

# A region of human chromosome 9p required for testis development contains two genes related to known sexual regulators

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**Deletion of the distal short arm of chromosome 9 (9p) has been reported in a number of cases to be associated with gonadal dysgenesis and XY sex reversal, suggesting that this region contains one or more genes required in two copies for normal testis development. Recent studies have greatly narrowed the interval containing this putative autosomal testis-determining gene(s) to the distal portion of 9p24.3. We previously identified *DMRT1*, a human gene with sequence similarity to genes that regulate the sexual development of nematodes and insects. These genes contain a novel DNA-binding domain, which we named the DM domain. *DMRT1* maps to 9p24.3 and in adults is expressed specifically in the testis. We have investigated the possible role of DM domain genes in 9p sex reversal. We identified a second DM domain gene, *DMRT2*, which also maps to 9p24.3. We found that point mutations in the coding region of *DMRT1* and the DM domain of *DMRT2* are not frequent in XY females. We showed by fluorescence *in situ* hybridization analysis that both genes are deleted in the smallest reported sex-reversing 9p deletion, suggesting that gonadal dysgenesis in 9p-deleted individuals might be due to combined hemizyosity of *DMRT1* and *DMRT2*.**

## INTRODUCTION

Sexual development in mammals requires formation and differentiation of the gonads. In males, the gonad produces the hormones Müllerian inhibiting substance (also called anti-Müllerian hormone) and testosterone, which together cause degeneration of the female Müllerian ducts and support the

development of male sexual characteristics (1,2). Surgical removal of the gonads in early embryogenesis causes both XX and XY animals to undergo female somatic development, demonstrating that the only essential role for the gonads in sex determination is to promote male development (3). This result also demonstrates that XY sex reversal would result either from failure to form the bipotential gonad or failure of sexual differentiation of the bipotential gonad once it has formed.

Molecular analysis of sex reversal syndromes has led to the identification of a number of genes involved in the formation of the bipotential gonad or its subsequent sexual development. At least two of these genes are on the sex chromosomes, *SRY* (4–7), the testis-determining factor on the Y chromosome, and the X-linked dosage sensitive sex reversal locus *DSS*, which contains the *DAX1* gene (8–10). Other genes are located on autosomes, including the steroidogenic factor 1 (*SF1*) gene on chromosome 9q (11,12), the Wilms tumor gene (*WT1*) on chromosome 11p (13) and the *SOX9* gene on chromosome 17q (14–16). Mutations in *SRY* affect only gonadal differentiation, while mutations in the other genes are associated with additional phenotypes in other organs. Mutations in known genes required for gonadal development account for a minority of cases of XY sex reversal (1,2). This clearly indicates that the majority of XY sex reversals must result from mutations at other chromosomal loci.

Deletions of chromosomes 9p and 10q have been associated with primary sex reversal of 46,XY individuals, indicating that these regions harbor genes essential for testicular development (17,18). In the case of 9p, relatively large deletions have been associated with a syndrome called 9p deletion syndrome, in which patients have phenotypes including mental retardation and craniofacial abnormalities (19). XY individuals with 9p deletion syndrome have a high frequency of partial or complete sex reversal, with female or ambiguous external and internal genitalia and partial or complete gonadal dysgenesis (17,20–23).

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Smaller 9p deletions can cause the same extent of sex reversal and gonadal dysgenesis without the other symptoms of 9p deletion syndrome (24–27). This suggests that gonadal dysgenesis and the other symptoms of 9p deletion syndrome are caused by the loss of different genes. Molecular and cytogenetic analysis of 9p deletions in sex-reversed patients has identified a range of deletion breakpoints from 9p21 to 9p24, with no clear correlation between the extent of the deletion and the severity of sex reversal. The wide range of deletion breakpoints suggests that the deletions are removing, rather than interrupting, a critical gene. The smallest sex-reversing 9p deletions described delete only a portion of the most distal region, 9p24.3 (25,27). This suggests that one or more genes within the 9p24.3–9pter interval is required for testis development. The size of the minimal critical region in 9p24.3 is estimated at ~250 kb based on the distance between D9S1779 (cosmid 67H3) and a subtelomeric cosmid clone (22E9) (28).

In many cases it has been possible to identify candidate genes involved in vertebrate development by studying invertebrate model organisms such as the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila*. In the case of sex determination this approach has been less successful, as most sex-determining genes in these two organisms are molecularly unrelated and appear not to have homologs involved in mammalian sex determination (29). Recently, however, we discovered an example of apparent evolutionary conservation in sex determination between nematodes and insects (30). We found that the *mab-3* gene of *C.elegans*, which is required for male sexual differentiation (31), encodes a protein with a novel DNA-binding motif that also is found in the *Drosophila* sexual regulator *doublesex* (*dsx*) (32–35). We named this motif the DM domain after *dsx* and *mab-3*. These two genes appear to be evolutionarily conserved based on four criteria. First, both genes encode proteins with DM domains. Second, both genes control analogous aspects of sexual development. Third, both genes act downstream in their respective sex determination pathways. Fourth, the male splice form of DSX can functionally substitute for the *mab-3* gene by restoring male-specific sense organ formation to a *mab-3* mutant *C.elegans* male (30). Thus, it is clear that DM domain proteins are essential for sexual development in the phyla Nematoda and Arthropoda. Based on the sex-determining roles of DM proteins in two species that diverged up to 500 million years ago, DM domain proteins are candidates to be involved in sex determination in other species as well.

