Gonadal sex reversal in mutant *Dax1* XY mice: a failure to upregulate *Sox9* in pre-Sertoli cells

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Summary

The nuclear receptor transcription factor *Dax1* is hypothesized to play a role in testicular development, although the mechanism of its action is unknown. Here, we present evidence that *Dax1* plays an early essential role in fetal testis development. We hypothesize that upregulation of *Sox9* expression in precursor somatic cells, a process required for their differentiation as Sertoli cells, depends on the coordinated expression of *Dax1*, *Sry* and another gene, *Tda1*. Our conclusion and model are based on the following experimental findings: (1) presence of a mutant *Dax1* allele (*Dax1*-) results in complete gonadal sex reversal in C57BL/6JEi (B6) XY mice, whereas testes develop in DBA/2J (D2) and (B6×D2)F1 XY mice; (2) B6-DAX1 sex reversal is inherited as a complex trait that includes the chromosome 4 gene *Tda1*; (3) B6 *Dax1*-/Y fetal gonads initiate development as ovaries, even though *Sry* expression is activated at the correct time and at appropriate levels; (4) upregulation of *Sox9* does not occur in B6 *Dax1*-/Y fetal gonads in spite of apparently normal *Sry* expression; and (5) overexpression of *Sry* in B6 *Dax1*-/Y fetal gonads upregulates *Sox9* and corrects testis development.

Key words: Testis development, *Nr0b1*, *Sry*, Fetal gonad, Autosomal modifier, Mouse

Introduction

Several genetic syndromes in humans are associated with abnormal gonadal development and differentiation (reviewed by Warne and Kanumakala, 2002). For example, XY humans with a duplication of a 160 kb region on chromosome Xp21 exhibit gonadal dysgenesis and develop ambiguous or female external genitalia (Bardoni et al., 1994), a condition known as dose-sensitive sex-reversal (DSS). Alternatively, a mutation within or a complete deletion of this chromosomal region causes X-linked adrenal hypoplasia congenita (AHC) in which XY individuals develop adrenal insufficiency and hypogonadotropic hypogonadism (HH), leading to delayed puberty (reviewed in McCabe, 2001). The gene responsible for these disorders is *NR0B1* (nuclear receptor subfamily 0, group B, member 1), also known as *DAX1* (DSS AHC critical region on the X chromosome, gene 1) (Muscatelli et al., 1994) (reviewed by Achermann et al., 2000). *DAX1*, an orphan receptor belonging to the superfamily of nuclear receptor transcription factors, is composed of two exons that encode a 470 amino acid protein. The N-terminal region of DAX1 is thought to serve as a DNA-binding domain and the C-terminal region contains a putative ligand-binding domain (Bae et al., 1996). In humans and mice, *DAX1/Dax1* is expressed throughout the hypothalamic-pituitary-adrenal/gonadal axis (Ikeda et al., 1996; Hanley et al., 2000; Ikeda et al., 2001). Although DAX1 represses the transcriptional activating potential of a number of proteins through protein-protein interaction (reviewed by Clipsham and McCabe, 2003), it is possible that DAX1 has additional functions, such as directly mediating gene expression by binding to DNA or RNA (reviewed by Ludbrook and Harley, 2004).

It was originally thought that *DAX1* was an ovarian determining gene, based on sex reversal data in humans (Bardoni et al., 1994; Swain et al., 1996). More recent studies, however, have challenged this view and suggested that *Dax1* is involved in testicular development. Three studies have dealt with loss of *Dax1* function. The first report investigated gonad development in 129Sv/J mice containing a *Dax1* exon 2 (ligand-binding domain) deletion (hereafter designated *Dax1*-). *Dax1*-/Y adult males had smaller than normal sized testes with impaired testicular germinal epithelial development and eventual germ cell loss (Yu et al., 1998). By contrast, homozygous *Dax1*- mice were fertile females and appeared to have no reproductive problems. A second study examined testicular development in fetal 129Sv/J *Dax1*-/Y mice (Meeks et al., 2003a). Testis development appeared to progress normally until E13.5, when some testis cords appeared disorganized and incomplete. The third study examined the effects of the *Dax1*- allele in mice containing a mixed genetic background and a *Mus domesticus poschiavinus* Y chromosome (Y^{POS}) (Meeks et al., 2003b). Combining a 'weaker' *Sry* (sex-determining region of chromosome Y) allele on Y^{POS} (Eicher et al., 1995) and *Dax1*- caused sex reversal in *Dax1*-/Y^{POS} mice.

Here, we report that *Dax1* has an early, essential role in fetal testis development. Experiments involving mice from the two inbred strains C57BL/6JEi (B6) and DBA/2J (D2) were used. The B6 strain was chosen because B6 XY mice are exceptionally sensitive to disturbances in the early events of testicular development and thus provide a sensitized genetic test system for identifying genes that are important for primary (gonadal) sex determination (Eicher and Washburn, 1986; Eicher et al., 1996; Eicher et al., 1982). The D2 strain was chosen because D2 XY mice develop normal testes under genetic circumstances in which B6 XY mice develop ovaries or ovotestes (Eicher et al., 1996). We found that ovaries developed in B6 *Dax1*-/Y mice (i.e. these mice are completely sex reversed), whereas testes developed in D2 and F1 *Dax1*-/Y mice. Multi-gene expression analysis indicated that *Sox9* expression was not upregulated in B6 *Dax1*-/Y fetal gonads even though *Sry* was expressed at the correct time and at appropriate levels. Ovarian development in B6 *Dax1*-/Y mice was prevented if *Sry* expression was increased by addition of a multicopy *Sry* transgene. Finally, experiments to map B6-derived genes involved in B6-DAX1 sex reversal identified a modifier gene located on distal chromosome (Chr) 4. We conclude that *Dax1* functions upstream of *Sox9* in the testis development pathway and hypothesize that DAX1, SRY and TDA1, a protein encoded by the Chr 4 gene (testis-determining autosomal 1, symbolized *Tda1*), are required for the upregulation of *Sox9* expression in the somatic supporting cell lineage precursors, a molecular event required for these cells to initiate differentiation as Sertoli cells.

Materials and methods

Mice

Two B6 congenic strains were produced, B6-$Dax1^{flox}$ and B6-*Dax1*-. To produce the B6-$Dax1^{flox}$ strain, the $Dax1^{flox}$ allele (Yu et al., 1998) was transferred from a 129 strain to the B6 strain using successive backcrossing. To produce the B6-*Dax1*- strain, we used a Zp3-Cre transgene (Tg93)-expressing Cre recombinase under control of the zona pellucida (*Zp3*) gene promoter (de Vries et al., 2000) to eliminate *Dax1* sequences located between two *loxP* sites in exon 2 [C-terminal region containing a putative ligand-binding domain] (Yu et al., 1998). This was accomplished by mating a B6 female carrying the $Dax1^{flox}$ allele to a B6 Tg93 male. Female offspring containing both $Dax1^{flox}$ and Tg93 were mated to normal B6 males and female offspring were identified that contained a *Dax1*- allele.

