GATA-1 testis activation region is essential for Sertoli cell-specific expression of *GATA-1* gene in transgenic mouse

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Abstract

Background: The erythroid transcription factor GATA-1 is also expressed in Sertoli cells of the testis. The testicular expression of *GATA-1* is regulated in a developmental and spermatogenic stage-specific manner. To further clarify the regulatory mechanisms of testicular *GATA-1* gene expression, we carried out transgenic reporter gene expression analyses.

Results: We found that *GATA-1* expression in Sertoli cells is markedly decreased concomitant with the emergence of elongated spermatids in the seminiferous tubules. Transgenic reporter mouse analyses revealed that a 15 kb *GATA-1* genomic region is sufficient to recapitulate the gene expression profile in Sertoli cells. While the *GATA-1* haematopoietic enhancer and the proximal first exon are included within the 15 kb genomic region, these regulatory elements are not essential for *GATA-1* expression in Sertoli cells. Further analyses using deletion constructs revealed that a 1.5 kb region 5′ to the GATA-1 haematopoietic enhancer is essential for gene expression in Sertoli cells and this region is referred to as the *GATA-1* testis activation region.

Conclusion: These results thus demonstrated that the *GATA-1* testis activation region is essential for Sertoli cell-specific expression of *GATA-1* gene. The 15 kb genomic region is applicable and useful for the expression vector system specific for adult Sertoli cells in stage VII to IX.

Introduction

GATA-1 is a founding member of the GATA family of transcription factors (Evans & Felsenfeld 1989; Yamamoto *et al*. 1990; Engel *et al*. 1991; Tsai *et al*. 1991). GATA factors share two conserved zinc finger domains and bind to a consensus WGATAR sequence. Necessary for cellular differentiation, GATA-1 is expressed predominantly in haematopoietic lineages such as erythroid cells, megakaryocytes, mast cells, and eosinophils (Harigae *et al*. 1998; Hirasawa *et al*. 2002; Ohneda & Yamamoto 2002). We previously reported that GATA-1 is also expressed in Sertoli cells of the testis

(Ito *et al*. 1993; Yamamoto *et al*. 1997), with expression being regulated in a developmental and spermatogenic stage-specific manner (Yomogida *et al*. 1994). Mouse testicular GATA-1 mRNA is transcribed from a testis-specific promoter and first exon (IT exon) located 8 kb 5′ to the haematopoietic first exon (IE exon). The remaining five exons encoding the entire translated region are shared by haematopoietic and testis transcripts (Ito *et al*. 1993).

During the development of mouse testis, the expression of GATA-1 is first identified in nuclei of Sertoli cells at 7-day-old-prepubertal mice (Yomogida *et al*. 1994). Thereafter, GATA-1 is expressed uniformly in all Sertoli cells of the seminiferous tubules until 3 weeks of age. From 5 weeks of age, Sertoli cells lacking the expression of GATA-1 start to appear in certain seminiferous tubules. In adult testis, GATA-1 expression is governed in a spermatogenic stage-specific manner and is restricted

to seminiferous tubules in stages VII, VIII and IX (Yomogida *et al.* 1994). During stages VII and VIII, the incidence of elongated spermatids is limited to the central region of seminiferous tubules. A contact between germ cell and Sertoli cell has loosened and elongated spermatids are released into the lumen at the following stage IX. These observations suggest that *GATA-1* gene expression in Sertoli cells is strictly regulated by a signal originating from germ cells. The results also suggest that *GATA-1* gene regulation in testis is different between juvenile and adult mice. Supporting this contention, in the testes of germ cell-deficient mutant mice, such as W/W^v and *jsd/jsd* mutants, the stage-specific expression profile of GATA-1 is lost, with GATA-1 being expressed uniformly in all Sertoli cells of all seminiferous tubules (Yomogida *et al.* 1994).

Regulation of *GATA-1* gene expression in haematopoietic cells has been studied extensively and characterized in detail (Onodera *et al.* 1997a; Nishimura *et al.* 2000; Shimizu *et al.* 2001; Ohneda *et al.* 2002). Through these efforts, the *GATA-1* gene *h*aematopoietic *e*nhancer (G1HE) and a couple of upstream promoter elements have been identified, demonstrating that *GATA-1* gene expression is regulated by GATA-1 or other GATA factors in conjunction with other yet uncharacterized transcription factors. However, virtually nothing is known about *GATA-1* gene regulation in Sertoli cells. In the present study therefore, we aimed to clarify the regulatory mechanisms of testicular *GATA-1* gene expression.

One technical difficulty in this study was the lack of suitable Sertoli cell lines available (Onodera *et al.* 1997b). Our previous attempt at exploiting primary Sertoli cell cultures was hampered because *GATA-1* gene expression was instantaneously lost after the start of culturing (Onodera *et al.* 1997b). We therefore utilized an *in vivo* reporter assay with the transgenic mouse system. We also examined the correlation between germ cell maturation and the testicular expression of GATA-1. This study delineated the regulatory requirements of *GATA-1* gene expression in Sertoli cells and identified a new activating regulatory element, which we named as *GATA-1 t*estis *a*ctivation *r*egion (G1TAR).

Results

### The presence of elongated spermatids correlates with repression of the *GATA-1* gene in Sertoli cells

In order to examine expression profile of the *GATA-1* gene during germ cell maturation, we first scored the emergence of elongated spermatids and GATA-1 immunoreactive seminiferous tubules in sections prepared from 5-week-old wild-type mouse testes (Fig. 1A–C). Out of the 1167 seminiferous tubules examined, no elongated spermatids were found in tubules positive for GATA-1 (Fig. 1C). In contrast, elongated spermatids were observed in 158 out of 218 GATA-1-negative tubules (asterisks in Fig. 1B,C). We previously observed that GATA-1 is expressed in all seminiferous tubules until 3 weeks of age (Yomogida *et al.* 1994), a stage during which seminiferous tubules lack elongated spermatids. Therefore, these results indicate that GATA-1 expression in Sertoli cells is down-regulated in the presence of elongated spermatids during the initial wave of spermatogenesis.

