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Reporter Mice Express Green Fluorescent Protein at Initiation of Meiosis in Spermatocytes

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Abstract

Transgenic mice were generated using a heat shock protein 2 (*Hspa2*) gene promoter to express green fluorescent protein (GFP) at the beginning of meiotic prophase I in spermatocytes.

Expression was confirmed in four lines by in situ fluorescence, immunohistochemistry, western blotting, and PCR assays. The expression and distribution of the GFP and HSPA2 proteins co-localized in spermatocytes and spermatids in three lines, but GFP expression was variegated in one line (F46), being present in some clones of meiotic and post-meiotic germ cells and not in others. Fluorescence-activated cell-sorting (FACS) was used to isolate purified populations of spermatocytes and spermatids. Although bisulfite sequencing revealed differences in the DNA methylation patterns in the promoter regions of the transgene of the variegated expressing GFP line, a uniformly expressing GFP reporter line, and the *Hspa2* gene, these differences did not correlate with variegated expression. The *Hspa2*-GFP reporter mice provide a novel tool for studies of meiosis by allowing detection of GFP in situ and in isolated spermatogenic cells. They will allow sorting of meiotic and post-meiotic germ cells for characterization of molecular features and correlation of expression of GFP with stage-specific spermatogenic cell proteins and developmental events.

Keywords

spermatogenesis; heat shock protein; variegated gene expression; fluorescence activated cell sorting

RESULTS AND DISCUSSION

Spermatogenesis is an ongoing developmental process that occurs in distinct mitotic, meiotic and post-meiotic phases. Each phase involves multiple steps, specific patterns of

gene and protein expression, and novel structural features and unique functional events. In the mitotic phase, spermatogonial stem cells (SSCs) divide to either self-renew or become differentiating spermatogonia. The meiotic phase begins with spermatogonia giving rise to spermatocytes, which undergo chromosomal synapsis and recombination and ends with two meiotic cell divisions to produce haploid spermatids. In the post-meiotic phase, dramatic changes in composition and morphology occur as spermatids transform into spermatozoa.

Germ cells in the three phases of spermatogenesis develop in tightly coordinated overlapping waves along seminiferous tubules, with successive cohorts of germ cells defining twelve specific stages of spermatogenesis in the mouse. Various methods have been used to fractionate the mixture of germ cells and somatic cells in the testis to allow in vitro and biochemical studies. They include centrifugal elutriation (Barchi *et al.*, 2009; Grabske *et al.*, 1975) and gravity sedimentation (Bellve *et al.*, 1977; La Salle *et al.*, 2009; O'Brien, 1993), but these require equipment not available in most laboratories. Antibodies to surface proteins have been used to isolate spermatogonia (Kubota *et al.*, 2003, 2004) but antibodies specific to surface proteins on meiotic or post-meiotic germ cells are not available. Fluorescence activated cells sorting (FACS) based on cell size differences and DNA staining also has been used to isolate populations of spermatogenic cells (Geisinger and Rodriguez-Casuriaga, 2010), but the potential genotoxic effects of DNA-binding fluorochromes is a limitation of this approach. While FACS has been used to isolate germ cells from transgenic mice expressing GFP in spermatogonia (Garcia and Hofmann, 2012) or spermatids (Reddi *et al.*, 1999b), GFP reporter mice are not available that allow isolation of separate populations of meiotic and post-meiotic germ cells by FACS.

Transgenic mice were generated using an *Hspa2* (previously *Hsp70-2*) gene promoter coupled to a green fluorescent protein (GFP) reporter (Fig. 1a). Transcription of the *Hspa2* gene and expression of the HSPA2 protein occur principally during spermatogenesis in the mouse and are rapidly up-regulated at the initiation of meiosis (Allen *et al.*, 1988a; Allen *et al.*, 1988b; Rosario *et al.*, 1992). Targeted disruption of the *Hspa2* gene resulted in male infertility due to arrest of meiosis at chromosome desynapsis and elimination of spermatocytes by apoptosis (Dix *et al.*, 1996).