We previously identified a human cDNA clone encoding a DM domain-containing protein, which we named *DMRT1* (Doublesex and MAB-3 related transcription factor 1; formerly called *DMT1*) (30). In adults, *DMRT1* mRNA is expressed only in the testis (30). Several recent studies have narrowed the smallest common region of overlap of 9p deletions associated with XY sex reversal to distal 9p24, suggesting that a gene or genes within this region is essential in two copies for testis development (24–27). Because *DMRT1* maps to distal 9p24, has sequence similarity to known sex-determining genes of two other phyla and is expressed specifically in the testis, it is an excellent candidate to be involved in sex determination.

In this study we investigate whether *DMRT1* or other DM domain-containing genes might play a role in human XY sex reversal. We show that the critical region of 9p24.3 contains not only *DMRT1*, but also a second DM domain-containing gene that we have named *DMRT2*. This suggests that sex reversal due

to 9p deletion might result from loss of both genes. Consistent with this possibility, sequencing of the entire coding region of *DMRT1* and the DNA-binding domain of *DMRT2* from a large number of XY females revealed only a single possible point mutation in *DMRT1*. By fluorescence *in situ* hybridization (FISH) analysis we show that the smallest sex-reversing 9p deletion reported thus far removes both *DMRT1* and *DMRT2*. Therefore, we suggest that XY sex reversal in 9p-deleted individuals may be due to hemizyosity of *DMRT1* and *DMRT2*.

## RESULTS

### Organization of the *DMRT1* gene

To determine the intron/exon organization and coding sequence of *DMRT1* (Fig. 1A), we determined the nucleotide sequence of a previously described *DMRT1* cDNA clone (30), used this cDNA as a probe to isolate clones from a human genomic DNA library and compared the cDNA and genomic sequences. To identify the 5'-end of the *DMRT1* mRNA, which is at least 140 nt upstream of the first in-frame methionine codon and missing from the cDNA clone, we used 5'-RACE. The 3'-UTR and poly(A) addition site are present in the cDNA clone. *DMRT1* is predicted to encode a 373 amino acid protein with a single DM DNA-binding domain near the N-terminus (Fig. 1A). Comparison of DMRT1 with two other DM domain-containing proteins, Doublesex (DSX) of *Drosophila* and MAB-3 of *C.elegans*, shows 67% identity to DSX and 50% identity to MAB-3 across the core C<sub>2</sub>H<sub>2</sub>C<sub>4</sub> region of the DM domain (30). The only similarity between these proteins outside the DM domain is a region rich in prolines and serines (Fig. 1A). The function of this region remains to be determined.

### Identification of a second DM domain gene in 9p24.3

We also sought other DM domain genes that might lie within the minimal sex reversal region of 9p. We used a degenerate PCR strategy (C.S. Raymond, J.R. Kettlewell, E.D. Parker, K.J. Seifert, B. Hirsch, V.J. Bardwell and D. Zarkower, manuscript submitted for publication) to isolate a DNA clone encoding a DM domain highly similar to that of *DMRT1*. We have named this new gene *DMRT2*. To determine the *DMRT2* coding sequence (Fig. 1B) we isolated a cDNA clone from a human adult testis library. Database searches also identified a partial *DMRT2* cDNA sequence from an adult female muscle library, suggesting that *DMRT2* expression is less spatially and sexually restricted than *DMRT1* expression, at least in adults. Comparison of the *DMRT1* and *DMRT2* protein sequences revealed that the two proteins are 80% identical in the core region of the DM domain. Both proteins also contain an additional 29 amino acid conserved DM domain region found in a subset of DM domain proteins, including DSX and MAB-3 (Fig. 1). The N-terminal portion of *DMRT2* is longer and more acidic than that of *DMRT1*, but both proteins have a number of conserved proline and glycine residues in the N-terminal portion. The C-terminal portion of *DMRT2* is shorter than that of *DMRT1* and is only ~20% identical to the corresponding portion of *DMRT1*. We used FISH to show that *DMRT2*, like *DMRT1*, maps within 9p24.3 (Fig. 2). Thus, the sex reversal region of 9p contains two genes that are expressed in testis and have sequence similarity to known invertebrate sex-determining genes, either of which might potentially be involved in 9p sex reversal.

**A**

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CCTCGCCACTCCAGCTGGCCCTCCGGCTCCAGCGCACAGCTCTCTCCGGCCTCTCTCCGGAGCGTGGCTGTCCTCGTGGGTTTCATCCCTCCGACGAGTCTCCAGCGGAGAGAGGGGGCC 120
      M P N D E A F S K P S T P S E A P H A P G V P P Q G R A G G 30
AGAGTGTCTCCGACTTCTCTAGGGGCCACCATGCCAACGACGAGGCAITTCAGCAAGCCCTTACACCGTCCGGAAGCCCTCCAGCCCCCGGGGTACCCGCCGACGAGAGCCGGGGGCT 240

