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Conserved Structure, Regulatory Elements, and Transcriptional Regulation from the GATA-1 Gene Testis Promoter[1]

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Transcription factor GATA-1 was first identified in erythroid cells, but was later shown to also be expressed in Sertoli cells of the mouse testis. GATA-1 transcription in testis initiates from a different first exon (exon IT) than the erythroid mRNA (transcribed from exon IE). To begin to address the question of how expression of GATA-1 might be differentially regulated in Sertoli and erythroid cells, we have cloned and determined the structure of the IT promoters of both the rat and mouse GATA-1 genes. The transcription regulatory mechanism(s) controlling the synthesis of exon IT-derived mRNA was investigated by transfection of wild-type and mutant reporter genes, with and without co-transfected GATA factor expression plasmids, into either fibroblasts or Sertoli cell lines. Two GATA binding sites in the IT promoter were found to be required for GATA factor-mediated activation in fibroblasts: GATA-IT-directed reporter gene expression was activated only after co-transfection with GATA-1, implying that transcriptional activation of GATA-1 in the testis might be at least partially mediated through these GATA regulatory elements. We also found that the endogenous GATA-1 gene was silent in primary culture and two different Sertoli cell lines, and that the repression of co-transfected GATA-IT reporter genes could not be relieved by forced expression of GATA-1 in Sertoli cells. Thus the GATA-IT promoter may be under the control of a regulatory network in Sertoli cells which involves both positive and negative regulation of transcription, and conserved GATA motifs found in the IT promoter may be required for transducing these effects.

Key words: GATA-1, promoter, Sertoli cell, testis, transcription factor.

The GATA factors consist of a small family of regulatory proteins which bind to "GATA" core consensus sequences through a highly conserved Zn finger DNA-binding domain (*1-7*). The GATA factor family was originally identified through the cloning of chicken homologues of the founding member of the family, GATA-1 (*5*). Later, each GATA factor was named (*e.g.*, GATA-1, GATA-2, and GATA-3) with a single letter prefix to denote species origin (*8*). The expression of GATA-1 has been found in erythroid, megakaryocytic and mast cells (*2, 9, 10*) and the function of GATA-1 has been shown to be essential for erythropoiesis, as embryonic stem cells harboring a targeted disruption of the GATA-1 locus failed to contribute to the erythroid lineage (*11-13*). GATA-2 was also found to be expressed in erythroid, megakaryocytic, and mast cells, and in addition, this factor is also expressed in undifferentiated hematopoietic progenitor cells as well as in several non-hematopoietic cell lineages (*5, 14*). Consistent with a more immature hematopoietic expression profile, targeted disruption of the GATA-2 gene resulted in a loss of all hematopoietic lineage cells (*15*).

We recently showed that GATA-1 is also expressed in Sertoli cells of the mouse testis (*16, 17*). GATA-1 mRNA in Sertoli cells is transcribed from a testis-specific first exon (IT) located approximately 8 kbp 5′ to the erythroid first exon (IE) (*16*). The remaining five exons which encode the mGATA-1 protein are used in common by the mature testis or erythroid mRNAs. The expression of GATA-1 in mouse 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 the expression of

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GATA-1 becomes highly restricted to spermatogenic stages VII, VIII, and IX in the adult mouse testis. In contrast, virtually every Sertoli cell in germ-deficient mice (W/W^v, jsd/jsd, or cryptorchid mutants, all of which lack significant number of germ cells) expresses GATA-1, indicating that the expression of GATA-1 is under the negative influence of the maturing germ cells (*17*).

Transcription of the mouse GATA-1 gene starts from multiple transcription initiation sites in erythroid cells. Two consensus GATA motifs (located approximately 600 bp 5′ to the cluster of erythroid initiation sites) were found to display modest transcriptional regulatory activity (*18, 19*). Functional GATA motifs were also found to effect both the chicken and human GATA-1 gene erythroid transcripts (*18, 20*). Once transcription of GATA-1 is initiated at exon IE, newly formed GATA-1 protein is thought to promote transcription from the IE promoter in a positive auto-regulatory loop. However, the trigger for initiation of GATA-1 transcription in erythroid cells has not yet been identified. In this regard, it is interesting to note that the expression of GATA-2 has been shown to precede the expression of GATA-1 in hematopoietic progenitor cells (*21, 22*). Ottolenghi and colleagues have also recently reported that GATA-1 transcription is initiated approximately equally from both the IT and IE exons in immortalized mouse hematopoietic progenitor cells when cultured in the presence of erythropoietin (*23*).

To examine the generality of the observation that the IT exon might be used for GATA-1 gene expression in other species, and to begin to define how Sertoli cell-specific expression of GATA-1 might be regulated, we cloned the mouse GATA-1 gene and determined its structure around the IT exon. Since the rat testis is the best characterized system for studying spermatogenic cycle-specific expression of Sertoli proteins (*24*), we also isolated and examined the rat gene as a comparative species in order to better define the structure and regulation of the IT promoter. The two rodent IT exon and promoter regions were found to be highly conserved, and in particular, we identified through this analysis two *cis*-regulatory motifs containing consensus GATA binding sites in both the mouse and rat IT promoters.

The activity of the mouse IT promoter, specifically with respect to the putative regulatory GATA binding sites, was examined by co-transfection assays into a fibroblast cell line (QT6) and into mouse Sertoli cell lines (TM4 and 15P1). Firefly luciferase (LUC) reporter constructs containing IT promoter elements were co-transfected along with expression plasmids to determine whether or not transcription factors GATA-1 or GATA-2 might be directly involved in the regulation of the GATA-IT promoter. The results show that mGATA-1 stimulates reporter gene expression markedly in fibroblasts, whereas it does not activate the same promoter after transfection into either Sertoli line, where endogenous GATA-1 expression is also repressed. These results suggest the presence of a complex regulatory network that acts to actively repress transcription from the IT promoter in Sertoli cells.

EXPERIMENTAL PROCEDURES

RNA Blot Hybridization Analysis—Male Wistar rats (Funabashi Experimental Animal Farm) were used for these studies. Total RNA was prepared from rat testis and bone marrow cells using the acid guanidine/phenol/chloroform method (*25*). Poly(A)$^+$RNA was selected with oligo (dT)-Latex beads (Takara Shuzo) and 3 μg of poly(A)$^+$ RNA was electrophoresed on 1.0% agarose gels containing 1.1 M formaldehyde. After transfer to a nitrocellulose filter (Hybond-C extra, Amersham), the RNAs were hybridized to ^{32}P-labeled rat erythroid-type GATA-1 cDNA probe [generously provided by Dr. C. Noda (*26*)].

5′-RACE Analysis—5′-RACE (Rapid Amplification of the cDNA Ends) analysis was carried out using the 5′-AmpliFINDER RACE procedure following the manufacturer's instructions (Clontech). The sequence and position of the two specific primers are as follows: rg131 primer (5′-GCAGGGTAGAGCGCCGTCTTGCTATA-3′) is complementary to the rat GATA-1 cDNA sequence corresponding to nt 408 to 433, whereas rg132 primer (5′-GGCATCCTCATGACTGGGACATACAG-3′) is complementary to that of nt 439 to 464 [numbering from the start site of the rGATA-1 cDNA clone (*26*)]. Using 2 μg of poly(A)$^+$RNA from 5-week-old rat testes or bone marrow, cDNA was synthesized using the rg132 primer. After adapter ligation, the first PCR cycle was carried out with the rg132 and adapter primers. Nested PCR amplification was then carried out with the rg131 and adapter primers. All amplified DNA was digested with *Eco*RI and *Sac*I, isolated from agarose gels, and ligated into pBluescript KS(+) (Stratagene).

Genomic DNA Blot Hybridization Analysis—High molecular weight DNA was prepared from the liver of a 2-week-old rat using standard procedures (*27*). DNA (20 μg) was digested with *Bam*HI and *Xba*I, subjected to electrophoresis on a 0.8% agarose gel, then transferred onto a Biodyne nylon membrane (Pall). The membranes were hybridized with two probes: one (the IT probe) was the *Eco*RI-*Sma*I fragment derived from the longest RACE clone pT28, which harbors the testis specific first exon (see "RESULTS"); and the other (IE probe) was an *Eco*RI-*Bam*HI fragment containing the IE exon (0.6 kb) (see Fig. 2). Hybridization and washing conditions were as described (*28*).