A D2-Y^{POS} consomic strain was constructed by mating a B6 XY^{POS} hermaphrodite to a D2 female. Successive backcrosses involving XY^{POS} males mated to D2 females resulted in the D2-Y^{POS} consomic strain. A D2-*Dax1*- congenic strain was produced by transferring *Dax1*- onto the D2 strain background using successive backcrossing.

A coisogenic B6 strain that is homozygous for the white-bellied agouti (A^W) allele at the agouti gene and carries the tabby-6J ($Eda^{Ta\text{-}6J}$) mutation at the X-linked ectodysplasin-A (*Eda*) gene was used to efficiently identify *Dax1*-/Y backcross females at weaning in linkage analyses crosses. (Hereafter, $Eda^{Ta\text{-}6J}$ is designated Ta^{6J}.) Ta^{6J}/+, A^w/A^w (or A^w/a) mice have a striped coat, whereas +/+ and +/Y, A^w/A^w (or A^w/a) mice have a non-striped coat. Thus, in the cross involving F1 *Dax1*-/+ females mated to Ta^{6J}/Y, A^w/A^w males, XY (i.e. *Dax1*-/Y) females can be distinguished from XX (i.e. Ta^{6J}/+) females because they lack a striped coat. (Males of the C57BL/6J-A^W $Eda^{Ta\text{-}6J}$ strain were provided by the Mouse Mutant Resource program of The Jackson Laboratory.)

Sry transgene rescue

To determine if increased *Sry* expression rescued gonadal sex reversal in B6 *Dax1*-/Y mice, two approaches were used. The first used B6 $XY^{AKR,Sxr}$ mice (Albrecht et al., 2003). The $Y^{AKR,Sxr}$ chromosome contains two copies of *Sry*, an endogenous (AKR/J strain-derived) copy located on the short arm and a second (RIII strain-derived) copy (i.e. Sxr) located in a duplicated segment of the short arm that is transposed distal to the pseudoautosomal region of the long arm of the Y chromosome. The second approach used B6 XY^{AKR} mice carrying a multicopy transgene, designated TgN(Sry-129)2Ei (Tg2) (Washburn et al., 2001), containing the 129-derived *Sry* gene (Koopman, 2001). Our unpublished data indicate that ~84 copies of *Sry* are present in Tg2 carriers and that the relative expression level of *Sry* in the testes of XY^{AKR} Tg2 fetuses is ~16-fold higher than in the testes of E12 XY^{AKR} fetuses. Of the possible genotypes obtained in these crosses, three were of interest: *Dax1*-/Y^{AKR}, *Dax1*-/$Y^{AKR,Sxr}$ and *Dax1*-/Y^{AKR} Tg2.

Genotyping

Genotyping was accomplished using lysate obtained by incubation of a small piece of tissue overnight in lysis buffer [0.05 M KCl, 0.05 M Tris at (pH 8.3), 0.1 mg/ml gelatin, 0.45% Nonident P-40, 0.45% Tween and 60 μg/ml Proteinase K] at 55°C. Primer sequences, PCR amplification cycles and generated amplicon sizes are available (see Table S1 in the supplementary material).

Staging of fetal gonads

Timed matings were performed to provide a rough estimate of fetal age. Because gonad development progresses rapidly in mice and the developmental stage of individual fetuses within a litter may differ, a more accurate assessment of fetal age was employed. Fetuses younger than embryonic day (E) 13 were staged by counting tail somites (ts) distal to the hindlimbs (e.g. ~28 ts corresponds to E12.5) (Hacker et al., 1995). Fetuses E13 and older were staged according to fore- and hindlimb morphology (Theiler, 1989).

Whole-mount immunohistochemistry

Whole-mount immunohistochemical analysis was performed as described previously (Albrecht and Eicher, 2001). Briefly, E12.5 and E13.5 gonad-mesonephros complexes were fixed overnight at 4°C in 4% paraformaldehyde, followed by a 24 hour incubation in blocking buffer (1% BSA, 0.1% saponin, 0.02% sodium azide in PBS) at 4°C. Samples were incubated with appropriate primary and secondary antibodies diluted in blocking buffer for 24 hours for each antibody. Primary antibodies included GATA4 (C-20, goat polyclonal, Santa Cruz Biotechnology; 1:500), PECAM1 (rat monoclonal, BD PharMingen; 1:100), WT1 (mouse monoclonal, DakoCytomation; 1:300), AMH (rabbit polyclonal, gift from Dr Natalie Josso (Rey et al., 1996); 1:250), SF1 (rabbit polyclonal, gift from Dr Ken-ichirou Morohashi (Morohashi et al., 1993); 1:1000) and SOX9 [rabbit polyclonal, gift from Dr Francis Poulat (Gasca et al., 2002); 1:1000]. Cy3-, Cy5-conjugated (Jackson ImmunoResearch; 1:500) or Alexa Fluor-488-conjugated (Molecular Probes; 1:750) secondary antibodies were used for visualization.

Fluorescently labeled samples were mounted in Slowfade-Light Antifade (Molecular Probes). Images were obtained using a Leica TCS-NT laser-scanning confocal microscope, and assembled using ImageJ software version 1.32v (http://rsb.nih.gov/ij/) and Adobe Photoshop v7.

Real-time RT-PCR

Gonads from fetuses ranging in age from E10 to E14 were used for real time RT-PCR analysis. At E10 and E10.5, tissue containing urogenital ridges was used. At E11.5 and E12, isolated gonad-mesonephros complexes were used. At E12.5, E13 and E14, gonads were carefully dissected free of the mesonephros and used. To prevent RNA degradation, tissues were collected using dissection instruments

cleaned with RnaseZap wipes (Ambion, Austin, TX). Tissues were homogenized in lysis buffer containing β-mercaptoethanol (Qiagen RNAeasy kit, Qiagen, Valencia, CA) and stored at –80°C until further use.

At each developmental time point analyzed, a minimum of three cDNA samples (each sample contained tissue from a single fetus) was analyzed for each genotype. RNA isolation and multigene real-time RT-PCR analysis was performed as previously described (Bouma et al., 2004).