F G K A S G A L V G A A S G T S A G G S S R G G G S G S G A S D L G A G S K K S 70
TTGGCAAGAGCTCTGGGGGCTAGTGGGGGGCCAGCGGCAAGAGCGCCCGGGGCGAGCAGCAGAGGAGGCGGCTCCGGCTCCGGGGGCTGGACCTGGGTGC CGGGGCAAGAGTCCC 360
      1
=====
P R L P K C A R C R N H G Y A S P L K G H K R F C M W R D C Q C K K C N L I A E 110
CGCGGCTCCCAAGTGCACGCTGCAGGAACCAACCGCTACCGCTCCGCGCTCAAGGGCCACAAGCGCTTCTGCATGTGGGGGACTGCCAGTGCAGGAAGTCAACCTATCCCGGAGA 480

R Q R V M A A Q V A L R R Q Q A Q E E E L L G I S H P I P L P S A A E L L V K R E 150
GGCAGCGGCTGATGGCCGCGAGGTGGCCCTGAGAAAGGCGAGCGCCAGGAGGAAATGGGTATCAGCCACCCCATCCACTCCAGTCCGAGTGGCGCGAGCTGTGTGTCAAAAGAGAGA 600
      Δ

N N G S N P C L M T E C S G T S Q P P P A S V P T T A A S E G R M V I Q D I P A 190
ACAATGGCAGTAACCGTGCCTCATGATGAGTGCAGTGGCACCTCTCAGCCACCCCGCCAGTGTCCCCACCAGTCCAGAGTTCAGAGGGACGTATGGTCAATCCAGGATATCTCTGCTG 720
      Δ

V T S R G H V E N T P D L V S D S T Y Y S S F Y Q P S L F P Y Y N N L Y N C P Q 230
TCACCAGCAGAGGCGCTGTGGAGAACACCTGACCTGGTTCAGACTCCACTACTACAGCAGCTTCTACCAGCCGCTCTGTCTTCTCTATACAAATCTATACACTGCCCGCAGT 840
      2

Y S M A L A A D S A S G E V G N P L G G S P V K N S L R G L P G P Y V P G Q T G 270
ACTCCATGGCTGGCTGCTGATTTCTGCTTCTGGGGAGTGGAAATCCCTCCGGGGATCCCTGTGAGAAACAGCCCTCCGGGCTCCCGGACCTTATGTGCTGTGACAGAGAA 960
      3      4

N Q W Q M K N M E N R H A M S S Q Y R M H S Y Y P P P S Y L G Q S V P Q F F T F 310
ACCAGTGCAGATGAAGAATCGAGAACCCGCTATGCAATGAGCTCCAGTACAGGATGCAITCTTACTACCGGCTCCCTCCCTTACCTGGCCAGAGCGTCCCGCAGTCTTCACTTTTG 1080
      Δ      5      6

E D A P S Y P E A R A S V F S P P S S Q D S G L V S L S S S S P I S N K S T K A 350
AGGATGCTCCCTCTACCCGGAAGCCAGGGGAGCGTATCTCCGCGCCAGCAGTCAAGATCTGGCTTGGTTCCCTCTCGAGCAGCTCTCTATTTAGTAAAGAGCACAAGGGCAG 1200
      Δ

V L E C E P A S E P S S F T V T P V I E E D E * 373
TGCITGAATGTGAGCCTGCGTCGGAGCCGACGCTTCACAGTCACTCCCGTCACTGAGGAGGACGAGTGCAGTGCCTGCTGCCGATGGCGGTTACCTGGAGTAACAGGCTTATTC 1320
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AGGTTCCGATGACATCTGCAATGATTTAAGTGTCTTACTACCGGAGTTTAAATAATAGTCTTCAITTTTAAATGACACTGGTTTTCATGAGTGTTCAGAAATAAAAGAAATCACTTC 1560
AAGTGAAGCCATTTGTGTGCTCTAAATGAGTCACTTAATGAGTGTACTTTTGTAGTTTAAATAAGAAATCTTAGTGCTTACGGGTTTFTTTTAAAGTATTTTAAAAACCTGC 1680
AAAGATATAAATTTAGCCAGTCTGACCGGCTGAGAGAAAGAGCAGGCAAAATAGTGTATTTTAAAGATCTGCTAAATGAGATATTTGTGTGTGTCTGTTGAAATAGGCC 1800
CACCCATCTCCCAATCCAAACGTAACGAAATATAATCAGAAACATTAAGCCCTGTAAATAAAATTTGCTGCTTTTGGTAAAGGTTGATATTAACAGTGCATTTAAAGTACC 1920
TTAGTGAAGCAGAGTCTGAGTGTGTTTGTAGAAATGAGTTTATCATACATCTTCTACTACTGAAATAAAATGAGTGCAGTCTGAGCTGTTGTAACCTAGCTGTAAATCTGATTTG 2040
GAAATAGTACATCTTAAAGTGTCTACAGATTTGAGTTCATGATTTTGTAAATAATTTGCTATGAGTACTTTGTATATAACAGAAACCTCTGAAATGAACTAGTCTAAATAAAT 2160
TCATGTCTTACTTAGTGCAGCTGACTGAAATAAATAATTTGATGACTGAAATAAATA 2225
    
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**B**

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DMRT1 1 M P N D E A F S K P . . . . . S T P S E A P H A P G V P P Q . G . . . . . R A G G F G K A S .
DMRT2 1 M A D P Q A G S A A G D W E I D V E S L E L E E D V C G A P R S T P F G P S P P A D G D C E D E D E D D D G V D E D A E