To further examine correlation between the *GATA-1* gene repression and the appearance of elongated spermatids, we used two germ-line mutant mouse strains, *jsd/jsd* (*j*uvenile *s*permatogonial *d*epletion) and Sl^{17H}/Sl^{17H} (Fig. 1D,E, respectively). These two mutants are unique in that the initial wave of spermatogenesis occurs normally, but further germ cell maturation is defective at the adult stage (Beamer *et al.* 1988; Mizunuma *et al.* 1992; Brannan *et al.* 1992). In the testes of adult *jsd/jsd* and Sl^{17H}/Sl^{17H} mice, expression of GATA-1 was observed uniformly in all seminiferous tubules (Yomogida *et al.* 1994 and data not shown). In contrast, during the first wave of spermatogenesis in these mutants, which occurs normally, GATA-1 was absent in seminiferous tubules containing elongated spermatids (asterisks in Fig. 1D,E). The correlation between GATA-1 repression and the emergence of elongated spermatids was further tested using mice with artificial cryptorchidism. Artificial cryptorchidism mice were prepared and then the cryptorchidism was surgically reversed to induce regenerative differentiation of mature germ cells from spermatogonia.

Figure 1 Suppression of *GATA-1* gene expression in the presence of elongated spermatids. (A) The outline of a section of 5-week-old C57BL/6 mouse testis examined for GATA-1 expression by immunohistochemical analysis. Each enclosure represents a section of seminiferous tubules. GATA-1-positive seminiferous tubules are shaded. 'e' represents seminiferous tubules with elongated spermatids. The central rectangular area corresponds to (B). (C) Out of 1167 seminiferous tubules, none with elongated spermatids expressed GATA-1. (D–F) GATA-1 immunostaining on sections prepared from the testes of 5-week-old *jsd/jsd* (D) and Sl^{17H}/Sl^{17H} (E) mutant mice and adult artificial cryptorchid mice after surgical reversal (F). In D–F, seminiferous tubules with elongated spermatids (asterisks) were negative for GATA-1 (brown nuclei). (G) Uniform expression of GATA-1 in all seminiferous tubules in ICR mice treated with antagonists of GnRH and androgen receptor. Scale bar: 100 μm.
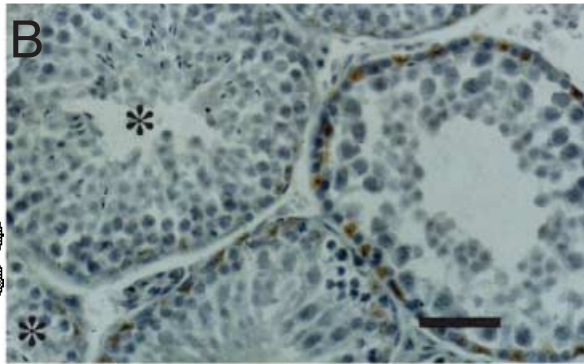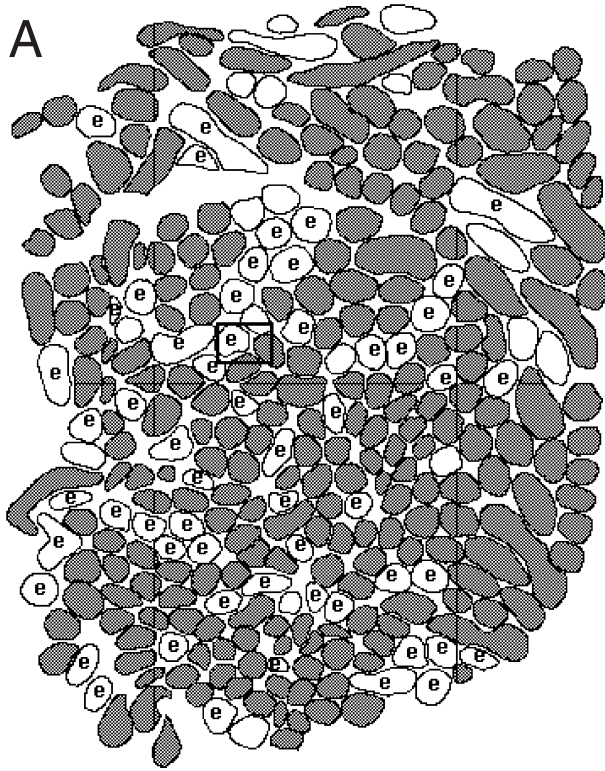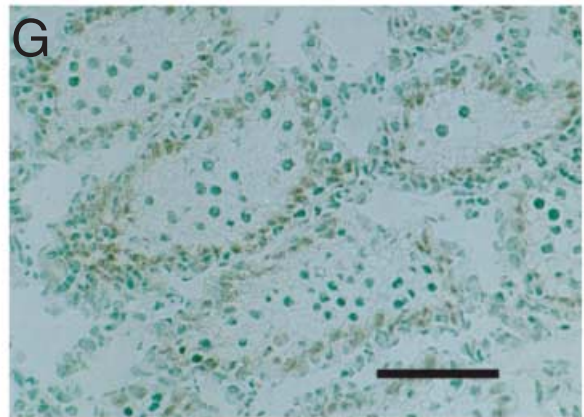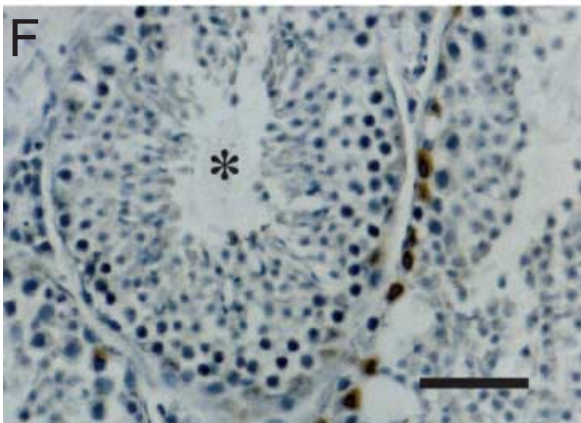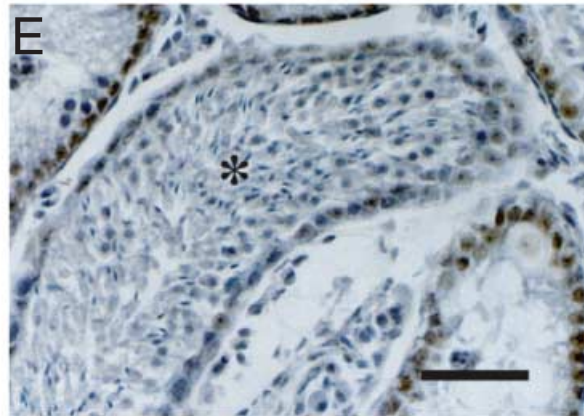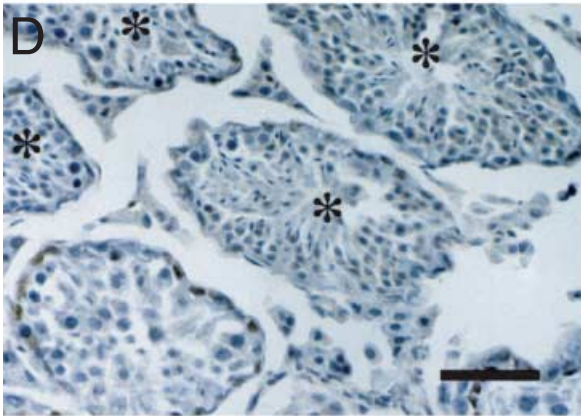
Since GATA-1 is expressed in all seminiferous tubules of mice with artificial cryptorchidism (Yomogida *et al.* 1994), testicular GATA-1 expression was examined 30 days following the surgical reversal, when elongated spermatids began to appear. Consistent with our observation in wild-type mice, repression of GATA-1 was reproducibly seen upon the emergence of elongated spermatids during regenerative differentiation (Fig. 1F).