Isolation of Genomic DNA Clones—Plaque hybridization screening was performed as described (*29*). A rat genomic DNA library in λEMBL3 (*30*) was screened with the full-length rat erythroid GATA-1 cDNA, resulting in the isolation of λEMBL3-1. The IT exon, however, was not found in this clone or in clones isolated from two other rat genomic libraries. We therefore constructed a size-restricted genomic library: high molecular weight rat genomic DNA was completely digested with *Bam*HI and, after selection by sucrose density gradient centrifugation of approximately 6-kb fragments, the DNA was ligated to λZAP expression vector (*Bam*HI predigested arms, Stratagene). The resulting recombinant phages were packaged *in vitro* using Gigapack II Gold packaging extract (Stratagene). This library was screened by using the pT28 probe. The clone λZBam6, containing the IT exon, was isolated from this "bookmobile."

The physical relationship of the λEMBL3-1 and λZBam6 clones was examined by PCR using rat high molecular weight DNA and specific primers. Primer bp1 (5′-AAGCTGAAGGTAAGGGGTAAGCCG-3′) corresponds to the 3′ end of λZBam6, and primer bp2 (5′-GTAT-

CATGGGCCTGTTCTATTCTC-3′) corresponds to the 5′ end of λEMBL3-1. The PCR-amplified 392 bp band was cloned into pBluescript and analyzed. DNA sequence determination was carried out by using Taq polymerase cycle sequencing system (ABI Japan). Isolation of the mouse genomic clone (λ11c) was described previously (*16*).

RNase Protection Analysis—A 372 bp *Xba*I-*Mbo*II fragment of the rat GATA-1 gene contains a part of the intron between exons IT and IE, the entire IT exon and the 5′ region of the IT exon. This fragment was cloned into pBluescript KS(+). After linearizing the plasmid by digestion with *Xba*I, antisense RNA probes were generated using phage T3 RNA polymerase in the presence of [α-^{32}P]-CTP. Total RNA (50 μg) from 3-week-old rat testes was hybridized to the probe for 16 h at 46°C in 30 μl (in 40 mM PIPES pH 6.4, 1 mM EDTA, 400 mM NaCl, and 80% formamide), then the sample was digested with RNases A and T1. The samples were then digested with proteinase K, extracted with phenol/chloroform and subjected to electrophoresis on a 6% polyacrylamide gel containing 7 M urea.

Plasmid Construction—Reporter plasmids used for evaluation of presumptive mouse exon IT promoter activity were constructed by the combined use of PCR amplification of genomic clones and subsequent subcloning into a master construct. A plasmid containing mouse exon IT (pBE0.8) was first prepared by subcloning a 0.8 kbp *Bgl*II-*Eco*RI fragment of mouse genomic clone λ11c into pBluescript KS(+). A fragment containing a part of the mouse IT promoter (−290 to +82) was generated by PCR amplification of pBE0.8 with PE-2 and T7 primers. PE-2 (5′-TGCC-TTCTGAGTACACAAATGACG-3′) corresponds to a sequence in the middle of mouse exon IT. This fragment was digested with *Sac*I (in the polylinker) and ligated into pBluescript KS(+) which had been digested with *Sac*I and *Sma*I to generate pGH0. pGH1 was then generated by removing the *Xba*I fragment (5′ polylinker to −115) from pGH0. Reporter plasmids pGL-A and pGL-B were generated by transferring the *Sac*I-*Hin*dIII fragments of pGH1 and pGH0 into pGL2-Basic (Promega), respectively.

To prepare other GATA-IT reporter constructs, an *Eco*RI-*Xba*I fragment of λ11c (nt −442 to −115) was subcloned into pBluescript (pXE0.3). This plasmid was digested with *Xba*I and *Kpn*I (in 3′ polylinker) and the fragment was isolated and ligated into pGL-A, resulting in pGL-C. pGL6 and pGL3 were prepared by inserting the −865 to −115 *Xba*I fragment into pGH1 and the −1440 to −865 *Sac*I-*Xba*I fragment along with −865 to −115 *Xba*I fragments into pGH1, respectively. pGL-D and pGL-E were generated by transferring *Hin*dIII-*Sac*I fragments from pGL6 and pGL3 into pGL2-Basic, respectively. Finally, pGL-F was generated by cloning the most gene-proximal 4 kb *Sac*I fragment into pGL-E.

Deletion mutants within the mouse IT promoter were produced as follows. Three PCR primers (all of which contained a *Sac*I linker for subsequent subcloning) were designed for this purpose; del1 (5′-TTTGAGCTCGCCCT-TATCTCAAGGCCAGCA-3′), del2 (5′-AGGGAGCTCAG-CATGTTCAATCAGGAATGC-3′) and del2f (5′-TTTGAG-CTCCTGGTCCGAATGCTTCTGG-3′). PCR amplification was carried out using these primers and GL primer 2 (Promega) with pGL-A as a template. pDEL-1 lacks the distal GATA motif, while pDEL-2 lacks both GATA motifs; pDEL-2f lacks 50 bp more sequence than pDEL-2. These constructs were generated by ligating the amplified fragments into pGL2-Basic. All the fragments generated in this study using PCR were verified by DNA sequencing analysis.

Expression plasmids pEFmGATA-1 and pEFmGATA-2 were prepared by ligating mGATA-1 or mGATA-2 into pEF-BssHII plasmid, a modified version of pEF-BOS (*31*). Molecular cloning of an active, full-length mGATA-2 cDNA will be described elsewhere.

Site-Directed Mutagenesis of the Reporter Plasmid pGL-A—The two GATA motifs in the IT promoter consist of two TTATCT sequences. A mutant reporter plasmid (mu-pGL-A) containing two TTAAAT motifs instead of the wild-type sequences was constructed using a PCR-based site-directed mutagenesis procedure. The primer used was muGATA (5′-GGGTTTAAAAACTGTTAAATCGGGGCCG-CCCTTAAATCAAGGCCAGCATGTTCAA-3′), which contains a *Dra*I linker. PCR was carried out with the muGATA primer and GL primer 2, again using pGL-A as the template. mu-pGL-A was generated by inserting this amplified fragment (after digestion with *Dra*I and *Hin*dIII) and the upstream *Sac*I-*Dra*I fragment (51 bp) derived from pGH1 into pGL2-Basic.

Co-Transfection/Transactivation Assay—Quail fibroblast cell line QT6 was maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (Cell Culture Laboratories). Mouse Sertoli cell line TM4 was maintained in DMEM/Ham's F-12 nutrient mixture (1:1) with 5% horse serum (Gibco BRL) and 2.5% fetal bovine serum. Another Sertoli cell line 15P1 (*32*) was maintained in DMEM with 10% fetal bovine serum. Transfection was carried out by standard $CaPO_4$ precipitation (*27*). QT6 cells were seeded in 24-well dishes 24 h before transfection at a density of 1×10^4 cells/well. Various reporter plasmids were transfected along with pEFmGATA-1, pEFmGATA-2, or pEF-BssHII. As an internal control, 50 ng of pENL (a plasmid constitutively expressing β-galactosidase) was included in all reactions.

TM4 cells were seeded in 6-well dishes 24 h before transfection at a density of 5×10^5 cells/well, and 2.5 μg of the various reporter plasmids were transfected along with 2 μg of pEFmGATA-1 or 2 μg of pEF-BssHII in the presence of 500 ng of pENL. Cytoplasmic extracts were prepared 36 h after transfection, and luciferase activity and β-galactosidase activity were determined using Pica-Gene (Toyo Ink) or β-galactosidase enzyme assay (Promega), respectively. 15P1 cells were transfected using the lipofection reagent DOTAP (Böehringer Mannheim). All transfection experiments reported here were performed on at least three separate occasions in duplicate. Relative LUC activity was determined by normalizing luciferase activity to β-galactosidase as the internal control.

Electrophoretic Gel Mobility Shift Assay (EGMSA) and Immunoblot Analysis—QT6 cells were transfected with pEFmGATA-1 or pEFmGATA-2 by standard $CaPO_4$ precipitation, and nuclear extracts were prepared as described (*33*). A mixture of protease inhibitors was added to the buffers in mGATA-2 analysis [final concentration of each inhibitor was 50 μg/ml of antipain-dihydrochloride, 10 μg/ml of (4-amidinophenyl)-methanesulfonylfluoride, 1 μg/ml of aprotinin, 100 μg/ml of chymostatin, 0.5 μg/ml of leupeptin, and 0.7 μg/ml of pepstatin]. Oligonucleotides

containing the GATA motifs of the mouse IT promoter (5′-CTGTTATCTCGGGGCCGCCCTTATCTCAAG-3′) and mouse α-globin promoter [MαP (*3*)] were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. EGMSA was carried out as described previously (*22*).

