Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10681–10685, November 1992
Genetics

Analysis of *Escherichia coli* β-galactosidase expression in transgenic mice by flow cytometry of sperm

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Communicated by Paul Berg, May 12, 1992

ABSTRACT **Alterations to the mammalian genome that occur during the development of germ cells, in particular during meiosis, can be introduced into the population upon fertilization. These alterations can occur through homologous recombination, genome rearrangement, or mutagenesis. Such events usually occur infrequently for any particular sequence. Because of the difficulty in analyzing a large number of offspring in a mammalian cross, we have developed a marker to detect these events in sperm, since a large number of these meiotic progeny are produced during male gametogenesis. We have expressed the *Escherichia coli lacZ* gene during spermatogenesis in transgenic mice and quantitated the levels of β-galactosidase activity in single sperm with the fluorescence-activated cell sorter and a fluorogenic substrate, 5-dodecanoylaminofluorescein di-β-D-galactopyranoside. Detection of rare positives was demonstrated in mixed sperm populations with as few as 0.01% positive sperm. Although the distribution of β-galactosidase activity in caudal epididymal sperm populations is bimodal, it appears that β-galactosidase, like other proteins that have been expressed postmeiotically, is distributed between transgene-positive and transgene-negative sperm.**

Molecular genetic analysis of rare meiotic events is simplified in lower eukaryotes such as *Saccharomyces cerevisiae* by the availability of a large number of meiotic progeny following sporulation (1). For mammalian systems, the study of meiotic and other germ-line events due to recombination, DNA rearrangement, and mutagenesis has been hampered by the reliance on crosses (2), since tissue culture lines undergoing meiosis do not exist. However, a large number of meiotic progeny are produced during spermatogenesis. In the mouse, $>10^7$ sperm can be collected from a single animal. The availability of a marker that could be detected in mouse sperm would allow the detailed study of these genetic events. Since sperm are terminally differentiated, a requirement for such a marker is that it be detectable at the single-cell level, ideally by flow cytometry. Flow cytometry with the fluorescence-activated cell sorter (FACS) can be used to both quantitate and physically sort marker-positive cells in a population. Herzenberg and coworkers (3, 4) have developed a FACS assay to measure *Escherichia coli* β-galactosidase activity in viable mammalian cells. Together with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and *o*-nitrophenyl β-D-galactopyranoside (ONPG) assays, the FACS assay makes β-galactosidase a highly versatile marker for molecular genetic studies.

We have expressed the *E. coli lacZ* gene during spermatogenesis in transgenic mice and have detected high levels of β-galactosidase activity in sperm by using a modified FACS assay. Reconstruction experiments were performed with transgenic and nontransgenic sperm in which we were able to detect as few as 0.01% transgenic sperm, indicating that this should provide a very sensitive method to detect rare germline events. Expression of the transgene was accomplished with the postmeiotic-specific mouse protamine 1 gene (*Prm-1*) promoter. It appears that β-galactosidase, like other proteins that have been expressed postmeiotically (5, 6), is distributed between transgene-positive and transgene-negative sperm in hemizygous mice.

MATERIALS AND METHODS

Recombinant DNA and Microinjection into Fertilized Mouse Eggs. Plasmid a-2 was constructed from plasmids pnlacF and mP1-lacF, which were kindly supplied by Jacques Peschon (Immunex, Seattle). Plasmid a-2 is identical to mP1-lacF except that it contains the simian virus 40 nuclear localization signal (7). The nuclear localization signal was derived from pnlacF, by replacement of the *Nco* I–Bgl II *lacZ* fragment of mP1-lacF with the *Nco* I–*Bgl* II *lacZ* fragment of pnlacF. The *Pst* I–*Pst* I *lacZ* fragment of a-2 was injected into the pronuclei of fertilized (C57BL/6 × CBA/Ca)F_2 mouse eggs, as described (8). Southern analysis and genomic DNA preparations were done according to the standard procedure (9), with the exception that 0.72 M 2-mercaptoethanol was added to the lysis buffer for the preparation of sperm DNA.