A common feature of *jsd/jsd* mutants and mice with cryptorchidism is a high expression level of FSH (Follicle-stimulating hormone) or testosterone resulting from lack of the negative feedback signal produced by mature spermatids (Barton *et al.* 1989; Shetty *et al.* 2001; Huckins *et al.* 1981; Mendis–Handagama *et al.* 1990). It was plausible that the constitutive expression of GATA-1 in Sertoli cells in these mutant mice might also be the result of direct activation of *GATA-1* gene transcription by these hormones. In order to test this possibility, the GATA-1 expression was examined in mice treated with antagonists of GnRH and androgen receptor, both of which suppress FSH and testosterone and inhibit germ cell maturation (Kangasniemi *et al.* 1996; Tohda *et al.* 2001; Tadokoro *et al.* 2002). Unexpectedly, the GATA-1 expression was observed in virtually all seminiferous tubules even in mice treated with these antagonists (Fig. 1G; see brown Sertoli cell nuclei). We therefore conclude that stage-specific expression of the *GATA-1* gene in Sertoli cells is not dependent on the hormonal regulation.

Genomic region regulating the specific expression profile of *GATA-1* in Sertoli cells

The stage-specific suppression of GATA-1 in Sertoli cells may be brought by transcriptional repressors, which bind directly to *GATA-1* genomic region or repress specifically the function of transcriptional activators through protein-level interactions. An alternative possibility is that repression is mediated by some post-transcriptional mechanisms. In order to delineate *cis*-acting elements directing *GATA-1* gene expression in Sertoli cells, we examined the testicular expression of the *LacZ* reporter gene under the control of various mouse *GATA-1* genomic regions in transgenic mice.

We first examined LacZ reporter expression in ITintIEintLacZ transgenic mouse lines (Fig. 2A, construct no. 1). This reporter construct contains 15 kb of the mouse *GATA-1* genomic locus spanning 1.5 kb 5′ of the IT exon to the second exon (Fig. 2A). We previously generated two lines of transgenic mice using this construct for testing *GATA-1* expression in haematopoietic cells (lines 114 and 226) (Onodera *et al.* 1997a). The

LacZ reporter expression completely recapitulated *GATA-1* gene expression in erythroid cells (Onodera *et al.* 1997a). Consistent with the GATA-1 expression revealed by immunostaining (asterisks, Fig. 2B), LacZ expression was observed in Sertoli cells of seminiferous tubules in stages VII to IX in both transgenic lines (asterisks, Fig. 2C). The expression was more evident in line 114 (Fig. 2C) than in line 226 (data not shown). LacZ reporter expression (blue in cytoplasm) coincided precisely with GATA-1 expression (brown in nuclei) in seminiferous tubules at stages VII, VIII and IX (Fig. 2D).

Comparing with our previous results of GATA-1 immunostaining in cryptorchidism (Fig. 2E, Yomogida *et al.* 1994), LacZ reporter expression was examined using a mouse with an artificial cryptorchidism by exploiting the ITintIEintLacZ transgenic mouse line. The ITintIEintLacZ transgene directed LacZ reporter expression in all Sertoli cells, recapitulating the expression of GATA-1 in cryptorchidism. These results indicate that the spermatogenic stage-specific expression of GATA-1 in Sertoli cells is regulated at the transcriptional level and that a particular 15 kb region seems sufficient to recapitulate *GATA-1* gene expression in Sertoli cells. The results also suggest that a region controlling *GATA-1* repression in the presence of elongated spermatids may reside within this genomic region.

The haematopoietic enhancer and proximal first exon are dispensable for *GATA-1* gene expression in Sertoli cells

