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Repression of kit Expression by Plzf in Germ Cells▽

Doria Filipponi,[1] Robin M. Hobbs,[2] Sergio Ottolenghi,[3] Pellegrino Rossi,[1] Emmanuele A. Jannini,[4] Pier Paolo Pandolfi,[2] and Susanna Dolci[1]*

Dipartimento di Sanità Pubblica e Biologia Cellulare, Università di Roma Tor Vergata, Rome, Italy[1]; Cancer Biology and Genetics Program, Department of Pathology, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10021[2]; Dipartimento di Biotecnologie e Bioscienze, Universit à Milano-Bicocca, Milan, Italy[3]; and Dipartimento di Medicina Sperimentale, Università dell'Aquila, L'Aquila, Italy[4]

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Male mice lacking expression of Plzf, a DNA sequence-specific transcriptional repressor, show progressive germ cell depletion due to exhaustion of the spermatogonial stem cell population. This is likely due to the deregulated expression of genes controlling the switch between spermatogonial self-renewal and differentiation. Here we show that Plzf directly represses the transcription of kit, a hallmark of spermatogonial differentiation. Plzf represses both endogenous kit expression and expression of a reporter gene under the control of the kit promoter region. A discrete sequence of the kit promoter, required for Plzf-mediated kit transcriptional repression, is bound by Plzf both in vivo and in vitro. A 3-bp mutation in this Plzf binding site abolishes the responsiveness of the kit promoter to Plzf repression. A significant increase in kit expression is also found in the undifferentiated spermatogonia isolated from *Plzf*$^{-/-}$ mice. Thus, we suggest that one mechanism by which Plzf maintains the pool of spermatogonial stem cells is through a direct repression of kit expression.

Spermatogenesis is a highly organized cyclic process with distinct phases: mitosis, meiosis, and spermiogenesis. During the mitotic phase, spermatogonia proliferate and continuously self-renew to give rise to two subpopulations of germ cells: the differentiated germ cells and the stem cells. One current model proposes that the A single (As) stem cells either renew themselves or divide into paired (Apr) daughter cells that remain connected by an intercellular bridge (20, 32). Apr spermatogonia divide further to form long chains of aligned (Aal) cells, which then generate the differentiating A1 to A4, intermediate, and type B spermatogonia. This population of spermatogonia starts expressing the receptor-coupled tyrosine kinase kit and becomes responsive to kit ligand (KL) (37). Thus, kit expression is the major biochemical feature which marks the process of spermatogonium differentiation.

The *kit (White spotting locus*) gene, encoding the transmembrane receptor of the cytokine stem cell factor/KL, regulates proliferation/survival and/or migration of several stem cell types such as the primordial germ cells (PGCs) (15, 18), the multipotent hematopoietic stem cells (HSCs) (9, 21), the neural crest (25), the intestinal Cajal cells (42), and spermatogonia (14, 17, 36). Many *kit* (*W*) or *KL* (*Steel* [*Sl*]) gene null mutations or deletions result in severe hematopoietic, germ cell, and pigmentation defects leading to in utero or perinatal death. During mouse development *kit* is expressed at a low level in the pluripotent inner cell mass (and in epiblast-derived embryonic stem [ES] cells in culture) (3) and, at relatively higher levels, in PGCs, in early hematopoietic progenitors, and other cells (for reviews, see references 3 and 4). In postnatal animals, *kit* is expressed in a variety of cell types including HSCs, immature hematopoietic progenitors and mast cells, melanocytes, and oocytes (3) and in the differentiating spermatogonia (40). The role of kit in the maintenance and proliferation of postnatal germ cells has been highlighted by the finding that a point mutation of kit, Y719F, responsible for phosphatidylinositol 3-kinase docking, affects spermatogonium proliferation during the prepubertal period, resulting in sterility (7, 23).

Plzf (promyelocytic leukemia zinc finger, also known as *Zfp145*) was initially identified as a fusion product with the gene *Rara* (retinoic acid receptor α) in chromosomal translocations of acute promyelocytic leukemic cells (13). The wild-type (wt) Plzf protein is a DNA sequence-specific transcriptional repressor that is characterized by nine *Krüppel*-like C_2-H_2 zinc fingers and an N-terminal BTB/POZ domain (26). By homodimerization, Plzf can exert local and long-range chromatin remodeling activity through the recruitment of DNA histone deacetylases and through the action of several nuclear corepressors (2). *Plzf* maps to the *luxoid* locus and plays a crucial role in patterning the developing limb and axial skeletal structures (1, 10). Plzf expression has been also identified in a particular subset of male germ cells, the spermatogonial stem cells (10, 12, 34). *Plzf*-null mice as well as *luxoid* mutants, besides skeletal abnormalities, show male sterility (10, 12). It has been hypothesized that inactivation of *Zfp145* causes an initial increased burst of proliferation, followed by the rapid exhaustion of the proliferative spermatogonial compartment, due to deregulated expression of genes controlling the tight balance between spermatogonial self-renewal and differentiation (12). Since kit is a hallmark of differentiating spermatogonia, we hypothesized that it might be a target of Plzf repression.

We recently defined minimal *kit* DNA regulatory elements responsible for the expression in early hematopoietic and germ cells (11). By fusing the promoter and enhancer elements from the first intron of the mouse *kit* gene to a green fluorescent protein (GFP) reporter gene, we obtained a construct that

* Corresponding author. Mailing address: Department of Public Health and Cell Biology, University of Rome Tor Vergata, Via Montpellier 1, Ed. E Nord, 00133 Rome, Italy. Phone: 390672596252. Fax: 390672596268. E-mail: dolci@uniroma2.it.

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correctly recapitulates *kit* expression throughout the development of both these lineages. Here we show that Plzf acts as a transcriptional repressor of kit expression and identify two Plzf binding sites in the kit promoter region. By deletion analysis we identify the critical regulatory DNA region that is responsible for the Plzf-mediated repression.

MATERIALS AND METHODS

Cell culture and transient transfection. Transient transfections using Hek293 cells, maintained in Dulbecco's modified Eagle's medium with 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM glutamine, were performed with Lipofectamine. The D3 ES cell line was grown in Dulbecco's modified Eagle's medium with 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM glutamine, 15% fetal calf serum, 1 mM sodium pyruvate, 0.5 μM mercaptoethanol, and 1,000 U leukemia inhibitory factor onto mitomycin-treated mouse embryonic fibroblasts. One passage of D3 cells was performed before transfection onto gelatin-coated plates. Before transfection, cells were trypsinized and resuspended in antibiotic-free Dulbecco's modified Eagle's medium. Pellets of 10^6 cells were resuspended in 50 μl of Lipofectamine transfection reagent (Life Technologies, Milan, Italy; 1.6 μg DNA/10 μl Lipofectamine) and incubated for 10 min at room temperature as reported by Ward and Stern (41).

Primary spermatogonia were obtained by differential enzymatic digestion of 7-day-postnatum (dpn) mouse testes from wt or transgenic p18 mice (11, 36). As for ES cells, spermatogonium transfections were performed in suspension with Lipofectamine. Plasmid concentrations were as follows: 1 μg for p13, p18, and all the constructs containing the kit regulatory regions; 1 μg pCMV-Plzf; 0.1 μg pCMV-TIR-myc plasmid (27); or 0.1 μg pRLuc was included in the Lipofectamine mixture as a control for transfection efficiency. One microgram of pCMV5 DNA was used as a carrier to equalize the total amount of transfected DNA, when pCMV-Plzf was omitted. In all cotransfection experiments, the total amount of transfected mammalian expression vectors was kept constant. All transfections were performed at least three times.

Mutagenesis of Plzf binding site. Plasmid p316 bis-wt was obtained by amplification of the 5′ kit flanking region using as forward (FW) primer the sequence AGGACCTATACAGTGGAGAGAAAGA and as reverse (RV) primer the sequence GTTCTGCTGGTAGTGGTCGG, obtained from the enhanced GFP (EGFP) coding sequence. The PCR product was subcloned in a TA cloning vector (Invitrogen, Milan) and then excised with EcoRI and NcoI and recloned in p316 after removal of the EcoRI-NcoI fragment containing the kit flanking regions. Plasmid p316 bis-mut was prepared with the same strategy by using as FW primer the sequence AGGACCTAT**CAG**GTGGAGAGAAAGA, containing a 3-bp mutation (bold) in the Plzf binding site. Both FW primers correspond to those used in the electrophoretic mobility shift assay (EMSA) experiments (see below).

