Development 120, 1549-1559 (1994)
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Expression of a mouse *Zfy-1/lacZ* transgene in the somatic cells of the embryonic gonad and germ cells of the adult testis

Brian P. Zambrowicz[1], James W. Zimmermann[2], Christopher J. Harendza[2], Elizabeth M. Simpson[3,*], David C. Page[3], Ralph L. Brinster[2] and Richard D. Palmiter[1,†]

[1]Howard Hughes Medical Institute and Department of Biochemistry, University of Washington SL-15, Seattle, WA 98195, USA
[2]Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA
[3]Howard Hughes Research Laboratories at Whitehead Institute, and Department of Biology, Massachusetts Institute of Technology, 9 Cambridge Center, Cambridge MA 02142, USA

*Present address: The Jackson Laboratory, Bar Harbor, ME 04609, USA
†Author for correspondence

SUMMARY

The *Zfy-1* and *Zfy-2* genes, which arose by gene duplication, map to the mouse Y chromosome and encode nearly identical zinc-finger proteins. *Zfy-1* is expressed in the genital ridge and adult testis and likely encodes a transcription activator. Although potential roles in sex determination and spermatogenesis have been hotly debated, the biological functions of *Zfy-1* remain unknown. To study the gene's regulation, transgenes with 21-28 kb of *Zfy-1* 5′ flanking DNA placed upstream of *lacZ* were constructed in plasmids or created by homologous recombination of coinjected DNA molecules. The resulting transgenic mice expressed β-galactosidase in the genital ridge of both males and females starting between embryonic day 10 and 11 (E10-E11), peaking at E12-E13 and then declining to low levels by E15, a pattern that matches *Zfy-1* mRNA as detected by RT-PCR. This *lacZ* expression in genital ridge was confined to somatic cells as demonstrated by its absence from the alkaline phosphatase-positive germ cells. It had been reported previously that *Zfy-1* mRNA was absent from the embryonic gonad of homozygous W^e embryos, which virtually lack germ cells. By contrast, we observed normal expression of the *Zfy-1/lacZ* transgene when introduced into the W^e background, suggesting that germ cells are not necessary for expression. In the adult, the *Zfy-1/lacZ* transgene is expressed abundantly in developing germ cells. Extragonadal (kidney, meninges, arteries, choroid plexus) expression of the transgene was also observed in embryos. A smaller transgene with only 4.3 kb of *Zfy-1* 5′ flanking DNA was expressed only in germ cells of adult mice. These results suggest that an enhancer for germ cell expression in the adult lies near the *Zfy-1* promoter and that an enhancer for expression in the somatic cells of the embryonic gonad is located further 5′.

Key words: *Zfy-1*, genital ridge, somatic cells, zinc-finger protein, mouse

INTRODUCTION

ZFY (zinc-finger gene on the Y chromosome) was identified because of its location within a genetically defined sex-determining region of the human Y chromosome (Page et al., 1987). Subsequent identification of *SRY* (gene from the sex-determining region of the Y chromosome) as the testis-determining factor eliminated *ZFY* as a candidate for that function (Sinclair et al., 1990; Gubbay et al., 1990; Koopman et al., 1991a). *Zfy-1*, a mouse homologue of human *ZFY* (Mardon and Page, 1989; Mardon et al., 1989; Ashworth et al., 1989), is a member of a multigene family including *Zfy-2*, also found on the Y chromosome, as well as *Zfx* and *Zfa* which are X-linked and autosomal, respectively (reviewed by Koopman et al., 1991b). All members of the family contain 13 zinc fingers, although differences in the third finger in *Zfy-2* and *Zfa* may prevent them from coordinating zinc. The zinc finger motif is found in many eukaryotic DNA- and RNA-binding proteins and these fingers interact with the nucleic acids (Miller et al., 1985; Pavletich and Pabo, 1991). Hence, these zinc-finger genes are thought to encode transcription factors; however, no target genes for any member of the family have been identified.

The expression patterns of the *Zfy*-like genes have been partially determined by northern blot hybridization and RT-PCR. *Zfx* is expressed in every organ examined (Mardon et al., 1990) while the other family members have more restricted patterns of expression. *Zfa* is expressed only in the adult testis (Ashworth et al., 1990). *Zfy-1*, but not *Zfy-2*, is expressed in mouse embryonic stem (ES) cells and blastocysts, while both *Zfy-1* and 2 are expressed in the germ cells of the adult testis (Koopman et al., 1989; Nagamine et al., 1989, 1990; Zwingman et al., 1993). In one study, both *Zfy-1* and *Zfy-2* mRNAs were detected by RT-PCR in the embryonic gonad (Nagamine et al., 1990) while in another study *Zfy-1* mRNA was detected but *Zfy-2* mRNA was below the level of detection of the RT-PCR assay (Koopman et al., 1989). The developmental timing of *Zfy-1* expression in the genital ridge is coincident with arrival of the germ cells, which occurs between E10 and E12 (Eddy et al.,

1981; Tam and Snow, 1981). Expression peaks at E13.5, a time when the primordial germ cell (PGC) population is also peaking in the embryonic gonad. *Zfy-1* gene expression was not detected in the genital ridge of W^e homozygous mice (Koopman et al., 1989) in which the testes form, but are virtually devoid of germ cells (Cattanach, 1978; Koopman et al., 1989). These data were interpreted to indicate that the early gonadal expression of *Zfy-1* is germ cell-dependent (Koopman et al., 1989, 1991b). Message levels for *Zfy-1* and *Zfy-2* increase in the testis between postnatal days 7 and 14 and high levels of transcript are found in isolated round spermatids (Nagamine et al., 1989, 1990). Because of the very low level of *Zfy-1* expression outside of the adult testis, embryonic expression studies have relied upon RT-PCR precluding determination of the cell types that express the gene. There are also indications from RT-PCR and immunohistochemistry, using a Zfy antibody, that *Zfy-1* is expressed in tissues other than the gonad during mouse development including the kidney and brain (Nagamine et al., 1990; Su and Lau, 1992). To characterize the expression pattern of *Zfy-1* during mouse development, including the cell types expressing the gene, we have identified the *Zfy-1* promoter and used it along with the 5′ flanking region to direct the expression of a *lacZ* reporter gene in transgenic mice. We report here that the *Zfy-1/lacZ* transgene is expressed in somatic cells of the genital ridge in both males and females.

MATERIALS AND METHODS

Constructs and transgenic mice

Constructs were made using standard cloning techniques (Sambrook et al., 1989). All *Zfy* family member DNAs were isolated previously (Simpson and Page, 1991). *Zfy-1* sequences were obtained from cEMS132. cEMS177 sequences were subcloned into plasmid pEMS406.1, and *Zfy-2* sequences near exon 1A were obtained from plasmid pEMS501.1, a subclone of cosmid cEMS212, while *Zfy-2* sequences around exon 2 were obtained from plasmid pEMS283, a subclone of cosmid cEMS142. Restriction fragments containing the DNA for microinjection were separated from other fragments by electrophoresis on agarose gels; they were isolated by electroelution into a dialysis bag, followed by phenol chloroform extraction and ethanol precipitation. Transgenic mice were generated as described (Brinster et al., 1985), and the presence of the transgene was identified by DNA hybridization. Coinjections of more than one DNA molecule were done with approximately equivalent numbers of the two DNA molecules. The structure of the integrated DNA was analyzed by probing a Southern blot of *Dra*III-digested liver DNA with a radiolabelled 1.1 kb *Xba*I-*Eco*RV fragment from placF (Mercer et al., 1991) containing the *E. coli lacZ* gene. Animals were examined by staining for β-galactosidase activity to ascertain transgene expression in various organs. Embryonic time points were obtained by mating mice for 1-2 hour periods and checking for vaginal plugs. The time of appearance of the vaginal plug was denoted as time E0.