We previously demonstrated that a region spanning 3.9 kb upstream of the haematopoietic IE exon to the second exon (IE3.9int) directs *GATA-1* gene expression in erythroid cells and this region is referred to as the *GATA-1 h*aematopoietic *r*egulatory *d*omain (*GATA-1 HRD*; Onodera *et al.* 1997a; Nishimura *et al.* 2000; Shimizu *et al.* 2001; Ohneda & Yamamoto 2002). The *GATA-1 HRD* contains the *GATA-1 h*aematopoietic *e*nhancer (G1HE), located between 3.9 kb and 2.6 kb 5′ of the IE exon. The ITintIEintLacZ construct (Construct no. 1), containing the entire *GATA-1 HRD*, directed *GATA-1* gene expression in both testis and erythrocytes (Fig. 3). We next examined whether *cis*-regulatory regions essential for the testicular expression of GATA-1 overlap with the *GATA-1 HRD*. To this end, we used IE3.9intLacZ (Construct no. 2), a construct lacking the IT exon and encoding only the *GATA-1 HRD* and 5.4IT[dHEIE]LacZ (Construct no. 3), a construct containing the IT exon, but lacking both G1HE and the IE exon.
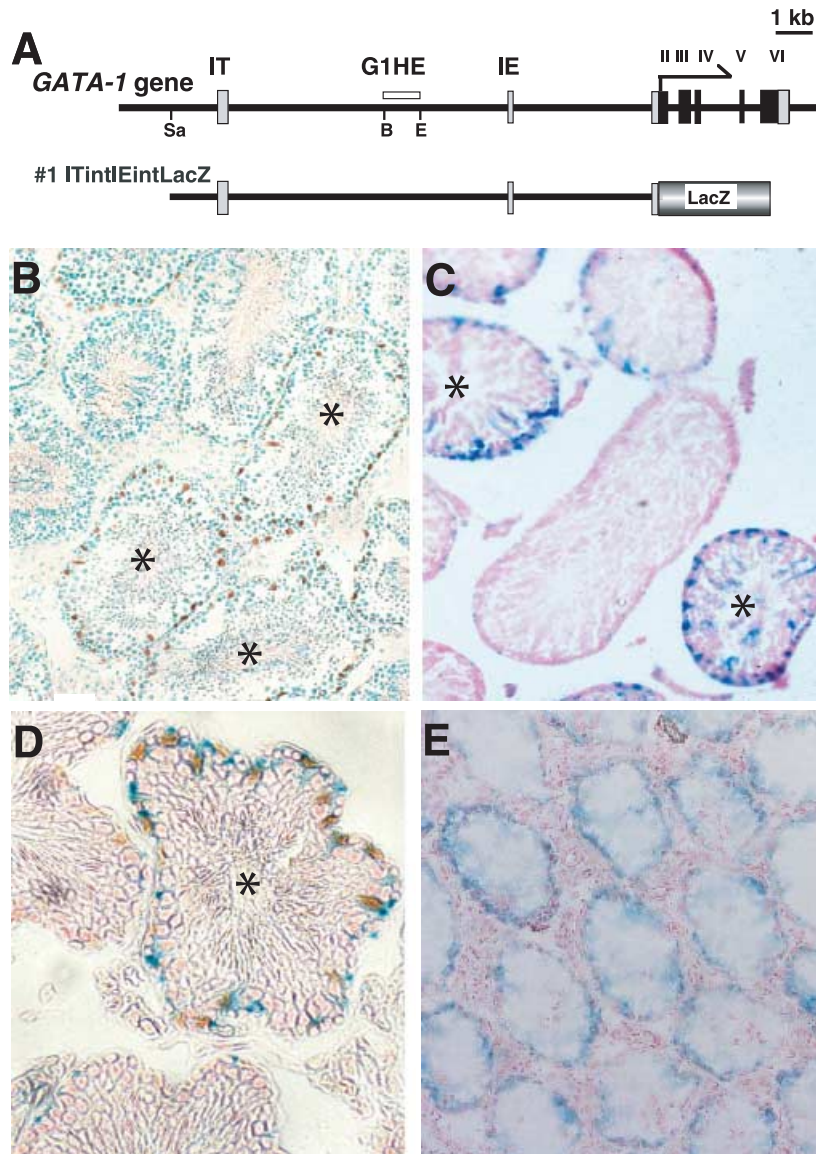
Figure 2 Mouse *GATA-1* genomic locus that recapitulates Sertoli cell- and spermatogenic cycle-specific *GATA-1* gene expression. (A) The mouse *GATA-1* genomic locus and structure of the ITintIEintLacZ (Construct no. 1) reporter gene. Exons are depicted as solid boxes (grey, untranslated; black, translated). Abbreviations of restriction enzyme sites: Sa; *Sac*I, B; *Bam*HI, E; *Eco*RI. (B) GATA-1 immunostaining in testis prepared from wild-type adult mouse. Tubules in spermatogenic stages VII, VIII and IX (asterisks) were positive for GATA-1 immunoreactivity. (C) X-Gal staining in the testis of adult ITintIEintLacZ transgenic mice (line 114). LacZ-positive seminiferous tubules were specifically observed in spermatogenic stages VII, VIII and IX (asterisks). (D) GATA-1- and LacZ-positive Sertoli cells overlapped in adult ITintIEintLacZ transgenic mice (line 226). Endogenous GATA-1 is stained with N6 antibody simultaneously with the X-Gal staining. Note the coincidence of the GATA-1 and LacZ reporter expression. (E) X-Gal staining in the testes of cryptorchid mice bearing the ITintIEintLacZ transgene (line 114). Reporter gene expression was detected in Sertoli cells of all seminiferous tubules.

Among three lines of transgenic mice bearing IE3.9intLacZ, no LacZ expression was detected in Sertoli cells at either postnatal day 14 or in 10–12-week-old-adults (Fig. 3). On the other hand, 5.4IT[dHEIE]LacZ specifically directed LacZ reporter gene expression in prepubertal and adult testes whilst preserving spermatogenic-stage specificity in 7 out of 9 transgenic mouse lines, but no expression was observed in haematopoietic
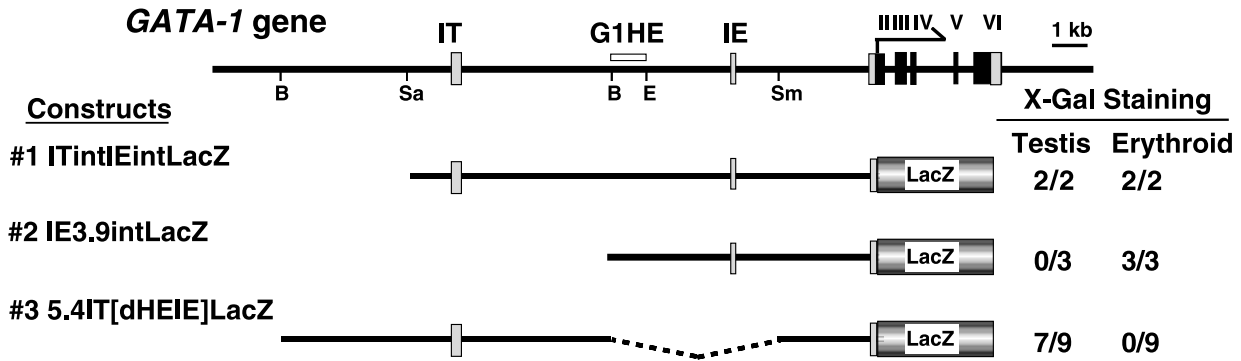
Figure 3 The *GATA-1* haematopoietic enhancer and the proximal first exon are not essential for reporter expression in the testis of transgenic mice. Structures of the reporter constructs (Constructs no. 1 to no. 3) are shown on the left and the results of X-Gal staining in testis and erythroid cells are shown on the right. Abbreviations of restriction enzyme sites: B; *Bam*HI, E; *Eco*RI, Sa; *Sac*I, Sm; *Sma*I.