The *Hspa2*-GFP transgene incorporated flanking copies of the *Acrv1* 53bp minimal insulator sequence (Abhyankar *et al.*, 2007; Reddi *et al.*, 1999a; Reddi *et al.*, 2003) in an attempt to enhance specificity of expression of the transgene. GFP was expressed strongly at the initiation of meiosis in spermatocytes in Stage VIII seminiferous tubules (Supplemental Fig. 1) in all of the transgenic lines, and to a lesser degrees in a few other tissues. This suggests the insulator element might have partially enhanced specificity of expression of the *Hspa2*-GFP transgene.

Of the six founder mice generated by pronuclear injection, two males failed to sire pups and one male and three females transmitted their transgenes to progeny in expected Mendelian ratios to establish four lines. Western blots of proteins from testes and four somatic tissues of male line F32 and wild-type (WT) litter-mate mice were probed with an antibody specific to GFP (Fig. 1b). GFP that migrated with a mass of ~32.5kDa was detected in testis and brain in F32, but not in WT mice. Transgene mRNA was identified by PCR in the testis in

all four lines, in testis only in two lines (F46 and F54), at low levels in brain, heart and spleen in one line (F49), and at low levels in brain in one line (F32) (Fig. 1c). The pattern of expression in line F32 mice was comparable to this reported previously (Inselman et al., 2010) and unless indicated otherwise, results were generated using F32 mice.

GFP and HSPA2 were seen by immunohistochemistry (IHC) to co-localize in serial sections of testis from an *Hspa2*-GFP transgenic mouse (Fig. 2), with GFP expression being restricted to meiotic and post-meiotic germ cells (Fig. 2b). HSPA2 (Fig 2c) but not GFP (Fig. 2d) was detected in serial sections of testis from a WT littermate, confirming the specificity of these antisera. GFP was detected by fluorescent light (506nm wavelength) in the intact testis (Fig. 2f). Comparable results were seen in F49 and F54 mice (data not shown). The pattern of expression of the transgene closely mimicked that seen for HSPA2 (Dix *et al.*, 1996; Rosario *et al.*, 1992; Inselman et al., 2010). An unexpected finding was variegated expression of GFP in line F46 seen by IHC (Fig. 2e) and in the intact testis under fluorescent light (Fig. 2g).

To determine if *Hspa2*-GFP reporter mice could be used to isolate specific populations of germ cells, testes were dissociated enzymatically (O'Brien, 1993) and subjected to FACS. Cells initially were sorted based on a combination of size as determined by side and forward light scatter parameters (Fig. 3a), GFP fluorescence (Fig. 3b), and DNA content as determined by Hoechst staining. This resulted in collection of cell populations that were enriched for spermatids (Fig. 3, left column) and spermatocytes (Fig. 3, right column), and a mixed subpopulation containing spermatocytes, multinucleated spermatids and somatic cells (Fig. 3, middle column). Residual bodies containing GFP but lacking DNA sorted with spermatids because of their small size (Fig. 3, arrow heads). The spermatid fraction was less than 2% contaminated with spermatocytes. The spermatocyte population captured more multinucleated spermatids resulting in 90% purity (Table 2). Because no substantial advantages were observed when DNA staining was included, only cell size and GFP fluorescence were used to sort germ cells in subsequent studies (Fig. 3 and Fig. 4, insert). This reduced processing and sorting time and avoided the potential genotoxic effects of the DNA stain.

The types of germ cells found within the three FACS populations were confirmed using qPCR. *Zbtb16*, *Spo11*, and *Prm1* were used as markers for spermatogonia, spermatocytes and spermatids, respectively, because of their gene expression profiles during postnatal testis development (Shima *et al.*, 2004). The relative expression level of those genes was determined by normalizing to *Rplp0* levels (Kim *et al.*, 2008) using a standard curve calculation. As expected, *Prm1* was highly expressed in the spermatid population and *Spo11* was expressed at the highest level in the spermatocyte population, as well as being relatively abundant in the mixed cell population (Fig. 4). The low level of expression of *Zbtb16* in all populations indicated that spermatogonia were a minor contaminant (Fig. 2b).

The variegated pattern of GFP expression in F46 males was seen in spermatocytes and spermatids (Fig. 2e) and was present in variable size patches of germ cells. It occurred in all 45 males examined by IHC in ten generations of F46 mice and was transmitted from both male and female parents mated with WT mice to offspring through multiple generations in

normal Mendelian ratios. Spermatogenic cells develop as clones derived from individual SSCs and remain interconnected by intercellular bridges that allow sharing of transcripts and proteins. This suggests that the patches of germ cells containing GFP or lacking GFP develop as separate clones with the variegation being due to silencing of transcriptional initiation in some patches of spermatocytes at the beginning of meiosis.