Anti-GATA-2 monoclonal antibodies (clone Nos. 67 and 71) cross-reactive to hGATA-2 were prepared by using cGATA-2 protein expressed in *Escherichia coli* as antigen (*22*). Immunoblot analysis of mGATA-1 expression in the nuclear extracts was performed as described previously by using N6 anti-mGATA-1 monoclonal antibody (*16*).

Primary Sertoli Cell Culture—Sertoli cells were isolated from 2-week-old C57BL/6 male mice as described by Cheng *et al.* (*34*) with slight modifications. Testes were removed and placed in a serum-free culture medium (Medium A) consist-ing of DMEM/Ham's F-12 mixture (1:1), containing 1.2 g/liter sodium bicarbonate, 15 mM HEPES, 10,000 U/liter penicillin, 100 mg/liter streptomycin, and 250 mg/liter amphtericin B. The testes were decapsulated and incubated for 15 min at 32°C with periodic pipetting in Medium A supplemented with 1 M glycine, 2 mM EDTA, 20 IU/ml DNase I, and 0.003% soybean trypsin inhibitor (w/v) to remove Leydig cells and other interstitial tissue. To remove the myoid cells, the seminiferous tubules were then incubated for 15 min at 32°C in a enzyme solution containing 0.05% collagenase/dispase (w/v) and 0.003% soybean trypsin inhibitor in Medium A. The tubules were then incubated one more time in the enzyme solution for 30 min.

The resulting cell suspension was diluted with Medium A, Sertoli cell clumps were settled by unit gravity and germ cells were left in the supernatant. The precipitate was resuspended in Medium A (Sertoli cell-enriched suspension), then passed through a 100 μm mesh cell strainer (FALCON 2360), which allowed 5-10 cell aggregates to pass through. Sertoli cell aggregates were then washed four times in Medium A (collected by mild centrifugation, 800 × *g*) and resuspended in Medium A for immunocytochemical analysis and primary culture. The Sertoli cell-enriched suspension consisted of more than 90% Sertoli cells when assessed by using Hoechst 33342 dye (*35*).

Sertoli cell aggregates were seeded in a chamber slide (Lab-Tek 177399, Nunc) at a density of 5×10^5 cells per chamber (1 ml) in Medium A supplemented with 10 μg/ml human insulin, 5 μg/ml human transferrin, and 2.5 ng/ml human EGF. After 2 days of incubation at 35°C with 5% CO_2, the residual germ cells were removed by hypoosmotic shock in 10 mM TrisHCl (pH 7.4) for 3 min. At this point, the Sertoli cell-enriched culture consisted of approximately 99% Sertoli cells. The chamber slides were incubated for one more day to allow the cells to recover. Immunocytochemical analysis was performed using indirect immunoperoxidase as described (*17*).

RESULTS

GATA-1 Temporal Expression in the Rat Testis—We first examined the expression profile of GATA-1 mRNA in rat testis during post-natal development by RNA blot hybridization using a full-length rat erythroid-type GATA-1 cDNA (*26*) as probe. We previously showed that murine GATA-1 mRNA was most abundantly expressed in the 2-week-old mouse testis, but that thereafter mGATA-1 expression declined and was expressed at only very low levels in the adult (*16*). The peak of rGATA-1 expression in the testis was at 3 weeks after birth and declined thereafter (Fig. 1), but expression of rGATA-1 mRNA was also readily detectable in the adult rat. The expression of GATA-1 in the rat testis coincides with the time of Sertoli cell maturation (*36*), as was the case for mouse Sertoli cell development (*17*). Taken together, these observations suggest that GATA-1 makes a specific functional contribution to vertebrate male reproduction.

Molecular Cloning and Structural Analysis of Rodent GATA-1T Promoters—While we previously showed that the expression of mGATA-1 is regulated by a specific first exon (IT) in mouse Sertoli cells (*16*), the presence of a IT exon had not been confirmed in other species, while the structure of the promoter region immediately flanking exon IT has not been defined in any species. In order to pursue the analysis of Sertoli cell-specific transcriptional regulation of GATA-1, we cloned the rGATA-1 gene for comparison with the mouse.

First, rat testis GATA-1 cDNAs were cloned using RACE (Rapid Amplification of the cDNA Ends; see "EXPERIMENTAL PROCEDURES"). Poly(A)$^+$RNA isolated from 5-week-old rat testes was reverse-transcribed, and an adapter was ligated to the synthesized first strand cDNAs. The cDNAs were then amplified by nested PCR using the rg132, rg131, and adapter primers. As a result, multiple bands ranging from 390 to 500 bp in size were found after agarose gel electrophoresis of the final PCR product (not shown), and these individual bands were then isolated and subcloned. Sequence analysis of several dozen independent clones

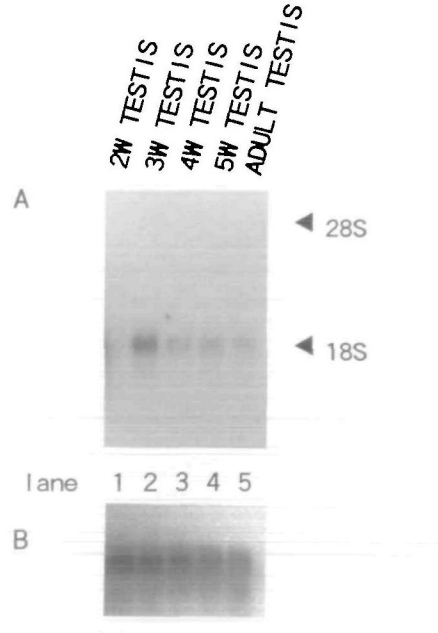


Fig. 1. **Expression of GATA-1 in the rat testis.** A: RNA blot hybridization analysis was performed using poly(A)$^+$RNAs isolated from the testes of 2-week-old (P14; lane 1), P21 (lane 2), P28 (lane 3), P35 (lane 4), and adult (lane 5) rats. RNAs were transferred to a nitrocellulose filter and the filter was hybridized to a rat GATA-1 cDNA probe. The filter was then washed and exposed for autoradiography. The positions of the 18S and 28S ribosomal RNAs are indicated with arrowheads. B: The same filter was washed and re-hybridized with a β-actin cDNA probe.

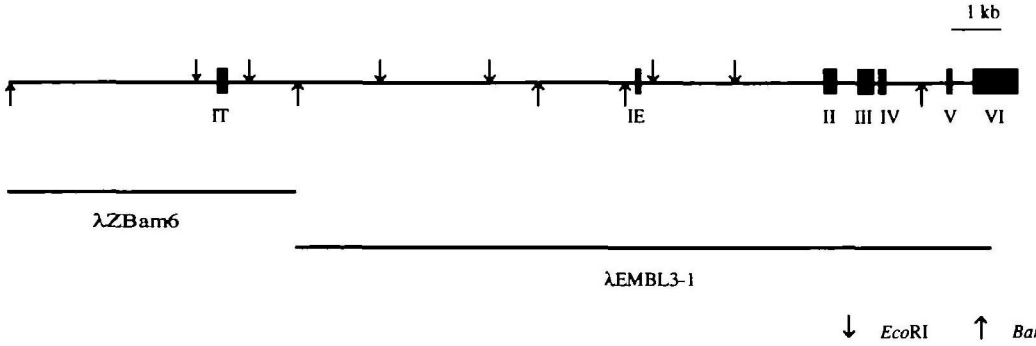


Fig. 2. **Structure of the rat GATA-1 gene.** Boxes show the relative size and position of each exon in the rat genome. Exons are numbered with Roman numerals. The two phage clones (λEMBL3-1 and λZBam6) were found (by PCR) to be immediately adjacent to one another, as described in the text.

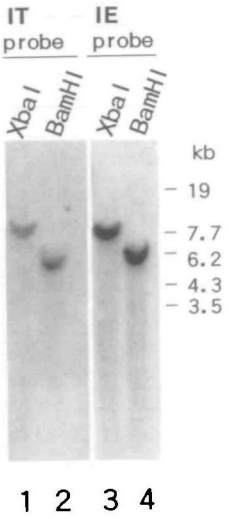


Fig. 3. **Genomic DNA blot hybridization analysis of the rat GATA-1 gene.** High molecular weight DNA from rat (20 μg/lane) was digested with *Xba*I (lanes 1 and 3) or *Bam*HI (lanes 2 and 4), electrophoresed on an agarose gel and transferred to a nylon membrane. The membrane was hybridized to the IT probe (lanes 1 and 2) derived from IT exon sequence of the longest 5′-RACE clone pT28, or to the IE probe (lanes 3 and 4) derived from the genomic subclone which contains exon IE. The sizes of the DNA fragments are indicated.

revealed that the amplified cDNAs have an overlapping sequence with high similarity to that of mouse exon IT, thereby indicating that rGATA-1 is expressed in rat testis through utilization of a testis-specific first exon.