Changes in relative gene expression between cDNA samples were determined using version 2 of the statistical algorithm 'Global Pattern Recognition' (GPR v2.0) (Akilesh et al., 2003). GPR v2.0 assigns a GPR score to each gene, indicating the fraction of normalizer genes to which the gene is found to be significantly different (P<0.05). Genes with a GPR score of at least 0.4 were considered expressed at significantly different levels between cDNA samples. In addition, GPR v2.0 uses a modified version of the recently published geometric averaging algorithm (geNorm) to calculate fold changes in relative gene expression (Vandesompele et al., 2002). Fold changes were determined based on the geometric mean of the 10 best normalizers (most stable) (Vandesompele et al., 2002), instead of calculating fold changes based on a single normalizer.

Modifier gene mapping

To map the modifier genes involved in B6-DAX1 XY sex reversal, B6 *Dax1*-/+ females were mated to D2 males and the resulting (B6×D2)F1 *Dax1*-/+ female offspring were backcrossed to B6 *Dax1*+/Y males. Fetal gonads were analyzed between E14.5 and E16 (Theiler, 1989), a developmental time period when ovarian and testicular tissue is easily distinguishable within single gonads (see Eicher et al., 1980; Eicher et al., 1996). Gross morphological inspection was accomplished by viewing and photographing each gonad using an inverted light microscope. Each gonad was classified as an ovary, a testis or an ovotestis (a gonad that contains both ovarian and testicular tissue). In addition, a small sample of somatic tissue was used to determine the *Dax1* genotype and presence of the Y chromosome. The remainder of each fetus was frozen for later DNA isolation. A total of 123 backcross fetuses were genotyped as *Dax1*-/Y and constitute the mice used for linkage analysis.

A genome scan was performed using a set of single nucleotide polymorphic (SNP) markers that differ between B6 and D2 and are located at ~10-20 Mb intervals along the length of each autosome (Petkov et al., 2004). SNP typing was performed by Kbiosciences (Hertfordshire, UK). (The list of SNPs used is available in Table S2 in the supplementary material.) Linkage analyses were carried out using the pseudomarker software package version 1.06 (Sen and Churchill, 2001) (http://www.jax.org/staff/churchill/labsite/software). Two versions of the data were analyzed. The first consisted of the 123 *Dax1*-/Y fetuses backcross mice, scored as 0, 0.5 and 1 for male, hermaphrodite and female, respectively. The classification of hermaphrodite included mice containing an ovary accompanied by an ovotestis, a testis accompanied by an ovotestis, or a pair of ovotestes. The second linkage analysis involved only the 19 male and 17 female fetuses, scored as a binary trait.

A three-stage analysis strategy (Sugiyama et al., 2001) was used to identify the genetic loci underlying sex reversal in B6 *Dax1*-/Y mice. We first employed interval mapping (Lander and Botstein, 1989) to identify QTLs with main effects and then carried out an exhaustive pair-wise search (Sen and Churchill, 2001) to identify epistatic interactions among loci. Permutation analysis (Churchill and Doerge, 1994) was used to establish significance thresholds and loci that exceed the 95% genome-wide adjusted level are reported. The third stage of analysis constructed a multi-locus regression model that includes simultaneous effects of all significant loci. We initialized the model construction by selecting all significant (P<0.05) and suggestive (P<0.63) QTLs and interactions, and then removed terms using a backward elimination strategy until each model term was significant at the P<0.001 level. The multi-locus regression model is available in Table S3 in the supplementary material.

Further confirmation for involvement of a distal Chr 4 locus in B6-DAX1 sex reversal was obtained by analyzing 32 additional *Dax1*-/Y females, produced by mating (B6×D2)F1 *Dax1*-/+ females to B6 or B6 Ta^{6J}/Y, A^{W}/A^{W} males. All offspring obtained from B6 males were analyzed for the *Dax1* alleles and the Y chromosome. In the case of offspring obtained from Ta^{6J}/Y males, only females lacking *Ta*-striping (i.e. that did not inherit the X chromosome from their father) were typed for *Dax1* and the Y chromosome. For both crosses, a *Dax1*-/Y mouse was classified as a female if both gonads had the shape and position (just below the kidneys) of normal ovaries, and the internal and external genitalia were consistent with normal female anatomy. In the cross involving normal B6 males, 10.5% of the offspring were *Dax1*-/Y females. In the cross involving B6 Ta^{6J}/Y, A^{W}/A^{W} males, 10.6% of the *Dax1*-/Y offspring were females. These results are similar to the 13.8% *Dax1*-/Y fetal females obtained in the initial mapping cross.

Results

Gonad development in *Dax1*-/Y mice

To determine if testis development was normal in B6 mice carrying the *Dax1*- mutation, we mated normal B6 males to B6 females carrying a $Dax1^{flox}$ allele (i.e. $Dax1^{flox}$ /+) and a transgene expressing Cre recombinase from the Zp3 promoter (Tg93). None of the offspring inherited the $Dax1^{flox}$ allele, whereas half inherited the *Dax1*- allele, indicating that Tg93 successfully deleted *Dax1* sequences between the *lox*P sites.

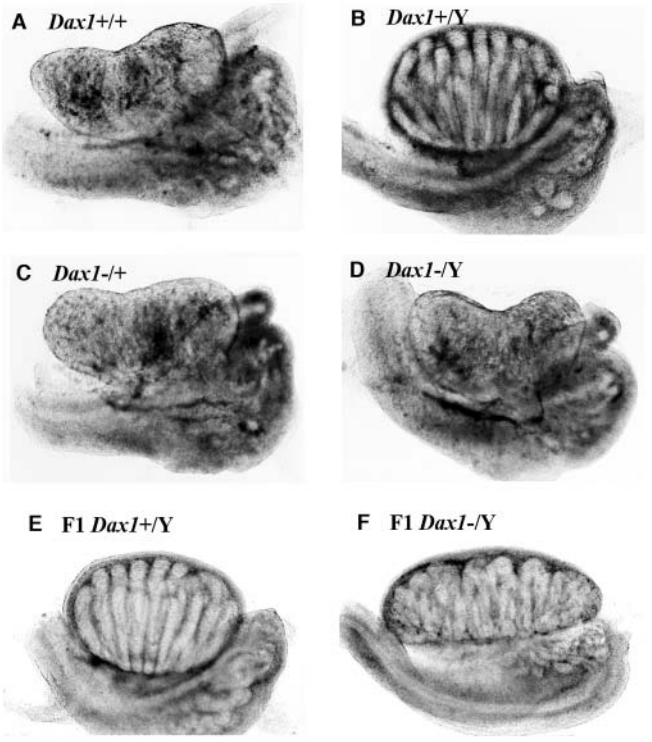

Fig. 1. Gross morphology of E14.5 gonad-mesonephros complexes. (A) B6 normal ovary. (B) B6 normal testis. (C) B6 *Dax1*-/+ ovary. (D) B6 *Dax1*-/Y ovary. (E) F1 *Dax1*+/Y testis. (F) F1 *Dax1*-/Y testis with abnormal cord development. In each image, the gonad is at the top and the mesonephros is at the bottom.

