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Identification of a short PIASx gene promoter that directs male germ cell-specific transcription in vivo☆

Henrikki Santti,[a] Laura Mikkonen,[a] Sirpa Hirvonen-Santti,[a] Jorma Toppari,[b] Olli A. Jänne,[a,c] and Jorma J. Palvimo[a,*]

[a] *Biomedicum Helsinki, Institute of Biomedicine, University of Helsinki, FIN-00014 Helsinki, Finland*
[b] *Departments of Physiology and Pediatrics, University of Turku, FIN-20520 Turku, Finland*
[c] *Department of Clinical Chemistry, University of Helsinki and Helsinki University Central Hospital, FIN-00014 Helsinki, Finland*

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Abstract

PIASx gene encodes two SUMO E3 ligases that are highly expressed in the testis. We have isolated and analyzed the promoter of the murine PIASx gene. Electrophoretic mobility shift assays with testicular nuclear extracts showed that the proximal promoter forms a major DNA–protein complex containing Sp1, Sp2, and Sp3 transcription factors. Reporter gene assays in cultured cells indicated that a fragment comprising nucleotides from −168 to +76 relative to transcription start site is sufficient for basal promoter activity in cultured cells, but these in vitro assays failed to reveal clear differences in promoter activity between testis- and non-testis-derived cell lines. Interestingly, however, the proximal promoter encompasses the elements necessary for a testis-specific transcription in vivo, as it directed β-galactosidase expression exclusively to male germ cells in transgenic mice. In conclusion, we have characterized the minimal PIASx promoter that can be used for highly specific targeting of transgene expression to male germ cells.

Keywords: PIASx; Transcription; Spermatogenesis; Testis; Male germ cell; GC box; Sumoylation

Protein inhibitor of activated STAT (PIAS) protein family is encoded by four different genes, namely the genes encoding PIAS1, PIAS3, PIASx, and PIASy proteins [1,2]. Moreover, the PIASx gene gives rise to two proteins (PIASxα/ARIP3 and PIASxβ/Miz1) through alternative splicing at the 3′-end of the transcript. The PIAS proteins share a high degree of structural similarity and they contain SAP (SAF-A/B, Acinus, and PIAS) motifs and RING finger-like domains [1,2]. In addition to inhibiting the function of STATs [3,4], PIAS proteins interact with and modulate transcriptional activities of several transcription factors, including the steroid receptors [5–10]. Recently, many PIAS proteins have been shown to possess E3 ligase activity in the protein sumoylation pathway [11–16]. Sumoylation is a distinct post-transcriptional modification that resembles ubiquitination, where small-ubiquitin-related modifier (SUMO) proteins are covalently attached to specific lysine residues of target proteins in a reversible manner [17,18]. At least two functional consequences of SUMO modification have been observed; sumoylation may block protein ubiquitination and thereby prevent degradation of the target protein, or sumoylation may influence subcellular or subnuclear localization of target proteins.

PIASx, PIAS1, and PIASy genes are highly expressed in the testis [5,6,8]. Their transcripts can be detected in Sertoli cells and in all germ cells. However, the expression pattern of the three genes is not identical in germ cells, since PIASx mRNA accumulates to high levels in spermatocytes, PIAS1 mRNA in spermatids, and PIASy mRNA in both spermatocytes and spermatids [19,20]. PIASx protein can be found in spermatogonia and

☆ *Abbreviations:* EMSA, electrophoretic mobility shift assay; nt, nucleotide(s); PIAS, protein inhibitor of activated STAT; Sp, promoter specificity protein; STAT, signal transducer and activator of transcription; SUMO, small ubiquitin-related modifier.

* Corresponding author. Fax: +358-9-19125302.
E-mail address: jorma.palvimo@helsinki.fi (J.J. Palvimo).

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pachytene spermatocytes up to stage XII of the seminiferous epithelium [20]. The PIAS protein homologue Su(var)2-10/Zimp in *Drosophila* has been, in turn, implicated in the maintenance of chromosome organization in interphase nuclei [21]. In view of this, PIAS proteins may be involved in the regulation of chromatin rearrangements during meiotic prophase of spermatogenesis.

Transcription in spermatogenic cells displays several differences compared to somatic cells. The differences are mainly due to major changes in cell morphology and chromosome structure in spermatogenesis. During the male germ cell development, the chromosome structure is changed by genetic recombination, reduction of chromosomal ploidy, and replacement of histones [22]. The unusual aspects of transcription during spermatogenesis include abundant expression of general transcription factors, the presence of testis-specific transcription factors, and the use of alternative promoters or transcription start sites [23]. Only a few testis-specific transcription factors have thus far been described and the cAMP-responsive element modulator (CREM) is the most extensively studied one. CREM interacts with TFIIA and selected TATA-binding protein-associated factors, and the lack of CREM leads to a block in the differentiation program at the first step of spermiogenesis [24]. However, CREM-binding sites are absent in many genes, suggesting that there are other, yet unknown testis-specific transcriptional regulators. In the present work, we have examined the factors governing the regulation of murine PIASx gene transcription in the testis. Our results indicate that the proximal promoter of the murine PIASx gene encompasses the *cis*-elements sufficient for targeting the transcription of this gene specifically to male germ cells and that Sp family proteins are likely to be important *trans*-acting factors for determining the male germ cell specificity of PIASx promoter activity.