β-galactosidase staining

Embryos or tissues were prepared for the staining of *E. coli* β-galactosidase by fixation for 1 hour in 4% paraformaldehyde in PBS (pH 7.3). They were rinsed 3× with gentle shaking in 0.1 M NaH_2PO_4 (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% NP-40. Staining was performed overnight at 37°C in the same buffer supplemented with 1 mg/ml X-Gal (Molecular Probes), 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide. After staining, tissues were stored in 70% ethanol. No embryonic gonad staining was observed in any of the hundreds of nontransgenic embryos examined.

Alkaline phosphatase staining

Alkaline phosphatase staining was done using a leukocyte alkaline phosphatase kit (Sigma). Embryos were frozen on dry ice in OCT embedding medium (Miles Scientific) and frozen sections were stained according to the Sigma protocol. Costaining for β-galactosidase activity and alkaline phosphatase activity was also done on the embryonic gonads from dissected embryos. Embryonic gonads were exposed by removal of the internal organs and subsequently stained for 5 hours at 37°C for β-galactosidase activity followed immediately by staining for 1 hour at room temperature in alkaline-dye mix (Sigma) with gentle agitation. Embryos were then stored in 70% ethanol. Litters contained transgenic and nontransgenic embryos.

Breeding of the transgene into the W^e background

Two C57B6/SJL hybrid transgenic mice were mated to produce a male mouse homozygous for the *Zfy-1* transgene (line 4592) and it was mated with 6 W^e heterozygous females. The resulting W^e heterozygous mice were identified by the white patches of fur on their heads and stomachs. All the offspring carried the transgene. These W^e heterozygous, *Zfy-1/lacZ* transgene-positive mice were bred to produce W^e homozygous mice carrying the transgene. The offspring of the matings were killed at E13 and stained for β-galactosidase activity. Placenta DNA was obtained from all mice and used for DNA dot hybridizations to identify the transgene carrying mice and for Southern blot hybridizations to identify the W^e homozygotes. Radioactivity was quantified and compared for DNA dot blots probed with a transgene probe verses an endogenous gene probe to determine hemi- versus homozygosity of the transgene. Southern blot hybridizations were carried out on *Bgl*II-digested placenta DNA, and were probed with a radiolabelled 4.3 kb *Eco*RI fragment of the murine c-*kit* cDNA (Koopman et al., 1991c).

RESULTS

Zfy-1/lacZ expression in transgenic mice

Cosmid clones were identified that contain the 5′ untranslated exons of *Zfy-1* (cEMS132 and cEMS189) and *Zfy-2* (cEMS212 and cEMS142) (Simpson and Page, 1991). The locations of the exon-intron boundaries were determined by comparison of the genomic sequences obtained from these cosmids with that of the published cDNAs (Mardon and Page, 1989; Ashworth et al., 1989). *Zfy-1* has several, alternatively spliced 5′ non-coding exons (Koopman et al., 1989 and our unpublished results) which complicates any nomenclature. In our nomenclature, exon 1A encodes the 5′ end of the *Zfy-2* transcript (Fig. 1A). This exon is spliced to exon 2, which is the same as exon A described by Koopman et al. (1989). Mapping data indicates that transcription of *Zfy-1* begins with exon 1B, which is then spliced to exon 2 (unpublished data). The unique *Eag*I site in exon 1B of *Zfy-1* was used to fuse approximately 4.3 kb of 5′ flanking sequence to a *lacZ* reporter gene (Fig. 1B, construct 2). This construct was either injected alone or coinjected with larger fragments obtained from the *Zfy-1* cosmid (cEMS132) in hopes that they would cointegrate or recombine to provide additional regulatory elements. The coinjected fragment was either (a) an approximately 28 kb portion of the genomic clone containing *Zfy-1* sequences 5′ of the unique *Eag*I site (Fig. 1B, construct 5), (b) an approximately 13 kb portion of the genomic clone containing sequences 3′ of the *Eag*I site (Fig. 1B, construct 4) or (c) the entire cosmid linearized with *Sal*I (Fig. 1B, construct 3).