Tissue Sections. Tissue was prefixed in 2% formaldehyde/0.8% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.3) for 5 min, rinsed in PBS, embedded in OCT (optimal-cutting-temperature compound, Miles), and frozen at −70°C. Cryostat sections were 5 μm thick. Staining was in PBS containing X-Gal (1 mg/ml), $MgCl_2$ (2 mM), $K_3Fe(CN)_6$ (1.64 mg/ml), and $K_4Fe(CN)_6 \cdot 3H_2O$ (2.12 mg/ml). Adult sections were stained for 40 min, and juvenile sections for 4 hr. After staining, the sections were fixed again for 10 min, then rinsed in PBS. The adults used for Fig. 2 were littermates derived from transgenic line no. 5, as were the juveniles.

Sperm Preparation and FACS Analysis. Sperm were squeezed from the caudal epididymis of adult males into 130 mM NaCl/20 mM Tris·HCl, pH 7.5 (TN buffer). In preparation for FACS analysis, the sperm were washed with PBS/10 mM Hepes/1% bovine serum albumin by centrifugation at 2000 rpm for 5 min. The C_{12}FDG reaction was done with 2×10^5 sperm in 100 μl of PBS with 33 μM 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG) (Imagene, Molecular Probes) at 25°C. The incubations were for 40 min (Fig. 3 *A* and *B*) or for 2.5 hr (Fig. 3*C*). In each case, 200 μl of PBS was added just prior to FACS analysis to increase the volume. Samples for FACS analysis were run on a Becton Dickinson FACScan and analyzed using Consort 30 software. The samples for FACS sorting were run on a Coulter EPICS Elite 730. Approximately 5×10^5 sperm were sorted from each peak. The integrity of the sorted sperm was checked by light microscopy.

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Abbreviations: FACS, fluorescence-activated cell sorter; C_{12}FDG, 5-dodecanoylaminofluorescein di-β-D-galactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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RESULTS

Expression of *lacZ* During Spermatogenesis in Transgenic Mice. Several genes have been described that are transcribed postmeiotically during spermatogenesis (10). Among these, the mouse protamine 1 gene (*Prm-1*) is highly transcribed, since protamines replace histones during the latter stages of spermatogenesis. Its promoter has been used to express a heterologous gene (human growth hormone) in transgenic mice, resulting in growth hormone-positive sperm (5). The *Prm-1* promoter was, therefore, chosen to drive *lacZ* expression in our transgenic mice.

Fig. 1*A* shows the transgene that was injected into fertilized mouse eggs. The 5′ *Prm-1* sequences have been shown to direct transcription in round spermatids, whereas the 3′ *Prm-1* sequences have been shown to delay translation until the elongating spermatid stage (5, 11, 12). In our transgene, the 3′ sequences also provide an intron and polyadenylylation site for correct processing of the mRNA. The *lacZ* coding region includes a nuclear localization signal from the simian virus 40 large tumor antigen (7). We included this to aid in retention of β-galactosidase, since much of the cytoplasm of developing sperm is lost during spermiogenesis.

Three founder mice that contained intact transgenes by Southern hybridization analysis of tail-tip DNA were derived (Fig. 1*B*). Two mice transmitted the transgene as a single locus to roughly 50% of their progeny. These founder mice, nos. 4 (female) and 5 (male), were estimated to contain approximately 10 and 40 copies of the transgene, respectively. The progeny of these mice, with one exception, all inherited the transgene locus intact. The third mouse, a male, did not transmit the transgene and was not studied further.

The transgene expression pattern was analyzed by X-Gal staining of frozen tissue sections from line no. 5. Whereas nontransgenic males showed no staining, adult transgenic