DMRT1 36 . . . . G A L V C A A S G T S A G G S S R G G . . . . . G S G S G A S D L G A G S K K S P R . . . . L
DMRT2 61 E E G D G E E A G A S P G M P G Q P E Q R G G P Q P R P P L A P Q A S P A G T G P R E R C T P A G G G A E P R K L S R T

DMRT1 74 P K C A R C R N H G Y A S P L K G H K R F C M W R D C C K K C H L I A E R Q R V M A A Q V A L R R Q A Q E E L G L
DMRT2 121 P K C A R C R N H G V V S C L K G H K R F C R H R D C C A N C L L V V E R Q R V M A A Q V A L R R Q A T E D K K C L

DMRT1 134 S H P I P L P S A A E L L V K R E N N G S N P C L M T E C S G T S Q P P P A S V P T T A A S E G R M V I Q D I P A V E S
DMRT2 181 S G . . . . . K Q N H . . . . . F . E R K A V T Q R . Q V R A P E L L A K S I L E V L L G L . F Y S Y

DMRT1 194 R G H V E N T P D L V S D S T Y Y S S F Y Q P S L F P Y Y N N L Y N C P Q Y S M A L A A D S A S G E V G N P L G G S P V
DMRT2 219 Y V Y I M N H L . . . . .

DMRT1 254 K N S L R G L P G P Y V P G Q T G N Q W Q M K N M E N R H A M S S Q Y R M H S Y Y P P P S Y L G Q S V P Q F F T F E D A
DMRT2

DMRT1 314 P S Y P E A R A S V F S P P S S Q D S G L V S L S S S S P I S N K S T K A V L E C E P A S E P S S F T V T P V I E E D E
DMRT2
    
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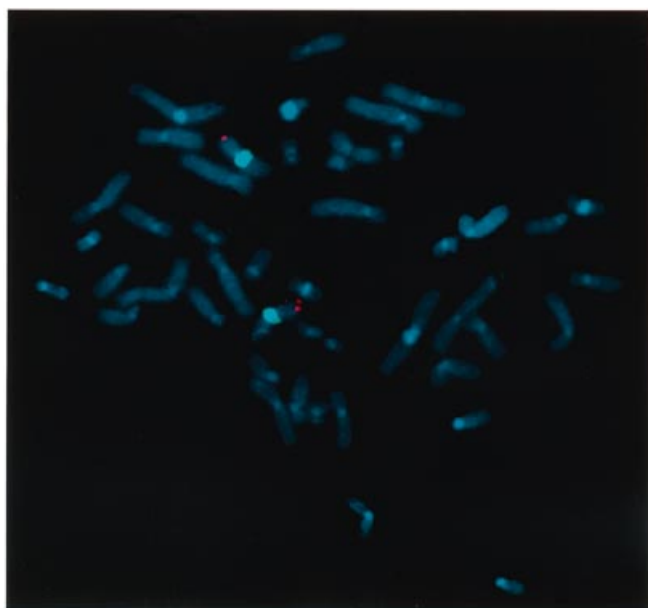
**Figure 1.** DMRT1 and DMRT2. (A) Sequence and exon organization of *DMRT1* cDNA. Coding region and 3'-UTR sequences are from a *DMRT1* cDNA clone (29) (GenBank accession no. AA412330). The 5'-UTR was isolated by 5'-RACE and the sequence was confirmed by comparison with genomic DNA sequence. The 5'-end of the mRNA has not been precisely determined, but is at least 140 nt 5' of the first in-frame AUG. The DM domain and proline/serine-rich region are indicated by overlining; the C<sub>2</sub>H<sub>2</sub>C<sub>4</sub> core region of the DM domain is overlined with double dashes. Exon-exon boundaries are indicated by Δ. Codons affected by nucleotide changes in XY females, XX males or controls are indicated by underlining and numbering. Numbers of sequence changes correspond to those in Figure 2A. (B) Comparison of DMRT1 and DMRT2 protein sequences. Amino acid identities are boxed in black and similarities are shaded gray. Amino acids are numbered relative to the first in-frame methionine codon. Alignments were performed using the ClustalW program. GenBank accession nos of cDNA sequences are AF130728 (*DMRT1*) and AF130729 (*DMRT2*).

**Sequencing of *DMRT1* and *DMRT2* from XY females and XX males**

If *DMRT1* or *DMRT2* mutations are a frequent cause of human XY sex reversal, one might expect to find point mutations in the coding region among a large population of XY females. To

test this possibility, we sequenced the entire coding region of *DMRT1* and the DM domain of *DMRT2* from a large number of sex-reversed individuals.