Table 1 Expression of LacZ reporter in testis and haematopoietic tissues of the transgenic mice

|	Transgenic		LacZ expression				
	line	Copy	E8.5-9.5	E15.5	2 week		Adult
Constructs	number	number	Yolk sac	Liver	Spleen	Testis	testis
No. 1	114	26	+	+	−	+	+
ITintIEintLacZ	226	3	+	+	+	−	+
No. 2	455	16	+	+	+	−	−
IE3.9intLacZ	508	4	+	+	+	−	−
	515	7	+	+	−	−	−
No. 3	0	33	ND	ND	−	+	+
5.4IT[dHEIE]LacZ	804	8	ND	ND	−	+	+
	647	1	−	−	−	+	+
	648	1	ND	ND	−	+	+
	649	1	ND	ND	−	−	−
	653	50	−	−	−	+	+
	658	25	ND	ND	−	−	+
	700	1	−	−	−	+	+
	501	2	ND	ND	−	−	−

ND, not determined.

cells (Fig. 3). These results thus unequivocally demonstrate that specific regulatory domains within the *GATA-1* gene regulate lineage-specific gene expression in erythroid and Sertoli cells. LacZ expression in the haematopoietic and testicular cells tested in this series of experiments during development of the transgenic mouse lines is shown in Table 1.

GATA-1 testis activation region (G1TAR)

Our next attempt was to narrow down the regulatory region essential for *GATA-1* expression in Sertoli cells. We noticed that the two constructs (ITintIEintLacZ

and 5.4IT[dHEIE]LacZ) which directed *GATA-1* gene expression in testis contained two regions of overlap: a region spanning from 1.5 kb 5′ of the IT exon to the 5′ end of G1HE (Region **a** in Fig. 4A) and a region between the *Sma*I site in the first intron and the translation initiation site in the second exon (Region **b**). We carried out X-Gal staining (for β-galactosidase detection; see Experimental procedures) in adult testes from lines of transgenic mice generated using a series of deletion constructs (Constructs nos. 4 to 12). Several constructs containing Region **b** but not Region **a**, such as IE3.9intLacZ, IT[dApHE]IElacZ and IT[dSmHE]IELacZ (Constructs nos. 2, 7 and 8, respectively) failed to direct reporter

Figure 4 G1TAR is essential for reporter gene expression in the testis of transgenic mice. (A) Overlapping *GATA-1* genomic regions between transgenic Constructs nos. 1 and 3 are indicated (Regions **a** and **b**). (B–D) Reporter gene constructs nos. 4 to 12 and results of X-Gal staining and EGFP expression in corresponding adult transgenic mouse testes. Note that a 623-bp region in G1TAR appears to be indispensable for LacZ reporter expression.
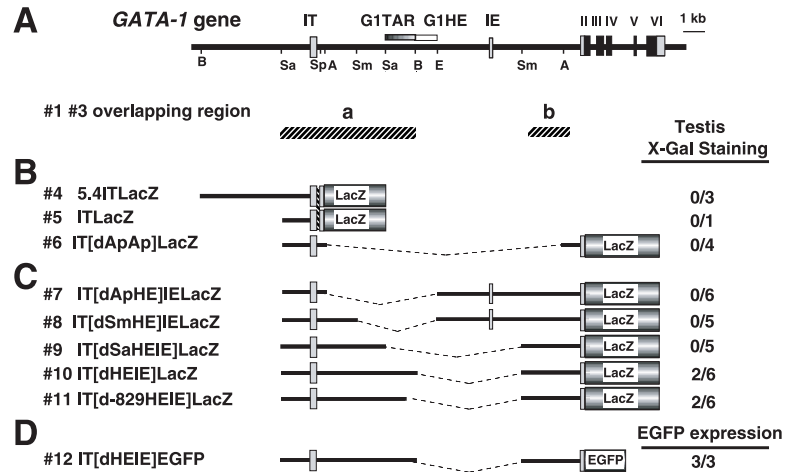
expression in Sertoli cells. We therefore dissected Region **a** to find critical regions for the gene expression in Sertoli cells.

The first series of constructs (Constructs nos. 4, 5 and 6) showed that a region upstream of the IT exon was insufficient for driving reporter gene expression in Sertoli cells (Fig. 4B). In the second series of experiment, we focused on the intergenic region spanning 3′ of the IT exon to the *Sma*I site in the first intron (Fig. 4C). Two out of six lines of IT[dHEIE]LacZ mice (Construct no. 10) showed positive X-Gal staining in the adult testis whereas no positive staining was observed among 5 lines of IT[dSaHEIE]LacZ transgenic mice (Construct no. 9), indicating that a *Sac*I—*Bam*HI fragment immediately 5′- to G1HE contains a critical region for directing reporter expression in Sertoli cells. This *Sac*I—*Bam*HI fragment was therefore designated G1TAR (*GATA-1 t*estis *a*ctivation *r*egion; Fig. 4A). We found that the transgenic expression of IT[dHEIE]LacZ (no. 10, 2/6) was less frequent compared to the expression of 5.4IT[dHEIE]LacZ (no. 3, 7/9), suggesting that additional *cis*-acting regions may exist within the upstream sequence of IT exon.

We then tested a construct with a 3′ deletion in the G1TAR, IT[d-829HEIE]LacZ (Construct no. 11), and observed LacZ reporter expression in Sertoli cells in 2 transgenic lines (Fig. 4C). This revealed that the most 5′ 623-bp region of the G1TAR is critical for the Sertoli cell–specific expression of *GATA-1* gene. Importantly, spermatogenic stage-specific expression of the reporter was conserved even in this truncated construct (data not shown). The IT[dHEIE] (Construct no. 10) region was able to direct reporter expression in Sertoli cells when the *LacZ* reporter gene was replaced with cDNA encoding enhanced green fluorescent protein (EGFP; Construct no. 12, Fig. 4D).