We next screened a rat genomic DNA library (*37*) to isolate the rat genomic GATA-1 locus (λEMBL3-1). While this clone spanned the region coding for exons IE to VI, it extended only 6 kb 5′ to exon IE, and lacked IT (Fig. 2). Screening of two additional rat genomic libraries also failed to identify genomic clones containing rat exon IT. The result of genomic DNA blot experiments using the IT screening probe showed that exon IT exists as a single copy in the rat genome (Fig. 3). As the analysis also showed the presence of exon IT within a 6 kbp *Bam*HI fragment (Fig. 3), we isolated and cloned this fragment directly (see "EXPERIMENTAL PROCEDURES"). A genomic clone containing the IT exon (λZBam6) was obtained from this final screen. Sequence analysis of PCR-amplified rat genomic DNA demonstrated that the two phage clones were contigu-

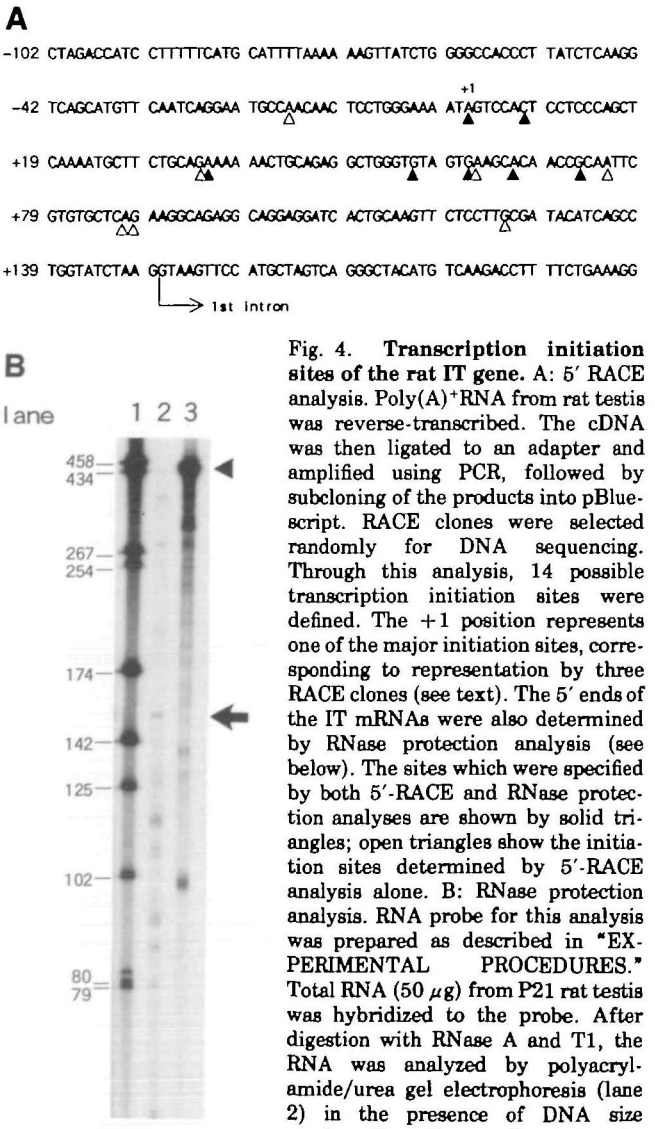


Fig. 4. **Transcription initiation sites of the rat IT gene.** A: 5′ RACE analysis. Poly(A)$^+$RNA from rat testis was reverse-transcribed. The cDNA was then ligated to an adapter and amplified using PCR, followed by subcloning of the products into pBluescript. RACE clones were selected randomly for DNA sequencing. Through this analysis, 14 possible transcription initiation sites were defined. The +1 position represents one of the major initiation sites, corresponding to representation by three RACE clones (see text). The 5′ ends of the IT mRNAs were also determined by RNase protection analysis (see below). The sites which were specified by both 5′-RACE and RNase protection analyses are shown by solid triangles; open triangles show the initiation sites determined by 5′-RACE analysis alone. B: RNase protection analysis. RNA probe for this analysis was prepared as described in "EXPERIMENTAL PROCEDURES." Total RNA (50 μg) from P21 rat testis was hybridized to the probe. After digestion with RNase A and T1, the RNA was analyzed by polyacrylamide/urea gel electrophoresis (lane 2) in the presence of DNA size markers (lane 1) or undigested probe (lane 3). The marker fragment sizes are indicated beside lane 1, the arrow indicates the position of the major protected band and the triangle indicates the position of the undigested probe. Note that various lengths of protected fragments were observed in this analysis, again indicating the presence of multiple initiation sites for IT transcription.

ous to one another over an intervening *Bam*HI site lying between the IT and IE exons (see Fig. 2).

These studies show that the sequence organization of the rGATA-1 gene is quite similar to that of mGATA-1; exon IT is located approximately 8 kbp 5′ to exon IE in the rat genome, and the locations of other exons are also well conserved in comparison to those of the mouse gene (Fig. 2). Thus, the presence and relative position of a testis-specific GATA-1 first exon is common within these two widely diverged rodent species.

Transcription of Testis-Type GATA-1 Starts from Multiple Initiation Sites—We next sought to determine the position of transcription initiation for testis-type GATA-1 transcript(s). Since the structure of the mouse and rat GATA-1 genes is similar and since it is significantly easier to obtain large quantities of RNA from rat than from mouse testis, we first determined the transcriptional initiation site(s) for the rat exon IT. From the previous 5′-RACE analysis we picked 33 random cDNA clones, which were then sequenced. Fourteen independent 5′ ends were identified as possible transcription start sites from this analysis (Fig. 4A).

In parallel to the 5′ RACE cloning and sequencing of rat exon IT, we carried out RNase protection analysis. To this end, a genomic DNA fragment spanning the presumptive rat exon IT was first subcloned. Radiolabeled antisense RNA was transcribed from this plasmid, and the resulting RNA probe (431 bp) was hybridized to total RNA isolated from 3-week-old rat testis. A number of unique protected fragments ranging in size from 79 to 151 nt (Fig. 4B) were identified in this analysis, while fragments larger than 151 nt corresponded to non-specific bands in the parallel undigested probe lane; of the *bona fide* protected fragments, the 151 bp fragment consistently displayed the most intense signal. Consistent with the intensity of the 151 bp band in the RNase protection analysis, 3 out of the