For *DMRT1*, we analyzed DNA from lymphoblastoid cell lines derived from 87 individuals with unexplained XY sex reversal (XY



**Figure 2.** A second DM domain gene maps to 9p24.3. FISH mapping of *DMRT2* to 9p24.3. Human metaphase chromosomes hybridized with a digoxigenin-labeled *DMRT2* genomic  $\lambda$  phage probe (red) and a chromosome 9-specific directly labeled centromere probe (green). Chromosomes are counterstained blue with DAPI.

females). We also analyzed DNA from 17 individuals with XX sex reversal (XX males) to test the possibility that dominant gain-of-function mutations in *DMRT1* can cause female-to-male sex reversal. While 80–90% of human XX males carry the *SRY* gene, those studied here had all been found to lack *SRY* (L.G. Brown and D.C. Page, unpublished data). The XY females all have an *SRY* gene based on PCR amplification; in most cases the HMG domain of *SRY* was sequenced and found to be normal (P. Beer-Romero and D.C. Page, unpublished data). All of the patients have normal karyotypes. We used PCR to amplify, from genomic DNA, each exon and ~100 bp of the adjacent intron or UTR sequence and directly sequenced the resulting DNA fragments using radiolabeled dideoxynucleotide terminator cycle sequencing. To differentiate between potential mutations and polymorphisms, DNA from a control population consisting of grandparents from the panel of Centre d'Etude du Polymorphisme Humain (CEPH) reference families was also sequenced.

We found nucleotide differences in *DMRT1* at five positions among the sex-reversed patients (Figs 1 and 3A). We also found a single silent mutation in a control individual (nucleotide change number 4; Figs 1 and 3A). All sequence differences were heterozygous. Four changes are clearly polymorphisms, as they occur at similar frequency among the control group. A fifth, which causes a Pro→Leu coding change in exon 4 (nucleotide change number 6; Figs 1 and 3), may be a loss-of-function mutation, as it was not found in 162 control chromosomes (81 individuals) sequenced. However, since the controls were not well matched by ethnic background to this patient, who is African-American, the possibility remains that the sequence difference is an inconsequential polymorphism. We also found a length polymorphism of 8 bp in intron 4 (data not shown). Further studies will be needed to determine if the Pro→Leu coding change has any effect on the function of *DMRT1*.

To test whether *DMRT2* point mutations might be a frequent cause of XY sex reversal, we sequenced genomic DNA from 54 XY females. Because the DM domain is the site of most loss-of-function mutations in the *mab-3* and *dsx* genes (30,34), we focused on the exon encoding the DM domain of *DMRT2*. We found a C→G polymorphism outside the DM domain in six patients (data not shown), but found no base changes in the DM domain. This suggests that, as with *DMRT1*, mutations in *DMRT2* may not be a common cause of XY sex reversal, although we cannot exclude the possibility of mutations elsewhere in *DMRT2*.

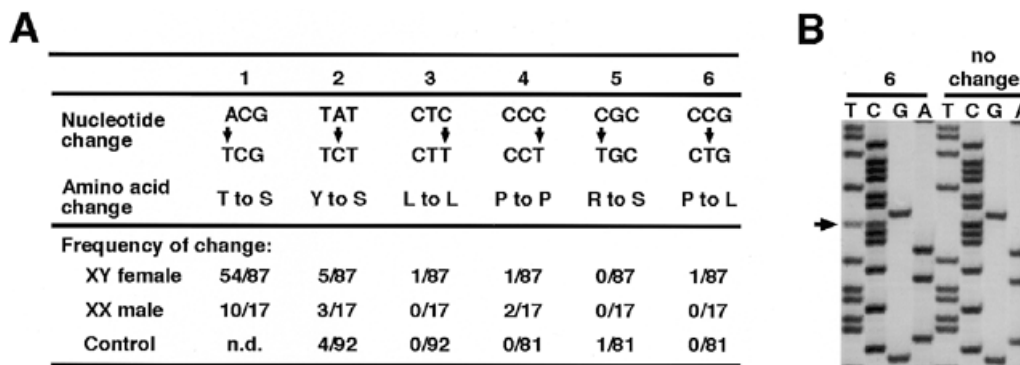
Our failure to find multiple mutations in *DMRT1* or *DMRT2* in a relatively large group of XY females suggests at least three possibilities. The first is that neither gene plays a role in sex determination. The second is that we may have sequenced an insufficient number of alleles or that mutations may occur outside the sequenced regions of either gene (see below). An alternative possibility is that the two genes have at least partially overlapping functions and sex reversal in 9p-deleted individuals results from hemizyosity of both genes.

### Sex-reversing 9p deletions remove both *DMRT1* and *DMRT2*

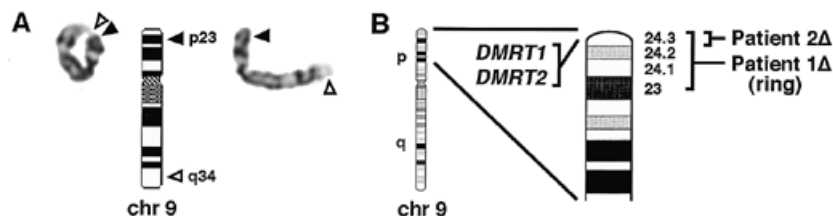
Based on the extremely distal positions of *DMRT1* and *DMRT2* in 9p24.3, both genes would be expected to be hemizygous in all previously reported XY females with 9p deletions. To test this prediction, we performed FISH analysis on cells from two 46,XY patients with 9p deletions. The extents of the deletions in these patients are indicated in Figure 4B.

Patient 1, who is newly reported here, presented with ambiguous external genitalia, including a normal size phallus, penoscrotal transposition, non-palpable testes and severe hypospadias. At the time of surgical repair, bilateral undescended testes were identified and characterized as very small and cystic in appearance. G-banded chromosome analysis of skin fibroblasts revealed a 46,XY male complement with one normal chromosome 9 and one ring chromosome 9, as shown in Figure 4A. Breakpoints within the ring were designated at bands 9p23 and 9q34.3, resulting in monosomy for the regions 9p23–9pter (Fig. 4B) and 9q34.3–9qter. FISH with probes to *DMRT1* and *DMRT2* confirmed a deletion of both genes within the ring chromosome (Fig. 5A).