Several causes of variegated transgene expression have been proposed. Transgenes often insert as concatemers and an inverse relationship between transgene copy number and transgene silencing was reported (Bao-Cutrona and Moral, 2009; Garrick *et al.*, 1998). However, repeat-induced gene silencing was not found to be the principal reason for transgene variegation by others (Williams *et al.*, 2008) and differences in transgene copy numbers did not correlate with variegated transgene expression in the present study (Supplemental Figs. 2a and 2b). Variegated transgene expression due to parent of origin imprinting also was described (Chaillet *et al.*, 1991; Kearns *et al.*, 2000), but the transmission of variegated GFP expression from both male and female F46 parents to their offspring through multiple generations makes this an unlikely cause. Epigenetic influences of the chromatin surrounding the transgene can lead to gene silencing (Elgin and Reuter, 2013) and variations in expression of randomly inserted transgenes in different founder lines were apparent even in early studies (Pinkert *et al.*, 1985). This may be the cause of differences in the tissue sites and levels of expression of GFP in the transgenic lines in the present study.

Bisulfite sequencing was used to determine if differences in methylation patterns in *Hspa2*-GFP transgene promoters were suggestive of epigenetic effects and correlated with variegated expression. The promoter of the variegated transgene was consistently hypermethylated in genomic DNA from tail biopsies and whole testis tissues compared to that in the uniformly expressing transgene (Fig. 5a, Supplemental Fig. 3). To examine the differences between transgenic lines, bisulfite sequencing of FACS isolated germ cells from F32 males showed the transgene to be hypermethylated in spermatids but not in spermatocytes (Fig. 5b). Furthermore, the F32 transgene promoter was hypermethylated in spermatids compared to the native *Hspa2* gene promoter (Fig. 5c; Supplemental Fig. 3). Since a population of cells from the uniformly expressing transgenic line was also hypermethylated, this suggests that the variegation was not linked to differences in methylation in this region of the transgene.

The *Hspa2*-GFP mice will provide a novel tool for investigators: 1) studying mutant mouse models of germ cell development affecting initiation or progression of meiosis in the male, 2) isolating spermatocytes for studies of meiotic chromosomal synapsis and recombination, 3) defining novel meiotic and post-meiotic processes essential for the genetic integrity of the species, 4) analyzing stage-specific programs of gene expression, and 5) characterizing transcriptional, translational and morphogenetic processes leading to formation of highly specialized spermatozoa. In addition, FACS with dissociated testis cells from postnatal days 10 to 20 (Bellve *et al.*, 1977) *Hspa2*-GFP mice or from adult *Hspa2*-GFP mice injected with WIN 18-446 and retinoic acid (Hogarth and Griswold, 2013) should allow isolation of synchronously developing populations of germ cells at specific phases of meiosis.

MATERIALS AND METHODS

Generation of Male Meiosis Reporter Transgenic Mice

Hspa2-GFP transgenic mice expressing a GFP reporter at the initiation of male meiosis were produced using a 907bp region of the *Hspa2* proximal promoter generated by PCR with primers designed to introduce restriction enzyme recognition sequences (Table 1). The same promoter region was used previously to construct a cre-expressing transgene (Inselman *et al.*, 2010). The PCR product was cloned into the *AscI*/*HindIII* sites of the *phrGFP II-C* vector (#240144, Agilent) upstream of the humanized recombinant green fluorescent protein (GFP) sequence, which eliminated the CMV promoter. A rabbit antiserum (#240141, Agilent) specific to the GFP protein encoded by this vector was used in these studies. A single copy of the 53 bp minimal insulator region from the *Acrv1* gene (Reddi *et al.*, 2003) was inserted on either side of the promoter/reporter cassette at the upstream *PciI* and downstream *PsiI* sites in an attempt to reduce positional insertion effects. Fragments were amplified with PFU polymerase by PCR (35 cycles at 20 sec at 94°C, 20 sec at 60°C, and 30 sec at 72°C). The *Hspa2*-GFP construct was recovered from the plasmid by digesting with *BciVI* restriction enzyme overnight at 37°C, the 2.9kb band was excised from an agarose gel, and the construct DNA was electro-eluted in Cellu-Sep H1 dialysis tubing (Membrane Filtration Products, Inc., Seguin, TX). The DNA then was washed twice with phenol/chloroform (1:1) and once with chloroform, precipitated with ethanol, washed with 70% ethanol, air dried, and suspended in TRIS/EDTA buffer (10mM Tris, 0.25mM EDTA, pH7.5).