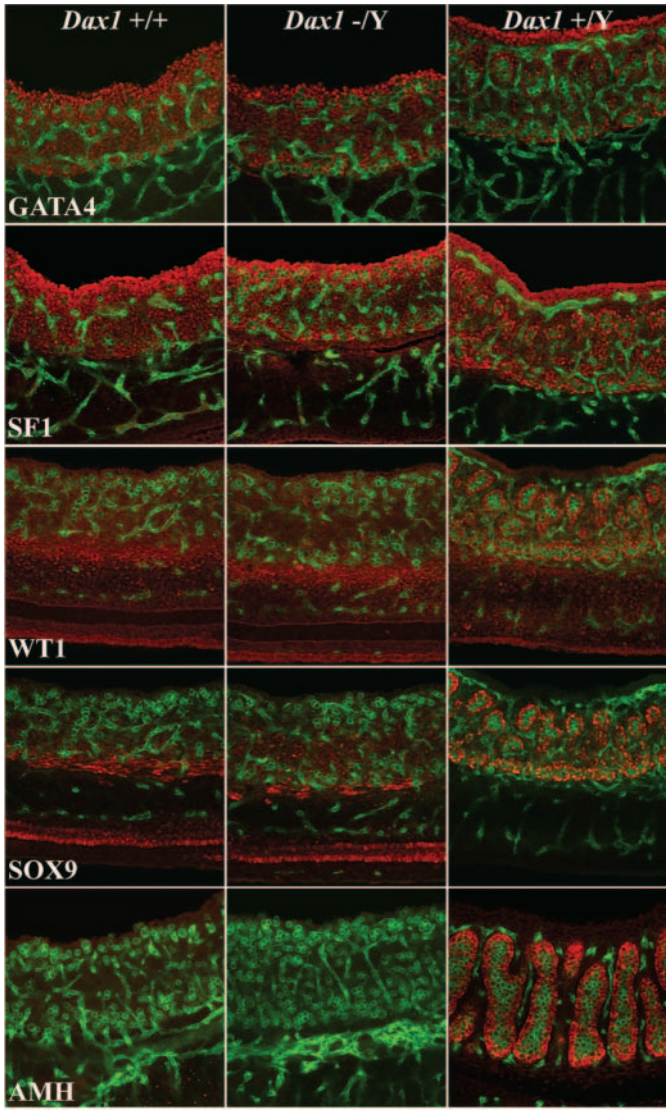

Fig. 2. Confocal and whole-mount immunohistochemical analyses of marker gene expression and gonad morphology in B6 E12.5 and E13.5 gonad-mesonephros complexes. In each case, both marker gene expression and gonad morphology of *Dax1*-/Y ovaries are indistinguishable from normal *Dax1*+/+ ovaries. The top four rows illustrate the expression of GATA4, SF1, WT1 and SOX9 in E12.5 gonads (red staining). The bottom row illustrates AMH expression in E13.5 gonads (red staining). PECAM expression on the surface of germ cells and vascular endothelial cells is shown in each image (green staining). The left, middle and right columns are B6 *Dax1*+/+ ovaries, B6 *Dax1*-/Y ovaries and B6 *Dax1*+/Y testes, respectively. In each image the gonad is at the top and the mesonephros at the bottom.

Our finding that all of the *Dax1*-/Y offspring presented as females was unexpected. Analysis of gonad development in the B6 *Dax1*-/Y fetal offspring revealed the presence of two ovaries (Fig. 1). We conclude that *Dax1*- causes sex reversal in B6 XY mice and refer to this as B6-DAX1 XY sex reversal.

To determine if *Dax1*- affected testis development in D2 mice, we transferred the *Dax1*- allele to the D2 strain background. In contrast to the findings in B6 *Dax1*-/Y mice, D2 *Dax1*-/Y mice developed as fertile males. Finally, we

Table 1. Examples of genes with significant changes in relative gene expression ($P<0.05$) between B6 *Dax1*+/Y, *Dax1*+/+ and *Dax1*-/Y fetal gonads

	Dax1+/Y versus *Dax1*+/+			*Dax1*+/Y versus *Dax1*-/Y		
Gene*	E12	E13	E14	E12	E13	E14
Genes involved in sex determination and differentiation						
Amh‡		20.23	150.88		19.56	74.72
Dhh‡	133.89	16.41	15.53	26.01	15.71	15.49
Sox9	3.45	5.15	2.97	2.43	5.73	3.24
Sry	229.37					–64.95†
Genes involved in steroidogenesis						
Cyp11a1‡		51.98	12.62		21.54	365.21
Cyp17a1‡		60.99	430.74		66.48	552.29
Cyp26b1‡		1.54	2.62			2.70
Hsd3b1		11.58	5.26		3.85	3.51
StAR			9.38		7.33	12.50
Genes preferentially expressed in testes						
Aard		28.52	5.11		8.63	3.02
Cbln1‡		22.65	11.83		20.59	44.23
Cbln4		2.68	1.78		4.38	3.21
Cst9‡		56.23	52.06		48.40	62.03
Dtna		9.05	5.35		8.64	7.30
Gpr73l1		22.60	11.47		21.98	19.14
Hhip		15.04	9.45		41.66	18.68
Ptgds		48.54	30.46		36.01	9.60
Ren1		11.36	89.25		10.97	31.34
Scarb1		1.69	5.02		1.83	5.27
Serpine2		3.53	2.53		5.63	3.47
Sostdc1		12.84	16.31		18.72	17.45
Tdl		40.94	22.45		12.68	9.55
Genes preferentially expressed in ovaries†						
Adamts19		–74.22	–9.10		–41.70	–17.03
Bmp2	–4.50	–9.97	–15.06	–3.09	–11.01	–15.91
Emx2		–6.72	–9.01		–4.35	–8.56
Fgfr2		–5.35	–7.21		–5.82	–8.26
Fst	–12.87	–504.18	–657.73	–9.47	–288.68	–560.68

*Gene names and PCR primer sequences used are given in Bouma et al. (Bouma et al., 2004).

†Negative value indicates the fold change direction when comparing *Dax1*+/Y with *Dax1*+/+ and *Dax1*-/Y fetal gonads.

‡Transcript levels are below the detection limit of the assay in B6 *Dax1*+/+ and B6 *Dax1*-/Y gonads.