ChIP. Proteins from isolated spermatogonia at 7 dpn were cross-linked to DNA by direct addition to the culture medium of formaldehyde at a 1% final concentration for 10 min at 37°C. After sonication, a ChIP assay was performed according to the method of Boyd and Farnham (8), and protein-DNA complexes were immunoprecipitated overnight in the presence of the specific anti-Plzf monoclonal antibody (Chemicon, Milan, Italy) or preimmune immunoglobulin G (IgG) (Sigma-Aldrich, Italy). A genomic region of 521 bp containing the Plzf binding site region 2 in the kit promoter was amplified by PCR using specific primers flanking the site Plzf-BS2 (primer A, FW, CCAACCTATCTACCT ACC; primer B, RV, GGGTGGACAGTAACAATC). PCR conditions were as follows: 94°C for 1 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s, and finally −72°C for 5 min. As controls, two genomic regions of a 506-bp sequence (primer C, FW, AGGTGGGAAGAGCGGCA; primer D, RV, CAGCACTGTGCACAAAGAGC) and a 717-bp sequence (primer E, FW, TTTGTGCACAGTGCTGGG; primer F, RV, CAGCGACACCTGGCAAA) within the kit first intron that do not contain any Plzf sites were amplified by PCR.

Immunofluorescence and immunohistochemistry. Testes were fixed in formalin and embedded in OCT compound or in paraffin for immunofluorescence or immunohistochemistry detection, respectively. For immunofluorescence, 5-μm tissue sections obtained from OCT-frozen testes or spermatogonium cell suspensions were made to adhere to poly-L-lysine-coated slides and permeabilized for 10 min in 0.1% Triton X in phosphate-buffered saline (PBS). After a 1-h block in 5% bovine serum albumin (BSA) in PBS, anti-Plzf (Chemicon, Milan, Italy) antibodies were added at a 1:100 dilution in 0.5% BSA in PBS and incubated overnight at 4°C. Cyanin-3 secondary antibodies were added to the sections or to the isolated cells for 1 additional hour. Nuclei were labeled by incubating the slides for 5 min in Hoechst 33349 (1 μg/ml). For immunohistochemistry, 3-μm serial sections were quenched for endogenous peroxidase activity by incubating them with 0.3% hydrogen peroxide. Sections were treated for 30 min at 98°C in EDTA antigen retrieval solution (DAKO, Milan, Italy) and then incubated at room temperature for 1 h with the primary antibody. Anti-Oct4 (Santa Cruz Biotechnology, Heidelberg, Germany), anti-CD114 (anti-human kit; DAKO, Milan, Italy), or anti-Plzf antibodies were all used at a 1:100 dilution in 0.5% BSA in PBS. Sections were washed three times in PBS and then incubated for 30 min at room temperature with a solution containing biotinylated secondary antibodies (anti-mouse and anti-rabbit IgG mixture) and StreptABComplex-horseradish peroxidase (DAKO, Milan, Italy). Sections were revealed by diaminobenzidine and counterstained with Gill's hematoxylin.

EMSA. Nuclear extracts were obtained from spermatogonia or from Hek293 mock-transfected or Plzf-transfected cells as previously reported (19). Complementary wt (FW, AGGACCTATACAGTGGAGAGAAAGA; RV, GTTCTTTCTCT CCACTGTATAGGTC) or mutated (FW, AGGACCTAT**CAG**GTGGAGAGAA AGA; RV, GTTCTTTCTCTCCAC**CTG**ATAGGTC; mutations indicated in bold) oligonucleotide probes obtained from region 2 of the kit promoter containing the second potential Plzf binding site were annealed, and the overhanging ends were extended with Klenow polymerase and [α-^{32}P]dCTP. Binding reactions were carried out in a volume of 10 μl. Ten micrograms of nuclear extracts in binding buffer (20 mM HEPES, pH 7.5, 1 mM $MgCl_2$, 10 μM $ZnCl_2$, 4% glycerol, 100 mg/ml BSA) was incubated on ice for 20 min, in the presence or absence of a 100× molar excess of unlabeled wt or mutated double-stranded oligonucleotides. Anti-Plzf antibodies or control mouse IgG antibodies were added at the concentrations of 0.4 and 4 μg/reaction mixture, respectively, and incubated at room temperature for 30 min. The mixtures were incubated for a further 20 min in the presence of 20,000 cpm of labeled probe at room temperature. DNA loading dye was added, and samples were electrophoretically separated through a 0.5× Tris-borate-EDTA–nondenaturing polyacrylamide gel before autoradiography.

EGFP fluorescence intensity and *Renilla* luciferase assay. The expression of EGFP was quantified in a microplate fluorescence reader (Cary-Eclipse; Varian, Germany) at 24 h after transfection. The *Renilla* luciferase assay was performed using the Dual-Luciferase Reporter System (Promega) on the cell extracts obtained from the microplates previously analyzed for fluorescence intensity. The luciferase activities were normalized for μg of protein. EGFP intensities were normalized to *Renilla* luciferase activities.

Western blotting. Cells were lysed in 1% Triton X-100–140 mM NaCl–10 mM Tris (pH 8) and then denatured by boiling them diluted in 4× sodium dodecyl sulfate (SDS) buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.25 M Tris [pH 6.9], 0.01% bromophenol blue). Proteins (10 μg from Hek293 and D3 cells, 40 μg from spermatogonia) were separated by SDS-10% polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Amersham, Milan, Italy) in 25 mM Tris-192 mM glycine buffer. The membrane was blocked in PBS-5% skim milk powder for 1 h. Incubation of the membrane with the primary antibody was carried out at 4°C overnight in PBS-5% BSA, the membrane was then washed three times in PBS, and the appropriate horseradish peroxidase-conjugated secondary antibody was added to PBS at the concentration recommended by the manufacturer (Santa Cruz Biotechnology, Heidelberg, Germany). Rabbit anti-EGFP antibodies and mouse anti-myc epitope were from Santa Cruz Biotechnology (Heidelberg, Germany). The horseradish peroxidase conjugate was detected by chemiluminescence with an ECL kit (Amersham, Milan, Italy) and autofluorography.

Northern blotting. Hek293 cells were cotransfected with p18 and *Renilla* luciferase plasmid in the presence of pCMV-Plzf or an empty plasmid. Cells were harvested at 24 h after transfection, and RNA was extracted with TRIzol (Invitrogen, Milan, Italy) from 10^7 cells. Total RNA (15 μg) was treated for 20 min with 20 U of RNase-free DNase to avoid plasmid contamination, separated by denaturing agarose gel electrophoresis, and blotted onto a nylon membrane (Hybond-N; Amersham, United Kingdom). Hybridization was carried out following the Quick Hybrid System instructions (Stratagene, CA) using an [α-^{32}P]dCTP-labeled EGFP cDNA. Ten thousand transfected cells were extracted for the *Renilla* luciferase assay.

Flow sorting of spermatogonia. Single-cell suspensions were isolated from the testes of *Plzf*$^{+/+}$ and *Plzf*$^{-/-}$ mice (1, 12) by enzymatic digestion essentially as described elsewhere (33). Cell samples were resuspended in PBS supplemented with 2% fetal bovine serum and stained with antibodies for 30 min on ice. Phycoerythrin-conjugated αv-integrin (CD51; clone RMV-7) and allophycocyanin-conjugated Thy-1 antibodies (CD90.2; clone 30-H12) were purchased from Biolegend (San Diego, CA). Flow sorting was performed on a MoFlo sorter (DAKO, CA) at the MSKCC Flow Sorting Facility using 4′,6′-diamidino-2-phenylindole (DAPI) for live/dead discrimination. For immunofluorescence analysis, sorted cells were spotted directly onto poly-L-lysine-coated microscope

slides, allowed to dry, and fixed in 4% paraformaldehyde for 15 min at room temperature. After being washed in PBS, cells were permeabilized in methanol at −20°C for 10 min and then washed again and processed for Plzf immunostaining as described above.