Transgenic mice were generated at the University of Rochester Transgenic Facility by pronuclear injection. Genomic DNA was extracted from tail biopsies of C57BL/6 pups using a DNeasy blood and tissue kit (QIAGEN, Valencia, CA) following the manufacturer's protocol. Mice carrying the *Hspa2*-GFP transgene were identified by PCR (35 cycles for 30 sec at 94°C, 30 sec at 62°C, and 1min at 72°C) with primers specific to the transgene (Table 1) using 5x buffer J (Invitrogen), and AmpliTaq DNA polymerase (Applied Biosystems), resulting in a 1200bp product. Six founders were generated and while two male founders failed to transmit the transgene, one male and three female founders transmitted the transgene to offspring. Colonies derived from each of the four founders were maintained by mating hemizygous transgenic males with wild-type (WT) C57BL/6 female mice unless indicated otherwise. One of the *Hspa2*-GFP transgenic lines has been transferred to the Jackson Laboratory (Bar Harbor, ME) to be made available to the research community (Tg(*Hspa2*-GFP)F32Eddy (MGI:5568248).

Real Time (RT) and Quantitative (q) PCR Analyses

Total RNA was isolated from brain, heart, liver, spleen, and testis tissues of transgenic mice using the RNeasy Midi kit (QIAGEN) according to manufacturer's instructions. It was treated with amplification grade DNase I (Invitrogen) for 5 min at 37°C to eliminate genomic DNA and then subjected to RT-PCR to generate cDNAs from each tissue, using MuLV reverse transcriptase (Applied Biosystems) and random hexamers (45 min at 42°C, 15 min at 70°C). Primers (Table 1) internal to the GFP reporter amplified a 173 bp RT-PCR product, while primers for positive control somatic glyceraldehyde-3-phosphate

dehydrogenase (*Gapdh*) generated a 327 bp band (30 cycles for 20 sec at 94°C, 20 sec at 62°C, 20 sec at 72°C). Duplicate RT-PCR products were generated without the MuLV enzyme as negative controls.

Total RNA was isolated using an RNAqueous-Micro kit (Ambion) and cDNA products were generated as above from spermatogenic cells separated by fluorescence-activated cell sorting (FACS). Gene markers to identify cell types present in the sorted germ cell populations were mouse zinc finger and BTB domain containing 16 (*Zbtb16*, previously *Plzf*), SPO11 meiotic protein bound to DSB homolog (*S. cerevisiae*) (*Spo11*), and protamine 1 (*Prm1*). Primers specific to these markers (Table 1) were used in qPCR assays with Power SYBR Green Master Mix (Applied Biosystems) and amplified using a 9700HT Fast PCR System (Applied Biosystems). Gene expression levels were estimated against a standard curve generated with cDNA from a pool of unsorted mixed germ cells and normalized to that of ribosomal protein, large, P0 (*Rplp0*) in triplicate. The relative mRNA expression levels of the transgene in the testes of line F32 and line F46 mice were determined using qPCR. Power SYBR Green Master Mix was combined with the same internal GFP primers used above and values were normalized to mouse beta actin (*Actb*).

Immunohistochemical Analyses

Immunohistochemistry on sections of testis fixed in Bouin's solution was performed with antibodies to GFP (#240141, Agilent) or HSPA2 (Rosario *et al.*, 1992) diluted 1:2500 and 1:1600, respectively, and incubated overnight at 4°C. This was followed using the Vectastain Elite rabbit IgG ABC kit (Vector Laboratories) according to the manufacturer's instructions. The horseradish peroxidase-conjugated secondary antibody was detected with 3,3'-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and sections were counterstained with Hematoxylin QS (Vector Laboratories).