Delayed testicular reporter gene expression in early postnatal development

In order to examine the onset of reporter gene expression in Sertoli cells, X-Gal staining was performed using ITintIEintLacZ transgenic mice at the early postnatal stage. Tubules stained positively by X-Gal were not detectable at day 8 (Fig. 5A), when GATA-1 immunoreactive tubules began to appear. X-Gal positive cells were first detectable, albeit at a very low level, in 11-day-old mice of line 114 (arrowhead, Fig. 5B). Most seminiferous tubules were X-Gal positive at day 14 (Fig. 5C). Semi-quantitative RT-PCR analyses revealed that endogenous GATA-1 expression in the testis was detectable as early as in 3-day-old postnatal mice and became more prominent in 7–10-day-old mice (Fig. 5D). Consistent with the X-Gal staining, the onset of LacZ expression was delayed and not detectable until postnatal day 14 in line 114 (Fig. 5D, upper panels). In line 226, the LacZ transcript at postnatal day 14 was faint but it was clearly detectable in adult (lower panels). These results indicate that the onset of ITintIEintLacZ transgene expression is delayed compared to endogenous GATA-1 expression. In order to test the possibility that the upstream region of IT is responsible for the immediate onset of the *GATA-1* gene during neonatal period, we examined LacZ reporter expression in Sertoli cells at 2 weeks of age using 5.4IT[dHEIE]LacZ (no. 3) transgenic mice. Similar to our observation in ITintIEintLacZ transgenic lines, LacZ expression in Sertoli cells were detected at low level at 2 weeks although the expression is clearly detectable in adult stage. These results indicate that the 5.4 kb upstream region is not sufficient for the reporter expression in early postnatal period.
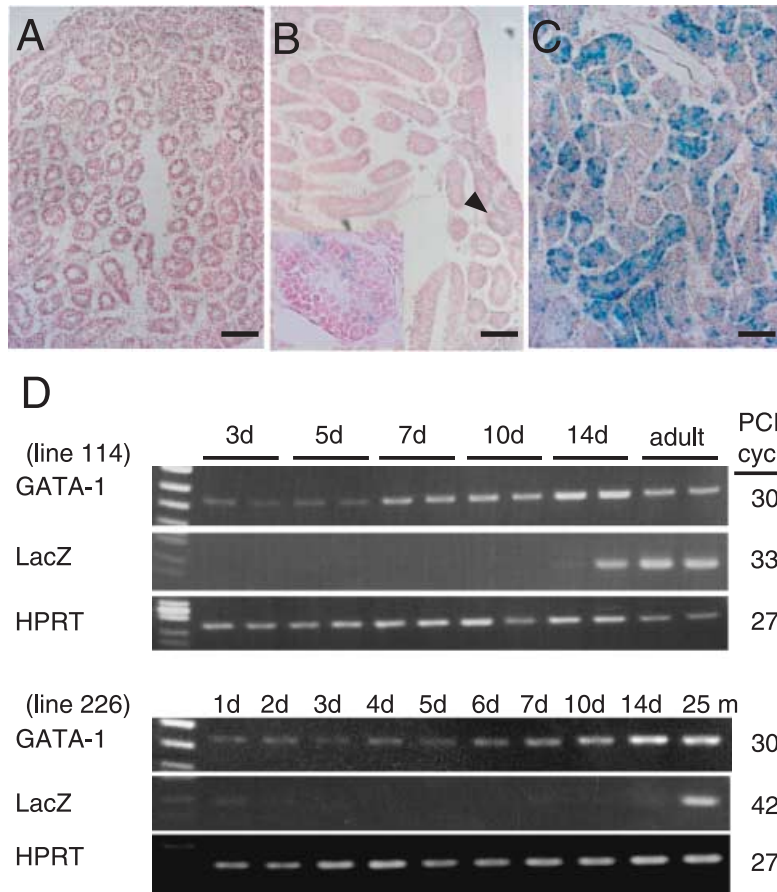
Figure 5 Expression of the ITintIEintLacZ transgene during postnatal development. (A–C) Transgenic *LacZ* reporter (LacZ) expression in testes of 8-day-old (A), 11-day-old (B) and 14-day-old (C) mice were analysed by X-Gal staining. The inset of Figure 5(B) is higher magnification of the LacZ positive seminiferous tubule (arrowhead). Scale bar: 100 μm (D) Endogenous GATA-1 and LacZ expression in testis were analysed by RT-PCR (top three panels, line 114; bottom three panels, line 226). The postnatal days are indicated at the top of the panels (d, day; m, month). The cycle of each PCR is shown on the right.

In summary, the ITintIEint region recapitulates *GATA-1* gene expression in a Sertoli cell- and spermatogenic cycle-specific manner in adult mouse, although it is insufficient for directing precisely the onset of *GATA-1* gene expression in the neonatal stage. The expression profile of endogenous *GATA-1* gene and *LacZ* reporter gene during the postnatal stage in the ITintIEintLacZ transgenic mouse is summarized in Fig. 6.

Discussion

In the present study, a tight correlation was demonstrated between the emergence of elongated spermatids and repression of *GATA-1* gene expression in Sertoli cells during the first wave of spermatogenesis and in the adult spermatogenic cycles. Our transgenic reporter expression study also revealed the presence of a new regulatory domain, G1TAR, directing the testicular expression of the *GATA-1* gene. In the mutant spermatogenesis mouse lines, GATA-1 was expressed in virtually all Sertoli cells, while in wild-type mice it was specifically
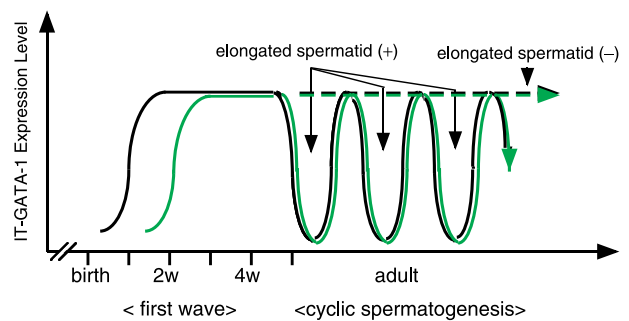

Figure 6 Schematic diagram showing GATA-1 and LacZ expression in testis during postnatal development. Endogenous GATA-1 and transgene mRNA expression are shown in solid black and green lines, respectively. Note that cyclic repression of GATA-1 and the LacZ reporter during spermatogenesis is observed exclusively in the presence of elongated spermatids in adults. In contrast, uniform expression of GATA-1 and LacZ reporter is observed in mice deficient of spermatogenesis (broken lines, elongated spermatid (–)).

expressed in Sertoli cells of seminiferous tubules in stages VII to IX. G1TAR with additional regulatory elements in the 15 kb *GATA-1* locus fully recapitulated the endogenous expression profile of GATA-1 in adult seminiferous tubules. The results unequivocally demonstrate that the spermatogenic stage-specific expression of GATA-1 is regulated at the transcriptional level. Thus, testicular expression of the *GATA-1* gene provides a unique model system for the regulation of stage-specific gene expression in Sertoli cells.