Quantitative reverse transcription-PCR (RT-PCR). Flow cytometry-sorted cells were collected directly into TRIzol (Invitrogen, Carlsbad, CA), and RNA was purified according to the manufacturer's suggestions. Contaminating genomic DNA was removed using DNase I Amplification Grade (Invitrogen), and cDNA synthesis was subsequently performed with the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative PCR was performed in duplicate for each cDNA sample using a Light Cycler and DNA Master SYBR green kit (Roche Diagnostics). Relative mRNA levels of the target gene were normalized to β-actin expression for each sample, and normalized standard deviations were calculated. Primer sequences were as follows: Plzf FW, CTCCGTAAGCGTCCCCTCTGC; Plzf RV, GGTGCAGGC TAGCACCGTCC; GFRα1 FW, CACTCCTGGATTTGCTGATGT; GFRα1 RV, AGTGTGCGGTACTTGGTGC; kit FW, GCCACGTCTCAGCCATCTG; kit RV, GTCGGGATCAATGCACGTCA; β-actin FW, GGCTGTATTCCCCTCCA TCG; β-actin RV, CCAGTTGGTAACAATGCCATGT.

Statistical analysis. The Student *t* test and analysis of variance (ANOVA) have been used to assess the significance, set at $P < 0.05$. All experiments were performed at least three times and at least in triplicate for each sample.

RESULTS

Plzf is detected in Oct4-positive but not in kit-positive spermatogonia. It has been previously shown that, in neonatal testis, Oct4 and Plzf are coexpressed in spermatogonial stem cells and undifferentiated spermatogonia (10). Using 12-day-postpartum (dpp) mouse testis sections, we confirm that Plzf is coexpressed in Oct4-positive cells, although interestingly, several Plzf-positive spermatogonia appear to be Oct4 negative (Fig. 1A, red arrows). These Plzf-positive, Oct4-negative cells were located in chains along the testis tubules, suggestive of the type Aal spermatogonia (Fig. 1A, red arrows), in contrast to the isolated Oct4-positive, Plzf-positive spermatogonia (type As spermatogonia, Fig. 1A, yellow arrows).

Since at this developmental age kit-expressing cells are present within the testicular tubules (37), we investigated if kit-expressing spermatogonia were also positive for Plzf. To this aim we used a transgenic line, which was obtained with a construct containing 6.9 kb of the *kit* promoter region and 3.5 kb of the first *kit* intron driving EGFP expression (construct p18). This transgenic line robustly expresses EGFP in the HSCs and in the germ line (11) including spermatogonia (Fig. 1B) (D. Filipponi et al., unpublished data). By immunofluorescence we found that Plzf-expressing cells did not correspond to EGFP-positive kit-expressing spermatogonia and that the nuclear morphology of these cells was different from that of the Plzf-expressing spermatogonia (Fig. 1B and C). Indeed, Hoechst 33349 identifies two classes of chromatin organization in p18-EGFP-isolated spermatogonia. Plzf is localized in nuclei less intensely labeled by Hoechst staining, while kit-expressing spermatogonia show nuclei with a more intense chromatin staining. Immunohistochemistry assays using anti-kit antibodies and anti-Plzf antibodies on serial testis sections confirmed that the Plzf-expressing cells are not the kit-expressing cells (Fig. 1D). In fact, we were never able to identify the same cell as positive for both the antigens in contiguous tubule sections, further confirming the results obtained using the EGFP-p18 transgenic line. Together, these observations support the hypothesis that the spermatogonial cell types expressing Plzf belonged to the precursors of the A1 spermatogonia (the first kit-expressing spermatogonia) (34). Moreover, the findings assign different molecular markers to each class of the morphologically different spermatogonial populations.

Plzf represses kit promoter activity. It has been recently reported that overexpression of Plzf in the human leukemia hematopoietic K562 cell line induces the downregulation of kit protein expression, as revealed by flow cytometry (35). Our observation that Plzf was not easily detectable in kit-positive spermatogonia led us to hypothesize a possible negative influence of this transcriptional repressor on the activity of the kit promoter.

To test this hypothesis, we took advantage of two previously characterized constructs (11), obtained by fusing the promoter or the promoter and enhancer elements from the first intron of the mouse *kit* gene to an EGFP reporter gene. The first construct contains about 6.9 kb of the *kit* promoter region and 3.5 kb of the first kit intron (p18, Fig. 2A). The second construct (p13, Fig. 2B) comprises only the promoter region. Among the transgenic lines obtained from these constructs, those that originated from p18 were found to express EGFP both in the hematopoietic compartment and in postnatal germ cells (11; Filipponi et al., unpublished) (also Fig. 1B), while those originating from p13 expressed low levels of EGFP only in PGCs. To find out which region(s) within these constructs potentially contained Plzf-responsive elements, p18 was cotransfected with a Plzf expression vector into kit-expressing cell types, such as D3 ES cells, Hek293 cells, or isolated mouse spermatogonia (Fig. 2A). Plzf expression strongly repressed kit reporter expression in all the cell types tested when we used the constructs containing the promoter region. The levels of the endogenous kit protein were also drastically reduced in Hek293 and D3 ES cells, even though only a small reduction of the levels of the endogenous kit protein was observed when Plzf was transfected in spermatogonia (see Discussion). EGFP reporter expression was dramatically reduced by Plzf cotransfection in Hek293 and D3 ES cells also when we used the p13 construct, lacking sequences from the first intron (Fig. 2B). A pCMV-EGFP construct, used in parallel to verify the specificity of the repressing activity of Plzf, was not repressed by Plzf cotransfection (Fig. 2C). Transfection efficiency was monitored by the coexpression of a fusion protein from a pCMV-TIR-myc plasmid (27), which did not interfere with kit or Plzf expression. Since both p18 and p13 constructs that were used in the transfection experiments were still sensitive to Plzf repression, the first *kit* intron appears to be unnecessary for Plzf responsiveness. We then scanned the 6.9 kb of the promoter to find out if Plzf-responsive elements were present. Two potential binding sites were found (Fig. 2B). The first binding site was located at around 6 kb (ATACAGT, position 1581874 of the *kit* contig, region 1), while the second was found at around 1.6 kb from the transcriptional start site (ATACAGT, position 1586134 of the *kit* contig, region 2). We then obtained a construct in which about 3 kb containing the first Plzf binding site was deleted. Transfections performed in Hek293 cells showed that the repression activity of Plzf was still maintained (Fig. 2D). Two constructs were then generated in which the second Plzf binding site was also deleted (Fig. 2E and F). The first construct (Ia) contained about 900 bp of the promoter while the second construct (Ib) contained about 400 bp of the promoter. After transfection in the absence of Plzf, both the constructs were independently able to activate EGFP expression