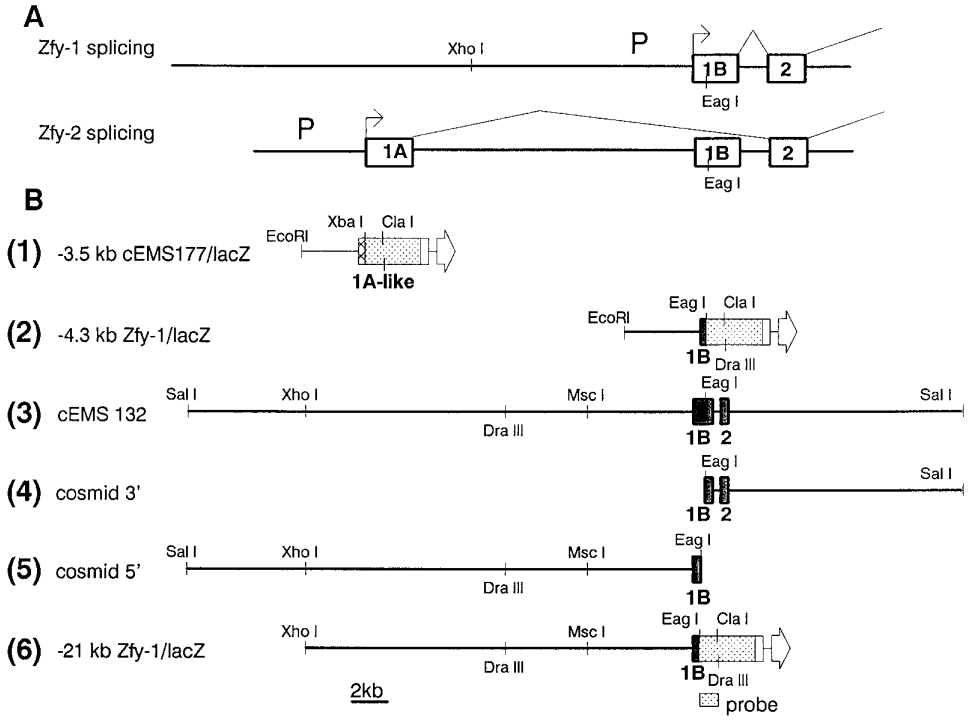

Fig. 1. (A) 5′ Splicing of *Zfy-1* and *Zfy-2*. The splicing patterns of the 5′ exons of the *Zfy-1* and *Zfy-2* genes are based on the comparison of cDNA sequences (Mardon and Page, 1989; Ashworth et al., 1989) and genomic sequences for the two genes. Sequences of *Zfy-1* and *Zfy-2* are nearly identical around exons 1B and 2, but diverge at some point greater than 2 kb 5′ of exon 1B. Exons are depicted as rectangles and splicing by thin lines. Arrows indicate the transcription start site. The drawing is not to scale. (B) Transgenes tested in transgenic mice. All fusions were made to an *E. coli* β-galactosidase reporter gene (dotted rectangle) that contains an intron and two exons (open rectangles and arrows) of the mouse Protamine-1 gene including the Protamine-1 polyadenylation signal. Closed rectangles depict exons of *Zfy-1* and are labelled as either exon 1B or 2. Construct 1 contains 3.5 kb of DNA 5′ of the cEMS177 exon 1A like sequences fused at the *Xba*I site to *lacZ* (the hatched sequences are the exon 1A-like sequences of cEMS177). Construct 2 contains 4.3 kb of *Zfy-1* 5′ flanking sequence fused at the unique *Eag*I site in exon 1B to *lacZ*. Constructs 3, 4 and 5 were coinjected with construct 2. Construct 3 is cosmid cEMS 132 linearized with *Sal*I. Construct 4 is the 3′ portion (*Eag*I-*Sal*I fragment) of the cosmid containing sequences 3′ of the *Eag*I site including *Zfy-1* exons 1B and 2 and intron 1B and part of intron 2. Construct 5 is the 5′ portion of the cosmid (*Sal*I-*Eag*I fragment) containing sequences 5′ of the *Eag*I site. Construct 6 contains 21 kb of *Zfy-1* 5′ flanking sequence fused at the *Eag*I site to *lacZ*. The *lacZ* probe used to analyze the structure of the coinjected transgenes is depicted below construct 6 (dotted rectangle) and contains the first 1.1 kb of *lacZ* sequence.

Founder animals were killed at E12-E13 and stained for β-galactosidase activity. Table 1 summarizes the results. When injected alone, the −4.3 kb *Zfy-1/lacZ* construct was expressed only in the adult testis as will be described later. Four of ten transgenic founder mice, produced as a result of coinjection with the 5′ cosmid fragment, stained for β-galactosidase activity in the embryonic gonad and four of seven transgenic mice produced using the linearized cosmid, stained in the embryonic gonad. However, none of the twelve transgenic mice produced using the 3′ cosmid fragment stained in the embryonic gonad. This suggests that sequences necessary for embryonic gonad expression lie 5′ of −4.3 kb. Therefore, stable transgenic mouse lines were produced by coinjection of the 5′ genomic fragment with the −4.3 kb *Zfy-1/lacZ* transgene. Three lines of mice were produced, two of which (4572 [TgN(*ZFY*1*lacZ*)217Bri] and 4592 [TgN(*ZFY*1*lacZ*)218Bri]) express β-galactosidase in the embryonic gonad.

Southern blot hybridization was carried out on genomic DNA obtained from these two lines of mice to examine the structure of the integrated transgenes produced by DNA coinjection. Fig. 2 shows the results obtained when DNA was digested with *Dra*III (unless otherwise stated) and hybridized

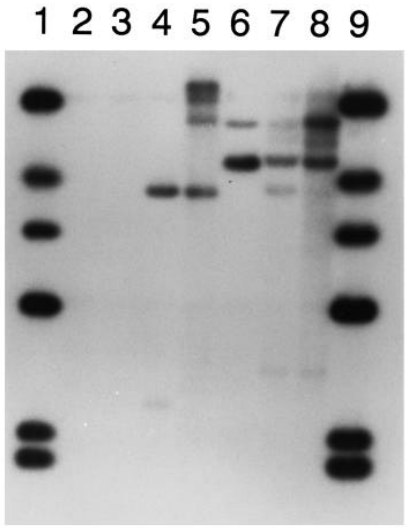

Fig. 2. Structure of the coinjected transgenes. Restriction enzyme (*Dra*III except for lane 4) digested DNA was subjected to Southern blot analysis with a *lacZ* probe (Fig. 1B, probe). The HdIII digested λ molecular mass standards are in lanes 1 and 9 (23.1, 9.4, 6.6, 4.3, 2.3 and 2.0 kb). Genomic DNA was obtained from a nontransgenic male mouse (lane 2), a nontransgenic female mouse (lane 3), a female mouse carrying just the −4.3 kb *Zfy-1/lacZ* construct (lane 5), and female mice from lines 4572 (lane 7) and 4592 (lane 8) both of which carry coinjections of construct 5 and construct 2. Lane 4 contains the 8 kb construct 2 that was used for microinjection. Lane 6 contains the *Dra*III digestion of a reference plasmid that contains 21 kb of *Zfy-1* 5′ flanking sequence fused directly to *lacZ* at the *Eag*I site (Fig. 1B, construct 6).

with a probe specific for *lacZ*. *Dra*III cuts once within the –4.3 kb *Zfy-1/lacZ* construct (Fig. 1B, construct 2) and also cuts approximately 12 kb 5′ of the *Eag*I site in the *Zfy-1* gene. Lanes 4 and 5 contain the –4.3 kb *Zfy-1/lacZ* transgene that was used in the coinjections as a size reference (This reference DNA was not digested with *Dra*III but was released from plasmid sequences by *Eco*RI and *Hd*III digestion) and genomic DNA from a line of mice carrying only the –4.3 kb *Zfy-1/lacZ* transgene, respectively. The 8 kb band in both these lanes indicates that multiple copies of the –4.3 kb *Zfy-1/lacZ* transgene integrated in head-to-tail tandem array. Lane 6 contains the *Dra*III digestion of a reference plasmid that contains 21 kb of *Zfy-1* 5′ flanking sequence fused at the *Eag*I site to *lacZ* (Fig. 1B, construct 6). Lanes 7 and 8 contain *Dra*III-digested genomic DNA obtained from animals in lines 4572 and 4592, respectively. In both of these lines, a 13 kb band was observed that is the same size as that generated from the reference plasmid demonstrating that homologous recombination of the coinjected DNA molecules had occurred. Both lines contain multiple copies of the transgene and it appears that the vast majority of the copies have undergone the recombination event. Both lines contain the 8 kb band which would be expected if some of the –4.3 kb *Zfy-1/lacZ* fragment also integrated as head-to-tail repeats. Digestion of DNA from the two lines with *Msc*I and *Cla*I confirmed that homologous recombination had occurred (data not shown).

To define a *Zfy-1* promoter that could be used as a tool for directing expression of transgenes to organs of endogenous *Zfy-1* expression without relying on recombination, a construct with approximately 21 kb of *Zfy-1* 5′ flanking sequence fused directly to *lacZ* was tested (Fig. 1B, construct 6). This transgene was used to produce 20 transgenic founder mice that were killed at E11.5-E12 and stained for β-galactosidase activity (Table 1). Five of the 20 founders expressed the *lacZ* gene in the genital ridge indicating that sequences that direct expression to the genital ridge reside within this 21 kb 5′ of *Zfy-1*.