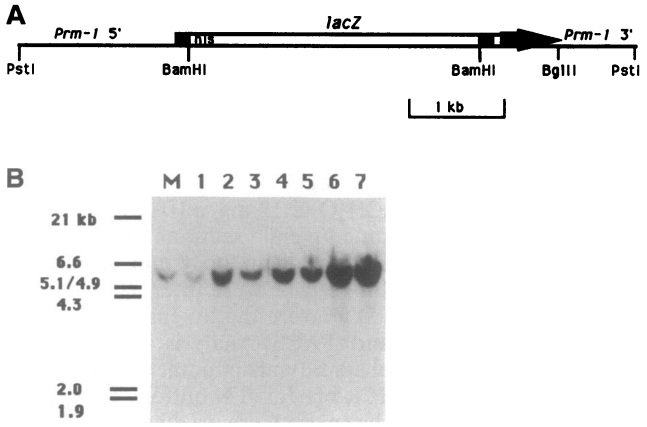


FIG. 1. (*A*) Restriction fragment injected into fertilized mouse eggs. The *E. coli lacZ* sequences (open bar) are inserted into the first exon of *Prm-1* (11). The 5′ *Prm-1* segment is 1.7 kilobase pairs (kb), which includes the tissue-specific promoter (thin line) and 92 base pairs of 5′ untranslated sequence from exon 1 (solid box). The 3′ *Prm-1* segment is 1.6 kb and consists of the remainder of exon 1 (solid box), the intron (open box), exon 2 (solid arrow), and untranscribed sequences (thin line). nls, Nuclear localization signal from simian virus 40. This 6.4-kb fragment was derived by *Pst* I cleavage of plasmid a-2 and was separated from plasmid vector sequences prior to injection. (*B*) Southern hybridization analysis of transgenic mice. Genomic tail-tip DNA was cleaved with *Bgl* II and probed with the 3.1-kb *lacZ Bam*HI fragment shown in *A*. Lanes 1–3, DNA from the G_0 mice numbered 4, 5, and 14, respectively (mouse 14 did not transmit the transgene); lanes 4–7, males from line 5 (lane 4, G_1 hemizygote; lane 5, G_2 hemizygote; lanes 6 and 7, G_2 homozygotes). Hemi- and homozygosity are based on Southern analysis and transmission of the transgene. Lane M, size markers (240 pg of plasmid a-2 cut with *Pst* I).

males exhibited deep blue staining in cells within the seminiferous tubules of the testis and in sperm within the epididymal lumen (Fig. 2 *A–C*). Similar results were obtained for line 4 (data not shown). No staining was visible in the liver or kidney of transgenic mice (data not shown).

Examination of sperm derived from the caudal epididymis revealed X-Gal staining over the head region and the midpiece, suggesting that β-galactosidase may be found associated with cellular compartments other than the nucleus, despite the presence of the simian virus 40 nuclear localization signal. The altered localization may be the result of differences in protein trafficking during spermatogenesis (12). In many cases, sperm that were curved and contained more cytoplasm stained a deeper blue with X-Gal (data not shown) and were more fluorescent with the fluorogenic substrate (see below) than were fully extended sperm. Differences in staining may be due in part to cytoplasmic loss that occurs during spermiogenesis (13).

The first round of spermatogenesis in the mouse is synchronous (14). To verify that the transgene was expressed during the predicted developmental stage, testis sections were prepared from prepubertal transgenic mice. No X-Gal staining was seen in 19-day-old mice, which contain spermatocytes but not haploid spermatids (Fig. 2*D*), whereas 28-day-old mice, which possess spermatids, exhibited X-Gal staining in cells at the seminiferous tubule lumen (Fig. 2*E*). Nontransgenic juveniles showed no staining at either age (Fig. 2*F* and data not shown). These results indicate that *lacZ* expression is restricted to postmeiotic cells.

FACS Analysis of Sperm from Hemizygous Transgenic Mice. Flow cytometry has been adapted to measure β-galactosidase activity in large numbers of individual viable mammalian cells (3, 4). In this assay, the substrate fluorescein digalactopyranoside is cleaved to produce fluorescein, which can be detected by the FACS. Because fluorescein can leak from cells, a related substrate has been developed, C_{12}FDG (15). The fluorescent product of the reaction of β-galactosidase with C_{12}FDG is dodecanoylaminofluorescein, which is retained in cellular membranes.

We have used C_{12}FDG to monitor β-galactosidase activity in transgenic sperm. Fig. 3*A* compares FACS profiles of sperm isolated from the caudal epididymis of a hemizygous transgenic mouse from each line and from a nontransgenic mouse. The sperm from the nontransgenic mouse showed little fluorescence. Approximately half of the sperm from the transgenic mice exhibited a high fluorescence intensity, whereas the other half was weakly fluorescent. The mean fluorescence intensity of the highly positive population was approximately 2 orders of magnitude higher than that from the nontransgenic mouse. The sperm from line 5, which has more transgene copies than line 4, showed a somewhat greater amount of β-galactosidase activity.

Fluorescence microscopy revealed some morphological differences between the bright and dull populations (data not shown). As with X-Gal staining, immature sperm, as judged by curvature and cytoplasmic content, appeared very intensely stained with C_{12}FDG, whereas dull sperm were often fully extended. However, these differences were not absolute and were difficult to quantify.