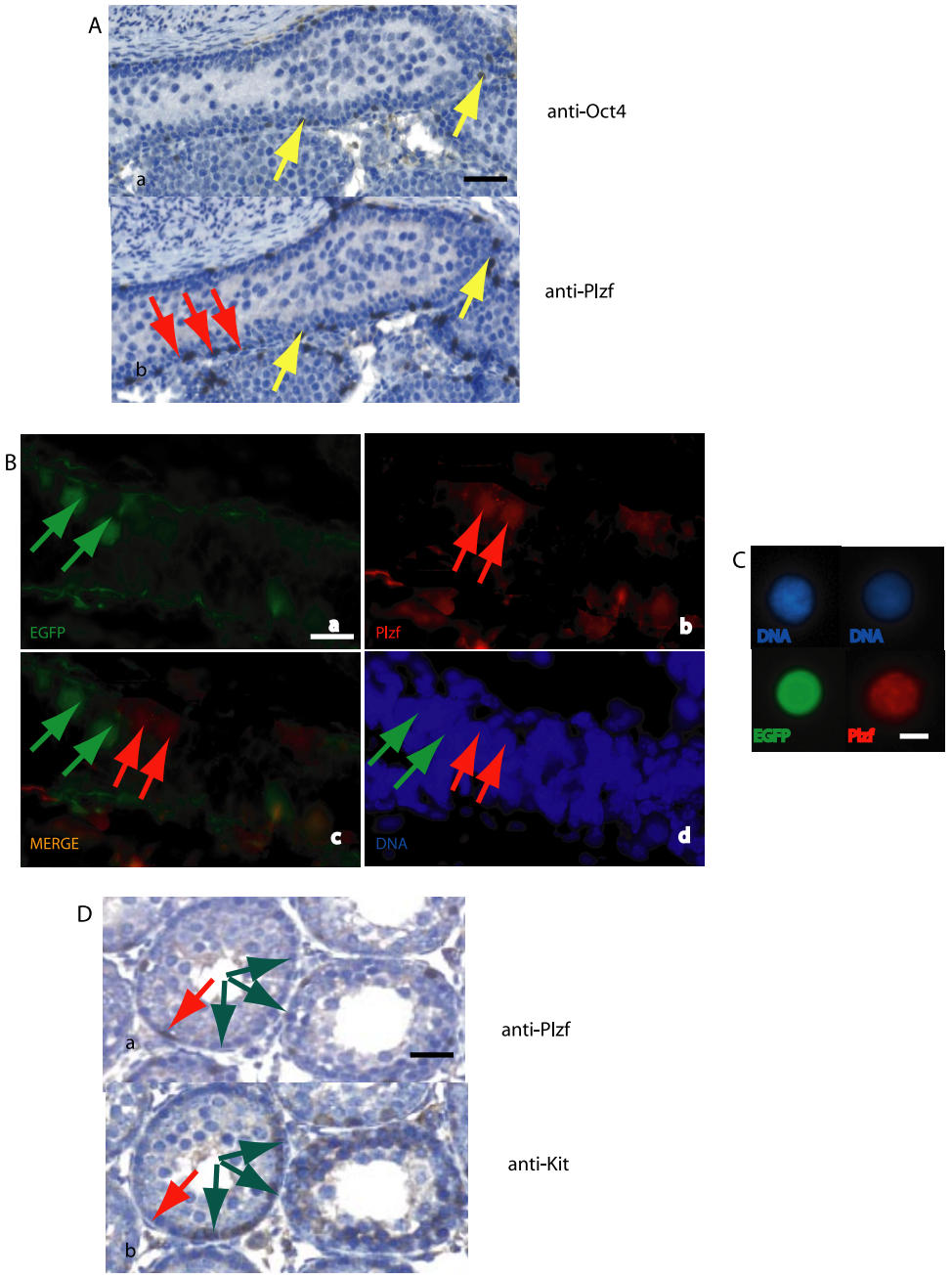

FIG. 1. Plzf colocalizes with Oct4 but not with kit in mouse spermatogonia. (A) Immunohistochemical detection of Plzf and Oct4 in contiguous paraffin sections of 12-dpp transgenic testes from p18-EGFP mice. Yellow arrows point to Oct4-positive spermatogonia (a) which are also Plzf positive (b); red arrows point to Plzf-positive but Oct4-negative spermatogonia (b). Bar, 50 μm. (B) Immunofluorescent detection of Plzf in frozen sections of transgenic testes from p18-EGFP mice. (a) Green arrows point to EGFP-positive spermatogonia (bar, 20 μm). (b) Red arrows point to anti-Plzf-positive spermatogonia. (c) Arrows indicate that EGFP (kit-expressing) and Plzf-positive cells do not match in the merged picture. (d) Green and red arrows in the Hoechst 33349-stained section point to the nuclear morphologies of kit- and Plzf-expressing cells. (C) Hoechst 33349 identifies two classes of chromatin organization in p18-EGFP isolated spermatogonia. Plzf is localized in nuclei less intensely labeled by Hoechst staining, while kit-expressing spermatogonia show nuclei with a more intense chromatin staining (bar, 10 μm). (D) Immunohistochemical detection of Plzf and kit in contiguous paraffin sections of 12-dpp transgenic testes from p18-EGFP mice. Red arrows point to Plzf-expressing cells which do not match with the kit-expressing spermatogonia (green arrows) in panels a and b. Bar, 50 μm.

in ES cells and Hek293 cells; however, Plzf responsiveness was completely lost (Fig. 2E and F). In order to quantitate the inhibitory effect of Plzf on the kit promoter, we performed a fluorimetric analysis after cotransfection experiments using *Renilla* luciferase to normalize for transfection efficiencies.

As shown in Fig. 3A, while pCMV-EGFP and Ia did not respond to the Plzf repressive effect, EGFP expression from plasmids p18 and p13 was inhibited between five- and threefold in both Hek293 and ES cells. Moreover, plasmid p316, which contains only the second Plzf binding site, was inhibited more

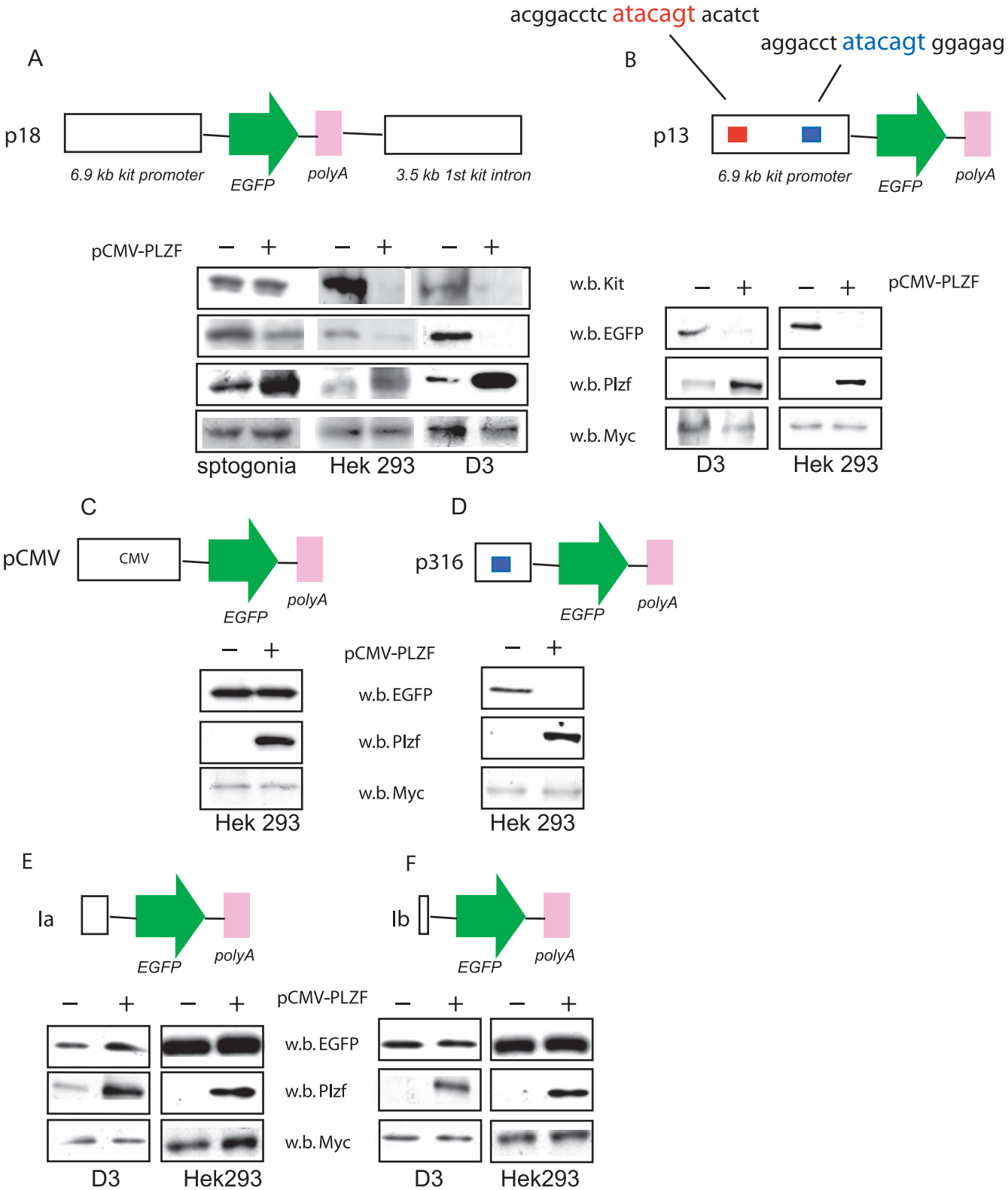

FIG. 2. Plzf overexpression represses the promoter activity of constructs containing *kit* regulatory regions. Repression of kit by Plzf requires one of the two potential binding sites in the kit promoter. (A) Schematic representation of plasmid p18, which was transfected in spermatogonia or D3 ES or Hek293 cells in the presence or absence of a Plzf expression vector. Cell extracts were probed by Western blotting for expression of kit, EGFP, Plzf, and a myc epitope from a cotransfected pCMV-TIR-myc plasmid. (B) Schematic representation of plasmid p13 in which two Plzf binding sites and sequences are indicated by red and blue squares and letters. p13 was transfected in D3 ES and/or Hek293 cells in the presence or absence of a Plzf expression vector. Transfection efficiencies were monitored by the coexpression of a fusion protein from a pCMV-TIR-myc plasmid (myc epitope). (C and D) Schematic representation of pCMV plasmid and plasmid p13 in which the first Plzf binding site was deleted. Hek293 cells were transfected in the presence or