Materials and methods

Cloning of the murine PIASx gene promoter. Murine PIASx promoter was isolated from *Kpn*I-digested 129/SvJ genomic DNA in bacterial artificial chromosome clone 33677 (Incyte Genomics, Palo Alto, CA) by hybridizing to digoxigenin-labeled 5′-untranslated region of rat PIASxα cDNA [5]. The promoter region comprising nt −418/+76 was PCR-amplified using primers 5′-GGTTTCCACATCACCCTTACTTG-3′ (complementary to nt −418 to −396, underlined) and 5′-AAGCTTCGCCGTCTCCTGCAACATCC-3′ (+76 to +57). The PCR product was cleaved with *Xho*I and *Hin*dIII and inserted in front of the firefly luciferase gene in pGL3-Basic vector (Promega, Madison, WI) giving rise to the promoter construct −349/+76. The promoter constructs −4221/+76, −2917/+76, −2473/+76, −2111/+76, and −1165/+76 were produced by inserting PIASx promoter fragments digested with *Kpn*I, *Bgl*II, *Hin*dIII, *Bgl*II or *Eco*RI and *Xho*I, respectively, to the −349/+76 construct. The construct −277/+76 was assembled by deleting the *Xho*I/*Xma*I fragment from the −349/+115 construct. The constructs −168/+76, −138/+76, −117/+76, −85/+76, −63/+76, and −22/+76 were obtained by using PCR with forward primers 5′-CCCGGGGGTAGGGACAGGCCACCAA-3′ (−168 to −150), 5′-CTCGAGTGGGTCGGGGGCGCGCTC-3′ (−138 to −121), 5′-CTCGAGCGGAAGACCGCGCGCGTT-3′ (−117 to −100), 5′-CTCGAGGCGAGGGGCGGGGCCAGC-3′ (−85 to −68), CTCGAGGCCGGGGGGCGGGACTCC-3′ (−63 to −46) or 5′-CCCGGGGGTGGAGGTGGTGGAGGTGT-3′ (−22 to −3), respectively, and the reverse primer 5′-AAGCTTCGCCGTCTCCTGCAACATCC-3′ (+76 to +57). The PCR products were cloned into the *Xma*I/*Hin*dIII or *Xho*I/*Hin*dIII site of pGL3-Basic. All constructs were sequenced by ALFexpress sequencing system (Amersham Biosciences, Uppsala, Sweden). The sequence of the longest promoter fragment has been deposited to GenBank (Accession No.: AF539748).

Promoter sequence analysis. Sequence of the proximal promoter of the murine PIASx gene was analyzed for potential transcription factor binding sites with Match program (BioBase, Wolfenbüttel, Germany) using Transfac 5.0 Public database.

Primer extension. Cy5-labeled antisense primers 5′-TCAGCCGTCACCGCTCCTCC-3′ (−41 to −22 relative to translation initiation site) and 5′-CTCAACTCCTCGAAATCCGCCAT-3′ (+1 to +23 relative to translation initiation site) were used in primer extension reactions. One-hundred nanograms of the primers was annealed with 20 μg murine testis RNA at 66 °C for 20 min in buffer containing 100 mM Tris–HCl (pH 8.3), 100 mM KCl, 20 mM $MgCl_2$, 20 mM dithiothreitol, 2 mM each of the four dNTPs, and 1 mM spermidine in a total volume of 11 μl. The reaction was allowed to cool to room temperature for 10 min. Extension reaction was carried out in a total volume of 20 μl in buffer containing 100 mM Tris–HCl (pH 8.3), 100 mM KCl, 20 mM $MgCl_2$, 20 mM dithiothreitol, 2 mM each of the four dNTPs, 1 mM spermidine, 2.8 mM sodium pyrophosphate, and AMV reverse transcriptase (Promega) at 42 °C for 30 min. The extension reaction mixture was combined with 20 μl of ALFexpress loading dye (Amersham Biosciences) and heated at 90 °C for 10 min. The primer extension products were subjected to electrophoresis under denaturing conditions on a 6% polyacrylamide gel parallel to the sequencing reaction and analyzed with ALFexpress DNA sequencer (Amersham Biosciences).

Cell culture and reporter gene assays. COS-1 (from American Type Culture Collection, Manassas, VA), MSC-1 (murine Sertoli cell line, obtained from Dr. Ilpo Huhtaniemi, University of Turku, Finland), and GC-1spg (murine spermatogonial cell line, from Dr. Jorma Keski-Oja, Univ. of Helsinki, Finland) cells were maintained as described [25–27]. The cells (5×10^4 cells/well) were seeded onto 12-well plates 24 h prior to transfection and transfected with 400 ng promoter constructs, empty pGL3-Basic or pGL3-Control (Promega) and 50 ng pCMVβ (Clontech, Palo Alto, CA) using FuGENE 6 reagent (Roche Molecular Biochemicals, Mannheim, Germany). Forty-eight hours after transfection, the cells were harvested, β-galactosidase activity was measured as described [28], and luciferase activity was determined using Luciferase Assay Reagent (Promega) and Luminoskan Ascent reader (Labsystems, Helsinki, Finland). The luciferase activities were related to the β-galactosidase activities. Activities of the promoter constructs are shown as relative luciferase units (RLU) relative to pGL3-Control (= 100).

Production of transgenic mice and β-galactosidase assay. Two PIASx promoter fragments, −4199/+76 and −168/+76, were cloned into the *Sma*I/*Hin*dIII site of the SDKlacZpA-derived vector that contains a Kozak consensus sequence fused in-frame to the lacZ coding region followed by an SV40 polyadenylation signal [29,30]. The transgene was removed from the plasmid backbone with *Sal*I digestion, and the isolated and purified fragment was injected into fertilized mouse oocytes of the FVB/N strain. The animals were housed under specific pathogen-free conditions and mated with wild-type FVB/N mice. Identification of the transgenic mice was performed from tail clip biopsy-derived genomic DNA by using PCR with primers 5′-AGGATGTTGCAGGAGACG-3′ and 5′-ACAACCCGTCGGATTCTC-3′.