Developmental expression of *Zfy-1/lacZ* in the gonad

We analyzed the developmental expression of the transgene in

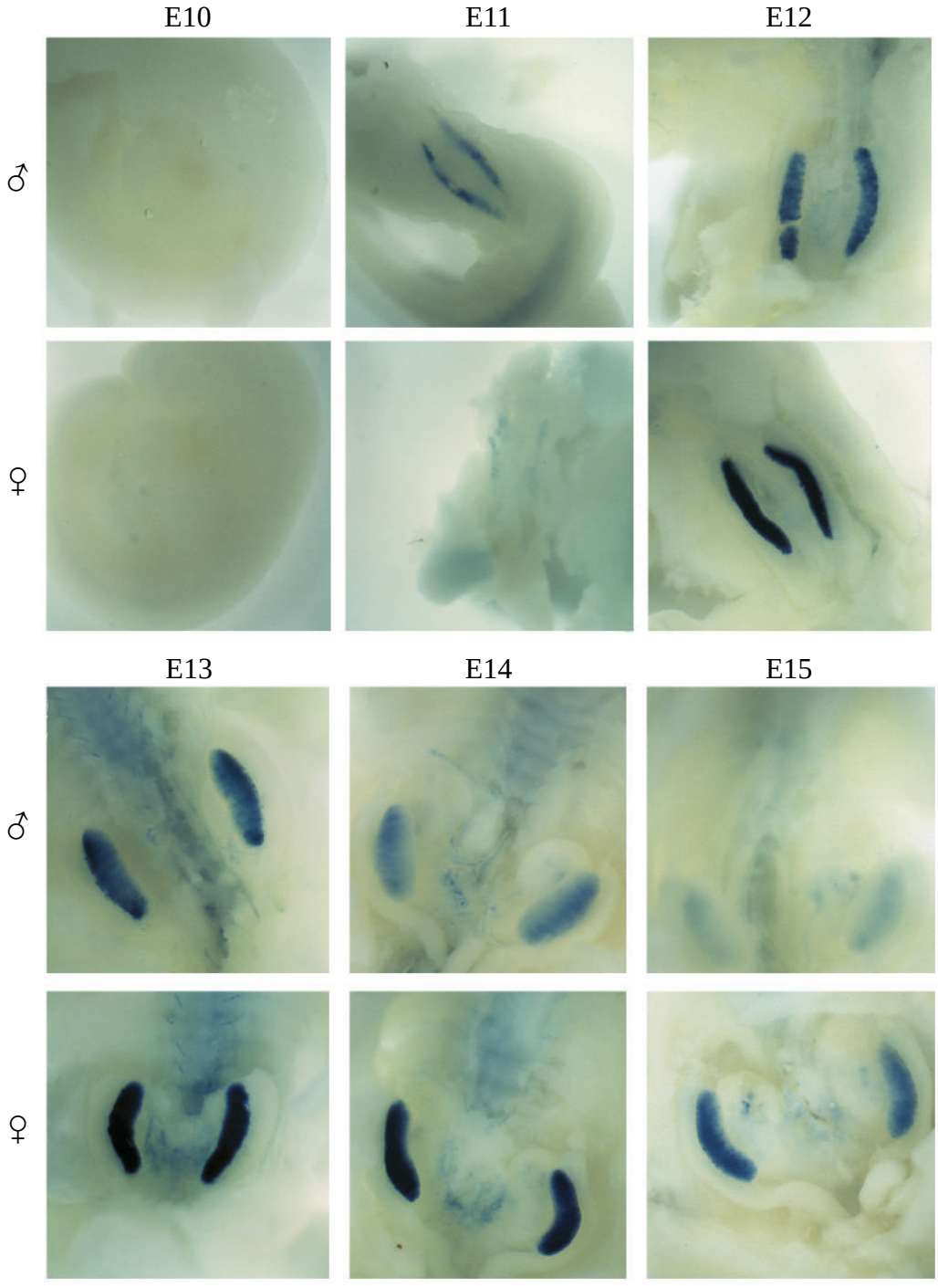

Fig. 3. Expression of the *Zfy-1/lacZ* transgene in the developing embryonic gonad. Embryos were obtained from line 4592 mice at E10 through E15 and stained with X-Gal to reveal β-galactosidase activity. Staining is compared for both males and females. All gonads were photographed at the same magnification.

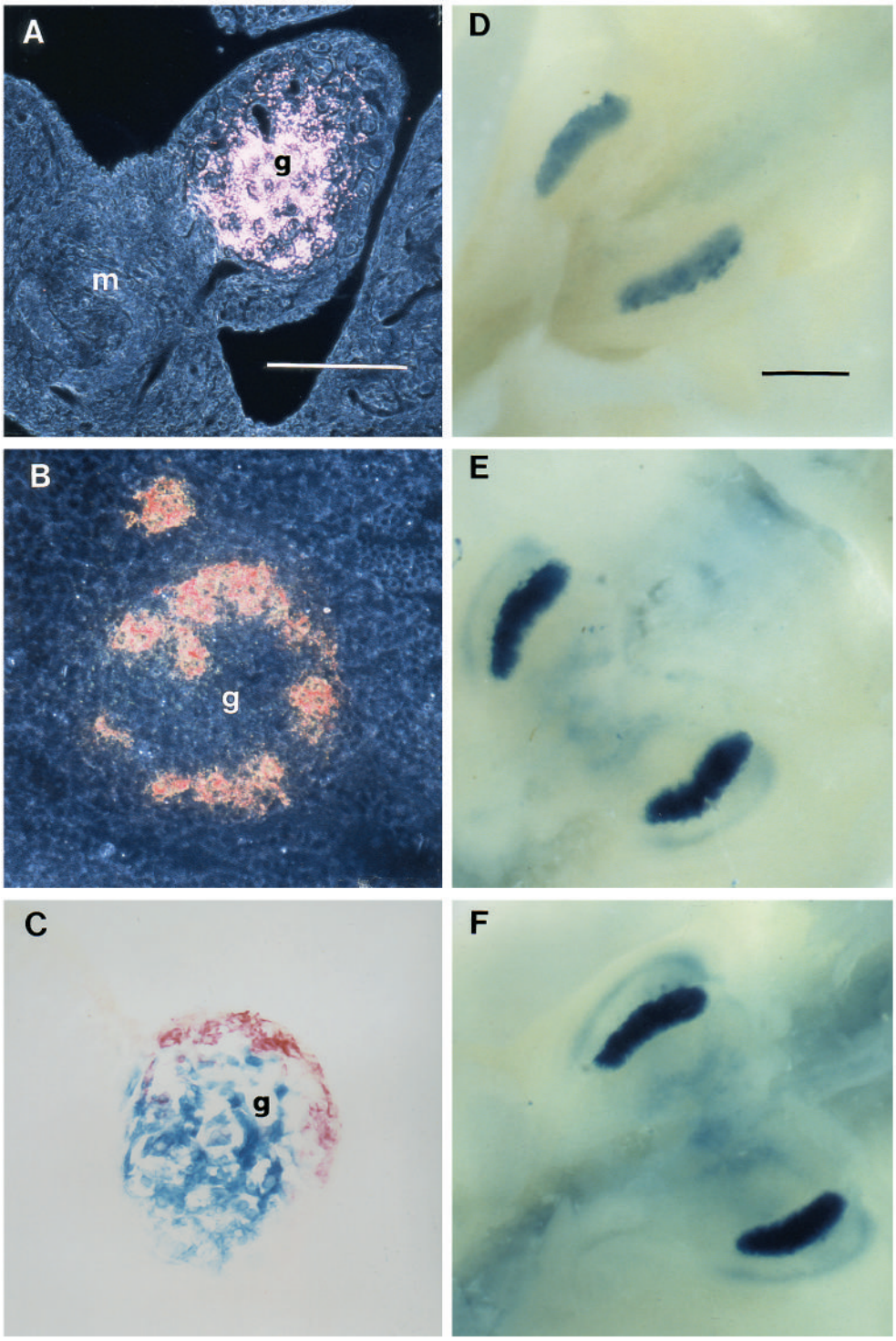

Fig. 4. Cellular localization of *Zfy-1/lacZ* transgene expression in the embryonic gonad. Female embryos were obtained at E12 from line 4592 and stained with X-Gal alone (A; pink staining under dark-field illumination) to reveal transgene expressing cells, for alkaline phosphatase activity alone (B; orange-red stain under dark-field illumination) to identify primordial germ cells, or stained for both cell types (C; blue for the transgene expressing cells and red for cells with high alkaline phosphatase activity under bright-field illumination). Male E12 gonad is identical in staining pattern, but stains less intensely for *lacZ* activity. Gonads in A,B, and C were photographed at the same magnification (bar in A indicates 200 μm). m and g denote the mesonephros and gonads, respectively. (D-F) X-Gal staining was done on E13 littermates resulting from the crossing of a W^e/+, *Zfy-1/lacZ* + (line 4592) male with a W^e/+, *Zfy-1/lacZ* + (line 4592) female. D-F depict the stained gonads of +/+ homozygous, W^e/+ heterozygous, and W^e/W^e homozygous littermates, respectively. All three animals are hemizygous for the transgene. Gonads in D,E, and F were photographed at the same magnification (bar in D indicates 1.0 mm).