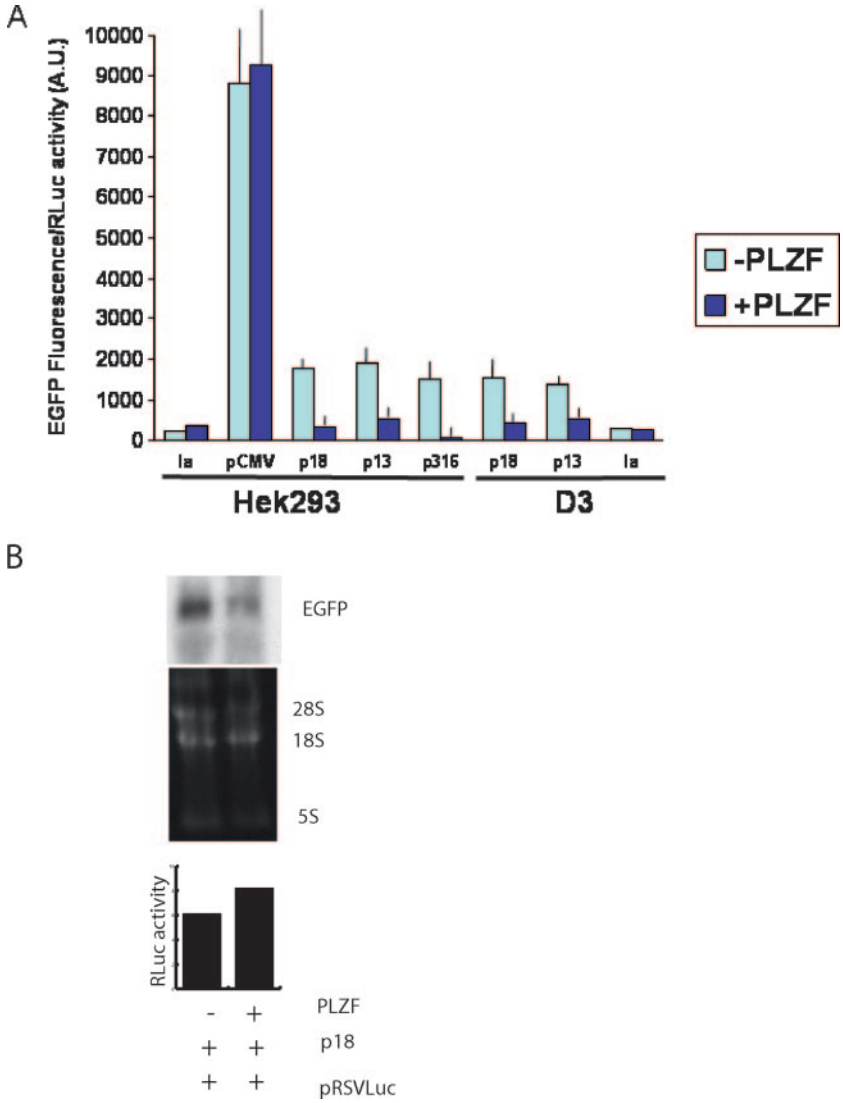

FIG. 3. Quantitative evaluation of the repressive effect of Plzf on kit promoter and evidence that it is exerted at the transcriptional level. (A) Hek293 and D3 ES cells were cotransfected with the plasmids reported (p18, p13, pCMV, p316, and pIa) and *Renilla* luciferase in the presence or absence of pCMV-Plzf. Bar graphs report the quantitative determination of gene expression by fluorimetric analysis, with results normalized to those for *Renilla* luciferase (A.U., arbitrary units). Standard deviations were obtained from three independent experiments. (B) Northern blot analysis of EGFP RNA from p18-transfected Hek293 cells in the presence or absence of pCMV-Plzf. Integrity and loading of RNA are shown in the middle panel. Bar graphs represent the activity of cotransfected *Renilla* luciferase to normalize for transfection efficiency (bottom panel).

than 10-fold in Hek293 cells. By performing a Northern blot analysis on Hek293 cells transfected with p18, we then confirmed that the Plzf repressive effect on the kit promoter was exerted at the transcriptional level (Fig. 3B).

kit promoter sequences containing a Plzf consensus are bound in vitro and in vivo by Plzf. Since we found that the sequence located at about 1.6 kb from the transcriptional start site was mediating Plzf repressing activity, we designed an oligonucleotide centered on the second Plzf binding site to use as a probe in EMSA experiments. Nuclear extracts from spermatogonia or from mock- or Plzf-transfected Hek293 cells were primed with the labeled probe (Fig. 4A). When either spermatogonia (lane 2) or Plzf-transfected Hek293 (lane 4) nuclear extract was used, a complex formed. Mock-transfected nuclear extracts also showed a less-intense retarded band (lane 3) due to endogenous expression of Plzf in Hek293 cells (Fig.

absence of a Plzf expression vector. Cell extracts were probed by Western blotting for EGFP, Plzf, and myc epitope expression. (D and E) Schematic representation of plasmid p13 in which the two Plzf binding sites were deleted. (E) Construct in which the deletion left about 300 bp of the promoter (pIA). (F) Construct in which the deletion left only 150 bp of the promoter (pIB). D3 ES and Hek293 cells were transfected in the presence or absence of a Plzf expression vector. Cell extracts were probed by Western blotting for EGFP, Plzf, and myc epitope expression.