All animal experiments were approved by the Institutional Review Board on animal experiments at the University of Helsinki. The tissues were collected from mice over 9 weeks of age and snap frozen in liquid nitrogen. For β-galactosidase assay, tissues were homogenized with a Polytron PT3000 homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in buffer containing 100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100, 1 mM dithiothreitol, and 1% protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO). The samples were centrifuged at 13,200*g* for 10 min, the supernatant was collected, heated at 48 °C for 1 h, and cleared by centrifugation at 13,200*g* for 5 min. β-Galactosidase activity was measured from 10 μl of supernatant using Galacto-Light (Applied Biosystems, Foster City, CA) and Luminoskan RT (ThermoLabsystems) according to the manufacturer's recommendations. β-Galactosidase activity in each sample was related to protein concentrations measured by Bradford's method (Bio-Rad, Hercules, CA).

β-Galactosidase staining. Testes were stained for β-galactosidase activity as described [31]. Briefly, testes were dissected out, decapsulated, rinsed in PBS, and fixed for 30 min in solution containing 0.1 M sodium phosphate (pH 7.3), 0.2% glutaraldehyde, 5 mM EGTA, 2 mM $MgCl_2$, and 2% formalin. After fixation, the tissues were rinsed in solution containing 0.1 M sodium phosphate (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 and stained for overnight at 22 °C in solution containing 1 g/L 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 0.1 M sodium phosphate (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide. After staining, the testes were dehydrated, embedded in paraffin, sectioned at 5-μm thickness, and counterstained with eosin.

Electrophoretic mobility shift assay. Nuclear extracts were isolated from Hannover–Wistar rat testis and liver as previously described [32]. Briefly, tissues were excised, snap-frozen in liquid nitrogen, and stored in −70 °C until use. Subsequently, tissues were rinsed with cold phosphate-buffered saline, minced with scissors, and homogenized with Potter–Elvehjem homogenizer in buffer A (10 mM Hepes, pH 7.6, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol, 0.5 mM dithiothreitol, 20 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride). The homogenate was loaded on top of the buffer A pad and centrifuged at 110,000*g* for 60 min. The pellet was resuspended into buffer B (10 mM Hepes, pH 7.6, 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 3 mM $MgCl_2$, 1 mM dithiothreitol, 20 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride) and homogenized with hand-held glass homogenizer. Subsequently, 1/10 volume of 4 M $(NH_4)_2SO_4$ was added, the sample was incubated in ice-water for 30 min with occasional mixing, and centrifuged at 155,000*g* for 60 min. The supernatant was transferred to a new tube, and 0.3 g of $(NH_4)_2SO_4$ per ml of supernatant was added. The samples were incubated in ice-water for 60 min with gentle shaking and centrifuged at 155,000*g* for 20 min. The pellets were resuspended in buffer C (25 mM Hepes, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) by gently shaking in ice-water for 60 min, dialyzed twice for 2 h against buffer C, and centrifuged at 13,000*g* for 2 min. The protein concentration was measured with Bradford Protein Assay.

Electrophoretic mobility shift assays (EMSAs) were carried out with 10 μg of nuclear extracts in buffer containing 0.5 μg of poly(dI-dC)$_2$ (Amersham Biosciences) [28]. The probes, encompassing nt −168/−69, −88/+10, −168/−69 or −100/−69, were ^{32}P-labeled with either the Klenow fragment of bacterial DNA polymerase (Amersham Biosciences) or T4 polynucleotide kinase (USB, Cleveland, OH). In competition assays, before the addition of radioactive probe, nuclear extracts were supplemented with a 100-fold molar excess of non-radioactive double-stranded oligomers comprising indicated regions of the murine PIASx promoter or with oligomers containing consensus site for the following transcription factors: Sp1 (E3231, Promega), AP-2 (E3211, Promega), Ets (sc-2549, Santa Cruz Biotechnology, Santa Cruz, CA) or USF (sc-2509, Santa Cruz Biotechnology). In supershift assays, nuclear extracts were preincubated with 2 μg of antibody against Sp1 (*sc-420*), Sp2 (*sc-643*), Sp3 (*sc-644*) or Sp4 (*sc-645*) (Santa Cruz Biotechnology) for 20 min on ice prior to the addition of radiolabeled probe.

Immunoblotting. Samples from the nuclear extracts (15 μg protein/lane) were resolved on SDS–PAGE and electrophoretically transferred onto Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membrane was blocked and washed according to the instructions of the manufacturer. Sp1 was detected with a polyclonal rabbit anti-Sp1 antibody (*sc-59*, Santa Cruz Biotechnology) using 1:2000 dilution. The immunocomplexes were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:10,000 dilution, Zymed Laboratories, South San Francisco, CA) and ECL detection system (Amersham Biosciences).