The bimodal distribution has consistently been observed with sperm from at least 30 mice from the two lines, with the percentage of sperm in each population varying from approximately 40% to 60%. In addition, a number of other transgenic lines derived from injections with modified *Prm-1*/*lacZ* genes also show a clearly bimodal pattern (data not shown). A bimodal staining pattern like that seen with C_{12}FDG was also observed after incubation of transgenic sperm with other fluorogenic substrates, although some other substrates gave more complicated staining patterns (data not shown).

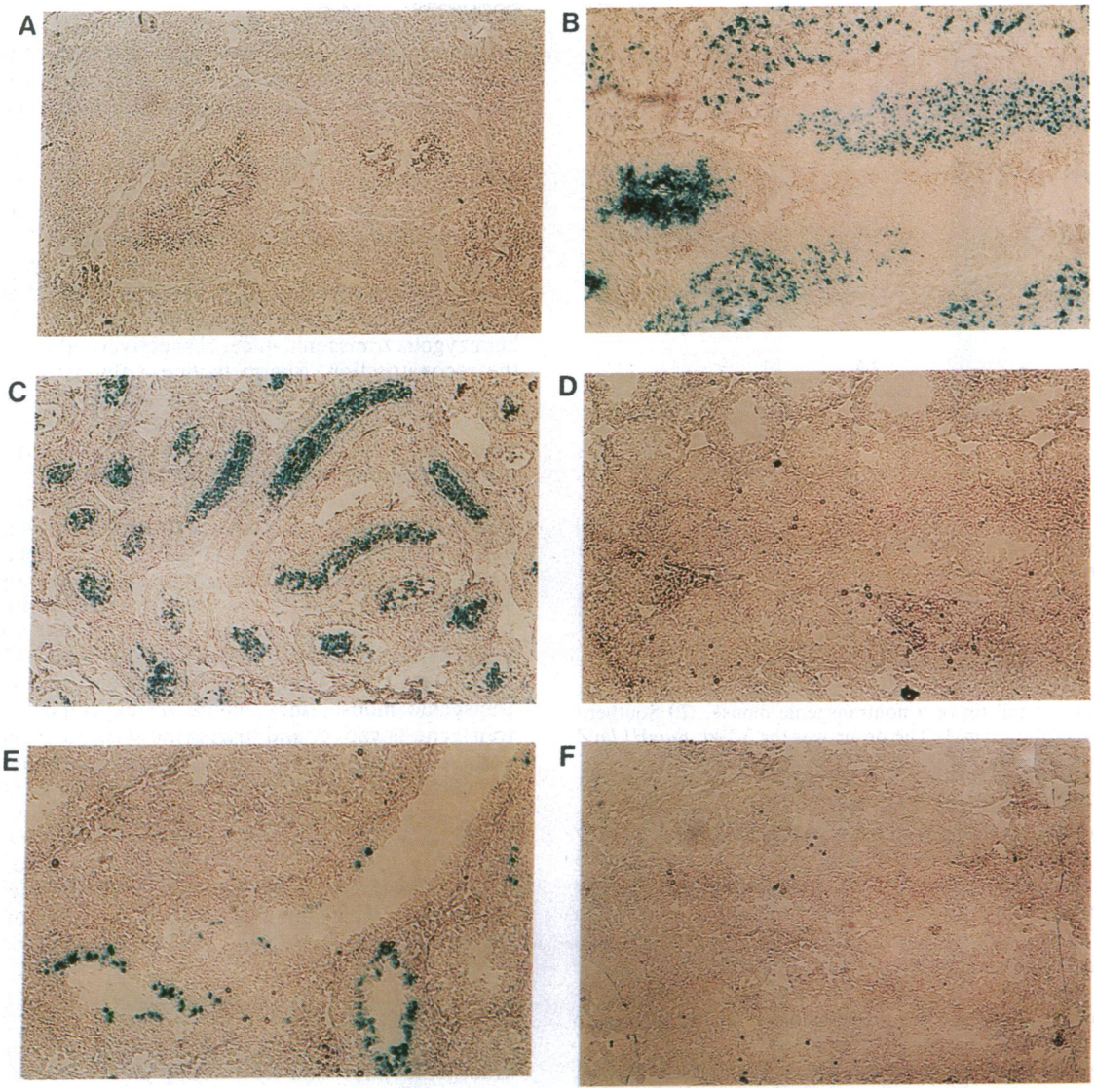


FIG. 2. Frozen tissue sections stained with X-Gal. (*A*) Nontransgenic adult testis. (*B*) Transgenic adult testis. (*C*) Transgenic adult caput epididymis. (*D*) Transgenic 19-day-old testis. (*E*) Transgenic 28-day-old testis. (*F*) Nontransgenic 28-day-old testis. (×240.)