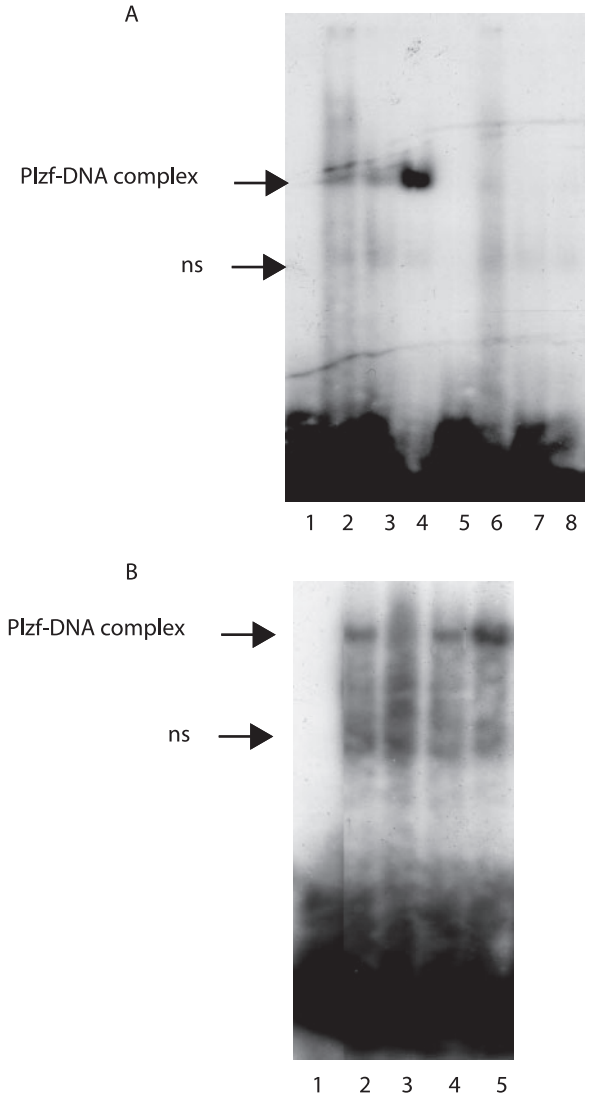

FIG. 4. Nuclear extracts from spermatogonia bind a Plzf consensus sequence present in the *kit* promoter. (A) EMSA of nuclear extracts from spermatogonia (lane 2) or mock-transfected (lane 3) or Plzf-transfected (lane 4) Hek293 cells with a radiolabeled oligonucleotide containing the Plzf binding site. The protein/DNA complexes show the same electrophoretic mobility either in spermatogonium extracts or in both mock- and Plzf-transfected Hek293 cells. A 100-fold molar excess of unlabeled oligonucleotide was able to displace the complexes in the presence of either spermatogonia or Plzf-transfected Hek293 nuclear extracts (lanes 7 and 8, respectively). A radiolabeled oligonucleotide, in which the Plzf binding site was mutated, was not able to produce any retarded band in the presence of Plzf-transfected Hek293 nuclear extracts (lane 6). Lanes 1 and 5 represent the mobility of the free wt or mutated oligonucleotides, respectively. (B) EMSA of nuclear extracts from spermatogonia with a radiolabeled oligonucleotide containing the Plzf binding site (lane 2), in the presence of 0.4 μg of anti-Plzf antibody (lane 3) or 4 μg of mouse IgGs (lane 4). As a control, Plzf-transfected Hek293 nuclear extracts were used (lane 5). Lane 1 represents the free probe. ns, nonspecific.

2A). The retarded bands obtained from spermatogonia and Plzf-transfected Hek293 nuclear extracts were displaced by a 100-fold molar excess of unlabeled probe (lanes 7 and 8, respectively). A mutated probe in which nucleotides ATACAGT were replaced by AT**CAG**GT was not able to produce any retarded band from Plzf-transfected Hek293 cells (lane 6). To demonstrate that the retarded band was actually due to Plzf binding, we performed an EMSA experiment using a specific anti-Plzf antibody. As shown in Fig. 4B, after preincubation of spermatogonium nuclear extracts with this antibody, formation of the specific retarded band was prevented, whereas a 10-fold-higher excess of mouse IgGs had no effect.

To determine whether Plzf was recruited to the endogenous *kit* promoter in vivo, we performed ChIP experiments by immunoprecipitating Plzf from formaldehyde cross-linked spermatogonium chromatin. By using primer pairs spanning the Plzf binding site found in region 2 of the *kit* promoter, immunoprecipitated chromatin was analyzed by PCR. The Plzf binding site of region 2 was amplified with primer pairs A and B (Fig. 5A) in anti-Plzf but not from control mouse IgG chromatin immunoprecipitates (Fig. 5B). In contrast, when two independent regions within the first 2.7 kb of the first c-*kit* intron were queried by PCR (primer sets C-D and E-F), we were not able to detect immunoprecipitated chromatin from either anti-Plzf or control IgGs (Fig. 5B). Consistent with the EMSA experiments, these results indicate that Plzf is associated with the region surrounding the Plzf binding site region 2 at the *kit* promoter in mouse spermatogonia.

The second Plzf DNA binding site of the kit promoter mediates the responsiveness to Plzf repression. In order to reconstitute the responsiveness to the repressing activity of Plzf, we cloned the Plzf-responsive region 2 (550 bp), which contained the second Plzf binding site, downstream of the EGFP gene in the construct Ia (pIaPBS). The construct was transfected in the presence or absence of the Plzf expression vector in Hek293 cells, and EGFP levels were assessed by Western blotting. As shown in Fig. 6A, Plzf responsiveness was restored when region 2 was cloned in construct Ia, even if it was positioned after the EGFP gene downstream from the promoter region. To verify if the repressive effect was actually mediated by the Plzf binding site, we performed transfection experiments in Hek293 cells using the two plasmids shown in Fig. 6B, i.e., plasmid p316 bis-wt, containing the 5′ kit flanking region starting 6 nucleotides upstream from the second Plzf binding site, and an identical plasmid containing a 3-bp mutation in the binding site (p316 bis-mut). Both quantitative evaluation of EGFP fluorescence versus *Renilla* luciferase activity (Fig. 6C) and Western blot analysis of EGFP protein levels (Fig. 6D) indicate that the 3-bp mutation in the Plzf binding site 2 abolishes the responsiveness of the kit promoter to the repressive activity of cotransfected Plzf.

Plzf-null spermatogonia express higher levels of *kit* transcript. Given the ability of Plzf to repress expression of the *kit* gene in vitro, we next set out to determine whether the absence of Plzf expression in vivo produced a corresponding derepression of kit levels. To perform this experiment, it was necessary to purify the undifferentiated Plzf-expressing spermatogonia and equivalent populations from $Plzf^{+/+}$ and $Plzf^{-/-}$ mice. Juvenile mice were used for purification prior to the germ cell degeneration observed in $Plzf^{-/-}$ testis (12). To isolate the undifferentiated spermatogonia from juvenile testis, we adapted cell surface markers found to select for the spermatogonial stem cell fraction in adult cryptorchid testis (24, 38). From these studies, flow-sorted spermatogonia, able to repop-

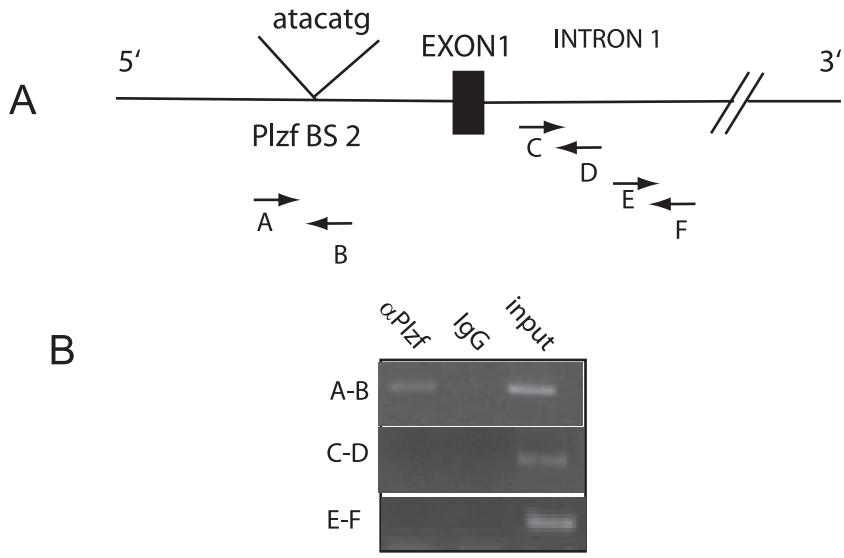

FIG. 5. Plzf binds to a consensus sequence in the kit promoter region in spermatogonium chromatin. (A) Schematic representation of part of the *kit* locus covering 3 kb of the promoter region, the first exon, and 3 kb of the first intron. The arrows are positioned to indicate the DNA regions amplified by PCR. (B) Spermatogonium chromatin was cross-linked by formaldehyde and immunoprecipitated by control IgGs or anti-Plzf antibodies. Primer sets A-B, C-D, and E-F were used to amplify the Plzf binding region in the promoter or two unrelated regions chosen within the first intron, respectively.

ulate recipient testis, were found to be present in the side scatter (SSC)-low population and were positive for the Thy-1 marker and yet had low or absent expression of αv-integrin. The juvenile mouse testis, similar to the cryptorchid, is relatively enriched in undifferentiated spermatogonia, and costaining for αv-integrin and Thy-1 revealed a discrete SSC-low, αv-integrin-negative/low, Thy-1-positive fraction (Fig. 7A) which was found to contain the Plzf-expressing spermatogonia in cells prepared from $Plzf^{+/+}$ testes (Fig. 7B). Plzf-positive cells were not found enriched in the other cell populations (Fig. 7B; data not shown). Cells within this fraction were highly enriched for Plzf mRNA (in the $Plzf^{+/+}$ case) and for that of GFRα1, the coreceptor for glial cell line-derived neurotropic factor, which is also expressed in undifferentiated spermatogonia (30). Importantly, in this fraction of undifferentiated spermatogonia, kit mRNA levels were significantly higher in $Plzf^{-/-}$ mice than in the wt controls (Fig. 7C). Thus, we confirm that the *kit* gene is misexpressed in the absence of Plzf in vivo.

