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 male mice were smaller than normal sized testes (reviewed by Ys1 et al., 1998). By contrast, homozygous Dax1-/-Y adult males had a more typical testis with impaired testicular germinal epithelial development and eventual germ cell loss (Yu et al., 1998). Testis development appeared to progress normally until E13.5, when some testis cords appeared disorganized and incomplete. The third study examined testicular development in fetal 129Sv/J Dax1-/-Y mice (Meeks et al., 2003a). Testis development appeared to progress normally until E14.5, 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 (F1) (Meeks et al., 2003b). Combining a ‘weaker’ Sry (sex-determining region of chromosome Y) allele on YPOS (Eicher et al., 1995) and Dax1- caused sex reversal in Dax1-/-YPOS mice.
Here, we report that Dax1 has an early, essential role in fetal testis development. Experiments involving mice from the two inbred strains C57BL/6J-Ei (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-Dax1flox and B6-Dax1-. To produce the B6-Dax1flox strain, the Dax1flox 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) express 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 on 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 Dax1flox allele to a B6 Tg93 male. Female offspring containing both Dax1flox and Tg93 were mated to normal B6 males and female offspring were identified that contained a Dax1- allele.

A D2-Ypos consomic strain was constructed by mating a B6 XYpos hermaphrodite to a D2 female. Successive backcrosses involving XYpos males mated to D2 females resulted in the D2-Ypos 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^T6-0.3) mutation at the X-linked ectodysplasin-A (Eda) gene was used to efficiently identify Dax1-/Y backcross mice at weaning in linkage analyses crosses. (Hereafter, Eda^T6-0.3 is designated Tb^6-0.3). Tb^6-0.3, 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 Tb^6-0.3/Y, A^W/A^W males, XY (i.e. Dax1+/Y) females can be distinguished from XX (i.e. Tb^6-0.3/+ ) females because they lack a striped coat. (Males of the C57BL/6J-A^W Eda^T6-0.3 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/AYKRstr mice (Albrecht et al., 2003). The Y^AYKRstr 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/YKR mice carrying a multicopy transgene, designated TgN(Sry-129)Ei (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/YKR Tg2 fetuses is ~16-fold higher than in the testes of E12 XY/YKR fetuses. Of the possible genotypes obtained in these crosses, three were of interest: Dax1-/Y^AYKR, Dax1-/Y^AYKRstr and Dax1-/Y^AYKR 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; 1:1000) 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<0.03) 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 *Ta6J*/Y, *A+*/A- males. All offspring obtained from B6 males were analyzed for the *Dax1* alleles and the Y chromosome. In the case of offspring obtained from *Ta6J*/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 *Ta6J*/Y, *A+*/A- mice, 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 loxP sites.

![Fig. 1. Gross morphology of E14.5 gonad-mesonephros complexes.](http://example.com/fig1.jpg)

(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.
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 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 XYPOS mice (Meeks et al., 2003b). Because genetic background influences whether sex reversal occurs in XYPOS mice (Eicher et al., 1995; Eicher et al., 1996), we tested if presence of D2-derived genes would influence testis development in Dax1-/-YPOS mice. We mated B6 Dax1-/-/+ females to D2 XYPOS males and analyzed the offspring at weaning: the 26 Dax1-/-YPOS offspring presented as females.

### 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

<table>
<thead>
<tr>
<th>Gene*</th>
<th>E12</th>
<th>E13</th>
<th>E14</th>
<th>E12</th>
<th>E13</th>
<th>E14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amh‡</td>
<td>20.23</td>
<td>150.88</td>
<td>19.56</td>
<td>74.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dhh‡</td>
<td>133.89</td>
<td>16.41</td>
<td>15.53</td>
<td>26.01</td>
<td>15.71</td>
<td>15.49</td>
</tr>
<tr>
<td>Sox9</td>
<td>3.45</td>
<td>5.15</td>
<td>2.97</td>
<td>2.43</td>
<td>5.73</td>
<td>3.24</td>
</tr>
<tr>
<td>Sry</td>
<td>229.37</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-64.95 †</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genes involved in sex determination and differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp11a1²</td>
</tr>
<tr>
<td>Cyp17a1²</td>
</tr>
<tr>
<td>Cyp26b1²</td>
</tr>
<tr>
<td>Hsd3b1</td>
</tr>
<tr>
<td>StAR</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Genes preferentially expressed in testes</th>
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<tbody>
<tr>
<td>Aard</td>
</tr>
<tr>
<td>Cbln1³</td>
</tr>
<tr>
<td>Cbln4³</td>
</tr>
<tr>
<td>Cst9²</td>
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<tr>
<td>Dna</td>
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<tr>
<td>Gpr731l</td>
</tr>
<tr>
<td>Hhip</td>
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<td>Igf2ds</td>
</tr>
<tr>
<td>Rtl1</td>
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<tr>
<td>Scrbel</td>
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<td>Serpine2</td>
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<tr>
<td>Sostdc1</td>
</tr>
<tr>
<td>Tdl</td>
</tr>
<tr>
<td>Tdrl</td>
</tr>
</tbody>
</table>

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

1Negative value indicates the fold change direction when comparing Dax1+/-Y with Dax1+/-+ and Dax1-/-Y fetal gonads.