Although FSH and testosterone are produced at high levels in adult *jsd/jsd*, W/W^v, and cryptorchid mice due to the lack of negative feedback regulation mediated by mature spermatids (Barton *et al.* 1989; Shetty *et al.* 2001; Mendis-Handagama *et al.* 1990; De Franca *et al.* 1994), antagonists of androgen receptor and GnRH failed to reduce *GATA-1* gene expression, suggesting that androgens may not be the primary determinant of *GATA-1* gene expression. Alternatively, we envisage that testicular GATA-1 expression may require a tight cell–cell contact between Sertoli cells themselves, since the GATA-1 expression in Sertoli cells increases simultaneously with the formation of tight junctions between Sertoli cells after birth (Cheng & Mruk 2002). In support of this hypothesis, our preliminary data indicates that a high cellular density is required for GATA-1 expression in primary cultures of Sertoli cells (K. Yomogida, unpublished). The stage specific repression of GATA-1 is observed only in the presence of tight cell–cell contact between elongated spermatids and Sertoli cells. These results imply that dynamics of cell contacts during spermatogenic cycles may play a critical role in the regulation of *GATA-1* gene expression. The important observation here is that the *cis*-acting regions responsible for the activation of *GATA-1* gene expression in Sertoli cells cannot be separated from those for the elongated spermatid-mediated repression of the *GATA-1* gene in our transgenic mouse studies. Based on this observation, we surmise that overlapping *cis*-acting regions may govern Sertoli-specific expression and spermatogenic stage-specific repression of the *GATA-1* gene.

Our present and previous transgenic mouse studies successfully dissected *cis*-acting regions responsible for the specific expression of the *GATA-1* gene in haematopoietic and Sertoli cells. Interestingly, whereas G1TAR locates immediately upstream of G1HE, these two regions specifically act on a different promoter, i.e. the IT and IE promoters, respectively. It has been well documented that GATA-1 itself plays a critical role in the regulation of haematopoietic *GATA-1* gene expression (Nishimura *et al.* 2000; Kobayashi *et al.* 2001; Vyas *et al.*

1999). A GATA box in the G1HE and a double GATA motif located in the upstream promoter region of the IE exon are both necessary for GATA-1 expression in erythroid cells (Nishimura *et al.* 2000; Ohneda *et al.* 2002). Similarly, we previously found a double GATA motif in the 5′-flanking region of the IT exon that is functionally important in a transient transfection analysis (Onodera *et al.* 1997b). However, our transgenic reporter analysis revealed that the 5′-flanking region of the IT exon itself is not sufficient to direct Sertoli cell-specific gene expression, indicating that testicular expression of the *GATA-1* gene is not dependent simply on its own gene product.

The region 5.4IT[dHEIE] (in Construct no. 3) directs gene expression efficiently (in 7 out of 9 transgenic lines tested) and exclusively in postnatal stage Sertoli cells. Furthermore, the reporter gene expression retains spermatogenic stage-specific expression of GATA-1. We therefore propose that this genomic region is applicable and useful for the expression vector system specific for adult Sertoli cells in stage VII to IX. Previous transgenic mouse studies indicate that the proximal promoter sequence of mouse Müllerian Inhibiting Substance (*MIS*) gene can direct Sertoli cell-specific gene expression (Giuili *et al.* 1997; Jeffs *et al.* 2001). However, since the expression of MIS is dramatically down-regulated after birth, this regulatory domain is not applicable for the gene expression in adult Sertoli cells. On the contrary, a 5.5 kb genomic fragment of rat androgen-binding protein (*ABP*) gene was reported to direct the gene expression specifically for adult Sertoli cells in transgenic mouse (Reventos *et al.* 1993; Esteban *et al.* 1997). Since this genomic region has not been dissected in detail, we could not compare and identify critical *cis*-acting motifs commonly used in G1TAR.

Recently, regulatory sequences of another Sertoli cell-specific gene, *Pem* homeobox gene, have been studied using reporter transgenic mouse system (Rao *et al.* 2003). The Pem protein is expressed specifically in stage IV to VIII seminiferous tubules partially overlapping the GATA-1 expression. The transgenic mouse study revealed that 0.6 kb 5′-flanking sequence of the *Pem* proximal promoter is sufficient for stage-specific expression of the gene in Sertoli cells. Interestingly, the 0.6 kb sequence contains multiple GATA binding sites, suggesting a possibility that GATA-1 contributes the stage-specific gene expression of the gene. However, unlike *GATA-1*, the *Pem* proximal promoter is dependent on androgen, indicating that the promoter is not entirely dependent on the GATA factors. The results as well as our data indicate that Sertoli cell- and

stage-specific expression of the *GATA-1* and the *Pem* gene is regulated through diverse molecular mechanisms. The 623-bp core region of G1TAR includes binding sites for SRY and the CCAAT/enhancer binding protein (C/EBP), and an E-box, a binding sequence for a basic helix-loop-helix (bHLH) type transcription factors (data not shown). These transcription factors have been reported to regulate gene expression in Sertoli cells (Swain & Lovell-Badge 1999; Dahle & Tasken 2002; Gronning *et al.* 1999; Kim & Griswold 2001). It is interesting to study whether the two genomic regions share a common, fundamental molecular basis governing stage specific-gene expression in adult Sertoli cells.