Patient 2, who has been described previously, presented at 3 years of age with female external genitalia and female internal structures, which included a uterus, vagina and dysgenic gonads, and has the smallest reported 9p deletion (25,28,36). The patient's father has a balanced 8;9 translocation (25). The patient inherited a normal maternal chromosome 9 and a paternal chromosome 9 in which the distal short arm is replaced by an extra copy of the distal short arm of chromosome 8. Previous cytogenetic and molecular analysis of DNA from this patient showed that the deletion breakpoint is in 9p24.3, distal to the marker D9S1779, narrowing the critical region for sex reversal to the terminal portion of 9p24.3. FISH with probes to *DMRT1* and *DMRT2* on EBV-transformed lymphoblastoid cells derived from the father showed hybridization of both probes to one chromosome 9 and one chromosome 8 (presumably the 8;9 derivative) (Fig. 5B). In cells derived from the patient, the probes hybridize to only one chromosome 9 (Fig. 5C). Thus, the father has two copies of *DMRT1* and *DMRT2*, while the 46,XY daughter is hemizygous for both genes.



**Figure 3.** Summary of *DMRT1* sequencing results. (A) Nucleotide changes found in *DMRT1*. DNA changes and resulting protein coding changes, if any, are indicated. For each change the frequency of its occurrence in XY females, XX males and normal controls is indicated. n.d., not done. (B) Exon 4 coding change in XY female WHT2795. (Left) Exon 4 sequence from XY female; (right) exon 4 sequence from control DNA. Mixed sequence (T+C) in DNA from XY female WHT2795 (arrow) indicates heterozygosity at this position. This coding change from proline to leucine corresponds to number 6 in (A) and in Figure 1.



**Figure 4.** Chromosomes 9 of patient 1 and idiogram of distal 9p. (A) G-banded chromosomes 9 of patient 1. This patient has a ring chromosome 9 (left) and a normal chromosome 9 (right). Band 9q34 is indicated by an open arrowhead and band 9p23 is indicated by a closed arrowhead. Karyotype of the patient is 46,XY,r(9)(p23q34.3). (B) 9p-deleted regions in patients 1 and 2 are indicated, based on G-banded chromosome analysis for patient 1 and on previously published conventional and molecular cytogenetic analyses for patient 2 (25,35). Positions of *DMRT1* and *DMRT2*, based on FISH mapping, are indicated. Based on metaphase FISH analysis we cannot determine whether *DMRT1* is proximal or distal to *DMRT2*.

## DISCUSSION

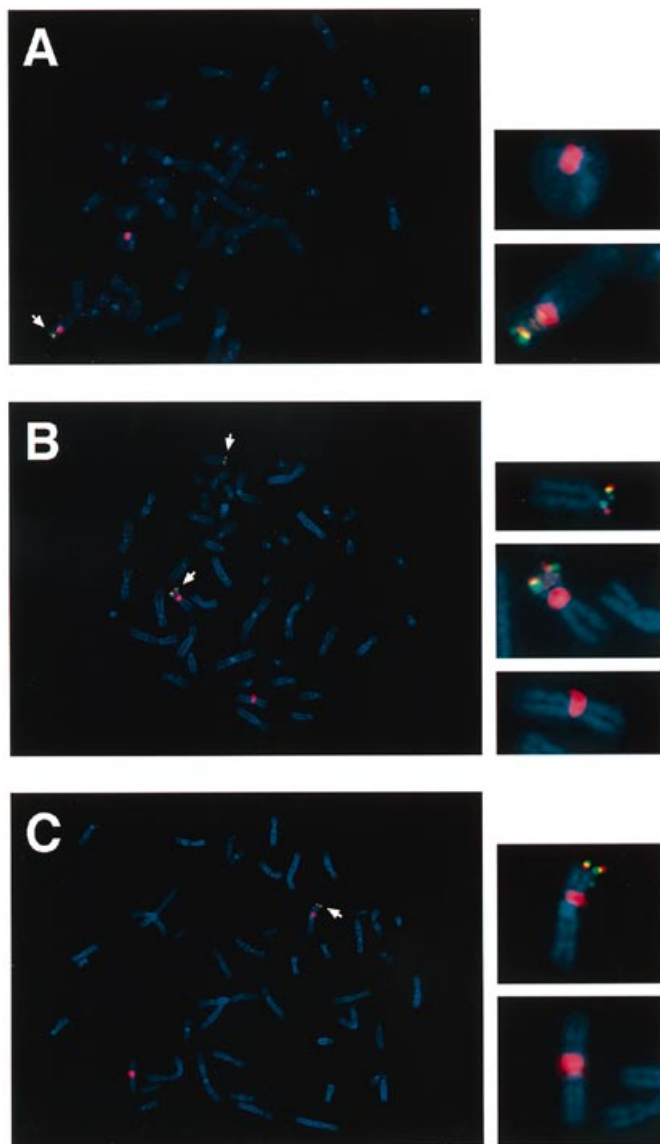
We have found that two DM domain-containing genes, *DMRT1* and *DMRT2*, map within 9p24.3, a region that is critical for testis development. Sequencing of the coding region of *DMRT1* from a large group of unexplained XY females identified only one potential point mutation. Similarly, sequencing of the DM domain of *DMRT2* failed to identify any point mutations. This result suggests that mutation of either *DMRT1* or *DMRT2* alone is not sufficient to cause XY sex reversal. Despite the lack of point mutations in *DMRT1* and *DMRT2*, FISH analysis of the smallest reported sex-reversing 9p deletion shows the absence of both genes, implying that all sex-reversing 9p deletions reported thus far remove both genes. Therefore, we suggest that sex reversal in 9p-deleted individuals might be due to hemizyosity of both *DMRT1* and *DMRT2*.