2Transcript 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.

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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+/- Y 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+/-+ testes, respectively. In each image the gonad is at the top and the mesonephros at the bottom.

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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).

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whereas the 23 Dax1+/YPOS offspring presented as males. We also backcrossed F1 Dax1+/- females to D2 XYPOS males and analyzed the offspring at weaning: the 17 Dax1+/YPOS offspring presented as females whereas the 15 Dax1+/YPOS offspring presented as males. We conclude that the combined presence of the SryPOS 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 (Adams19, Bmp2, Emx2, Fgrf2 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, Csf9, 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+/+ and Dax1-/Y gonads until E12.5, after which Sry expression was downregulated in Dax1+/+ 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.

**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 (YAKR,Sxr) that contains two copies of Sry (Albrecht and Eicher, 1997). Four Dax1-/YAKR and five Dax1-/YAKR,Sxr offspring were examined at weaning and all were female. The finding that the Dax1-/YAKR,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 XYAKR Tg2 mice (data not shown). We mated B6 Dax1+/- females to B6 XYAKR Tg2 males and analyzed the offspring at weaning. The 12 Dax1-/YAKR 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-/YAKR 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-/YAKR 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-/YAKR Tg2 mice at E13.5 using whole-mount immunohistochemistry and confocal microscopy (Fig. 4). Gonad morphology in B6 Dax1-/YAKR Tg2 testes was indistinguishable from control B6 Dax1+/YAKR testes. We conclude that testis development in B6 Dax1-/YAKR mice is rescued if multiple copies of Sry are present.
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, (B6/11003D2)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.

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 YPOS chromosome to B6 causes...
ovarian development in XY individuals. B6-YPOS sex reversal is hypothesized to be caused, at least in part, by the inability of B6 autosomal genes to regulate Sox9POs 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’ Sox9POs allele causes XY sex reversal (Meeks et al., 2003b). In their study, however, it was unclear if the observed sex reversal in Dax1POs mice was influenced by the genetic background of the mice used or the combined presence of Dax1 and Sox9POs. To test if the genetic background was important, both Dax1POs and Sox9POs were tested in B6 (B6×D2)F1 and backcross Dax1POs mice (produced by mating Dax1+/F1 females to D2POs males). In all three cases, the Dax1POs mice were female. We conclude that SpyPOs is unable to initiate testicular development in Dax1POs 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-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 may be 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 Dax1POs mice (abnormal testes and ovaries, respectively), however, suggest that factors, such as genetic background (Wnt4POs 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-Wnt4POs 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 Dax1POs 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 Spy, was not observed. Significantly, Spy expression was initiated at the correct time and at normal levels in Dax1POs 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 Spy are co-expressed in pre-Sertoli cells with SOX9 expression initiating slightly after Spy expression (Sekido et al., 2004). Taken together, these data suggest that Dax1 participates either in parallel with, or immediately downstream of, Spy to activate Sox9 expression and the male sex-determination genetic cascade. Absence of Sox9 upregulation in B6 Dax1POs gonads allows the supporting cell precursors to differentiate as granulosa cells.

Normally, Spy 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, Spy transcript levels were not downregulated in E13 and E14 B6 Dax1POs gonads. Recent results suggest that the upregulation of Sox9 in pre-Sertoli cells is responsible for downregulation and eventual silencing of Spy (Chaboissier et al., 2004; Morais da Silva et al., 1996). Because Sox9 expression is not upregulated in Dax1POs gonads, the finding that Spy is not immediately downregulated in these gonads is consistent with this idea. It is not clear how Spy expression is eventually extinguished in Dax1POs gonads. Perhaps in the absence of Sox9, Spy expression is downregulated by itself, given that Sox genes

### Table 2. Inheritance of four distal Chr 4 markers in Dax1POs backcross females

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<thead>
<tr>
<th>Females</th>
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Numbers indicate fetal (F) and weanED (W) Dax1POs females. B, B/B and D, B/D. Four distal Chr 4 SNP markers are listed.
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\textsuperscript{129} allele. [The open reading frame of the Sry\textsuperscript{129} allele is identical to that of Sry\textsuperscript{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, Sox9 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 Sox9 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-YP\textsuperscript{POS} (Meeks et al., 2003b) (this study) and B6 XY\textsuperscript{YP\textsuperscript{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\textsuperscript{POS} allele also will prevent the upregulation of Sox9 in Dax1-/-YP\textsuperscript{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 Sox9 action (Swain et al., 1998). 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\textsuperscript{YP\textsuperscript{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 information for this article is available at http://dev.biologists.org/cgi/content/full/132/13/3045/DC1

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