Western Blotting Analyses

Protein lysates of brain, heart, liver, spleen, and testis from transgenic and WT littermate males were prepared by mechanically homogenizing the tissues in lysis buffer (20 mM HEPES, 140 mM NaCl, 1 mM DTT, 0.1% Triton X100) containing a proteinase inhibitor cocktail with EDTA (Roche Applied Science, Indianapolis, IN). Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL). Approximately 10 µg of protein per sample were separated on a 12% pre-cast Ready Gel (Bio-Rad Laboratories) and transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked overnight with 5% skim milk in PBS at 4°C prior to being probed with antibody to GFP (1:5000, #240141, Agilent) for one h at room temperature. After multiple washes, HRP-conjugated anti-rabbit secondary antibody (1:20,000, catalog #A-0545, Sigma-Aldrich) was added for one h and subsequently visualized using an ECL western blot detection kit (Amersham Biosciences) according to the manufacturer's protocol. The tissue blot was stripped for 15 min at 37°C with Restore Buffer (Thermo Scientific), blocked again overnight, and probed with an antibody to GAPDH (1:5000, catalog #G8795, Sigma-Aldrich) as a loading control. The product was detected using an HRP-conjugated anti-mouse secondary antibody (1:20,000, catalog #A2304, Sigma-Aldrich). To compare expression levels between the transgenic lines,

western blots with protein lysates from testes of lines F32 and F46 mice were probed with the antibody to GFP. These blots were stripped with Restore Buffer and then probed with antibody to HSPA2 (1:1000) as a loading control.

Fluorescence-Activated Cell Sorting (FACS) of Mixed Germ Cells

Mixed germ cells were prepared from testes of adult *Hspa2*-GFP mice as described (O'Brien, 1993). Approximately 5×10^7 cells were placed in 100 mm petri dishes containing 10 ml of MEM α medium supplemented with 25 mM NaHCO₃, 25 mM HEPES, 0.3 mM penicillin, 0.15 mM streptomycin, 0.3% sodium lactate, and 5% fetal bovine serum and incubated overnight at 32°C in a 5% CO₂ atmosphere. The cells were collected, washed once in a modified Enriched Krebs-Ringer Bicarbonate medium containing 0.5% BSA, 0.4 μ g/ml DNase I, and 1% Polyvinyl Pyrrolidone (40 kDa), and suspended in that medium at 5×10^6 cells/ml.

Cells expressing GFP were examined and sorted using a FACS Aria II flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with BD FACSDiVa software. The gates to collect single cells were initially set based on forward-scatter (area) vs. forward-scatter (height) dot plot using a 525/50 filter to detect GFP expression and a 450/50 filter to detect cells stained with 1mM Hoechst (H33342). However, the Hoechst stain was eliminated without loss of specificity, which increased efficiency of the sort. Samples of cells sorted by GFP and size alone were placed on slides in Vectashield mounting medium (Vector Laboratories) and imaged using a Zeiss Axioplan microscope by Nomarski contrast or using fluorescent illumination to visualize GFP and DNA stained with DAPI (4', 6-diamidino-2-phenylindole).

Cells were counted and purities of fractions determined manually. Multiple images captured under fluorescent light from four separate experiments were visually inspected and the numbers of spermatids, spermatocytes, and multinucleated spermatids counted. Cells were categorized based on the pattern seen with DAPI staining.

DNA Methylation Analysis

Genomic DNA was prepared for analysis using the MethylCode Bisulfite Conversion kit according to the manufacturer's protocol (Invitrogen). PCR products were amplified from the DNA using primers specific to the *Hspa2*-GFP transgene or endogenous *Hspa2* (Table 1) and cloned into a TOPO pCR2.1 vector (Invitrogen). The inserts were sequenced using a BigDye Terminator Cycle kit (Applied Biosystems) and sequences from treated and untreated DNA samples were compared to identify cytosine residues resistant to bisulfite conversion.

Transgene Copy Number Estimation

Genomic DNA isolated from tail biopsies of mice in four transgenic lines (F32, F46, F49, F54) was digested with restriction enzymes (PciI, SpeI, XmnI) for five h at 37°C and separated by electrophoresis on 1.25% agarose gels, along with samples containing known dilutions of construct DNA to compare for estimation of transgene copy numbers. Following separation, samples were capillary blotted to Hybond-N+ nylon membrane (Amersham

Biosciences). The samples were cross-linked to the membrane with UV light and baked for one h before being probed with a ^{32}P -dCTP radio-labeled PCR product specific to the GFP reporter.