DISCUSSION

The main conclusion that can be drawn from the present results is that a major role played by Plzf in the negative regulation of spermatogonium stem cell differentiation might be the direct inhibition of kit transcription. Recent reports on the generation of $Plzf^{-/-}$ mice have shown that Plzf not only plays a crucial role in patterning the developing limb and axial skeletal structures (1) but also is essential for the normal balance between maintenance and differentiation of the stem cells in the testis (10, 12). Plzf knockouts or *luxoid* mutants show a progressive lack of spermatogonia in the tubules, an impaired spermatogenic process, and consequently decreased production of mature sperm. It has been suggested that the inactivation of *Plzf* induces an increased burst of spermatogonium proliferation followed by a rapid exhaustion of the proliferative spermatogonial compartment. It is well known that spermatogonium proliferation is tightly controlled by the expression of the tyrosine kinase-coupled receptor kit and its ligand KL (36) and that point mutations of the phosphatidylinositol 3-kinase docking site (Tyr 742) essentially block spermatogonium proliferation and result in male sterility (7, 23).

The positive controlling elements in the *kit* locus regulating its expression in the different lineages have been identified by the production of transgenic animals carrying a reporter gene under the control of kit genomic regions or bacterial artificial chromosome reporter constructs (3, 5, 11) or by the identification of DNase I-hypersensitive sites in the vicinity of the *kit* transcription start site (5, 11). *kit* expression in germ cells as well as in melanoblasts follows a bimodal pattern: its onset is very early in embryogenesis (at around 7.5 days postcoitum) (28), and it is turned off after midgestation in germ cells at around 13.5 days postcoitum (6, 28). In the germ cell lineage it is first expressed during fetal development, in a narrow window between 7.5 and 13.5 days postcoitum in PGCs, while after birth, in the male, it is reexpressed at the onset of spermatogenesis in differentiating spermatogonia (type A1) at around 6 dpn and in female germ cells at birth (28). In the male, postnatal expression of *kit* is confined to type A1 through type B spermatogonia, mediates the last rounds of mitotic division, and marks the transition from undifferentiated spermatogonia to spermatogonia committed to meiosis. The evidence of such a bimodal pattern of expression, at least in the germ line, would suggest the involvement of a repressor or a combination of a repressor and a tissue-specific transcription factor, which could fine-tune *kit* expression only in a subset of spermatogonia.

Several studies have indicated that the Plzf protein can bind DNA in a sequence-specific manner and function to repress transcription from promoters containing Plzf binding sites (2,

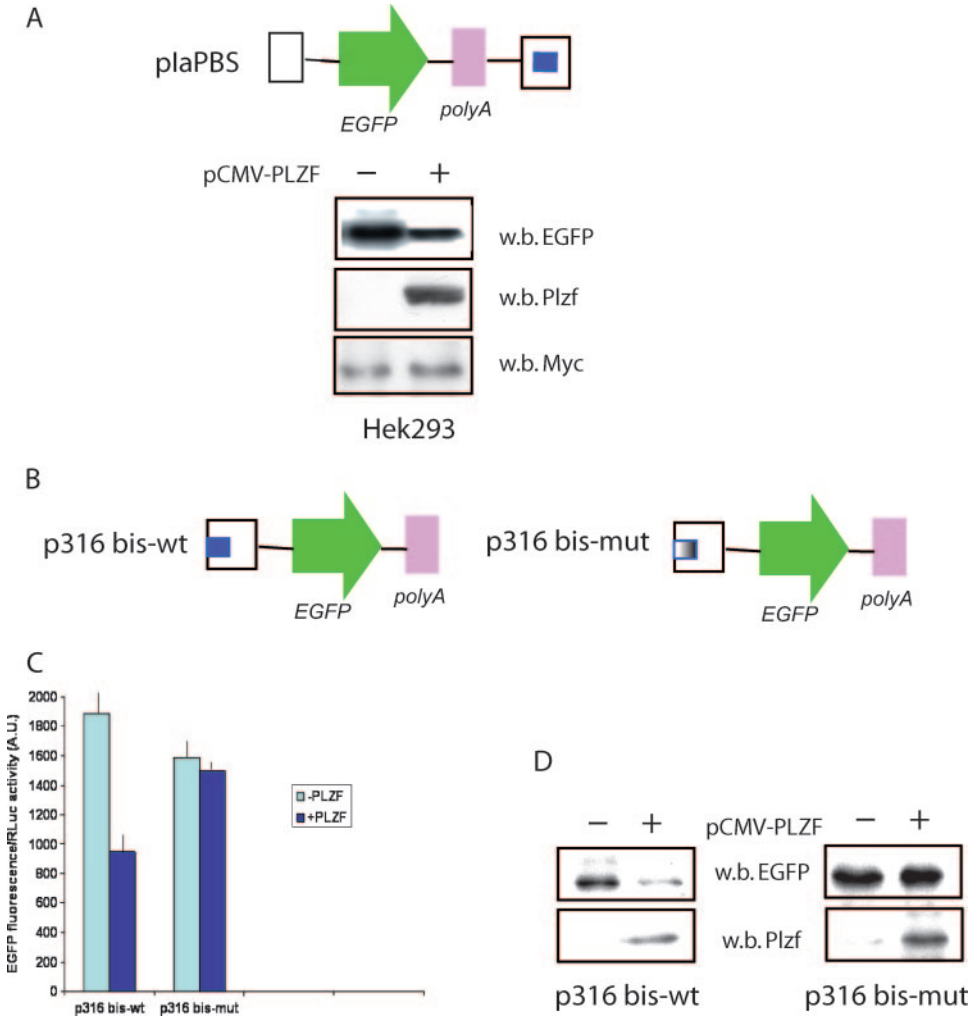

FIG. 6. Plzf binding site restores sensitivity to Plzf repressing activity, which is lost with a 3-bp mutation in the kit flanking region. (A) Schematic representation of plasmid pIa in which one of the two Plzf binding sites (indicated by the blue square) was cloned downstream of the EGFP gene (pIaPBS). Hek293 cells were transfected in the presence or absence of a Plzf expression vector. Cell extracts were probed by Western blotting for EGFP, Plzf, and myc expression, respectively. (B) Schematic representation of plasmids p316 bis-wt and p316 bis-mut, in which the second Plzf binding site (indicated by the blue square), was mutated (shadowed square). (C) Hek293 cells were cotransfected with the plasmids described in panel B and *Renilla* luciferase in the presence or absence of pCMV-Plzf. Bar graphs report the quantitative determination of gene expression by fluorimetric analysis normalized to *Renilla* luciferase (A.U., arbitrary units). Standard deviations were obtained from three independent experiments. (D) Cell extracts from the experiments reported in panel C were probed by Western blotting for EGFP and Plzf.

22, 43). If Plzf were to act as a transcriptional repressor for kit, its expression should be highest in the kit-negative germ cells. Indeed, in contrast to the kit expression pattern in the male gonad, Plzf is first detected a few days before birth in the germ cell compartment and is then maintained in the slowly cycling spermatogonia of the adult testis (12). We find that, in agreement with the data of Buaas et al. (10), Plzf is present in Oct4-positive spermatogonial stem cells but is not detectable in the kit-expressing spermatogonia. By immunohistochemistry assays with three different markers, Oct4, Plzf, and kit, we have been able to identify at least three classes of spermatogonia in the prepubertal testis: Oct4- and Plzf-double-positive spermatogonia as single cells (As), Plzf-positive spermatogonia in chains (Aal), and kit-positive spermatogonia (from A1 through the B stage). Heterogeneity in the expression of markers specific for undifferentiated spermatogonia, such as Oct4 and Plzf, has previously been noted and may represent functional differences among this immature germ cell population (31).

The observation that Plzf expression inversely correlates with kit expression in spermatogonia suggests that it potentially can act as a transcriptional repressor for kit expression. Since one of the major developmental events which marks the transition from a stem cell to a differentiated germ cell during spermatogenesis is the appearance, within the tubules, of kit-expressing spermatogonia, we explored the possibility that Plzf regulates kit expression by directly acting on the transcriptional activity of the *kit* gene.