mouse lines 4572 and 4592, but because both lines revealed nearly identical developmental expression pattern in all organs, we only show data for the 4592 line that expresses at a higher level. The only difference in expression between the two lines was that the lower level of expression in the 4572 line resulted in a loss of detectable staining in the embryonic gonad after E14 in male mice. Fig. 3 shows the profile for β-galactosidase staining of the developing gonad. Staining was seen in both males and females, but females expressed at a higher level and expression persisted longer, whereas it was repressed in males shortly after the formation of testis cords. This demonstrates that other genes on the Y chromosome are not necessary for *Zfy-1/lacZ* transgene transcription and that testis differentiation may inhibit *Zfy-1* expression. Previously it was reported that *Zfy-1* mRNA was detected in the embryonic gonads of XY mice, which develop as females due to a mutation in the *Sry* gene (Gubbay et al., 1990). The staining we observed in the genital ridge was not detected at E10, but was faintly detected

Table 1. Staining patterns of transgenic lines

Transgene	No. founders tested	Embryonic gonad	Meninges pattern	Testis	Ectopic
−4.3 kb Zfy-1/lac Z	27	0/27	0/27	6/6 Sp ++ RS +	N.D.
cosmid 5′ + −4.3 kb Zfy-1/lac Z	10	4/10	4/10	2/2 Sp ++ Se + In + RS +	1/10 FL,E,Ey 1/10 Fb 1/10 T,GP,D
cosmid 3′ + −4.3 kb Zfy-1/lac Z	12	0/12	0/12	N.D.	1/12 M 1/12 B,S 1/12 Ni,W,N,FT,S,U,B 1/12 GP,M 1/12 LJ,S
cEMS 132 + −4.3 kb Zfy-1/lac Z	7	4/7	3/7	N.D.	1 C
−21 kb Zfy-1/lac Z	20	5/20	10.20	N.D.	1 W
−3.5 kb cEMS 177/lac Z	10	0/10	0/10	10/10 RS ++++	1/10 L 1/10 L,Lu 2/10 U,B 1/10 B 1/10 CP 1/10 CP,CC

Numbers indicate the number of founder animals exhibiting a staining pattern in the organ indicated.

Meninges pattern indicates staining in the meninges of the brain and spine. Abbreviations: Sp, spermatocytes; RS, round spermatids; Se, Sertoli cells; In, interstitial cells; N.D., not determined; FL, forelimb; E, ear; Ey, eye; Fb, forebrain; T, tongue; GP, genital papillae; D, digits; M, mesonephros; B, brain; S, spine; Ni, nipples; W, whiskers; N, nostril; FT, fingertips; S, stomach; U, ureter; LJ, lower jaw; C, cheek; L, liver; Lu, lung; CP, caudate putamen; CC, cerebral cortex. The level of expression for cell types in the testis is given as; low (+), medium (++), and very high (++++).

at E11 and increased and peaked at E12-E13 followed by a decrease to low levels by E15. This is the same pattern that was observed for *Zfy-1* mRNA as detected by RT-PCR (Koopman et al., 1989) and a *Zfy* antibody detected only minimal amounts of protein in E13 testis and was unable to detect any *Zfy* protein by E15 (Su and Lau, 1992). The similarity between transgene expression and *Zfy-1* mRNA and protein expression suggests that all the correct regulatory elements are present within this construct.

When RT-PCR was carried out on mRNA derived from the embryonic gonad of W^e homozygotes, no *Zfy-1* message was detected (Koopman et al., 1989). W^e homozygotes are virtually devoid of germ cells in the gonad due to a mutation in the c-*kit* receptor; therefore, Koopman et al. (1989) suggested that *Zfy-1* expression is germ cell dependent. Surprisingly, when we examined cross-sections of the stained genital ridges, we found the transgene was expressed in the somatic cells rather than the germ cells (Fig. 4A). To confirm this, E12 gonads were either stained just for alkaline phosphatase activity, which reveals germ cells (Chiquone et al., 1954) (Fig. 4B) or costained with X-Gal, to detect the transgene-expressing cells (Fig. 4C). The two stains clearly identify separate populations of cells, demonstrating that the transgene is expressed in the somatic cells and not the germ cells. Staining was carried out at E12 because there is less mixing of the somatic and germ cells prior to the formation of the testis cords. The gonadal staining pattern at this time is indistinguishable in males and females.

Because we observed somatic cell expression of the *Zfy-1* transgene, but Koopman et al. (1989) were unable to detect *Zfy-1* message by RT-PCR in W^e gonads, we hypothesized that *Zfy-1* is normally expressed in the somatic cells of the embryonic gonad, but requires the presence of germ cells to be activated. To test this hypothesis, the *Zfy-1/lacZ* transgene (line 4592) was introduced into the W^e homozygous background and the resulting embryos were stained for β-galactosidase activity at E13. The transgenic mice were identified by placental DNA dot hybridization and placental DNA was also used to identify W^e homozygotes using RFLP analysis. The W^e allele cosegregates with an RFLP of approximately 13.5 kb that is detected by digestion with *Bgl*II and hybridization of Southern blots with a mouse c-*kit* probe. Male and female heterozygous W^e mice that carried the transgene were mated and the RFLP analysis of their offspring allowed us to identify 9 W^e homozygous mice that carried the *Zfy-1/lacZ* transgene. These 9 W^e homozygous embryos all expressed the transgene at a normal level in the genital ridge (Fig. 4D-F for homozygous wild type, heterozygous, and homozygous W^e littermates, respectively, stained for β-galactosidase activity). These results demonstrate that the transgene is expressed in the developing gonad of W^e mice.

Zfy-1/lacZ expression in adult testis

The developmental time when each spermatogenic cell type first appears in the prepuberal mouse has been well defined (Bellve' et al., 1977). Thus, for example, spermatocytes first appear in the mouse testis 9-10 days after birth and haploid round spermatids first appear after day 19. By examining both the timing of a gene's expression in the prepuberal mouse testis and the position of the expressing cells in the seminiferous tubule, it is possible to establish what cell types express the gene. When the −4.3 kb *Zfy-1/lacZ* transgene was tested alone, expression was not detected in the genital ridge in any of 28 founder mice (Table 1), but expression was observed in the adult testis. There was no expression at day 1 (data not shown) or day 7 (Fig. 5C) after birth, but pachytene spermatocytes

clearly stained at day 17 (Fig. 5D) and both spermatocytes and spermatids stained in the adult (Fig. 5E). The −4.3 kb *Zfy-1/lacZ* testis expression was examined in 6 lines including line 3287 [TgN(Zf*lacZ*)219Bri] and 3283 [TgN(Zf*lacZ*)220Bri].