Distribution of β-Galactosidase Activity Between *lacZ*⁺ and *lacZ*⁻ Sperm. As a result of chromosome segregation during meiosis, half of the sperm from hemizygous mice should carry the transgene. In support of this expectation, we see transmission of the *lacZ* transgene to half of the progeny in crosses with nontransgenic females. Since the mice analyzed in Fig. 3*A* were hemizygous, the possibility existed that the highly fluorescent sperm carried the transgene locus, whereas the weakly fluorescent sperm did not.

To test this, the two sperm populations from a transgenic mouse were sorted from each other by the FACS. DNA was isolated from each population and compared to tail-tip DNA

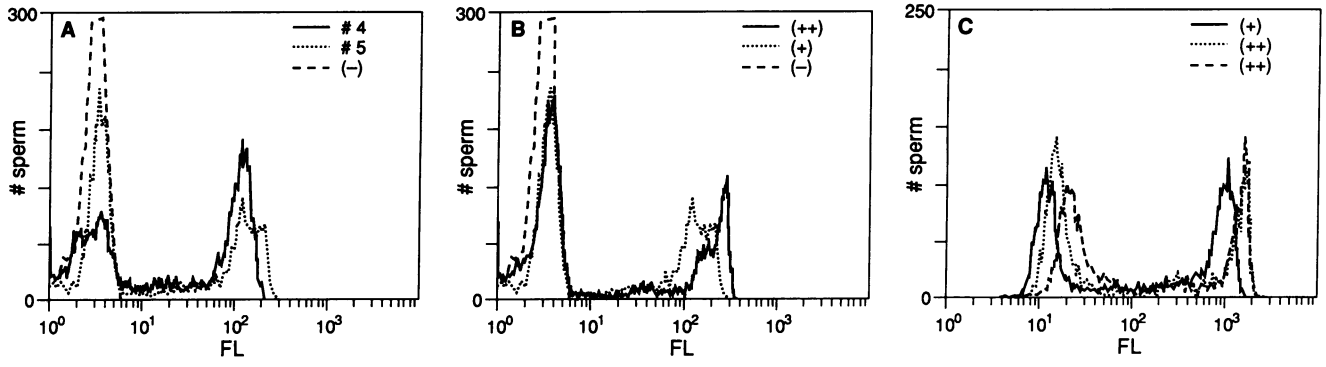


FIG. 3. FACS analysis to detect β-galactosidase activity in transgenic mouse sperm. The fluorescence (FL) intensity is on the *x* axis; the number of sperm is on the *y* axis. (*A*) Hemizygous transgenics from lines 4 and 5, plus a nontransgenic littermate (−) from line 5. (*B*) Hemizygous transgenic (+) and nontransgenic (−) littermates from line 5, as shown in *A*, plus a homozygous transgenic (++) littermate. (*C*) Hemizygous transgenic (+) from line 4, plus two homozygous transgenic (++) littermates.

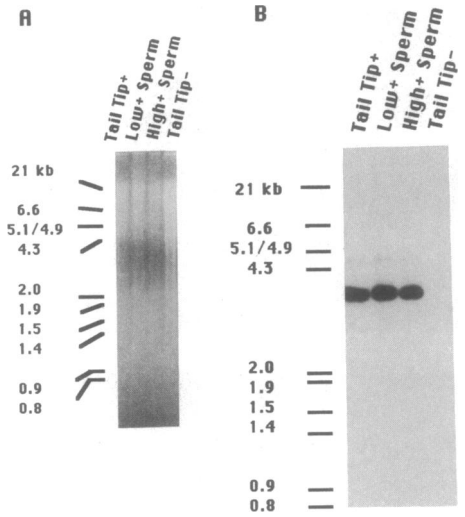


FIG. 4. Southern hybridization analysis of sorted sperm. (*A*) Ethidium bromide-stained agarose gel of *Bam*HI-digested genomic DNA. The first three lanes contained DNA from a hemizygous transgenic mouse from line 5. Each lane contained approximately 0.8 μg of genomic DNA from either the tail tip or the sorted sperm. Low + sperm, population in the left peak in the FACS analysis; high + sperm, population in the right peak. The fourth lane contains 3.2 μg of DNA from the tail tip of a nontransgenic mouse. (*B*) Southern hybridization of the gel in *A*. The probe was the 3.1-kb *Bam*HI *lacZ* fragment shown in Fig. 1*A*.