We recently characterized the genomic region of the *kit* locus, involved in the regulation of kit expression in the germ line (11; Filipponi et al., unpublished). Here we demonstrate that when Plzf is overexpressed in kit-expressing cells such as spermatogonia, ES cells, or Hek293 cells, it is able to strongly

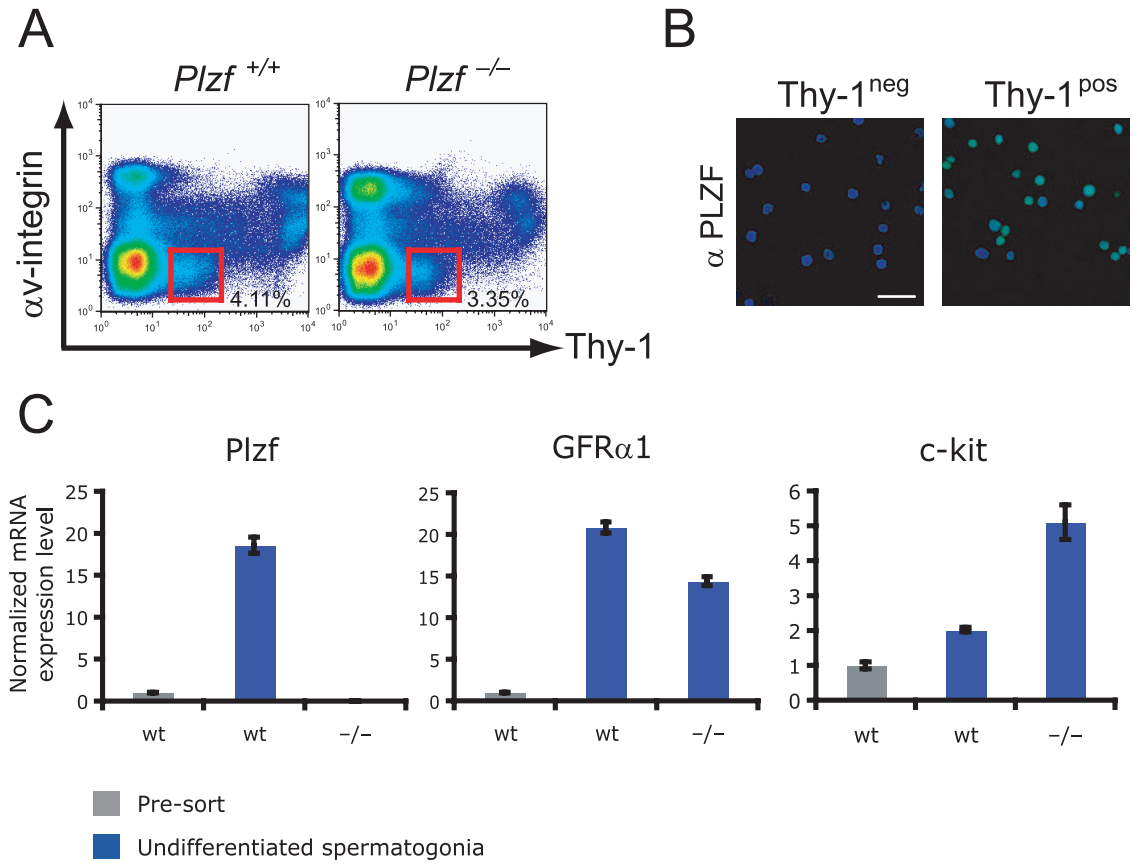

FIG. 7. Overexpression of kit in *Plzf*$^{-/-}$ immature spermatogonia. (A) Undifferentiated spermatogonia were isolated from the pooled testis cells of two *Plzf*$^{+/+}$ and two *Plzf*$^{-/-}$ juvenile mice (2 weeks postnatum) by flow sorting using the indicated markers. Panels show profiles of the SSC-low population and are gated on the αv-integrin-negative/low, Thy-1-positive fraction with indicated population percentages. (B) Spermatogonia from wt juvenile mice were sorted using the cell surface markers in panel A and spotted onto microscope slides for immunofluorescence analysis. Images show the αv-integrin-negative/low, Thy-1-negative and -positive fractions stained for Plzf in green plus DAPI nuclear counterstaining in blue. Bar, 50 μm. (C) Total RNA from the undifferentiated spermatogonium populations sorted in panel A was analyzed by quantitative RT-PCR to assess expression of the indicated genes (wt and −/− indicate samples from *Plzf*$^{+/+}$ and *Plzf*$^{-/-}$ mice, respectively). The bar graphs show gene expression normalized to β-actin mRNA levels with standard deviations. Included in the graphs is an analyzed testis cell sample isolated prior to flow sorting to demonstrate relative enrichment of gene expression in the undifferentiated fractions.

reduce the expression of a reporter gene (EGFP) by directly acting on the *kit* genomic regions which are driving EGFP expression. In transfected Hek293 and D3 cells, we also found a strong decrease of the endogenous kit levels, further supporting a role for Plzf as a kit repressor. Reduction of endogenous kit levels was less evident in transfected primary spermatogonia. This could be explained by the high stability of kit protein in spermatogonia (Filipponi et al., unpublished) and by the fact that spermatogonia isolated from 7-dpp mice consist of a heterogeneous population of mitotic germ cells, in which kit-negative spermatogonia are significantly represented (our unpublished observations) (also Fig. 1B). Furthermore, the transfection efficiency of primary spermatogonia was very low (less than 10%) compared to that of immortalized cell lines (note that, in order to obtain a signal comparable to that of the normalizing myc epitope in Fig. 2A, we loaded a fourfold-higher amount of proteins from spermatogonia with respect to Hek293 and D3 cells).

To date, only a few genes have been identified as the direct targets of Plzf repression, such as the cyclin A2 (43), c-*myc* (29), HoxD11 (2), Pbx1 (39), and VLA4 (35) genes. We found that responsiveness to Plzf was located within the first 7 kb of the kit promoter.

We found two direct Plzf binding sites (ATACAGT) within the first 7 kb of the promoter, which we used in the transfection experiments. The first is located at around 5.8 kb, and deletion of 3 kb containing this sequence did not abolish the repressing activity of Plzf. The second binding site is located at around 1.6 kb, and deletion of 500 bp containing the ATACAGT sequence restores the ability of Plzf to repress the promoter activity. While we cannot exclude the possibility that the first Plzf binding site 1 in the kit promoter is bound by Plzf in vivo, its deletion does not affect the Plzf responsiveness of the kit promoter in transfection experiments. EMSA indicated that nuclear extracts from spermatogonia or Hek293 mock- or Plzf-transfected cells were able to produce a specific retarded band when we used a 20-mer double-strand oligonucleotide containing a canonical Plzf binding site, corresponding to Plzf binding site 2. Plzf was found to directly associate with this responsive element, in vivo, as assessed by ChIP experiments. Cloning the genomic region containing the Plzf binding site 2 back into the deleted plasmid was able to restore sensitivity to the repressing

activity of Plzf. Finally, a discrete 3-bp mutation in the Plzf binding site 2 completely abolishes Plzf-mediated repression of EGFP reporter gene expression driven by the kit promoter.

The Plzf ability to repress kit expression in vivo was confirmed by the analysis of *kit* transcript levels in undifferentiated spermatogonia from $Plzf^{+/+}$ and $Plzf^{-/-}$ testes. By quantitative RT-PCR, we detected low levels of *kit* transcripts in undifferentiated spermatogonia. However, at least a threefold increase of kit mRNAs was found in *Plzf*-null undifferentiated spermatogonia. Indeed, though prepubertal testes from $Plzf^{+/+}$ and $Plzf^{-/-}$ mice had similar numbers of proliferating cells per tubule, more proliferating tubules were found in $Plzf^{-/-}$ mice (12), as could be expected if premature kit expression was inducing proliferation in undifferentiated spermatogonia. Since Plzf profoundly represses kit expression, loss of Plzf can lead to dysregulation of the differentiative cycle of spermatogonial stem cells, accounting for the progressive depletion of the stem cell compartment observed in the absence of Plzf. Indeed, kit misexpression has been previously reported in Wsh and Ph mutant mice. In these mice, mutation in the negative upstream kit control elements induces kit ectopic expression, which causes an impairment of early melanogenesis and pigment deficiency (16).

Identification of Plzf target genes has been critical to understanding both the role that Plzf plays in differentiation and the effect of translocation in acute promyelocytic leukemia. The description of *kit* as a target for Plzf repression in the germ cells as well as in hematopoietic cells (35) will enhance our understanding of the normal function of Plzf and will help to explain how dysregulation of Plzf can be so deleterious for development.

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