Values are fold changes based on a minimum of 10 normalizers.

determined if F1 *Dax1*-/Y mice developed normal testes. We mated B6 *Dax1*-/+ females to normal D2 males and analyzed the gonads in 17 *Dax1*-/Y F1 fetuses. All of these F1 fetuses contained two testes, indicating that presence of D2-derived genes promotes testis development in *Dax1*-Y mice. Although testes were present, abnormal cord development was observed in 11 of the 34 gonads, indicating that testis development is compromised in F1 *Dax1*-/Y mice as early as E14.5 (Fig. 1). This finding is consistent with the findings reported by Meeks et al. that testicular cord development is abnormal in *Dax1*-/Y mice (Meeks et al., 2003a).

Meeks and co-workers had reported that the combined presence of a *Dax1*- allele and a 'weaker' *Sry* allele causes sex reversal in XY^{POS} mice (Meeks et al., 2003b). Because genetic background influences whether sex reversal occurs in XY^{POS} mice (Eicher et al., 1995; Eicher et al., 1996), we tested if presence of D2-derived genes would influence testis development in *Dax1*-/Y^{POS} mice. We mated B6 *Dax1*-/+ females to D2 XY^{POS} males and analyzed the offspring at weaning: the 26 *Dax1*-/Y^{POS} offspring presented as females,

whereas the 23 *Dax1*+/Y^{POS} offspring presented as males. We also backcrossed F1 *Dax1*-/+ females to D2 XY^{POS} males and analyzed the offspring at weaning: the 17 *Dax1*-/Y^{POS} offspring presented as females whereas the 15 *Dax1*+/Y^{POS} offspring presented as males. We conclude that the combined presence of the *Sry*^{POS} allele and the *Dax1*- allele cause sex reversal in XY mice.

Protein expression and gonad morphology in B6 *Dax1*-/Y fetal gonads

Whole-mount immunohistochemistry and confocal microscopy were used to examine the expression of protein markers and gonad morphology in E12.5 and E13.5 B6 *Dax1*-/Y gonads. Three classes of proteins identifying different cell types were used: (1) NR5A1 (SF1), WT1 and GATA4, which are present in gonadal somatic cells in both ovaries and testes, but increased in Sertoli cells during testis cord development; (2) SOX9 and AMH, which are present exclusively in testicular Sertoli cells; and (3) PECAM1, which is present in both ovarian and testicular germ and vascular endothelial cells (Armstrong et al., 1993; Ikeda et al., 1994; Ketola et al., 2002; Martineau et al., 1997; Morais da Silva et al., 1996; Pelletier et al., 1991; Rey et al., 2003; Stallings et al., 2002; Viger et al., 1998). As shown in Fig. 2, the localization pattern of these proteins in B6 *Dax1*-/Y fetal gonads was indistinguishable from the localization pattern observed in B6 *Dax1*+/+ fetal ovaries. Furthermore, the morphology of B6 *Dax1*-/Y fetal gonads was indistinguishable from B6 *Dax1*+/+ fetal ovaries. These data indicate that B6 *Dax1*-/Y fetal gonads fail to differentiate as testes, but rather initiate development as ovaries.

Multi-gene expression profiling

Real-time RT-PCR analysis was performed to examine the gene expression profile of B6 *Dax1*-/Y fetal gonads compared with normal B6 XX and XY gonads between E10 and E14 (see Materials and methods). With the exception of *Sry*, gene expression in gonads of fetal B6 *Dax1*-/Y mice followed the ovarian developmental pattern (Table 1). For example, five genes (*Adamts19*, *Bmp2*, *Emx2*, *Fgfr2* and *Fst*), normally expressed at higher levels in B6 XX gonads compared to XY gonads (Bouma et al., 2004; Menke and Page, 2002), were expressed in B6 *Dax1*-/Y gonads at levels comparable with those in B6 XX gonads. Alternatively, 12 genes (*Aard*, *Amh*, *Cbln1*, *Cbln4*, *Cst9*, *Cyp11a1*, *Cyp17a1*, *Cyp26b1*, *Dhh*, *Hhip*, *Sox9* and *Tdl*), normally expressed at higher levels in B6 XY compared with XX gonads (Bouma et al., 2004; Menke and Page, 2002), were expressed at levels comparable with those in B6 XX gonads. *Sry* expression levels were similar in B6 *Dax1*+/Y and *Dax1*-/Y gonads until E12.5, after which *Sry* expression was downregulated in *Dax1*+/Y testes but not in *Dax1*-/Y ovaries (Fig. 3). Whereas *Sry* was below the detection limit of our assay in E14 *Dax1*+/Y testes, *Sry* expression in E14 *Dax1*-/Y ovaries remained unchanged relative to the expression level measured in E12.5 and E13 *Dax1*-/Y ovaries. *Sry* transcripts were not detected in the ovaries of B6 *Dax1*-/Y newborn mice (data not shown), indicating that *Sry* expression was extinguished between E14 and birth. We conclude that B6 mice require *Dax1* for *Sry* to initiate testicular development.

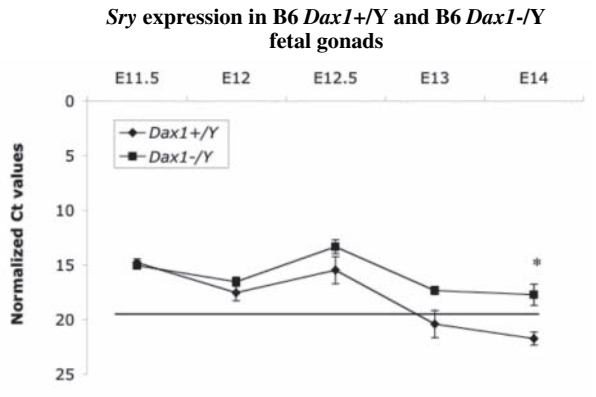

Fig. 3. Relative *Sry* expression in B6 *Dax1*-/Y and *Dax1*+/Y gonads as determined by real time RT-PCR analysis. *y*-axis indicates 18s RNA normalized Ct values. Black line illustrates the normalized threshold detection limit of the real time RT-PCR assay (Ct=37.5 – mean Ct 18s RNA). Values below this line indicate the gene is not expressed. An asterisk indicates a significant difference between B6 *Dax1*+/Y and B6 *Dax1*-/Y fetal gonads at $P \leq 0.05$.