Estimates of copy number insertions determined by Southern blotting were corroborated by qPCR using Power SYBR Green Master Mix. Internal GFP primers (Table 1) were used to estimate copy numbers in 100 ng samples of genomic DNA from the four transgenic mouse lines. Values from triplicate sample preparations were compared to a standard curve generated with dilutions of transgene construct DNA added to 100 ng of genomic DNA from WT mice to estimate the number of transgene copies in the genomic DNA of each transgenic line.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HSPA2	heat-shock protein 2
GFP	green fluorescent protein
hrGFP	humanized recombinant GFP
WT	wild-type
FACS	fluorescence activated cell sorting
UV	ultraviolet

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FIG. 1. *Hspa2*-GFP transgene structure and expression. **(a)** Schematic diagram of transgene construct with the 907bp *Hspa2* promoter, GFP reporter and bovine growth hormone polyA signal flanked by core *Acrv1* insulator components (arrows indicate orientation). Restriction enzymes sites are indicated by *Pci*I, *Xmn*I, and *Spe*I. **(b)** Upper panel is a western blot with antibody to GFP identifying a 32.5kDa band in testis and brain in an F32 male and not in a WT littermate. Lower panel is a western blot with antibody to GAPDH and the same samples as loading control. **(c)** Tissue distribution of the transgenic message was examined

in four somatic tissues and the testis using RT-PCR and primers specific to the GFP reporter. Detectable levels of expression can be seen in the testis of all four mouse lines, while some expression in somatic tissues is evident in lines F32 and F49. Lanes contain cDNA products from brain (Br), heart (He), liver (Li), spleen (Sp), and testis (Te). Gene specific primers to *Gapdh* were used as a positive control. Duplicate samples were prepared either with (+) or without (–) reverse transcriptase.

FIG. 2.

The HSPA2-GFP transgene expression pattern is mouse line dependent.

Immunohistochemistry performed on serial sections of Bouin's fixed testes from either a GFP-positive F32 male (**a** and **b**) or its WT littermate (**c** and **d**) showed that the transgene is expressed in primary spermatocytes. Sections were probed with either anti-HSPA2 (**a**; **F32** and **c**; **WT**) or anti-GFP (**b** **F32** and **d**; **WT**). Scale bar: 25µm. (**e**) Immunohistochemistry using an anti-GFP antibody shows variegated expression of the transgenic protein in an adult testis section from a line F46 mouse (scale bar: 25µm). GFP fluorescence is readily visualized in whole testis tissue under UV light. (**f**) Transgenic testis with epididymis attached from line F32 (right) and WT littermate (left) mice. (**g**) Transgenic testis showing the variability of variegated GFP expression typically found in a line F46 mouse.

FIG. 3.

Sample images of FACS processed germ cells show isolated spermatocytes and spermatids. Preparations of enzymatically dissociated testicular cells were sorted on a BD FACSaria II using cell size and GFP expression as parameters to capture cells. FACS collected cells are seen by (a) Nomarski contrast microscopy or fluorescence microscopy to visualize (b) GFP expression and (c) DAPI staining. The columns left to right show cells from populations containing spermatids, a mixture of multi-nucleated spermatids and spermatocytes, and spermatocytes. Arrows indicate residual bodies devoid of DNA. Scale bar: 25 μ m.

FIG. 4.

Quantitative real-time PCR shows relative purity of the cell populations harvested. Gene markers with abundant transcripts in spermatogonial (*Zbtb16*, formerly *Plzf*), spermatocyte (*Spo11*), or spermatid (*Prm1*) germ cells were chosen to identify which cell-types were present in the sorted populations. The bar graph shows relative expression levels of *Zbtb16* (black bars), *Prm1* (white bars), and *Spo11* (gray bars) normalized to ribosomal protein *Rplp0* from the spermatid, mixture, and spermatocyte pools of collected germ cells. Values are mean $\pm$ SEM (n=3). The inset shows the location of the gates set in relation to forward scatter or size (FSC-A) and GFP fluorescence intensity (GFP FITC-A). The average Pearson correlation coefficient (R^2) values from three intra-assay experiments were 0.9678 for *Zbtb16*, 0.9849 for *Spo11*, 0.9921 for *Prm1*, and 0.9959 for *Rplp0*.