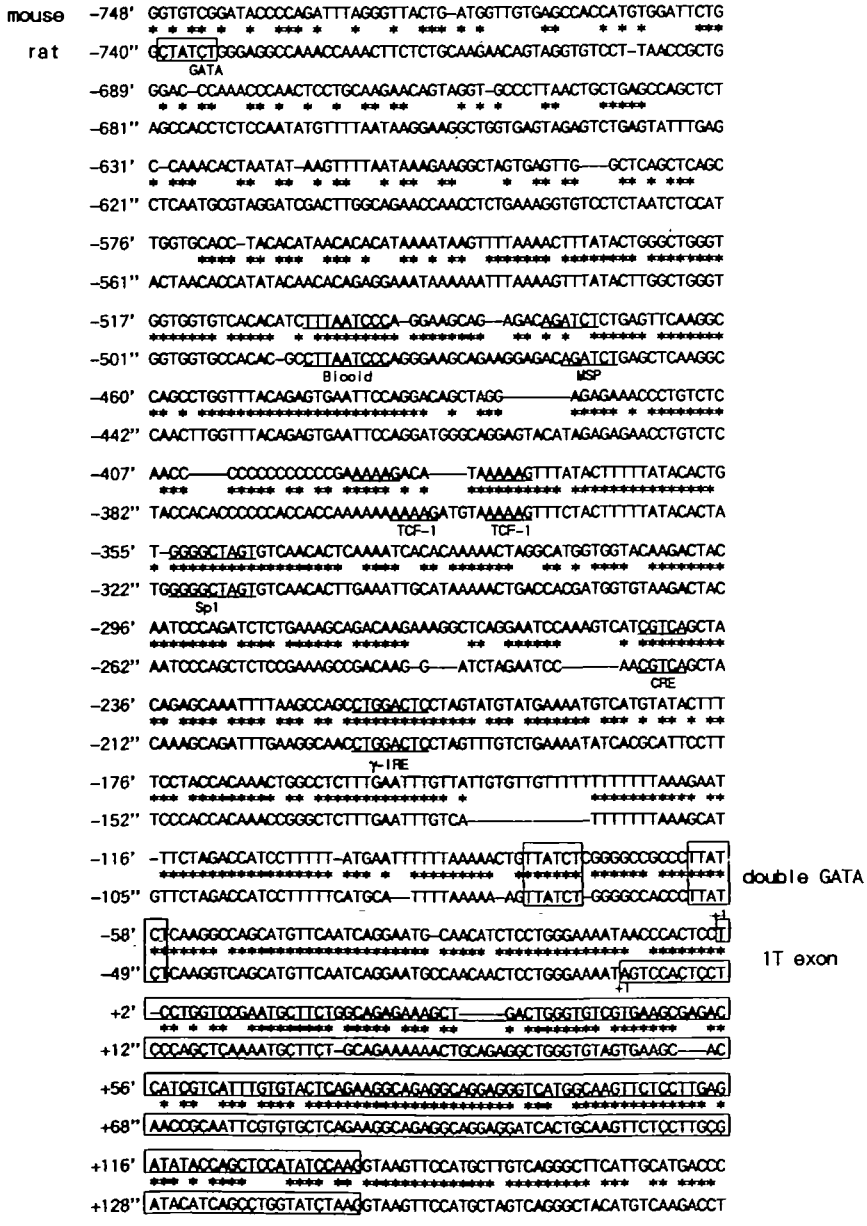


Fig. 5. **Sequence comparison between mouse and rat IT promoter and upstream regions.** Sequences of the mouse (upper) and rat (lower) IT exons, 5′ flanking regions and parts of the first intron are shown. The asterisks indicate nucleotide identities. The +1 position corresponds to the major transcription initiation sites determined by RNase protection and 5′ RACE analyses (rat; Fig. 4) or primer extension analysis (mouse), respectively. GATA motifs are shaded, while the IT exons are boxed. Putative transcription factor binding sites that are conserved in both mouse and rat sequences are underlined.

33 5′-RACE clones also had 5′ ends that corresponded to this position, and we therefore assume that this is the principal start site. Other positions determined by 5′-RACE and RNase protection analyses also showed a high degree of coincidence (see Fig. 4A). We therefore concluded, based on a summary of the RNase protection and RACE results, that transcription from IT starts from multiple initiation sites, but displays clear preferences. We also determined the transcription start site of mouse exon IT using these same methods, and the largest protection products showed excellent coincidence with that of the most prominent rat IT exon start site (data not shown). We therefore assigned that position as the major start site for mouse IT transcription initiation (see below).

Sequence Comparison of the Mouse and Rat GATA-IT Promoters—The sequences flanking the mouse and rat IT promoters were sequenced (from nt positions −1588 to +513 and from −822 to +586, respectively). When the two promoters were compared with one another (Fig. 5), they showed strong sequence similarity in the −540 to +160 region (nucleotide numbering corresponding to the mouse sequence); the homology score in this region is approximately 80%, while the two diverge significantly outside of this region. Like the IE promoters, the IT promoters lack a canonical TATA box. One potentially important finding from the sequence comparison was the discovery of two highly conserved consensus GATA motifs [*i.e.*, two TTATCT sequences at −69 and −53 (rat) or

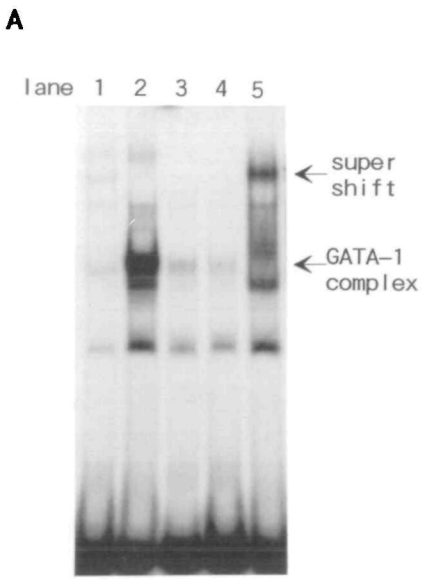
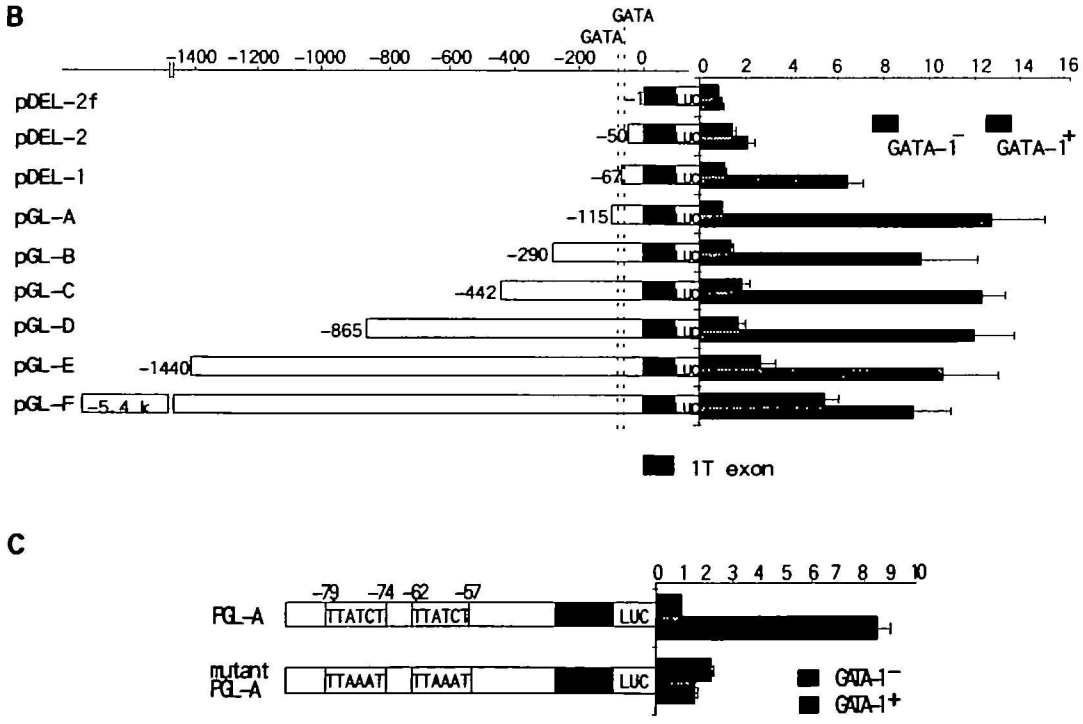


Fig. 6. ***Trans*-activation of the GATA-IT promoter in QT6 cells by exogenous mGATA-1.** A: Expression of exogenous mGATA-1 in QT6 cells. QT6 cells were transfected with pEFmGATA-1 (lanes 2–5) and nuclear extracts were prepared. The extracts were incubated with ^{32}P-labeled oligonucleotide containing the rat double GATA motif in the absence (lane 2) or in the presence of a 50-fold (lane 3) or 200-fold (lane 4) molar excess of unlabeled probe. An anti-mGATA-1 monoclonal antibody (N6; Ref. *16*) was also added to a reaction mixture containing only the extract and probe (lane 5). The mixtures were subjected to electrophoresis and followed by autoradiography. The DNA:protein complex containing mGATA-1 and oligonucleotide probe, and the supershifted band produced after inclusion of the monoclonal antibody, are indicated by arrows. Lane 1 shows an experiment with the nuclear extract from mock-transfected cells. B: Various mGATA-IT promoter/luciferase reporter gene constructs (100 ng) were transfected into QT6 cells along with 50 ng of pEFmGATA-1 (GATA-1$^+$) or control pEF-BssHII vector (GATA-1$^-$). LUC reporter activities were quantitated 36 h after transfection. All experiments were performed in duplicate, and the values shown represent an average of three transfection experiments. Increases in activation are shown relative to comparison with pGL-A (GATA-1$^-$). Standard deviations are indicated by bars. Schematic diagrams of the reporter constructs are shown on the left side of the figure. Numbers indicate the sequence position endpoint of the deletion relative to the major transcription initiation site (Fig. 5). The position of the double GATA motif is indicated by dotted lines. Note that pDEL-1 lacks one of the two sites of the double GATA motif, while pDEL-2 and pDEL-2f lack both sites. C: LUC reporter activities in the mutant reporter constructs. The TTATCT to TTAAAT mutations were introduced into the double GATA motif of pGL-A.



−79 and −62 (mouse)] lying very close to the major transcription initiation sites of the two IT promoters. This suggested that the transcription of exon IT, as has been demonstrated for exon IE, could be regulated by GATA factors.