for transgene content by Southern hybridization analysis. The ethidium bromide-stained gel is shown in Fig. 4*A*. A very similar amount of genomic DNA is present in each of the lanes derived from the transgenic mouse (lanes 1–3). The autoradiogram of the Southern transfer of this gel is shown in Fig. 4*B*. The amount of hybridization with the *lacZ* probe is nearly identical in each of the lanes, indicating that transgene content is very similar between the weakly and highly fluorescent sperm. Thus, the bimodal pattern does not appear to be due to the genotype of the sperm.

FACS Analysis of Homozygous Transgenic Mouse Sperm. Sperm derived from homozygous transgenic mice were also analyzed for their β-galactosidase activity by the FACS assay. Mice were identified as homozygotes by Southern analysis (Fig. 1*B*) and by transmission of the transgene to all of their progeny. Homozygotes from line 5 and line 4 are shown in Fig. 3 *B* and *C*, respectively, together with their hemizygous littermates. The homozygotes from both lines, like the hemizygotes, exhibit a bimodal expression pattern. Therefore, the level of β-galactosidase activity can be low in some *lacZ*$^+$ sperm. For each line, the mean fluorescence intensity of the right peak is slightly higher in the homozygotes than in the hemizygotes, as would be expected from twice the transgene copies.

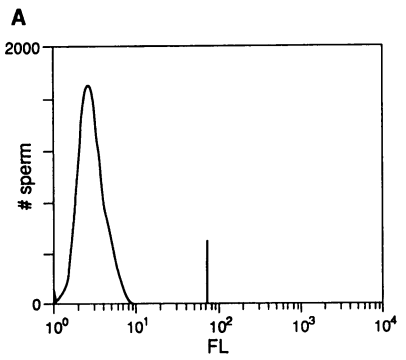
Identification of Rare β-Galactosidase-Positive Sperm by Flow Cytometry. Key to the premise that β-galactosidase is a useful marker for analyzing rare events in a population is the ability to detect a small number of positive sperm in a large background of negative sperm with the FACS. We performed reconstruction experiments to test this. Fig. 5 *A* and *B* show the FACS profiles of sperm from the nontransgenic and hemizygous transgenic mice, respectively, that were used in the reconstruction. Sperm having a fluorescence intensity greater than that marked with the vertical line were considered positive in the mixtures. With the nontransgenic mouse, there were no false positives, whereas approximately half of the transgenic mouse sperm were positive.

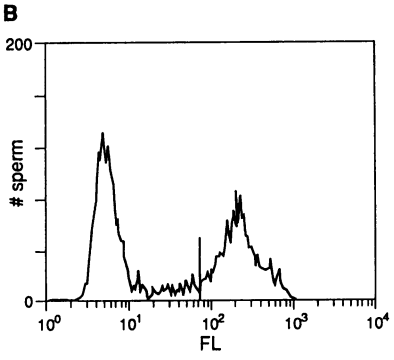
Fig. 5*C* shows results of mixtures of sperm from transgenic and nontransgenic mice. As few as four β-galactosidase-positive sperm (from as little as 0.025% of the sperm from the transgenic mouse) were detected within the population of 40,000 negative sperm that were analyzed. This approximates what is predicted, since about half of the sperm from the transgenic mice fall within the positive range. Although the four positive sperm would be expected to be derived from the transgenic mouse, they would be expected to consist of transgene-negative and transgene-positive sperm. FACS sorting of sperm would lead, therefore, to a substantial enrichment for molecular events that result in *lacZ* expression and β-galactosidase activity, although not a complete purification. The data on the 40,000 sperm in each mixture in Fig. 5*C* were acquired in <2 min of FACS time. Continued data acquisition with the FACS over the course of an hour would allow the examination of ≈10^7 sperm from one mouse epididymis.

DISCUSSION

We have expressed *E. coli lacZ* during spermatogenesis in transgenic mice and detected β-galactosidase activity in sperm. High levels of β-galactosidase activity were detected by flow cytometry after incubation with a fluorogenic substrate. Reconstruction experiments demonstrated this assay to be a very sensitive method to quantitate rare positive cells in the population.