Results

Sequence analysis of the murine PIASx gene promoter

Since the murine PIASx gene is expressed in a testis-specific fashion [19], we sought to define the factors

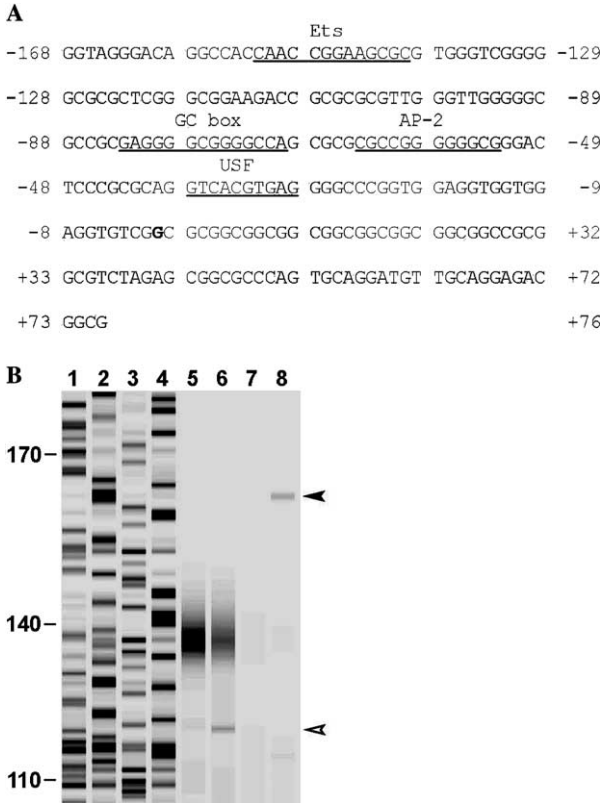

Fig. 1. Sequence of the murine PIASx gene proximal promoter. (A) The sequences of Ets-, AP-2-, and USF-binding sites and GC box are underlined, and the transcription start site is bolded. (B) Primer extension analysis was performed from testicular total-RNA with two different fluorescently labeled antisense primers starting from −22 and +23 from the translation start site (*lanes 6* and *8*, respectively). As a control, primer extension was carried out without RNA (*lanes 5* and *7*). A sequencing reaction was used to determine lengths of the products (*lanes 1*, *2*, *3*, and *4*). Open arrowhead and solid arrowhead correspond to products produced by primers starting from −22 and +23, respectively.

governing its transcriptional regulation. As a first step, we cloned the 5′-flanking region of the murine PIASx gene that extends 4.3 kb upstream of translation initiation site by using 5′-untranslated region of PIASx mRNA as a hybridization probe. The sequence of the putative proximal promoter is very GC rich (79%) and it lacks TATA or CAAT boxes (Fig. 1A). Search for potential transcription factor-binding sites revealed putative sites for members of the Ets protein family and for Sp, AP-2, and USF proteins at locations −152 to −140, −83 to −70, −64 to −53, and −38 to −29, respectively (Fig. 1A). Transcription start site (marked as +1) of the PIASx gene, located at nt 141 upstream of translation initiation site, was determined by primer extension analysis using murine testicular RNA (Fig. 1B).

Functional analysis of PIASx gene promoter in vitro

To study transcriptional activity of PIASx promoter sequences, we performed reporter gene assays in cultured cells. Different fragments from the 5′-flanking region of the PIASx gene, ranging from nt −4221 to −22 (relative to transcription start site), were cloned in front of the luciferase reporter gene and transiently transfected into cell lines that originate from kidney (COS-1),