In contrast, staining was observed at all developmental time points in the 4592 line that contains 28 kb of 5′ flanking DNA. Staining was restricted to Sertoli and Leydig cells at days 1 and 7 (Fig. 5F,G). Staining was abundant in spermatocytes at day 17 (Fig. 5H) and persisted at similar levels in spermatids of the adult testis (Fig. 5I). In the 4572 line, no staining was detected between E14 and the onset of meiosis (day 10 after birth), but staining resembled that of the 4592 line and the −4.3 kb *Zfy-1/lacZ* line at later stages (data not shown). This difference probably reflects the generally lower level of expression in the somatic cells of the 4572 line compared to the 4592 line. These results indicate that the enhancer responsible for adult germ cell expression resides within 4.3 kb upstream of exon 1B, whereas the enhancer for genital ridge expression lies 5′ of −4.3 kb. In all of the *Zfy-1/lacZ* transgenic mice, staining in round spermatids did not appear to increase in intensity relative to the staining seen in spermatocytes. This is in contrast to the large increase in *Zfy* (the probe used could not distinguish between *Zfy-1* and *Zfy-2* message) mRNA in spermatids as detected by northern blot (Nagamine et al., 1990). This difference will be discussed later. The 4592 line of mice also expressed the transgene in theca and granulosa cells of the ovary (data not shown). It is noteworthy that these cells derive from the same genital ridge precursors as the Sertoli and Leydig cells.

In the process of looking for exon 1A of *Zfy-1* (using probes complementary to *Zfy-2*), cosmid cEMS177 was isolated. This cosmid contains a sequence that is clearly homologous to exon 1A of *Zfy-2*. There is 83% sequence identity over exon 1A and this identity extends at least 250 bp 5′ of exon 1A(unpublished data). This exon 1A-like sequence from cEMS177 and 3.5 kb of 5′ flanking DNA was fused to *lacZ* (Fig. 1B, construct 1) and the resulting construct was used to generate transgenic mice. When these mice were analyzed for expression, X-Gal staining was observed exclusively in round and elongating spermatids of the adult testis. Hence, staining was observed at day 25 and in adults (Fig. 5B) but not at day 17 (Fig. 5A). We postulate that expression of the *cEMS177/lacZ* transgene resembles that of *Zfy-2*.

Extragonadal expression of *Zfy-1/lacZ*

Lines 4572 and 4592 (containing 28 kb of coinjected Zfy-1 5′ flanking DNA) also express the transgene in the kidney, brain and spine prior to birth. The expression in the brain and spine was lower than that seen in the gonad as shown by the whole E12 embryo stained for 6 hours (Fig. 6A). The expression in E14 kidney was high in the parietal cells and the Bowmans capsule of glomeruli (Fig. 6B, larger arrow). Faint staining was also detected in cells lining the ducts (smaller arrow). *Zfy* protein was also detected in the ducts of the embryonic kidney (Su and Lau, 1992). Mature glomeruli are not present in the mouse kidney until about E14 and we did not detect strong kidney expression until about this time. Expression in the brain was found in the meninges (Fig. 6C, largest and smallest arrows) as well as in the choroid plexus (middle-sized arrow). Spine expression was localized at E14 to the meninges and the walls of some arteries (Fig. 6D). RT-PCR had detected *Zfy-1* transcript in E12 brain and kidney and immunohistochemistry identified *Zfy* protein in the kidney (Nagamine et al., 1990; Su and Lau, 1992). Ten of 20 founder mice carrying the −21 kb *Zfy-1/lacZ* transgene, 4 of 10 mice carrying the 5′ cosmid coinjection and 3 of 7 mice carrying the entire cosmid coinjection also stained in the brain and spine with the same pattern described for lines 4572 and 4592 (Table 1). All founders or lines except one that stained in the embryonic gonad also stained in the brain and spine.

DISCUSSION

The developmental timing of *Zfy-1/lacZ* fusion gene expression in both the embryonic gonad and in the testis after birth mimics the mRNA levels as detected by RT-PCR and northern blot analysis (Koopman et al., 1989; Nagamine et al., 1990). RT-PCR had also detected *Zfy-1* mRNA in the E12 kidney and brain (Nagamine et al., 1990), organs where we detect the expression of our *Zfy-1/lacZ* transgenes. The only tissue in which *Zfy-1* mRNA was detected in which we failed to demonstrate a corresponding expression of the transgene was the E12 liver (Nagamine et al., 1990). The remarkable resemblance of mRNA localization and transgene expression suggest that most if not all of the regulatory elements required for the normal expression of *Zfy-1* lie within 28 kb 5′ of the start of *Zfy-1* transcription.

The restriction of transgene expression to the somatic cells of the genital ridge was surprising as it had been suggested that *Zfy-1* expression in the developing gonad is germ cell-dependent (Koopman et al., 1989, 1991b; Ashworth et al., 1991). In fact, this was one of the pieces of data suggesting that *Zfy-1* could not be the sex-determining gene on the Y chromosome. Simultaneous staining of germ cells for alkaline phosphatase and of transgene-expressing cells for β-galactosidase leaves little doubt that transgene expression is confined to the somatic cells in the genital ridge. Expression of the transgene in the E13 gonad of W^e homozygotes indicates that the transgene can be expressed in gonads deficient in germ cells. The question remains as to why the *Zfy-1* mRNA was not detected by RT-PCR in gonads of W^e mice (Koopman et al., 1989). There are several possibilities. Koopman et al. (1989) identified W^e homozygotes by paleness of the liver at E14.5, which is a manifestation of acute anemia. As many W^e homozygotes die before birth, it is reasonable to assume that those with overtly pale livers at E14.5 are already very sick. Consequently, *Zfy-1* transcription may already have ceased or transcription in general may be lower. The only control for this was the amplification of HPRT as a positive control and it is noticeably lower in W^e homozygotes compared to wild-type littermates. We stained at E13 because by E14.5 transgene expression level falls significantly in normal animals and in line 4572, expression is no longer detectable. In addition, Su and Lau (1992), using an antibody directed against *Zfy* protein, detected only minimal protein in the E13 testis and were unable to detect any *Zfy* protein by E15. Perhaps *Zfy-1* transcript levels are so low at E14.5 that even a small reduction in *Zfy-1* transcript levels could place *Zfy-1* mRNA below a level detectable by RT-PCR. Another possibility is that the transgene expression in the somatic cells is ectopic and germ cells actually do express the endogenous *Zfy-1* gene. This possibil-