Through this analysis, we also identified a number of consensus transcription factor binding sites for other transcription factors that are conserved between the mouse and rat promoters; *e.g.*, CRE [cAMP responsive element (*38*)], Bicoid (*39*), TCF-1 [T cell-specific transcription factor (*40*)], Sp1 (*40*), γIRE [interferon-γ response element (*41*)], and a major sperm protein (MSP) binding site (AGATCT at −479) (*42*) were identified (Fig. 5). The specific functional contribution of these putative transcription factor binding sites to Sertoli cell-specific transcription from the mGATA-IT promoter was not investigated further here.

Exon IT Is Transcribed in Bone Marrow Cells—Tsai *et al.* (*19*) previously reported that transcription from mouse exon IE starts from multiple initiation sites. Consistent with this observation, 5′-RACE analysis using primers corresponding to the rGATA-1 sequence used in common by testis- and erythroid-type transcripts and substrate poly(A)$^+$RNA isolated from rat bone marrow cells showed the presence of many distinct 5′ ends of rat exon IE (data not shown).

To our surprise, when we analyzed the sequence of 5′-RACE clones isolated in this analysis, we found exon IT sequences among these clones, in addition to the (anticipated) IE clones. Among 29 recombinants isolated from the 5′-RACE cloning of rat bone marrow RNA, there were 2 clones harboring exon IT sequence, 26 clones containing the IE exon and 1 clone starting from within the second exon. While it seems possible that the testis RNA preparation could be contaminated with blood, it seemed exceedingly unlikely that the converse might be true. This result therefore shows that the IT promoter is also used in rat bone marrow cells, albeit perhaps only infrequently. Conversely, we also found exon IE utilization in the testis after thorough analysis of the 5′-RACE clones obtained from that RNA (see above). Among 33 rat testis 5′-RACE clones analyzed, 4 contained exon IE sequences, 26 corresponded to IT sequence, and 3 clones started from within the second (common) exon. Thus, utilization of the IT and the IE promoters does not appear to be exclusive within either Sertoli or erythroid cells, but rather dictates a preference.

The mGATA-IT Promoter Is Activated by GATA-1—To assess whether the GATA motifs found by comparative sequence analysis in the IT promoter might be functionally required for transcription, we first examined the binding of GATA-1 to these sequences. To this end, EGMSA was carried out using nuclear extracts prepared from quail fibroblast cells transfected with an mGATA-1 expression plasmid ("EXPERIMENTAL PROCEDURES") and a synthetic oligonucleotide probe corresponding to the mGATA-IT promoter double GATA motif. A band containing the GATA-1/DNA complex was clearly observed (Fig. 6A, lane 2); this gel shift band was specifically competed by unlabeled probe (lanes 3 and 4) and was ablated by including anti-mGATA-1 monoclonal antibody (lane 5). These results demonstrate that GATA-1 can bind *in vitro* to the exon IT promoter GATA site(s).

We next examined the activity of the mGATA-IT promoter in functional co-transfection assays. For this purpose, reporter plasmids were constructed by fusing the firefly luciferase downstream of nucleotide +82 within exon IT, leaving various IT promoter fragments to drive expression of the fusion gene (see Fig. 6B). The reporter constructs were transfected into QT6 cells along with either pEFmGATA-1 (expressing GATA-1) or control plasmid pEF-BssHII.

As shown in Fig. 6B, pGL-A to pGL-F (all containing the double GATA motif) show increased luciferase activity when co-transfected with pEFmGATA-1. The longest construct (pGL-F) shows the highest level of basal activity, and a similar GATA-1-induced activity to all the others, whereas maximally induced expression levels (10- to 14-fold) were achieved using the smallest promoter examined in this series, containing only 115 bp of the GATA-IT promoter (pGL-A). This activation is completely abolished after deleting the mGATA-IT promoter sequence to −50, which deletes both of the IT GATA sites (pDEL-2), while retention of one GATA site (in pDEL-1) leads to a 6-fold higher reporter activity in the presence of the

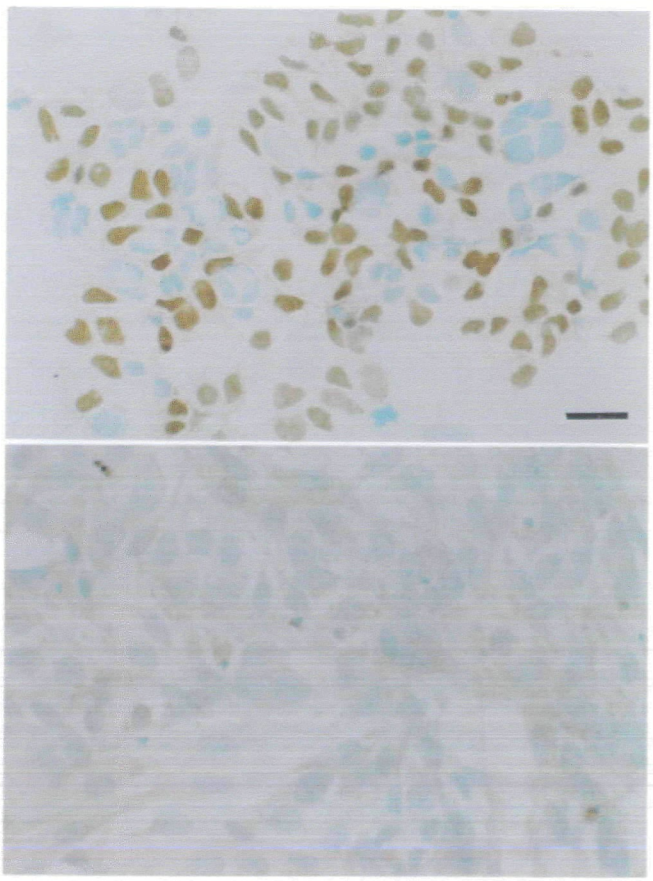


Fig. 7. **Extinction of GATA-1 expression in primary cultures of Sertoli cells.** Primary Sertoli cell culture was initiated using a Sertoli cell-enriched fraction of P14 mouse testis cells. GATA-1 expression was examined at the beginning of the culture (top) and after 3 days (bottom), using immunocytochemical staining by the anti-mGATA-1 antibody. Diaminobenzidine was used as a chromogen, so that positive signals are seen as brown color. Sertoli cell nuclei were stained with methyl green. Scale bar corresponds to 25 μm.

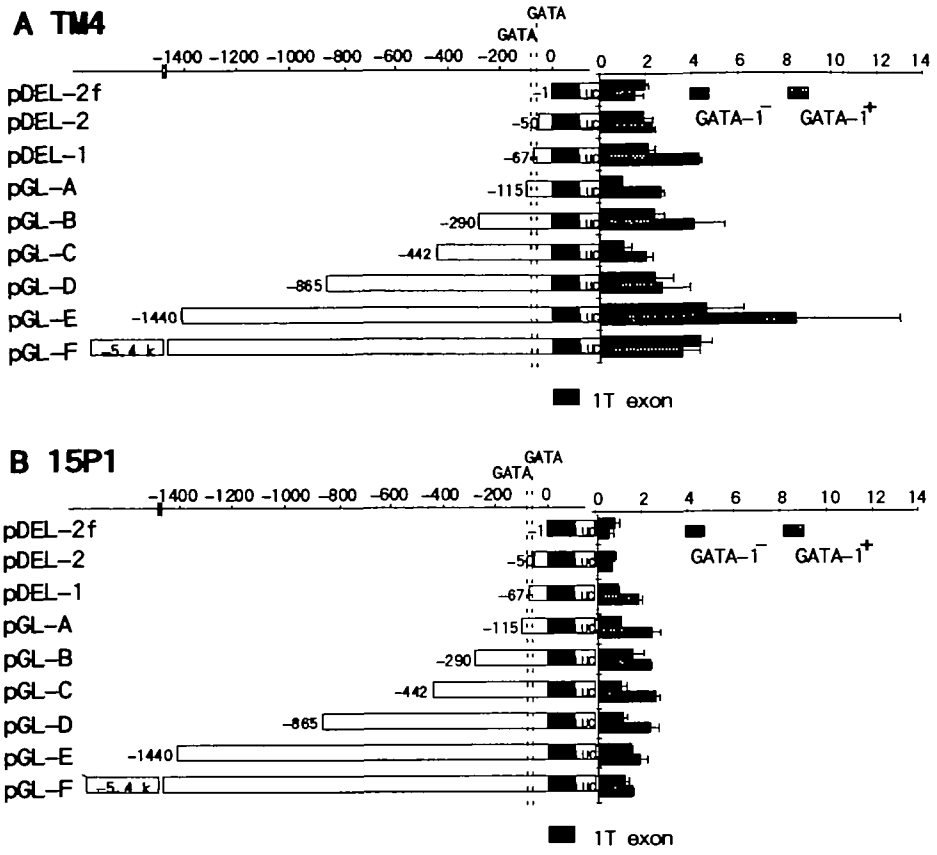


Fig. 8. **Activation of the IT promoter in TM4 and 15P1 cells is repressed.** A and B: Activity of the IT promoter is repressed in TM-4 and 15P1 cells. The same set of IT promoter-LUC constructs as described in Fig. 6B were transfected into TM4 (A) or 15P1 (B) cells with either pEFm-GATA-1 (GATA-1$^+$) or with a control plasmid (GATA-1$^-$). All transfections were performed in duplicate and values represent the average of three transfections.