Bimodal β-galactosidase activity profiles were consistently observed for sperm from several mice from different transgenic lines. For hemizygous mice, FACS sorting of the highly





C

% sperm from transgenic	predicted no. positive	actual no. positive
1.0	200	215
0.5	100	91
0.25	50	46
0.1	20	16
0.05	10	10
0.025	5	4
0	0	0

FIG. 5. FACS analysis of a dilution series of transgenic mouse sperm with a large number of nontransgenic mouse sperm. (*A*) Nontransgenic mouse sperm. (*B*) Transgenic mouse sperm. The vertical line in *A* and *B* is the marker that was set to delimit the positive sperm. Sperm were derived from mouse line 5. FL, fluorescence. (*C*) Table of dilution series of transgenic mouse sperm. Predicted no. positive, total number of sperm analyzed (40,000) multiplied by the percent sperm added from the transgenic divided by 2. The division by 2 was performed because roughly half the transgenic mouse sperm are to the right of the marker; actual no. positive, number of sperm with a fluorescence intensity above the marker as detected by the FACS.

fluorescent population from the weakly fluorescent population suggested that both *lacZ*$^-$ and *lacZ*$^+$ sperm were present within each population, so that some *lacZ*$^-$ sperm were highly β-galactosidase-positive and some *lacZ*$^+$ sperm were very weakly β-galactosidase-positive.

Since intercellular bridges connect haploid spermatids in a syncytium during spermatogenesis (16, 17), it has been proposed that although sperm are genotypically haploid, they are phenotypically diploid for gene products synthesized postmeiotically (5). Sharing of both *Prm-1* transcripts (18) and human growth hormone [produced from a gene fusion with 5′ *Prm-1* sequences (5)] has been demonstrated among postmeiotic spermatids. Our transgene consists of 5′ sequences from *Prm-1* that direct transcription postmeiotically in round spermatids and 3′ sequences from *Prm-1* that can delay translation until the elongating spermatid stage (12). Therefore, it is likely that *lacZ*$^-$ sperm can become β-galactosidase-positive as a result of sharing of transgene transcripts or β-galactosidase itself from *lacZ*$^+$ to *lacZ*$^-$ spermatids through the intercellular bridges.

Converse to *lacZ*$^-$ sperm being β-galactosidase-positive, *lacZ*$^+$ sperm can also exhibit very low levels of β-galactosidase activity. Two observations support this. First, FACS analysis of sperm from homozygous mice gave a bimodal distribution similar to that seen with hemizygous mice. Second, FACS sorting of sperm derived from hemizygous mice indicated that weakly β-galactosidase-positive sperm also carried the *lacZ* transgene. We have also observed that *lacZ*$^+$ sperm exhibit reduced levels of β-galactosidase staining following *in vitro* capacitation, a process of sperm maturation (data not shown). Low β-galactosidase activity in approximately half of the sperm may be due to a number of factors, including moribundity of some of the sperm or lack of expression of the transgenes in all of the spermatids. Several examples of transgenes, including *lacZ*, that become inactivated in some fraction of tissues or animals exist in the literature (19, 20). Alternatively, given that *in vitro* capacitation results in reduced staining, β-galactosidase may be lost during sperm maturation as a result of cytoplasmic loss.

We anticipate that expression of *lacZ* during spermatogenesis followed by flow cytometry of sperm will be useful for quantitative analysis of a number of cellular processes. By making appropriate alterations to the *lacZ* expression vector, it should be possible to study infrequent meiotic events such as homologous recombination and DNA rearrangements in greater detail than has been possible. Given the lack of a perfect correlation of β-galactosidase activity with *lacZ* genotype, the sensitivity of the assay is somewhat reduced. However, with the sorting capacity of the FACS, a substantial enrichment for events leading to β-galactosidase expression would be achieved. Fluorescent sperm could be separated and DNA isolated from them, as in Fig. 4, in order to analyze events at the molecular level. The use of the polymerase chain reaction would allow the analysis of events at the single-cell level as has been done with human sperm (21).

We are indebted to Liz Lacy and Kay Higgins for instructions and assistance for creating transgenic mice and to Jacques Peschon and Richard Palmiter for providing plasmids mP1-lacF and pnlacF. We thank Len Herzenberg, Debra Wolgemuth, Pat Saling, and Mitch Eddy for helpful discussions, as well as members of the Jasin lab. This work was supported by a Beckman Foundation Young Investigators Grant, the Frederick R. Adler Chair, and a National Institutes of Health Developmental Core Grant (P30-CA-08748-26).

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