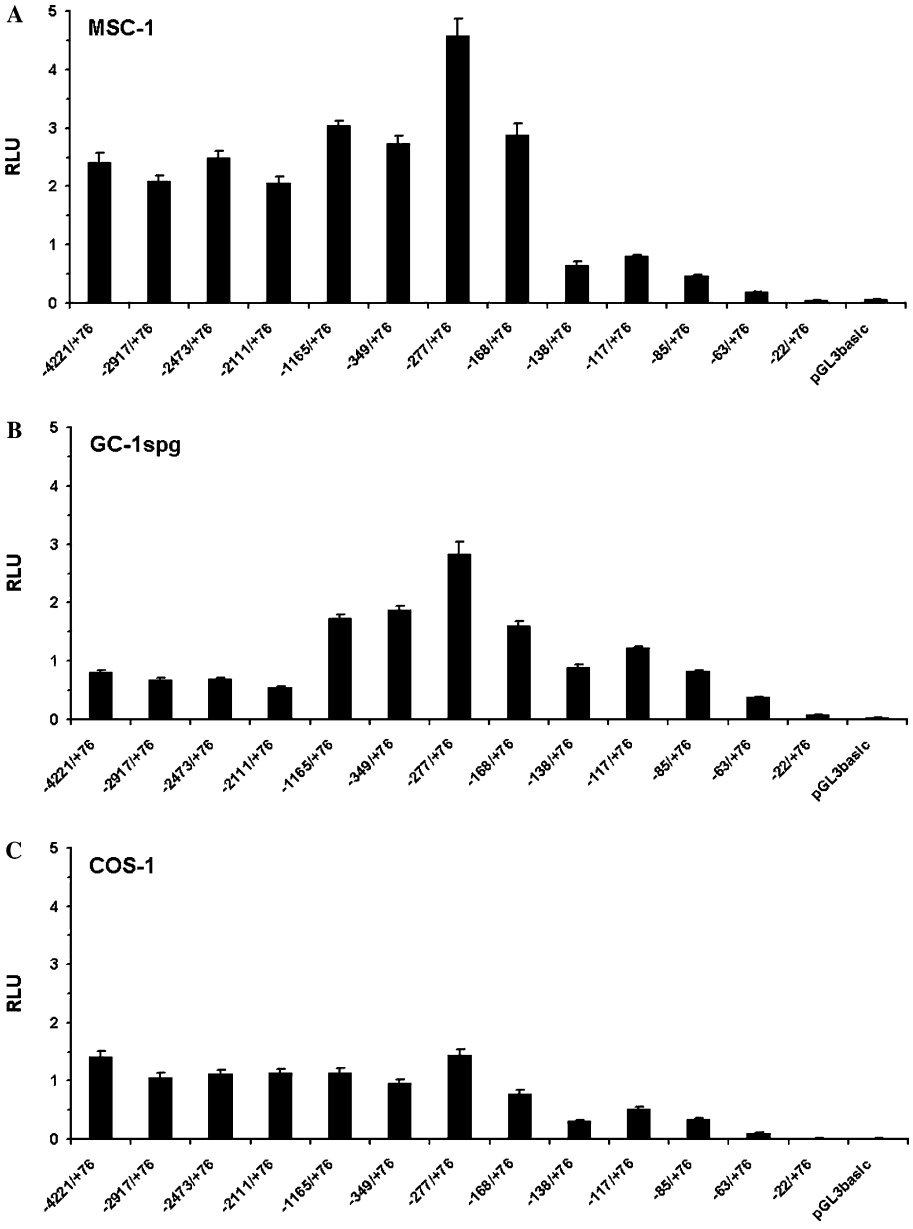

Fig. 2. Analysis of the PIASx gene promoter activity in cultured cells. The activities of the indicated luciferase constructs were measured in triplicates in transiently transfected MSC-1 (A), GC-1spg (B), and COS-1 (C) cells. The promoter activities are shown as relative luciferase units (RLU) relative to the pGL3-Control (= 100). The means ± SEM values of at least three experiments are shown.

Sertoli cells (MSC-1), and spermatogonia (GC-1spg). Of these cell lines MSC-1 and to a lesser extent GC-1spg express PIASx mRNA, whereas COS-1 is devoid of PIASx mRNA (data not shown). Luciferase activities were normalized with β-galactosidase activity to take into account transfection efficiency and subsequently compared to the activity of pGL3-Control (a luciferase construct driven by SV40 promoter and enhancer). In all cell lines studied, the construct extending to nt −277 exhibited the highest activity. Transcriptional activities of the −277/+76 construct were 4.6%, 2.8%, and 1.4% of that of pGL3-Control in MSC-1, GC-1spg, and COS-1 cells, respectively (Fig. 2). In MSC-1 cells that showed the highest transcriptional activity relative to pGL3-Control, the −168/+76 construct exhibited similar activity to that of the longest construct −4221/+76 (Fig. 2A). Interestingly, deletion of region from −168 to −138 resulted in ∼80% decline in the reporter gene activity, and additional deletions of region from −85 to −22 gradually abolished the transcriptional activity of the PIASx promoter in MSC-1 cells. Likewise, the transcriptional activity of −168/+76 in COS-1 cells was similar to the construct −4221/+76, and truncation of the promoter from −168 to −22 gradually eliminated the luciferase activity (Fig. 2C). In GC-1spg cells (Fig. 2B), truncation constructs down to −85/+76 possessed transcriptional activity similar to or higher than that of the longest construct, and further deletions stepwise blunted the activity. In sum, these results show that although PIASx promoter constructs exhibited somewhat higher activity in the MSC-1 and GC-1spg cells than in the non-testis-derived cell line COS-1, the magnitude of difference is lower than would have been expected on the basis of PIASx expression between testis and other tissues in vivo [5,19,20].

The proximal PIASx promoter directs reporter gene expression to male germ cells in vivo

Since the preceding assays failed to reveal high promoter strength in testis-derived cell lines in vitro, we next determined by in vivo experiments whether this was due to a lack of binding elements in the promoter fragments or the absence of specific transcription factors in the cell lines examined. To this end, we produced two transgenic mouse strains containing β-galactosidase gene fused to PIASx promoter sequences, extending either to nt −4199 or nt −168 from transcription start site. In the 4199-nt and 168-nt promoter fragment-harboring strains, three of the five and one of the three lines, respectively, expressed the transgene. In both strains, β-galactosidase activity in the testis of transgenic animals was ∼10,000-fold higher than that in other 11 tissues studied, such as in the liver, in which β-galactosidase activity did not differ between transgenic and wild-type mice (Fig. 3 and data not shown).

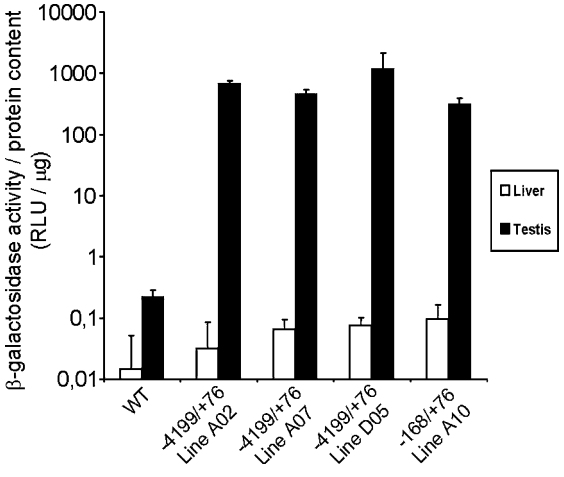

Fig. 3. PIASx promoter directs testis-specific expression of β-galactosidase in mice. The activity of the β-galactosidase reporter gene was measured in duplicates from liver (*open bars*) and testis (*solid bars*) homogenates by chemiluminescence-based assay from −168/+76 and −4199/+76 promoter mice. The means ± SEM values of at least two mice per bar are shown.