In contrast to the well-assigned roles of GATA-1 during haematopoiesis, the roles of testicular GATA-1 have not yet been clarified. Importantly, at least two other GATA family members, GATA-4 and GATA-6, are also expressed in Sertoli cells (Ketola *et al.* 1999). GATA-4 is expressed both embryonic and postnatal stage and the expression is independent of spermatogenic cycle, whereas GATA-6 expression is observed from late foetal to adult stage. In addition, FOG-1 and FOG-2, multizinc finger proteins that interact with N-terminal zinc finger of GATA factors are expressed in Sertoli cells (Ketola *et al.* 2002; Robert *et al.* 2002). Of interest, recent report has shown that FOG-1 co-localizes with GATA-1 in adult Sertoli cells in a spermatogenic stage-specific manner, indicating that GATA-1—FOG-1 interaction contributes expression of stage-specific genes in adults (Ketola *et al.* 2002).

GATA motifs have been identified in the regulatory regions of several Sertoli-specific genes, such as the FSH receptor, Inhibin α-subunit, Inhibin/Activin β-B-subunit and the Müllerian inhibiting substance (MIS) (Kim & Griswold 2001; Feng *et al.* 1998, 2000; Beau *et al.* 2000). Both transactivation of reporter genes by GATA-1 in transient transfection studies using Sertoli cell lines and GATA-1 binding to GATA motifs using testicular cell extracts have been executed nicely in these reports. The absolute requirement of GATA-1 in the regulation of target genes, however, has not been demonstrated rigorously *in vivo* yet. It is still possible that the GATA motifs are occupied by other testicular GATA factors. In developmental stage, impaired Sertoli cell differentiation is observed in mice with *GATA-4* mutation that abrogates the interaction with FOG as well as in a null mutant mouse of *FOG-2* (Tevosian *et al.* 2002). Taken together, it seems likely that the GATA and FOG combinations play unique roles in developmental stage- and spermatogenic cycle-specific gene expression in Sertoli cells.

Experimental procedures

Animals

C57BL/6, ICR, and W/W^v mice were supplied from the Shizuoka Experimental Animal Farm. BDF1 mice were supplied from CLEA Japan. Sl^{17H}/Sl^{17H} and *jsd/jsd* mice were maintained in the Experimental Animal Facility in the Research Institute for Microbial Diseases at Osaka University. The artificial cryptorchidism mice were prepared from adult C57BL/6 mice and transgenic mice as described (Nishimune *et al.* 1978).

Hormone treatment

The Gonadotropin-Releasing Hormone (GnRH) antagonist Nal-Glu ([Ac-D-D^2-Nal^1, D^{4}Cl-Phe^2, D^3-Pal^3, Arg^5, D-Glu^6(AA), D-Ala^{10}]GnRH) was kindly supplied by Dr H. K. Kim of the Contraceptive Development Branch of the NICHD, Bethesda, MD. It was suspended in distilled water at a concentration of 8 mg/ml and given to ICR mice through mini-osmotic pump (ALZET Model 2004; ALZA Corporation, Palo Alto, CA, USA). The pump was placed under the back skin and delivered a dose of 2500 µg/kg/day. Flutamide, an androgen-receptor antagonist, was given by subcutaneous implantation of a pellet delivering 1.2 mg/day (Innovative Research of America, Sarasota, FL, USA). The Nal-Glu-Flutamide treatment was continued for 4 weeks (Kangasnieni *et al.* 1996; Tohda *et al.* 2001; Tadokoro *et al.* 2002).

Immunohistochemical analysis

Anti-GATA-1 monoclonal (N6) antibody was used for GATA-1 immunostaining (Yomogida *et al.* 1994; Onodera *et al.* 1997a). Diaminobenzidine was used as chromogen, while nuclei were counterstained with haematoxylin to identify elongated spermatids. In some experiments, methyl green was used for counterstaining.

Reporter construction and generation of transgenic mouse lines

Various reporter genes were constructed using restriction enzyme sites in the regulatory regions of the *GATA-1* gene. IT[d-829HEIE]LacZ (Construct no. 11) was constructed using a Kilo-Sequencing Deletion Kit (TaKaRa Biomedicals). DNA fragments were purified from the plasmid DNA and transgenic mice were generated by standard strategies (Hogan *et al.* 1994). Founder mice were screened by PCR with a set of primers, beta-gal1 (5′-ACCGACTACACAAATCAGCG-3′) and beta-gal2 (5′-CAAC-CACCGCACGATAGAGA-3′) designed to detect LacZ. Founder mice were crossed with BDF1 and male offspring were examined for further analyses. The copy numbers of the transgenic lines were estimated by real-time PCR using an ABI PRISM 7700 Sequence Detector (Perkin Elmer). The PCR amplicon of genomic DNA prepared from heterozygotes of a LacZ-knock-in mouse was used as a standard (one copy number).

X-Gal staining of tissues and embryos

For X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactosidase) staining, tissues (livers, spleens, and testes from embryos and 2-week-old mice) were fixed with 1% formaldehyde, 0.2% glutaraldehyde, and 0.02% Nonidet P-40 in phosphate-buffered saline (PBS; pH 7.3) for 30 min at room temperature, except for adult testes that were fixed for 2 h. After washing with PBS, tissues were incubated in PBS containing 20% sucrose at 4 °C overnight. Samples were embedded with Tissue-Tec 4583 OCT compound (Sakura Finetechnical, Tokyo, Japan) and rapidly frozen. Cryosections were stained overnight with X-Gal staining reagent (2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 1 mg/ml X-Gal in PBS) at 37 °C. Nuclei were counterstained with Kernechtrot staining solution.

RT-PCR analysis

Total RNA from the testes of ITintIEintLacZ transgenic mice was prepared by ISOGEN (Nippon Gene, Toyama). The cDNAs were synthesized with Superscript II reverse transcriptase (Life Technologies). IT primer (5′-CGTGAAGCGAGACCATCGTC-3′) along with G1exIIIR primer (5′-CCGTCTTCAAGGTGTC-CAAGAACGT-3′) were used for GATA-1 mRNA expression, whereas IT primer along with LacZR-2 primer (5′-GCAAC-GAAAATCACGTTCTTGTTGG-3′) were used for LacZ reporter expression. The hypoxanthine guanine phosphoribosyl transferase (HPRT) amplicon was used to standardize the amount of cDNAs used.

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