Sry transgene rescue of B6-DAX1 sex reversal

Although the testis-determining pathway was not activated in B6 *Dax1*-/Y gonads, despite normal *Sry* expression, the possibility remained that increased *Sry* expression prevents B6-DAX1 sex reversal. We used two approaches to investigate this. The first involved mating B6 *Dax1*-/+ females to B6 males carrying a specially constructed AKR/J Y chromosome (Y^{AKR,Sxr}) that contains two copies of *Sry* (Albrecht and Eicher, 1997). Four *Dax1*-/Y^{AKR} and five *Dax1*-/Y^{AKR,Sxr} offspring were examined at weaning and all were female. The finding that the *Dax1*-/Y^{AKR,Sxr} mice develop as females indicates that presence of two copies of *Sry* does not prevent ovarian development in B6 *Dax1*-/Y mice.

The second approach used an *Sry* transgene (Tg2) (Washburn et al., 2001) derived from the 129 *Sry* gene (Koopman et al., 1991). Tg2 contains all the necessary cis-acting sequences required for testicular tissue development because all XX Tg2 mice develop exclusively testicular tissue. Tg2 contains 84 copies of *Sry* and results in a 16-fold increase in *Sry* expression in the fetal gonads of E12 B6 XY^{AKR} Tg2 mice (data not shown). We mated B6 *Dax1*-/+ females to B6 XY^{AKR} Tg2 males and analyzed the offspring at weaning. The 12 *Dax1*-/Y^{AKR} offspring lacking Tg2 developed as females, which agreed with the previous finding that the AKR Y chromosome does not correct B6-DAX1 sex reversal. By contrast, the 20 B6 *Dax1*-/Y^{AKR} Tg2 offspring presented as males, and the subset of males tested were fertile. Real-time RT-PCR analysis indicated that *Sox9* is upregulated in E12 B6 *Dax1*-/Y^{AKR} Tg2 and *Dax1*+/+ Tg2 fetal gonads, and the relative expression levels in both are similar to expression levels found in B6 *Dax1*+/Y fetal gonads (data not shown).

We also examined GATA4 and PECAM1 expression in gonads of B6 *Dax1*-/Y^{AKR} Tg2 mice at E13.5 using whole-mount immunohistochemistry and confocal microscopy (Fig. 4). Gonad morphology in B6 *Dax1*-/Y^{AKR} Tg2 testes was indistinguishable from control B6 *Dax1*+/Y^{AKR} testes. We conclude that testis development in B6 *Dax1*-/Y^{AKR} mice is rescued if multiple copies of *Sry* are present.

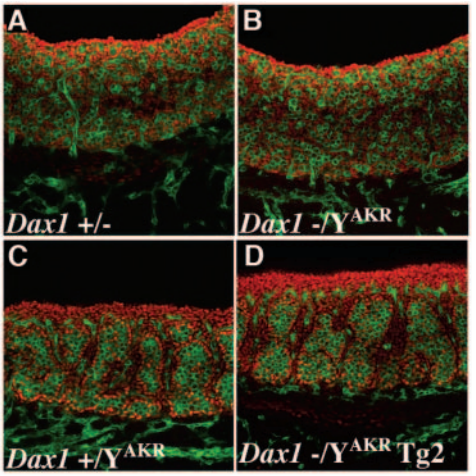

Fig. 4. Confocal and whole-mount immunohistochemical analyses of GATA4 (red) and PECAM1 (green) expression in B6 E13.5 gonad-mesonephros complexes: *Dax1*-/+ (A), *Dax1*-/Y^{AKR} (B), *Dax1*+/Y^{AKR} (C) and *Dax1*-/Y^{AKR} Tg2 (D). *Dax1*+/Y^{AKR} and *Dax1*-/Y^{AKR} Tg2 gonads have normal testis morphology and upregulated GATA4 expression in Sertoli cells compared with *Dax1*-/+ and *Dax1*-/Y^{AKR} gonads. PECAM expression is visible on the surface of germ cells and vascular endothelial cells. In each image, the gonad is at the top and the mesonephros at the bottom.

Identification of modifier loci involved in B6-DAX1 XY sex reversal

To map the chromosomal location of B6-derived genes involved in B6-DAX1 XY sex reversal, (B6×D2)F1 *Dax1*-/+ females were mated to B6 males, and the gonadal phenotype of 123 *Dax1*-/Y fetuses was assessed at E14.5 to E16: 19 (13.8%) contained exclusively testicular tissue, 17 (15.5%) contained exclusively ovarian tissue and 87 (70.7%) contained both ovarian and testicular tissue (ovotestis), with 22 containing an ovary and an ovotestis, 52 containing two ovotestes and 13 containing a testis and ovotestis. A genome scan involving 160 SNP (single nucleotide polymorphic) loci detected two chromosomes with peak LOD scores that approached the 0.05 genome-wide threshold (threshold LOD=3.15) (Fig. 5A; see Table S2 in the supplementary material). Chromosome (Chr) 4 contained a single peak (LOD=3.06) distal to marker rs3718220. The Chr 1 LOD profile was bimodal with highest peak (LOD=2.61) proximal to maker rs3697376 and a second peak (LOD=2.19) distal to marker rs3664528. Suggestive peaks also were noted on Chrs 5, 6, 16, and 17.

A second genome scan (Fig. 5B) was carried out using only the subset of 17 female (two ovaries) and 19 male (two testes) *Dax1*-/Y backcross fetuses. The genome wide 0.05 threshold for this scan was LOD=2.97. The Chr 4 peak (LOD=3.66) exceeded the significance threshold. The Chr 1 LOD profile again was bimodal with peaks (LOD=2.91 and LOD=2.67) in the same locations.

To confirm the Chr 4 location of a major B6-derived modifier, we obtained 32 additional *Dax1*-/Y backcross adult females (see Materials and methods). A genome scan again identified a Chr 4 locus located between markers rs4224709 and rs371820. A summary of data for these 32 females and the original 17 *Dax1*-/Y fetuses is presented in Table 2.