We next localized the cell types that exhibit PIASx promoter activity by histochemical staining for β-galactosidase activity in testis sections. In 168-nt promoter fragment-harboring mice, β-galactosidase activity was detected in pachytene spermatocytes, round spermatids, and elongating spermatids (Figs. 4A–D). No staining was seen in the seminiferous tubules of wild-type mice (Figs. 4E and F). Together, these results indicate that the strong promoter activity of the PIASx gene is confined to the testis, and that the *cis*-elements in proximal promoter are sufficient for targeting of the reporter gene specifically to male germ cells.

Sp1, Sp2, and Sp3 bind to the proximal PIASx promoter

To identify potential *trans*-acting factors responsible for the testis-specific activity of the PIASx promoter, we used EMSA with two probes, encompassing nt −168/−69 and −88/+10, to cover the proximal promoter region. The probes were incubated with nuclear proteins derived from rat liver (Fig. 5A, *lanes 1* and *5*) or testis (Fig. 5A, *lanes 2–4* and *6–8*). With both probes, we detected a major testicular protein–DNA complex (Fig. 5A, *solid arrowhead*) that was competed for by an excess of unlabeled probe (Fig. 5A, *lanes 3* and *7*), a GC box-containing oligomer extending from nt −100 to −69 (Fig. 5A, *lanes 4* and *8*) or an Sp1 consensus site-containing oligomer, whereas an excess of AP-2 or USF consensus site oligomers did not influence DNA–protein complex formation (data not shown). Additionally, minor complexes that were abolished by an excess of unlabeled probe, but not with GC box-containing oligomer, were evident (Fig. 5A, cf.

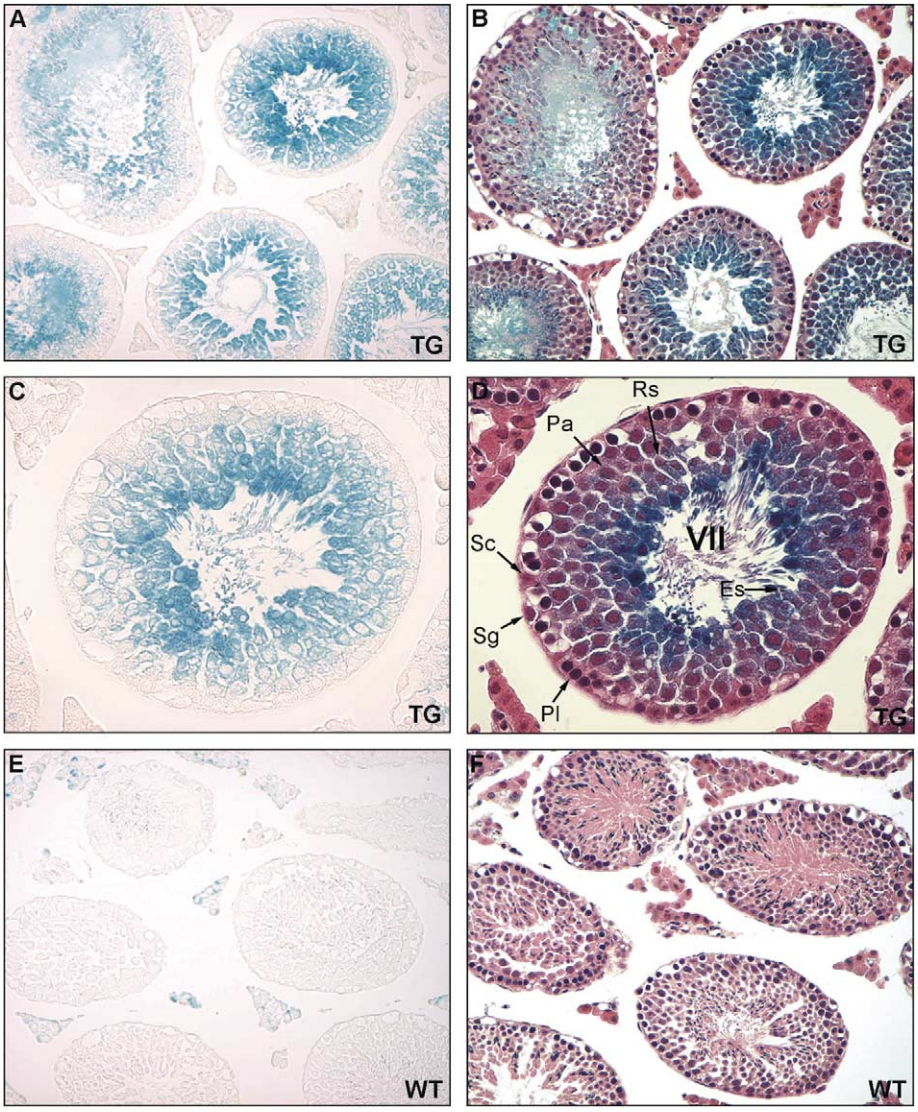

Fig. 4. Localization of the reporter gene in mouse testis by β-galactosidase staining. Testes were stained for β-galactosidase activity, sectioned, and counter-stained. (A–D) Microphotographs from the transgenic (TG) mouse and (E and F) from the wild type (WT) mouse. Images without counter-staining are shown in the left and images counter-stained with eosin are shown in the right. *Es*, elongating spermatid; *Pa*, pachytene spermatocyte; *Pl*, preleptotene spermatocyte; *Rs*, round spermatid; *Sc*, Sertoli cell; and *Sg*, spermatogonium.

lanes 3 and *4*, and *lanes 7* and *8*). To identify some of the testicular nuclear proteins residing in the major complex, we used antibodies against Sp proteins in supershift assays. As shown in Fig. 5A, antibodies against Sp1, Sp2, and Sp3 (Fig. 5A, *lanes 11*, *12*, and *13*), but not Sp4 (Fig. 5A, *lane 14*), either supershifted or disrupted the major DNA–protein complex. Interestingly, only very weak DNA–protein complexes were formed with nuclear proteins from liver. A similar, albeit less marked, difference in binding efficiency between hepatic and testicular nuclear extracts was also seen with an Sp1 consensus binding site-containing probe (Fig. 5B). Interestingly, this difference was not simply due to lower amounts of Sp1 in hepatic nuclear extracts as shown by immunoblotting (Fig. 5C).