The model we suggest for 9p deletion sex reversal involves at least partial functional redundancy between *DMRT1* and *DMRT2*. The two proteins are highly similar in the DM domain, but less similar in their N- and C-terminal domains. One might expect two functionally redundant proteins to be highly similar throughout. However, comparison of the DSX and MAB-3 proteins illustrates that this need not be the case. Despite substantial differences in protein sequence, MAB-3 and DSX bind to similar DNA sequences *in vitro* and *in vivo* and are functionally interchangeable *in vivo* (30,37). From these results it is clear that DM domain proteins need not be

highly similar in primary sequence to perform related functions *in vivo*. Similarly, the SRY protein is evolutionarily conserved only in the HMG domain; the rest of the protein appears to have evolved rapidly (38).

Several possible mechanisms have been proposed for XY sex reversal associated with 9p deletions, including haploinsufficiency of the deleted gene(s) or the uncovering of a recessive mutation on the remaining chromosome. The relatively high frequency of XY sex reversal observed in 9p deletion syndrome suggests haploinsufficiency, because otherwise the frequency of pre-existing mutant alleles would have to be correspondingly high. If *DMRT1* and/or *DMRT2* are involved in sex reversal associated with 9p deletions, our sequencing data and FISH analysis are also consistent with haploinsufficiency. It is clear that the process of sex determination is highly sensitive to gene dosage and activity. For example, the same mutant allele of *SRY* can be present in a fertile male father and an XY female offspring, demonstrating that there is a sensitive threshold for *SRY* activity that may vary between individuals. Similarly, of the other genes and loci known to be required for gonad formation and sex determination, there is evidence of sensitive activity thresholds of *SOX9*, *WT1* and *DSS*, as well as the gene(s) on 9p (1,6,10,16,39–42).

9p deletions are associated with variable sexual phenotypes and some 46,XY individuals with 9p deletions are phenotypic males. There are several possible explanations for this variability



**Figure 5.** Hemizyosity of *DMRT1* and *DMRT2* in sex-reversing 9p deletions. (A) FISH analysis of patient 1 for *DMRT1* (small red signal) and *DMRT2* (small green signal). *DMRT1* and *DMRT2* hybridization (arrow) is detected on the normal chromosome 9 but not on the ring chromosome 9. Large red signals represent hybridization of a chromosome 9 centromeric probe. (B) FISH analysis of the father of patient 2 for *DMRT1* (small red signal) and *DMRT2* (small green signal). *DMRT1* and *DMRT2* hybridization (arrows) is detected on one chromosome 9 (large red signal from chromosome 9 centromeric probe) and one chromosome 8 (as previously determined by G-banding). The other chromosome 9 is hybridized only by the centromeric probe (large red signal). (C) FISH analysis of patient 2 for *DMRT1* (red) and *DMRT2* (green). *DMRT1* and *DMRT2* hybridization (arrow) is detected only on one chromosome 9. The close proximity of *DMRT1* and *DMRT2* on chromosome 9 often results in an overlapping (yellow) signal on metaphase chromosomes.

ity. First, as discussed above, if hemizyosity of *DMRT1* and *DMRT2* or other genes is responsible, there may be a sensitive threshold for the activity of the gene(s), resulting in incomplete penetrance, with some individuals unaffected. We favor this possibility. Second, variability in the extent of sex reversal could be due to deletion of a variable number of sex-determining genes. While this is possible, it is unlikely, because there is

no apparent correlation between the extent of 9p deletions and the severity of XY feminization. For example, of the two patients described in this study, the more completely feminized patient has the smaller 9p deletion. Third, variability due to imprinting is possible, but is unlikely, as there is no correlation between the parental origin of the affected chromosome 9 and the severity of sex reversal (17,43). In addition to these possibilities, some cases of 9p deletion that do not cause sex reversal may prove upon further analysis to be interstitial deletions that retain subtelomeric loci, including *DMRT1* and *DMRT2*.

The DNA sequencing data presented here suggest that point mutations in the *DMRT1* and *DMRT2* coding regions are not a frequent cause of XY sex reversal. We cannot, however, exclude the frequent occurrence of small deletions or regulatory mutations in *DMRT1* or *DMRT2* in 46,XY females: if a deletion removed one or both PCR primer-binding sites, we would fail to amplify the affected allele and as a result would sequence only the unaffected exon. Likewise, we cannot exclude as a cause of sex reversal the occurrence of cytogenetically undetectable lesions that remove the entire *DMRT1* or *DMRT2* gene. It is important to note that the patients examined here are fully or nearly fully feminized. Thus, it remains possible that point mutations in *DMRT1* or *DMRT2* might cause a less severe intersexual phenotype. It is also possible that homozygous mutations in either gene have a more pleiotropic phenotype.