GATA-1 expression plasmid than in its absence. Since GATA-1 exhibits strong binding to the double GATA motif (above), and as there are no other consensus GATA sequences present in the −50 to −115 region of the promoter describing the uninducible *versus* fully inducible phenotypes, these results taken together strongly suggest that GATA-1 binds to the double GATA motifs to transcriptionally activate the mGATA-IT promoter.

The direct functional contribution of the double GATA motif to IT promoter activity was finally examined by introducing site-specific mutations into the two GATA consensus binding sites. The GATA sites (sense strand TTATCT) in the reporter plasmid pGL-A were changed to TTAAAT, and a co-transfection/transactivation experiment was performed, again using QT6 cells. The mutant pGL-A plasmid showed essentially identical activity to that of an irrelevant control plasmid (Fig. 6C) and was not affected by co-transfection with the GATA-1 expression vector, conclusively demonstrating that the two GATA motifs in the mGATA-IT promoter are essential *cis*-regulatory elements for activation of the IT promoter.

Expression of GATA-1 Is Repressed in Sertoli Cells— The previous results clearly demonstrated the contribution of the promoter GATA sequences to the transcriptional regulation of GATA-IT. To address the question of how the IT promoter is regulated in Sertoli cells, we first attempted to identify a Sertoli cell line that expressed GATA-1. However, we repeatedly failed to identify such a GATA-1-positive Sertoli cell line among several cell lines surveyed (data not shown). We therefore attempted to generate primary Sertoli cell cultures using 2-week-old mouse testis, where all Sertoli cells express GATA-1 (*17*), and found, as expected, that almost all of the primary explanted Sertoli cells displayed GATA-1 staining immediately after isolation (Fig. 7, top). However, all of these primary explants lost the capacity to express GATA-1 within only three days of culture (Fig. 7, bottom), thereby precluding their further use for transfection studies. These results indicate that the expression of GATA-1 gene is strictly inhibited in primary Sertoli cell culture, and suggest that both the Sertoli cell lines and the primary cultures probably display properties representative of either immature Sertoli cells or of adult spermatogenic stages where GATA-1 is normally not expressed (*17*).

To attempt to define how GATA-1 gene expression might be regulated from the IT promoter in Sertoli cells, we performed co-transfection/*trans*-activation assays using the mouse Sertoli cell lines TM4 or 15P1 (*32*) as transfection recipients. In both TM4 and 15P1 cells, the GATA-IT reporter constructs all displayed essentially basal activity whether they were co-transfected with the GATA-1 expression vector or a control (Fig. 8, A and B). We also found that GATA-1 accumulated abundantly in the cells transfected with the expression construct (not shown).

We found that GATA-2 mRNA is expressed in TM4 and 15P1 cells as well as in 2-week-old mouse testis (data not shown). Based on this observation, we speculated that GATA-2 might occupy the double GATA binding sites but may not be able to induce an activating signal, in essence blocking GATA-1 binding and therefore auto-activation of the IT promoter. We tested this hypothesis by co-transfecting an mGATA-2 expression plasmid into fibroblasts

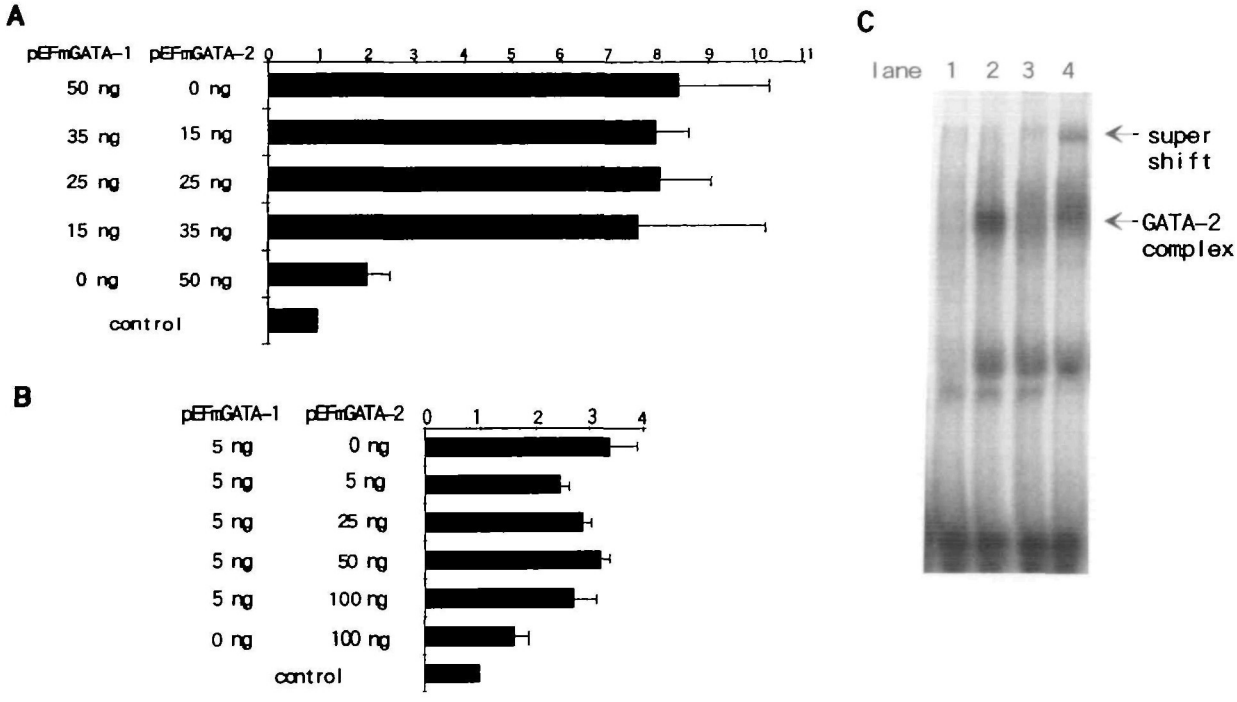


Fig. 9. **mGATA-2 does not activate the IT promoter or inhibit IT *trans*-activation by mGATA-1.** A: 100 ng of pGL-A was transfected into QT6 cells with various concentrations of pEFmGATA-1 and pEFmGATA-2 (total 50 ng) as indicated in the figure. The control shows the LUC activity of cells transfected with 100 ng of pGL-A and 50 ng of control plasmid pEF-BssHII. Activation is shown relative to LUC activity in the control transfection. B: 100 ng of pGL-A was transfected into QT6 cells with incrementally increasing amounts of pEFmGATA-2. For this analysis, 5 ng of pEFmGATA-1 was co-transfected to accentuate the initial LUC activity. The total amount of effector plasmids included in all of the transfections was adjusted to 100 ng using pEF-BssHII. The control was the final LUC activity in cells transfected with 100 ng of pGL-A plus 100 ng of pEF-BssHII. C: QT6 cells were transfected with pEFmGATA-2 and nuclear extract was prepared. The extract (lane 2) and mock-transfected extract (lane 1) were incubated with ^{32}P-labeled MαP probe and subjected to EGMSA. The authenticity of the GATA-2:DNA complex was confirmed by coincubation with two clones of anti-cGATA-2 monoclonal antibody: #67 is a supershift antibody (lane 4), while #71 interferes with the binding of mGATA-2 to the probe (lane 3).

along with the pGL-A and varying amounts of mGATA-1 expression plasmid.