FIG. 5.

Bisulfite sequencing shows different patterns of CpG methylation in transgenic tissues from the F46 and F32 lines of mice and endogenous *Hspa2*. Genomic DNA in the 5' portion of the transgene corresponding to the *Hspa2*-GFP promoter contains 29 cytosines potentially affected by bisulfite treatment. Each row is a separate clone and grey boxes are methylated cytosines that resisted treatment and conversion to uracil. **(a)** Multiple clones from both testis and somatic tissues from F46 males are consistently hypermethylated compared to those of F32 animals. **(b)** CpG methylation patterns indicate the transgene incorporated into the F32 line of mice is transcriptionally regulated in FACS prepared spermatids but not spermatocytes. **(c)** A graphical representation of methylation patterns for endogenous *Hspa2* found in spermatid and spermatocyte genomic DNA from F32 mice. *Hspa2*-GFP becomes methylated after meiosis in round spermatids, which contrasts sharply with *Hspa2*, whose transcriptional expression does not seem to be regulated by CpG methylation in either spermatids or spermatocytes.

Table 1
Oligonucleotides Used in These Experiments

Primers used in these experiments are listed. Bases underlined indicate restriction enzyme recognition sites.

Name	Orientation	Sequence 5' to 3'
AscI promoter	forward	<u>GGCGCGCC</u> AGGAAAGCCGAGGGAGAAAGTT
HindIII promoter	reverse	CTGACGCTTTCGTCCTAACGTT <u>AAGCTT</u>
PciI insulator	forward	<u>ACATGTT</u> TGAAGCTACCCCTAACAC
PciI insulator	reverse	CAGAAAATGCTCTTCACTAG <u>ACATGT</u>
PsiI insulator	forward	<u>TTATAA</u> CTAGTGAAGAGCATTTTCTG
PsiI insulator	reverse	GTGTTAGGGGGTAGCTTCAATT <u>TATAA</u>
Transgene detection	forward	TTGAAGCTACCCCTAACACACTAT
Transgene detection	reverse	CATGGTGAACACGTGGTTGT
hrGFP reporter	forward	TGATCCTGGTGTACCGCCTGAACA
hrGFP reporter	reverse	GATGGCGGTCTCGTGCTGCTCTAC
<i>Gapdh</i>	forward	GCCGGTGCTGAGTATGTCGT
<i>Gapdh</i>	reverse	ATCACGCCACAGCTTTCCAGAGGG
<i>Zbtb16</i>	forward	AAAGCCTTGTGTGTGATCAATGC
<i>Zbtb16</i>	reverse	CTGCAAATATAGCTGCGCACGCCT
<i>Spo11</i>	forward	TGCCTGCCTTCACAATAGACAA
<i>Spo11</i>	reverse	ATGGACAATACTTTCAGAATCA
<i>Prm1</i>	forward	CAGACGGAGGAGGCGATGCT
<i>Prm1</i>	reverse	GGCGAGATGCTCTTGAAGTC
<i>Rplp0</i>	forward	AAAGACTGGAGACAAGGTGGGA
<i>Rplp0</i>	reverse	AGTAAGTGGGAAGGTGTACTCAG
Transgene CpG	forward	GTTATTTTTTGATTTTGTGGATAAT
Transgene CpG	reverse	GTTATAGGAATAGTAGGGGTAGGGA
<i>Hspa2</i> CpG	forward	GGGAGTTAGAGGGGTGATATTG
<i>Hspa2</i> CpG	reverse	GTTATAGGAATAGTAGGGGTAGGGA

Table 2**Purity of Cell Populations**

Percentages of the purity of FACS cell populations are listed. Purity was calculated based on manual cell counts from images captured from four different experiments. Values shown include SEM.

Fraction	Cell Count	% Spermatocytes	% Spermatids	% Multinucleated
Spermatids	456	1.97 ± 0.81	98.03 ± 0.76	6.58 ± 0.79
Spermatocytes	316	90.19 ± 0.37		9.81 ± 0.46