There are now three lines of circumstantial evidence that make *DMRT1* and *DMRT2* likely candidates to be involved in mammalian sexual development. First, both are related to proven sex-determining genes that are conserved between insects and nematodes. Second, both genes are located in a very short (~250 kb) chromosomal region that is essential for testis development. Currently these are the only candidate genes identified in the critical region. Third, both genes are expressed in the adult testis. To definitively establish the roles of *DMRT1* and *DMRT2* in sex determination it will be necessary to disrupt these two genes in the mouse, individually and together; these experiments are underway.

## MATERIALS AND METHODS

### Library screening

A human placental genomic DNA  $\lambda$  library (Clontech, Palo Alto, CA) was screened with a  $^{32}\text{P}$ -labeled *DMRT1* cDNA clone (GenBank accession no. AA412330). Single positive plaques were isolated and DNA was prepared from liquid lysates. Human *DMRT2*-containing genomic clones were isolated by screening the human genomic DNA library at low stringency using a mouse *Dmrt2* cDNA fragment (C.S. Raymond, J.R. Kettlewell, E.D. Parker, K.J. Seifert, B. Hirsch, V.J. Bardwell and D. Zarkower, manuscript submitted for publication) as a probe. A human *DMRT2* cDNA clone was isolated by probing a testis  $\lambda$  phage cDNA library (Clontech) with a genomic DNA fragment encoding the DMRT2 DM domain. Database searches using this cDNA sequence identified an expressed sequence tag (EST) sequence (GenBank accession no. Z24950) from a human female skeletal muscle cDNA library.

## FISH analysis

FISH (44) was performed using *DMRT1* and *DMRT2* genomic  $\lambda$  phage clones labeled by nick translation with digoxigenin-11-dUTP or biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN) and detected with rhodamine-anti-digoxigenin or fluorescein-conjugated avidin, respectively. Signals were further amplified using rabbit anti-sheep and rhodamine-anti-rabbit (for the digoxigenin-labeled probe) or anti-avidin and fluorescein-conjugated avidin (for the biotin-labeled probe). A directly labeled Spectrum Orange or Spectrum Green (VYSIS, Downers Grove, IL) chromosome 9 centromere probe (D9Z1) was also applied. Chromosomes were counterstained with 4,6'-diamidino-2-phenylindole. In each case at least 10 metaphase cells were examined using fluorescent microscopy and images were captured using CytoVision software (Applied Imaging, Santa Clara, CA).

## DNA amplification and sequencing

Genomic DNA from lymphoblastoid cells of 87 XY females, 17 XX males and 92 normal controls was used as a template to amplify each *DMRT1* exon and immediate flanking sequence by PCR. PCR reactions were incubated at 94°C for 1 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. Reactions included 10% DMSO. PCR products were purified using the High Pure PCR Product Purification kit (Boehringer Mannheim) and analyzed on 1% agarose gels. The PCR products were directly sequenced using the Thermo Sequenase radiolabeled dideoxy nucleotide terminator cycle sequencing kit (USB, Cleveland, OH). Sequence reactions were run on 6% denaturing polyacrylamide gels and were autoradiographed overnight.

For each exon of *DMRT1* a genomic DNA fragment encompassing the exon was amplified and directly sequenced as follows. Exon 1 was amplified with primers E1FA and E1RA and sequenced on one strand with two internal primers (E1FS and CR76). Exons 2 and 3 were amplified and both strands were sequenced using the same primers (E2FS, E2RS, E3FS and E3RS). Exons 4 and 5 were amplified with E4F and E4R and with E5F and CR64, respectively, and sequenced with E4F or E5F. Primer sequences are from introns and 5'- and 3'-UTRs, allowing sequencing of the full coding region and all splice junctions. In all cases where both strands were sequenced, polymorphisms detected on one strand were also detected on the other. *DMRT2* was amplified with primers JK7 and JK8 and sequenced with JK10. Primer sequences were as follows. Primers for *DMRT1*: E1FA, CTCCGAGCGTCGCTGTCCGTCGG; E1RA, GAGCCAAGATCGCGCCACTACACTGC; E1FS, TCCCTGGCAGCAGTCTCCAGGCGAG; E1RS, AGCCTGGC-AACCGAGCGAGACTC; E2FS, GTGTTTTGGCAAAGCT-GATTCTGG; E2RS, TGCAACCTTCGGCCTCCCTCATGC; E3FS, AGAAGTAAAGTTTCGTGGACTAAC; E3RS, TGCA-CATGCATGTGGCTTTCACAC; E4F, CACTGTGCCAGCC-TGTTACCTTG; E4R, AAGCCATTAGACACAGCTAATGAC; E5F, GAATAATGAATGTATAAAGACCAGCCAC; CR64, CAGCCTCTGGACTAAACTCTAAGG; CR76, GGCAAAG-CGTCTGGGGCGCTAGTG. Primers for *DMRT2*: JK7, GTGGACGAAGACGCGGAAGAAGAG; JK8, GCACCTCG-GTGGCCTGCTGCCTCC; JK10, CGACGGCGAGGAGGCAG-GCGCGTC.

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