Sequence analysis predicted the presence of an Ets-protein binding element near the 5′-end of the proximal promoter. Since also Ets proteins have been implicated in transcriptional regulation of spermatogenesis [33,34], nuclear protein binding to region −168/−136 was assessed by EMSA experiments. The Ets binding site-containing oligomer, nt −168/−136, competed for DNA–protein complexes migrating more slowly than the principal DNA–protein complex (Fig. 5D, *lanes 3* and *5*, *arrow*), without interfering with the latter complex formation. The probe covering nt −168/−136 produced a single DNA–protein complex, the intensity of which did not, however, differ between liver and nuclear extracts (Fig. 5B, cf. *lanes 7* and *8*). The DNA–protein complex was competed out with an excess of unlabeled

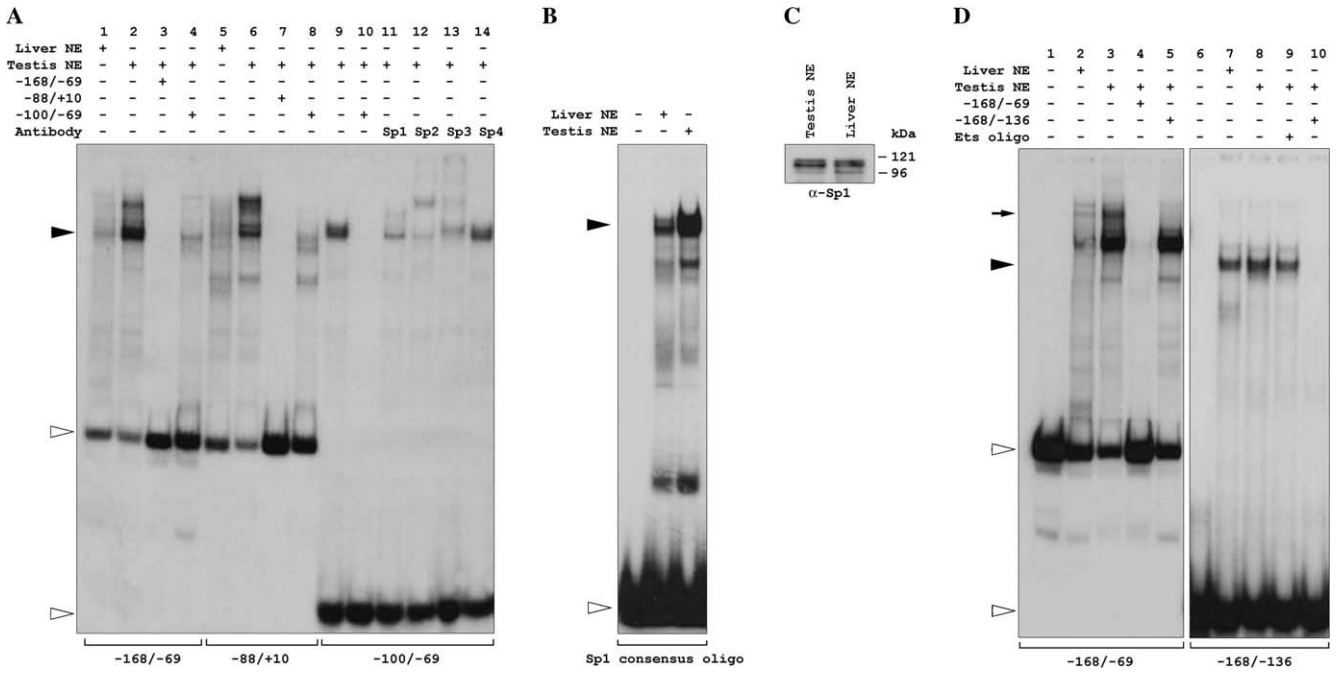

Fig. 5. EMSA of nuclear proteins binding to the proximal promoter of PIASx gene. (A) EMSA was performed with ^{32}P-labeled probes extending −168/−69 (*lanes 1–4*), −88/+10 (*lanes 5–8*) or −100/−69 (*lanes 9–14*) from transcription start site of the PIASx gene. The probes were incubated with liver (*lanes 1* and *5*) and testis (*lanes 2–4* and *6–14*) derived nuclear extracts. Binding of the probe was competed with unlabeled −168/−69 (*lane 3*), −88/+10 (*lane 7*), and −100/-69 (*lanes 4*, *8*, and *10*) probes. Nuclear extracts were preincubated with antibodies against Sp1 (*lane 11*), Sp2 (*lane 12*), Sp3 (*lane 13*), and Sp4 (*lane 14*). *Solid arrowhead* depicts the major testicular protein–DNA complex and *open arrowheads* depict free probes. (B) EMSA was performed with ^{32}P-labeled Sp1 consensus oligonucleotide. The probe was incubated with liver (*lane 2*) and testis (*lane 3*) nuclear extracts. *Solid arrowhead* depicts the major protein–DNA complex and *open arrowheads* depict free probe. (C) Testis and liver nuclear extracts were immunoblotted with anti-Sp1 antibody. (D) EMSA was performed with ^{32}P-labeled probes −168/−69 (*lanes 1–5*), or −168/−136 (*lanes 6–10*). The probes were incubated with liver (*lanes 2* and *7*) and testis (*lanes 3–5* and *8–10*) nuclear extracts. Binding of the probe was competed with unlabeled −168/−69 (*lane 4*), −168/−136 (*lanes 5* and *10*) probes, and Ets consensus oligomer (*lane 9*). *Arrow* depicts complex competed out with −168/−136 oligomer and *solid arrowhead* indicates complex formed by the same oligomer. *Open arrowheads* depict free probes.