As anticipated, forcibly expressed GATA-2 was found to activate the GATA-IT reporter gene only marginally in QT6 cells (Fig. 9A). The expression of mGATA-2 in QT6 cells was confirmed by EGMSA, where a band containing mGATA-2 protein could be clearly identified in nuclear extracts prepared from QT6 cells after transfection with pEFmGATA-2, and the EGMSA band could be supershifted by the inclusion of anti-GATA-2 monoclonal antibody (Fig. 9C). However, co-transfection of mGATA-2 and mGATA-1 together does not affect the *trans*-activation potential of mGATA-1 acting on the mGATA-IT promoter (Fig. 9A). Furthermore, while the cotransfection of as little as 5 ng of pEFmGATA-1 can activate the IT promoter in QT6 cells, incremental addition of mGATA-2 does not affect GATA-1 *trans*-activation from the IT promoter (Fig. 9B). These data, taken together, indicate that mGATA-2 does not significantly affect the activity of the IT promoter (in either Sertoli or fibroblast cells), and therefore suggests that mGATA-1 is probably the key effector controlling regulation of testis-type GATA-1 gene expression.

DISCUSSION

The cells in the testis that express GATA-1 have been identified as Sertoli cells, which provide support for the growth and differentiation of germ cells (*17*). We show here that the expression of GATA-1 in Sertoli cells of both the rat and mouse initiates at a testis-specific promoter (IT), indicating the general use of an alternative first exon for this gene in the testis of animals. We also found a highly conserved double GATA motif in the rat and mouse IT promoter region. The transcriptional activity of the IT promoter was functionally addressed by co-transfection/*trans*-activation assays using reporter gene constructs plus plasmids expressing either mGATA-1 or mGATA-2, and testing the activity of the IT promoter in fibroblast and Sertoli cell lines. The results of analysis of these normal and mutant regulatory regions in fibroblasts show that transcription of GATA-1 from the IT promoter is activated by GATA-1 binding to a double GATA motif in the promoter, but the same GATA-1 expression plasmid failed to activate gene expression of the reporter after transfection into cultured TM4 or 15P1 Sertoli cells, suggesting that GATA-1 expression is negatively regulated in these cells. Since GATA-1 is not expressed in any of the Sertoli cells examined here, and since forced expression of GATA-1 in these same cells did not confer any demonstrable phenotype on the IT-directed reporter genes, we were not able to explicitly test the hypothesis that the GATA-IT promoter is under direct negative regulatory influence in Sertoli cells.

In the rat testis, proliferation of Sertoli cells is most

active shortly before birth and then declines (*36*). Sertoli cell functions (*e.g.*, formation of the blood-testis barrier, production of androgen-binding proteins, transferrin and seminiferous tubule fluid) initiate at approximately 15 days after birth (P15) and stabilize by about P35. Sertoli cells cease division approximately 3 weeks after birth, which corresponds well to the initiation of GATA-1 gene expression in the rat testis, and then meiosis of germ cells begins at approximately P24 (*43*). The relative number of Sertoli cells declines concomitantly with a massive proliferation of germ cells. Thus, the expression of GATA-1 mRNA declines as the first wave of spermatogenesis is initiated (see Fig. 1). In the mature mouse, GATA-1 is expressed in Sertoli cells only at stages VII, VIII, and IX of the spermatogenic cycle (*17*).

In contrast to the adult expression pattern, Sertoli cells during the embryonic period play a specific role in the formation of the male genital organ by secreting Müllerian duct inhibiting substance (MIS). A highly conserved GATA motif was found in a functionally important region of the MIS gene promoter (see Fig. 1 of Ref. *44*), suggesting that the MIS gene may be one of the earliest targets for GATA factor function in embryonic Sertoli cells. We have, however, recently found that GATA-2 is also expressed in newborn mouse Sertoli cells, and that the timing of GATA-2 expression precedes that of GATA-1 (unpublished observation). The expression of GATA-2 in the testis sharply declines after birth, and is undetectable in P14 testis by RNA blot analysis. Instead, the expression of GATA-1 is induced concomitantly with the first wave of spermatogenesis. A similar relationship between GATA-1 and GATA-2 expression (*i.e.*, GATA-2 expression in progenitor cells which gradually shifts to elevated GATA-1 expression during cell differentiation) has also been demonstrated in the erythroid (*21, 45*) and mast cell lineages (our unpublished observation). Thus, a parallel hypothesis to GATA factor regulation in the hematopoietic system could be raised: GATA-2 may activate one set of early Sertoli genes that are regulated by GATA motifs in embryonic cells and, as the cells differentiate, GATA-1 may control another, perhaps overlapping, set of mature Sertoli genes whose products are important to the growth and differentiation of germ cells.

The mouse, chicken and human GATA-1 erythroid first exons (IE) have been shown to be activated by GATA-1 through GATA binding sites in their promoter or upstream regulatory regions. The GATA motifs described in the IE regulatory regions have therefore been suggested to mediate positive feedback regulation of gene expression in erythroid cells (*18-20*). We show here that, as in the IE-flanking regions, a double GATA motif exists in the IT promoters of the mouse and rat GATA-1 genes, suggesting that the IT promoters may also be under the positive autoregulatory influence of GATA-1. Indeed, the co-transfection/*trans*-activation experiments in fibroblasts demonstrated that the double GATA motifs are essential for the expression of a GATA-IT-directed reporter gene. However, the cell-type specificity of the IT and IE promoters cannot be explained solely by this feedback loop, since both gene regulatory regions contain functional GATA motifs.

In contrast to the results using co-transfected GATA-1, co-transfection of the reporter genes plus a GATA-2 expression plasmid into fibroblasts did not activate transcription of the GATA-IT-directed reporter gene, thereby demonstrating specificity in transcription factor requirements. Furthermore, reporter gene activation was only marginal in a mouse Sertoli cell line which expresses abundant endogenous GATA-2, but does not express GATA-1. Based on these results, we initially speculated that GATA-2 might bind to the double GATA motifs in early stage Sertoli cells to block activation of the IT promoter by GATA-1 during that stage. However, co-transfection of mGATA-2 with the reporter gene into fibroblasts showed that the mere presence of mGATA-2 protein does not affect IT-directed transcription. These results suggest that GATA-2 does not compete effectively for binding to the double GATA motif within the GATA-IT promoter, and therefore that this initial hypothesis was invalid. The results rather suggest an alternative possibility: that *trans*-activation of the reporter by GATA-1 is specifically suppressed in the TM4 and 15P1 Sertoli cell line. Although the mechanism of this speculated repression is not clear at the present time, such a mechanism may well employ the same controls which normally repress GATA-1 transcription in Sertoli cells of the adult testis during the majority of the spermatogenic cycle, where the GATA-1 gene is normally inactive.

Recent studies have demonstrated that comparable levels of GATA-1 transcription are initiated from the IT and IE promoters in temperature-sensitive SV40 T antigen-transformed early hematopoietic progenitor cells when the cells are cultured in the presence of erythropoietin (*23*). Consistent with this observation, the detailed 5′-RACE analysis reported here demonstrates the presence of transcripts originating from the IT promoter in rat bone marrow blood cells. Thus the regulatory mechanisms by which GATA-1 is activated in hematopoietic progenitor cells may need to be re-evaluated from these emerging observations. The possibility exists that, in addition to the demonstrated important contribution by the IE promoter, the IT promoter may also make a functional contribution to GATA-1 gene expression in earlier stages of hematopoietic cellular differentiation.

The regulation of GATA-1 gene expression in Sertoli cells provides a good model system to investigate the functional interaction between stem cells and support cells. There is now ample evidence showing that germ and Sertoli cells functionally interact with one another. For example, when Sertoli cells are seeded onto a culture dish, the secretion of androgen-binding protein, transferrin and inhibin is minimal, whereas the secretion of these proteins increases when germ cells are added back to the culture (*46-48*). The presence of the c-kit receptor on the surface of germ cells, and the presence of its ligand (stem cell factor) on the surface of Sertoli cells, also suggests the presence of productive interactions between germ and Sertoli cells (*49*). We have previously demonstrated that transcription from the IT promoter is repressed by signals originating from germ cells (*17*). This negative regulation of GATA-1 gene expression could be a consequence of the physical contact of germ cells, at certain stages of differentiation, with Sertoli cells. Current experiments suggest that this signal may be transduced from the elongated spermatids to Sertoli cells to turn off GATA-1 transcription (unpublished observation).

One major limitation to further functional definition of germ-Sertoli interactions is the present lack of cultured Sertoli cell lines that express GATA-1. Even in primary Sertoli cell cultures, GATA-1 expression is quickly extinguished after testis disruption and seeding into culture. Identification of such a Sertoli system will be necessary to address several questions raised by the present studies: whether or not, for example, GATA-1 plays any role in the negative regulation of the GATA-IT promoter in Sertoli cells, as suggested by the fibroblast transfection studies, or whether negative regulation of the GATA-IT promoter is controlled by an entirely different mechanism. For further analysis of IT gene regulation, therefore, it will be necessary to establish a Sertoli cell culture model system which retains GATA-1 expression, and studies focused on this goal are now in progress.

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