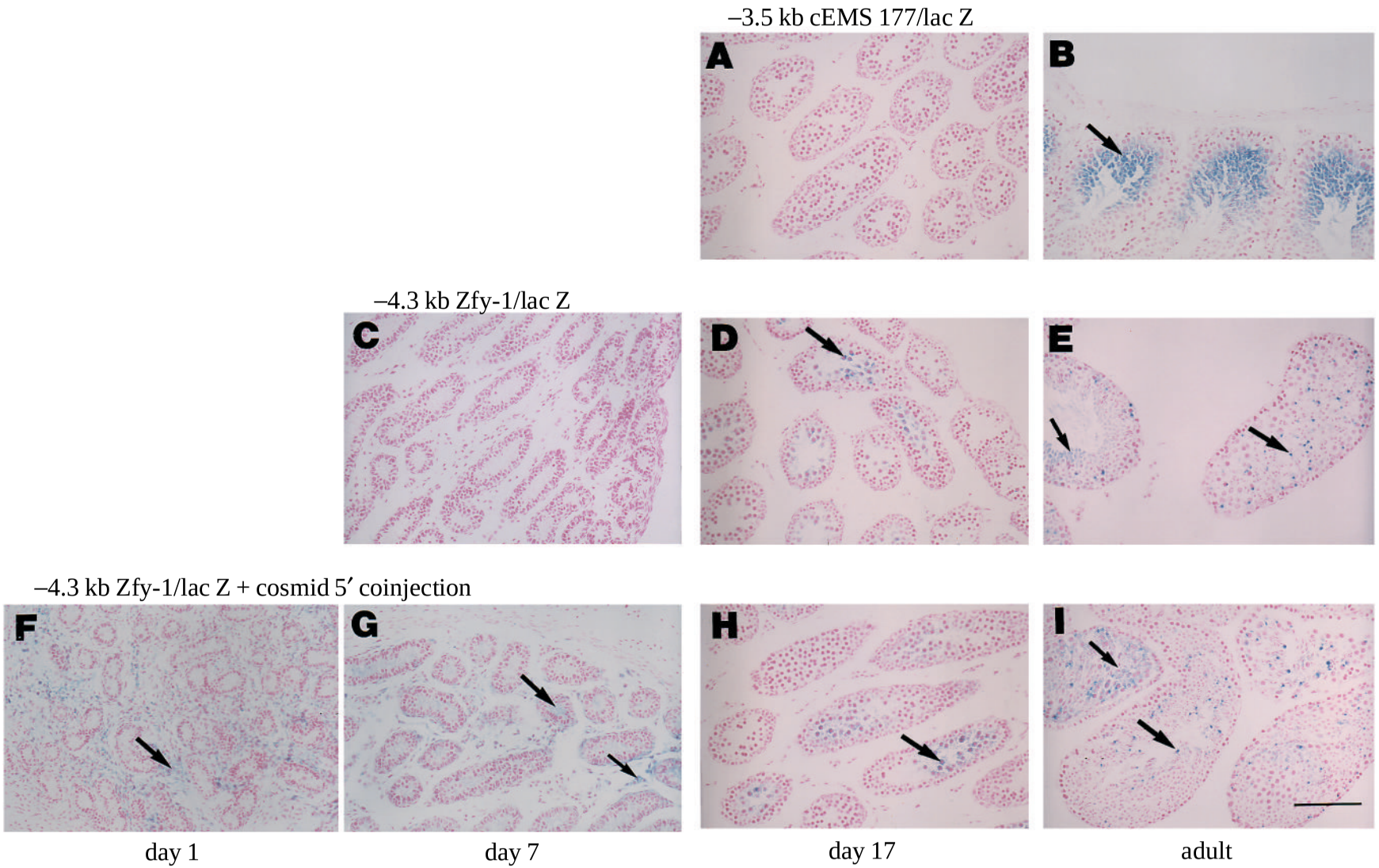

Fig. 5. Developmental expression of *Zfy/lacZ* transgenes in the testis after birth. Testes were isolated from animals containing the –3.5 kb *cEMS177/lacZ* construct (A,B). The –4.3 kb *Zfy-1/lacZ* transgene (C-E), or the coinjection of the 5′ cosmid fragment with the –4.3 kb *Zfy-1/lacZ* construct (line 4592) (F-I) at the times after birth indicated and stained with X-Gal. Cross sections were 10 μm and all sections were photographed at the same magnification (bar in I indicates 100 μm). All sections were counterstained with nuclear fast red. The –3.5 kb *cEMS177/lacZ* testis does not stain at day 17 (A), but intense blue staining is clearly seen in round and elongating spermatids in the adult animal (B, arrow). The -4.3 kb *Zfy-1/lacZ* testis does not stain at day 7 (C), but staining is clearly present in spermatocytes at day 17 (D, arrow) and persists in both spermatocytes (E, larger arrow) and spermatids (E, smaller arrow) in the adult. The line 4592 testis stains well in the interstitial cells (F, arrow) and extremely faintly in Sertoli cells at day 1. Sertoli cell (G, larger arrow) and lower level interstitial cell staining (G, smaller arrow) remains at day 7. At day 17 staining is seen clearly in spermatocytes (H, arrow) and staining persists in spermatocytes (I, larger arrow) and spermatids (I, smaller arrow) in the adult.

ity seems unlikely because of the close resemblance of the transgene expression profile to the endogenous mRNA profile not only in the developing gonad but also in extragonadal tissues. The evidence suggests that *Zfy-1* is expressed in the somatic compartment of the genital ridge regardless of the presence or absence of germ cells.

The expression of *Zfy-1* in the somatic cells of the genital ridge is probably in the same cells that express *Sry*. These cells are the precursors for the Sertoli cells of the adult testis and granulosa cells of the adult ovary. The enhancer for somatic expression of *Zfy-1* apparently lies between –4.3 and –21 kb, whereas the enhancer for expression in spermatocytes and ES cells is located within the proximal 4.3 kb (our unpublished observations). *Sry* is also expressed in germ cells of the adult including spermatocytes and spermatids (Rossi et al., 1993), and blastocysts (Zwingman et al., 1993) which emphasizes the similarity in expression of the two genes. Perhaps the enhancers for *Zfy-1* and *Sry* may be similar and bind at least some of the same transcription factors. None of the factors required for embryonic gonad expression appear to be encoded by the Y chromosome because expression of the *Zfy-1/lacZ* and *Sry* transgenes (Koopman et al., 1991a) occurs in both males and females.

The *cEMS177/lacZ* transgenic mice may provide data concerning the regulation of the *Zfy-2* gene because cEMS177 and *Zfy-2* have homologous exon 1A and 5′ flanking sequences (83% sequence identity over 500 bp). We now know that cEMS177 is derived from an autosome (E.M. Simpson, unpublished observations). Considering that there are no sequences resembling exon 1A upstream of Zfy-1, further mapping of cEMS177 may provide clues regarding the absence of this exon and suggest how *Zfy-1* and *Zfy-2* evolved. *cEMS177/lacZ* transgene expression was restricted to round and elongating spermatids; hence we predict that regulatory elements in the corresponding region of the *Zfy-2* promoter may direct expression to round spermatids. This would explain why the *Zfy-1/acZ* transgenes do not demonstrate increased expression levels in round spermatids relative to spermatocytes, whereas there is a dramatic increase in total *Zfy* mRNA detected between these two stages, by northern blot hybridization (their probe could not differentiate between *Zfy-1* and *Zfy-2* mRNAs; Nagamine et al., 1990). We hypothesize that before birth, *Zfy-1* and possibly *Zfy-2* are expressed at low