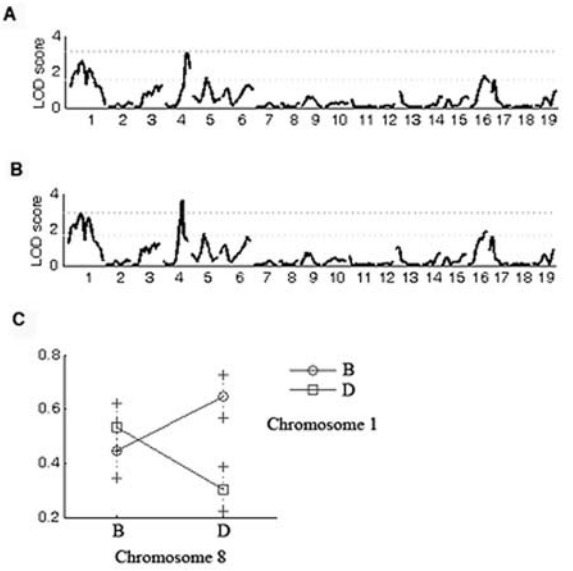

Fig. 5. Genome scan of 123 backcross *Dax1*-/Y fetuses scored as 0 for male, 1 for hermaphrodite and 2 for female (A). Genome scan including only 17 male and 19 female backcross *Dax1*-/Y fetuses (B). (A,B) The *y*-axis provides the LOD score and the *x*-axis indicates the genomic location in genetic map units (cM) with chromosome numbers indicated. An allele interaction plot for Chrs 1 and 8 (C). The *y*-axis is scaled such that 0 represents male, 0.5 represents hermaphrodite and 1 represents female. The effect of Chr 8 on the tendency to produce females differs depending on the allelic stage of Chr 1, and Chr 1 has an effect on the sex ratio only when the Chr 8 locus is heterozygous. The lines in the plot indicate homozygosity B (B; open circles) and heterozygosity B/D (D; squares) for the Chr 1 locus.

A genome-wide analysis of locus pairs (Sen and Churchill, 2001) revealed a significant pair-wise interaction between loci on Chr 1 and Chr 8 (Fig. 5C, see Table S3 in the supplementary material), and the likelihood of ovarian development in *Dax1*-/Y backcross fetuses. The effect of the locus on Chr 8 on the tendency to produce females occurs only when it is heterozygous (B6/D2) and is dependent on the allelic state of a locus on Chr 1. For example, there is a greater probability that *Dax1*-/Y backcross fetuses will develop ovarian tissue if the Chr 8 locus is heterozygous (B6/D2) and the Chr 1 locus is homozygous B6 (Fig. 5C).

We conclude that a gene located on distal Chr 4 is involved in B6-DAX1 sex reversal. The data also indicate that additional modifier genes are involved, including two genes on Chr 1 and an interaction effect between a gene on Chr 1 and a gene on Chr 8.

Discussion

Studies from our laboratory indicate that the B6 genetic background is sensitive to changes affecting genes involved in early testicular development, and thus B6 mice are a useful model with which to examine the function of genes during fetal gonad development and to identify novel genes involved in gonadal sex determination and differentiation (Eicher et al., 1996; Eicher et al., 1982). For example, transferring the *Mus domesticus poschiavinus* Y^{POS} chromosome to B6 causes

Table 2. Inheritance of four distal Chr 4 markers in *Dax1*-/Y backcross females

	Females		RS number			
	F	W	4224709	3708285	3718220	3680364
	1	1	B	D	D	D
	2	1	B	B	D	D
	0	3	B	B	B	D
	10	21	B	B	B	B
	1	2	D	B	B	B
	1	1	D	D	B	B
	0	0	D	D	D	B
	2	3	D	D	D	D
Total	17	32				
Females		B	39	41	39	36
		D	10	8	10	13

Numbers indicate fetal (F) and weanED (W) *Dax1*-/Y females.
B, B/B and D, B/D. Four distal Chr 4 SNP markers are listed.

ovarian development in XY individuals. B6-Y^{POS} sex reversal is hypothesized to be caused, at least in part, by the inability of B6 autosomal genes to regulate Sry^{POS} transcription correctly (Albrecht et al., 2003).

Dax1 is required for fetal testis development in B6 mice

Data presented here indicate that a mutant *Dax1* gene causes ovarian development in B6 XY mice. By contrast, presence of the same mutant *Dax1* gene causes no major impact on testicular development in (B6×D2)F1 XY mice, given that these males are fertile. Together, these findings demonstrate that *Dax1* is crucial for B6 fetal testis differentiation and suggest that B6-derived autosomal genes play a role in B6-DAX1 sex reversal.

Previously, Meeks and collaborators reported that the mutant *Dax1*- allele in combination with the 'weaker' Sry^{POS} allele causes XY sex reversal (Meeks et al., 2003b). In their study, however, it was unclear if the observed sex reversal in *Dax1*-/Y^{POS} mice was influenced by the genetic background of the mice used or the combined presence of *Dax1*- and Sry^{POS}. To test if the genetic background was important, both *Dax1*- and Sry^{POS} were tested in B6 (B6×D2)F1 and backcross *Dax1*-/Y^{POS} mice (produced by mating *Dax1*-/+ F1 females to D2-Y^{POS} males). In all three cases, the *Dax1*-/Y^{POS} mice were female. We conclude that Sry^{POS} is unable to initiate testicular development in *Dax1*-/Y^{POS} mice even when D2 autosomal genes are present.

Identification of an important modifier locus

Genetic mapping revealed that a locus on distal Chr 4 and two loci on Chr 1 are involved in B6-DAX1 sex reversal. In addition, an interactive effect was observed between Chr 1 and Chr 8. The finding of a QTL (quantitative trait locus) on distal Chr 4 is significant. We previously mapped an autosomal testis-determining locus (*Tda1*) to distal Chr 4 in studies involving B6-Y^{POS} sex reversal (Eicher et al., 1996) and the results involving B6-DAX1 sex reversal suggest that both inherited sex reversals involve the same Chr 4 locus. Clearly, one or more genes located within this Chr 4 QTL play a major role in sex determination and differentiation.

The nature of the interaction between Chr 1 and Chr 8 on the sex reversal phenotype is a classical 'masking' epistasis in which the effect of Chr 1 is only observed when the Chr 8 locus carries a D2 allele. This observation may help in the further dissection of this complex trait by narrowing the field of candidate genes to those with products that can manifest such interactions.

Interestingly, the *Wnt4* gene resides in a region of Chr4 that overlaps with *Tda1*, thus *Tda1* maybe *Wnt4*. In addition, recent experiments show that *Wnt4*, which initially was demonstrated to play an important role in ovary development, is also needed for testis development (Vainio et al., 1999; Jeays-Ward et al., 2003; Jeays-Ward et al., 2004; Yao et al., 2004). Moreover a number of studies have implicated *Wnt4*, acting through β-catenin and SF1, in the regulation of *Dax1* expression (Jordan et al., 2001; Jordan et al., 2003; Mizusaki et al., 2003). The phenotypic differences observed between $Wnt4^{-/-}$ XY and B6 *Dax1*-/Y mice (abnormal testes and ovaries, respectively), however, suggest that factors, such as genetic background ($Wnt4^{-/-}$ mice contained a mixed 129Sv, CBA, B6 genetic background) or primary cell type affected [*Wnt4* is involved in steroidogenic and endothelial cell migration from the mesonephros (Jeays-Ward et al., 2003)], are responsible for these phenotypic differences. Future experiments using B6-*Wnt4*- mice will address these issues.