probe (Fig. 5D, *lane 10*), but not with an Ets consensus site-containing oligomer (Fig. 5D, *lane 9*), indicating that the binding protein is not a typical member of the Ets family. Taken together, we identified Sp1, Sp2, Sp3, and an Ets-like element-binding protein as major testicular *trans*-acting proteins capable of binding to the PIASx promoter.

Discussion

This study shows that a short (244 nt) GC-rich promoter fragment of the murine PIASx gene is sufficient for male germ cell-specific transcription in vivo. A significantly longer, 4.2-kb PIASx promoter fragment also directs reporter gene expression to the same cells; pachytene spermatocytes, round spermatids, and elongating spermatids (data not shown). The expression pattern of the reporter gene was not identical with those of PIASx mRNAs and proteins in the rat [20] or mouse PIASx mRNAs [19], in that the β-galactosidase activity was very low or undetectable in Sertoli cells, spermatogonia, and early spermatocytes, whereas the reporter was expressed to high levels in spermatids that show only very low levels of endogenous PIASx protein [20]. The latter disparity may be due to differences in the translation and stability of β-galactosidase and PIASx mRNAs, and/or in the stability between the β-galactosidase and PIASx proteins. The lack of reporter gene expression in Sertoli cells may, in turn, be due to the fact that the elements required for transcription of the PIASx gene in somatic cells in vivo lie outside the promoter fragments studied. It is of note, however, that the reporter gene was efficiently expressed in the pachytene spermatocytes that also show the highest levels of PIASx mRNA [19,20].

The Sp family proteins Sp1, Sp2, and Sp3, from testis-derived nuclear extracts were responsible for the in vitro formation of the major DNA–protein complex at the GC box (−83 to −70) within the proximal PIASx promoter. Interestingly, the general GC box-binding activity in liver nuclear extracts was markedly weaker than in testis, even though the immunoreactive level of Sp1 protein was similar. Whole testis nuclear extracts were used in the binding studies and these results reflect the overall complement of Sp proteins in different cell

types of rat testis. Sp1 has been shown to be present in Sertoli cells and in germ cells up to pachytene spermatocytes [35] and Sp3 has been demonstrated in primary spermatocytes and early spermatids [36]. Thus, at least Sp1 and Sp3 are likely to be involved in the regulation of the PIASx promoter in vivo.

The lack of testis cell specificity in cell culture-based reporter assays cannot be due to the absence of Sp transcription factors in cell lines, as these factors are ubiquitously expressed in cultured cells [37]. However, it is very likely that the relative abundance of different Sp proteins varies considerably between testis cell-derived cell lines and germ cells in the testis. Sp1 and Sp3 are known to differ with respect to their transcriptional activity; Sp1 functions as an activator, whereas Sp3 usually acts as a repressor [37]. Since Sp1 and Sp3 are capable of binding to the same element, they may interfere with each other. Thus, a proper ratio of Sp proteins in testicular cells may be needed for activation of the PIASx promoter. Although Sp proteins formed the major DNA–protein complex at the PIASx proximal promoter in vitro, it is very likely that their cooperation with other transcription factors on the promoter is essential for tissue-specific transcriptional regulation in vivo. Interestingly, the PIASx proximal promoter harbors an Ets-type binding site (−152 to −140) the presence of which was important for the promoter activity in reporter gene assays in vitro. This element formed a specific complex in EMSA that was not, however, competed for by Ets consensus site-containing oligomer. Previously, putative Ets protein-binding sites have been shown to be important for transcription of testis-specific phosphoglycerate kinase 2 (PGK2) and β1,4-galactosyltransferase-I genes, although respective proteins have not been identified [33,34]. Similar to our case, protein binding to β1,4-galactosyltransferase-I gene promoter was not affected by excess of unlabeled Ets consensus oligomer [34].

Similar to the PIASx gene, surprisingly short promoters of other testis-specific genes, such as PGK2, lactate dehydrogenase C, pyruvate dehydrogenase α-subunit (Pdhα-2), and protamine 1 genes, have been shown to confer testis-specific transcription in vivo [38–41]. The proximal promoter of the testis-specific genes is often GC-rich and CpG methylation within the core promoters may represent a mechanism for their repression in somatic cells, as exemplified by the Pdhα-2 promoter [40]. In view of this, it is of interest to note that Sp1-binding sites play a key role in protecting CpG islands of the adenine phosphoribosyltransferase gene from de novo methylation in transgenic mice [42,43]. Since the proximal PIASx promoter is GC-rich and contains several potential methylation sites, promoter methylation offers another plausible mechanism, in addition to transcription factor availability and cooperativity, by which the activity of the PIASx promoter could be regulated in a cell-type-specific fashion.

In conclusion, we have identified the region of the murine PIASx gene that governs the *cis*-acting elements required for strong germ cell-specific transcription in vivo. Our results show that this promoter can be used for specific targeting of transgene expression to male germ cells.

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