expressed in Sertoli lineage cells and that the expression of GATA-1 in Sertoli cells is induced concomitantly with the first wave of spermatogenesis in prepubertal mouse testis. Sertoli cells are known to proliferate most actively in the fetal testis shortly before birth (reviewed in Russell et al., 1990; Pelliniemi et al., 1993; Gondos and Berndtson, 1993). The rate of the Sertoli cell growth declines linearly thereafter and this proliferation ceases entirely by about 3 weeks after birth. In contrast, development of mature Sertoli cell function (e.g. the formation of the blood-testis barrier, the expression of androgen-binding protein and the production of seminiferous fluid) begins in the testis at about 2 weeks of age in the mouse (Gondos and Berndtson, 1993). The testis of 5-week-old mice contains fully differentiated, mature Sertoli cells exhibiting all these functions. Thus, the timing of the Sertoli cell expansion and acquisition of developmental function shows excellent correlation with the emergence of GATA-1 expression during mouse testis development.

Several lines of evidence suggest that there is normally active communication between germ and Sertoli cells, and that this communication may constitute an important part of the regulation of spermatogenesis (reviewed in de Krester, 1990; Russell, 1993). For example, germ cells can affect Sertoli cell function in in vitro co-culture systems: the synthesis of transferrin in cultured Sertoli cells is known to be influenced by the presence of germ cells (Griswold, 1988) and the secretion of androgen-binding protein from Sertoli cells is stimulated in vitro by direct contact with pachytene primary spermatocytes (Le Magueresse and Jegou, 1988). The present study demonstrates that GATA-1 is expressed exclusively in stages VII, VIII and IX of spermatogenesis in the adult mouse testes, but also that GATA-1 expression is suppressed during other stages; analysis of germ-deficient mouse strains (*W/W^v*, *jsd/jsd* and cryptorchid animals) indicates that this suppression is likely due to a negative feedback signal generated by the germ cells.

In this regard, it is interesting to note that seminiferous tubules lacking GATA-1-positive cells are first observed in approximately 5-week-old mouse testis (see Fig. 2F), and a majority of the elongated spermatids of the first wave appear at the same time. This coincidence further underscores the possibility that the signals that repress fully differentiated Sertoli cell function may be generated by the elongated spermatids. An alternative possibility is that the repressive signals may be generated in Sertoli cells through phagocytosis of residual bodies, which takes place at stage IX of spermatogenic cycle. We are presently testing: (1) whether or not, and if so which, specific stages of germ cells are responsible for the negative regulation of GATA-1 expression in Sertoli cells; and (2), whether or not such negative regulation is mediated by humoral factors or by direct contact of the germ and Sertoli cells.

The most likely targets for GATA-1 activity in Sertoli cells are genes that are specifically transcribed in this lineage. We indeed find consensus GATA-binding sites (Ko and Engel, 1993; Merika and Orkin, 1993) in the promoters of the genes encoding mouse and human transferrin (Idzerda et al., 1989; Gullo et al., 1991), mouse and rat α-inhibin (Su and Hsueh, 1992; Pei et al., 1991), rat androgen-binding protein (Joseph et al., 1988) and the chicken and rat FSH receptors (Wakabayashi and Nishimori, personal communication). These are all major Sertoli cell-specific or -restricted genes, and the expression of these gene products are known to be essential for Sertoli cell-specific function. An intriguing aspect of the expression of these genes is that, in Sertoli cells, they are regulated in a spermatogenic cycle-specific manner. The expression of transferrin mRNA is at the lowest level at the stages IX to X (Morales et al., 1987). The androgen-dependent changes in secretion of a group of proteins (androgen regulated proteins, ARPs) occur specifically at stages VI to VIII of the spermatogenic cycle, and this regulation is largely dependent on a normal germ cell complement to in vitro Sertoli cell culture system (McKinnell and Sharpe, 1992). Thus, several spermatogenic cycle-dependent changes of Sertoli-specific gene expression correlate well with that of GATA-1 expression.

In so far as the regulatory sequences for (at least a subset of) these genes have been defined, GATA-binding sites are found within the boundaries of deletion constructs where their absence abrogates correct transcriptional regulation of these genes (Idzerda et al., 1989; Pei et al., 1991; Su and Hsueh, 1992). Inhibin α genes in the mouse and rat contain two conserved GATA sequences at −117 (proximal) and −151 (distal) (Pei et al., 1991; Su and Hsueh, 1992). The analysis of transcriptional activity of mouse inhibin α promoter deletion constructs transfected into rat granulosa cells (supportive cells in ovary in which α-inhibin and FSH receptor are also expressed) revealed that the region harboring the two GATA sequences (−165 to −98) is essential for basal transcriptional activity of this promoter (Su and Hsueh, 1992). In contrast, Pei et al. (1991) mutated a putative cAMP responsive element (CRE) in the rat inhibin α promoter which lies immediately 5′ to the proximal GATA site (−122; CTGCGTCA) to a GATA consensus sequence (CTGTATCA). Upon transfection of the mutant reporter construct into the rat granulosa cells, they

found not only that the expression of the reporter gene was completely unresponsive to forskolin (due to mutation of the CRE), but also that basal expression of the reporter gene was substantially reduced (to approximately 10% of the parental reporter construct activity). One interpretation of this experiment is that a GATA factor in granulosa cells acts as a negative regulator of α inhibin-directed reporter gene expression by binding to this newly created GATA-binding site. The expression of inhibin α and β-B mRNAs is lowest in stages VII to VIII of the spermatogenic cycle (Bhasin et al., 1988) in mouse Sertoli cells, whereas expression of GATA-1 is restricted to stages VII-IX. Thus, the results are consistent with the possibility that GATA-1 negatively regulates the expression of the inhibin α gene in Sertoli cells. This and other aspects of potential regulation by GATA factors in the testis may become more complex as we learn more about the specific expression pattern of GATA-4, which is also expressed in, but not yet definitively localized within, this organ (Arceci et al., 1993). Whether any or all of these Sertoli-restricted genes are indeed directly controlled by GATA-1 activity is the subject of current investigation.

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