B6-DAX1 sex reversal is due to lack of *Sox9* up-regulation

Whole-mount immunohistochemical and real time RT-PCR analyses revealed that B6 *Dax1*-/Y fetal gonads initiate development as ovaries rather than testes. Moreover, these data suggest that the testis determining pathway is interrupted at a very early stage because expression of testis-specific genes, other than *Sry*, was not observed. Significantly, *Sry* expression was initiated at the correct time and at normal levels in *Dax1*-/Y fetal gonads. Expression of *Sox9* is necessary for Sertoli cell differentiation and accumulating evidence suggests that *Sox9* is the immediate downstream target of SRY (reviewed by Canning and Lovell-Badge, 2002; Lovell-Badge et al., 2002). For example, expression of *Sox9* driven by *Wt1* regulatory sequences initiates testicular differentiation in XX mice (Vidal et al., 2001), and SOX9 and *Sry* are co-expressed in pre-Sertoli cells with SOX9 expression initiating slightly after *Sry* expression (Sekido et al., 2004). Taken together, these data suggest that *Dax1* participates either in parallel with, or immediately downstream of, *Sry* to activate *Sox9* expression and the male sex-determination genetic cascade. Absence of *Sox9* upregulation in B6 *Dax1*-/Y gonads allows the supporting cell precursors to differentiate as granulosa cells.

Normally, *Sry* expression is downregulated after E12.5 in XY gonads (Hacker et al., 1995; Jeske et al., 1995), including B6 XY gonads (Bouma et al., 2004). However, *Sry* transcript levels were not downregulated in E13 and E14 B6 *Dax1*-/Y gonads. Recent results suggest that the upregulation of *Sox9* in pre-Sertoli cells is responsible for downregulation and eventual silencing of *Sry* (Chaboissier et al., 2004; Morais da Silva et al., 1996). Because *Sox9* expression is not upregulated in *Dax1*-/Y gonads, the finding that *Sry* is not immediately downregulated in these gonads is consistent with this idea. It is not clear how *Sry* expression is eventually extinguished in *Dax1*-/Y gonads. Perhaps in the absence of *Sox9*, *Sry* expression is downregulated by itself, given that Sox genes

appear to recognize similar DNA-binding sites (Bergstrom et al., 2000; Bowles et al., 2000).

B6 *Dax1*-/Y ovarian development is prevented by the addition of multiple copies of the Sry^{129} allele. [The open reading frame of the Sry^{129} allele is identical to that of Sry^{B6} (Albrecht and Eicher, 1997).] Although the exact mechanism behind this sex reversal prevention is unclear, multiple copies of *Sry* are sufficient to upregulate *Sox9* expression in B6 fetal gonads even in the absence of *Dax1*. We envision that *Sry* overexpression bypasses DAX1 deficiency in B6 XY mice, a possibility stemming from evidence that DAX1 can act post-transcriptionally by directly binding to mRNA and transporting it from the nucleus (Lalli et al., 2000). In the absence of DAX1, *Sry* transcripts, although present at normal levels in B6 mice, may not be exported from the nucleus and translated into SRY protein. Thus, by significantly increasing the expression of *Sry* in B6 *Dax1*-/Y mice, a sufficient number of *Sry* transcripts could escape the nucleus and be translated. Alternatively, increasing *Sry* transcript levels may lower the threshold needed for *Sox9* transcriptional activation.

Model for upregulation of *Sox9* in pre-Sertoli cells

We propose that the upregulation of *Sox9* in XY mice depends on the correct dose (i.e. expression level) of *Sry*, *Dax1* and one or more autosomal genes, including *Tda1* located on Chr 4 (Fig. 6). We suggest that testicular development in B6 mice is sensitive to perturbations in the expression levels of these genes, and that alterations in the expression of these genes interfere with the upregulation of *Sox9*, leading to failure of testicular development. The findings that complete gonadal sex reversal occurs in B6 *Dax1*-/Y (this study), *Dax1*-/Y^{POS} (Meeks et al., 2003b) (this study) and B6 XY^{POS} (Eicher et al., 1996) mice are compatible with this hypothesis, given that in each case two of the three genes (i.e. *Sry*, *Dax1* and *Tda1*) either malfunction and/or are expressed at suboptimal levels. For example, if the protein encoded by the B6-derived *Tda1* gene is less efficient at facilitating the upregulation of *Sox9*, even when expression levels of *Sry* are normal, homozygosity for the B6 *Tda1* allele together with the absence of DAX1 will fail to increase *Sox9* expression to the levels needed for Sertoli cell differentiation and the supporting cell precursors will initiate development as granulosa rather than Sertoli cells. Similarly, absence of DAX1 and presence of a 'weak' Sry^{POS} allele also will prevent the upregulation of *Sox9* in *Dax1*-/Y^{POS} mice, leading to granulosa rather than Sertoli cell differentiation. The importance of correct dose is further apparent from an investigation using transgenic mice carrying extra copies of *Dax1*: depending on the *Sry* allele present, increasing *Dax1* expression in XY mice caused delayed testicular cord development or partial development of ovarian tissue by antagonizing *Sry* action (Swain et al., 1998).

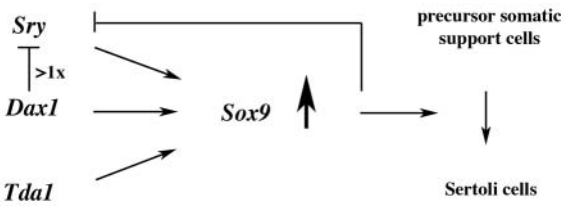

Fig. 6. Model for regulation of *Sox9* expression in XY precursor somatic support cells. We hypothesize that upregulation of *Sox9* is dependent on the correct expression levels of *Sry*, *Dax1* and a gene (or genes) on distal Chr 4 referred to as *Tda1* (testis-determining gene, autosomal 1). Perturbations in the expression level of any two of these three genes interferes with the upregulation of *Sox9*, and can result in the failure of the precursor somatic support cells to differentiate into Sertoli cells.

In conclusion, our findings provide evidence that *Dax1* is essential for normal B6 XY fetal testis development. Furthermore, the importance of B6 mice as a genetic model for identifying novel gonadal sex-determining genes is demonstrated by the identification of an important locus on distal Chr 4 (*Tda1*) involved in at least two mouse XY sex reversal models, B6 *Dax1*-/Y and B6 XY^{POS}. Finally, a model is presented in which correct doses of *Dax1*, *Sry* and *Tda1* are required for upregulation of *Sox9* in precursor somatic support cells, an event essential for fetal testis development and differentiation.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/13/3045/DC1

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