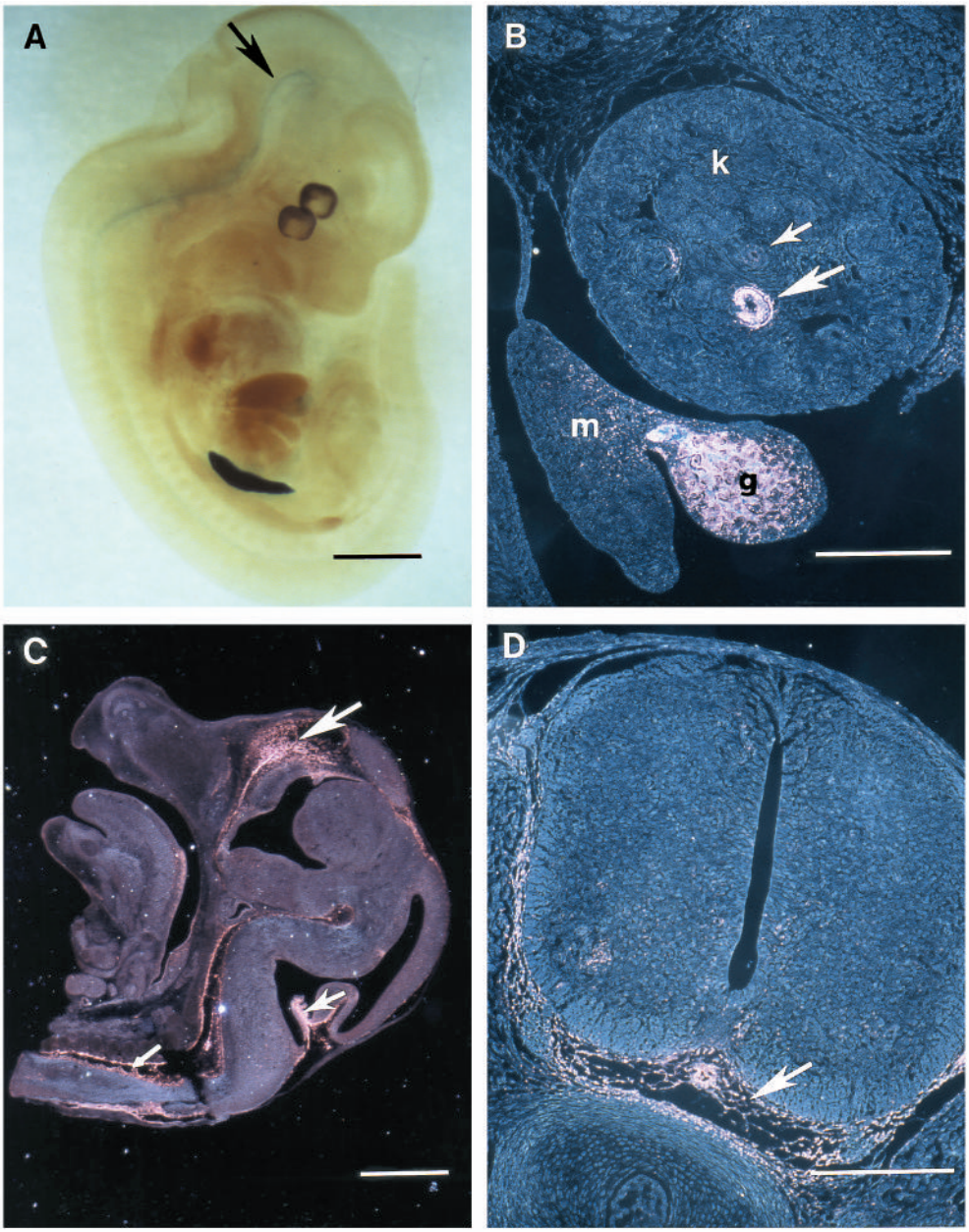

Fig. 6. Meninges and kidney expression of the *Zfy-1/lacZ* transgene. (A) An E12 mouse embryo (line 4592) was stained for 6 hours with X-Gal, revealing that the neural expression (A, arrow) is of a lower level than that seen in the embryonic gonad. Line 4592 transgenic mice were obtained at E14 (B-D) and stained overnight with X-Gal to reveal β-galactosidase activity. Kidney expression is found in the parietal cells and Bowman's capsule of glomeruli (B, larger arrow) and very faintly in the cells surrounding the ducts (B, smaller arrow). k and m denote kidney and mesonephros, respectively. Expression in the brain (sagittal section) is found in the meninges (C, largest and smallest arrow), in the walls of some arteries and in the choroid plexus (C, middle-sized arrow). Spine expression is found in the meninges (D, arrow) and in the walls of some arteries. The bars in A and C indicate 1 mm and in B and D indicate 200 μm. B-D are under dark-field illumination (β-galactosidase staining appears pink).

levels in the somatic cells of the gonad, *Zfy-1*, and likely *Zfy-2* (Nagamine et al., 1990), are expressed in germ cells at the onset of meiosis, and *Zfy-2* subsequently is expressed at high levels in round spermatids. Verification of this hypothesis requires identification and testing of the *Zfy-2* promoter. Interestingly, both cEMS177 and *Zfy-2* contain multiple CRE-like half-site sequences within 200 bp 5′ of exon 1A (unpublished data). CRE-like sequences have been implicated in the spermatid-specific transcription regulation of the mouse protamine 1 gene (Zambrowicz et al., 1993) and the gene encoding the mouse testis-specific isoform of the angiotensin-converting enzyme (Howard et al., 1993). CRE half-sites can be bound by CREB in vitro and split CREs are capable of activating transcription with the orientation and distance between half-sites having little effect upon this activation (Fink et al., 1988). A testis-specific protein has been identified that appears after day 12 and binds to the CRE-like sequence of the mouse protamine 1 gene (Zambrowicz and Palmiter, 1994). Hence, the CRE-like sequences in cEMS177 and the *Zfy-2* gene may also serve to regulate spermatid transcription of these genes.

Our data also provide clues to the potential function of *Zfy-1*. It has been suggested that *Zfy-1* could be the *Spy* gene (Simpson and Page, 1991; Koopman et al., 1990b), a gene required for normal spermatogenesis, which is lost in the deletion from the Sxr^a region that produces Sxr^b (Burgoyne et al., 1986; Sutcliffe and Burgoyne, 1989). *Spy* was identified genetically because XO Sxr^b mice have a more severe phenotype than XO Sxr^a mice. In contrast to XO Sxr^a mice, XO Sxr^b mice almost totally lack meiotic and post meiotic germ cells and *Spy* is therefore thought to be required for the proliferation of differentiating type A spermatogonia (Sutcliffe and Burgoyne, 1989). Levy and Burgoyne (1986) have demonstrated that XO germ cells in XO/XY chimeras show a similar if not identical defect in spermatogonial divisions to that seen in XO Sxr^b mice. If we assume that this phenotypic similarity is entirely due to the absence of *Spy* in both cases, then that suggests *Spy* functions in a cell autonomous fashion within germ cells. The *lacZ* expression described here indicates that the only gonadal expression of *Zfy-1* prior to the *Spy* arrest is in somatic cells. The germ cell autonomy of *Spy* expression and the lack of *Zfy-1* expression in germ cells (prior to *Spy* arrest) argue against their being one and the same. If *Zfy-1* has a function in the embryonic gonad, it must be subtle, as germ cells appear to develop normally to the spermatogonial stem cell stages in XO Sxr^b mice and testes appear to form normally in XX *SRY* transgenic male mice (Koopman et al., 1991a), both of which lack an intact *Zfy-1* gene. It is difficult to determine the function of *Zfy-1* in the germ cells during spermatogenesis because no mutations have been identified that affect only the *Zfy-1* gene. All known mutations in *Zfy-1* are part of larger chromosomal deletions. Targeted disruption of the *Zfy-1* gene will be required to obtain a clear understanding of *Zfy-1* function.

We thank Robert Braun for constructive discussions and support during the course of this work. We also thank Glenda Froelick for her histology work, Raj Kapur and Carol Quaife for helping analyze expression, and Mary Avarbock for DNA microinjection. We are also grateful for the W^e mice provided by Robin Lovell-Badge and the mouse c-*kit* probe provided by Peter Besmer. This work was supported in part by NIH grants HD-09172, HD-23657, and HD-22532. B. Z. was supported by PHS NRSA T32 GM-07270 from NIGMS. E. M. S. was the recipient of a Medical Foundation/Charles A. King Trust Fellowship.

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(Accepted 9 March 1994)