Heterozygous HESX1 mutations associated with isolated congenital pituitary hypoplasia and septo-optic dysplasia

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We have previously shown that familial septo-optic dysplasia (SOD), a syndromic form of congenital hypopituitarism involving optic nerve hypoplasia and agenesis of midline brain structures, is associated with homozygosity for an inactivating mutation in the homeobox gene HESX1/Hesx1 in man and mouse. However, as most SOD/congenital hypopituitarism occurs sporadically, the possible contribution of HESX1 mutations to the aetiology of these cases is presently unclear. Interestingly, a small proportion of mice heterozygous for the Hesx1 null allele show a milder SOD phenocopy, implying that heterozygous mutations in human HESX1 could underlie some cases of congenital pituitary hypoplasia with or without midline defects. Accordingly, we have now scanned for HESX1 mutations in 228 patients with a broad spectrum of congenital pituitary defects, ranging in severity from isolated growth hormone deficiency to SOD with panhypopituitarism. Three different heterozygous missense mutations were detected in individuals with relatively mild pituitary hypoplasia or SOD, which display incomplete penetrance and variable phenotype amongst heterozygous family members. Gel shift analysis of the HESX1-S170L mutant protein, which is encoded by the C509T mutated allele, indicated that a significant reduction in relative DNA binding activity results from this mutation. Segregation analysis of a haplotype spanning 6.1 cM, which contains the HESX1 locus, indicated that only one HESX1 mutation was present in the families containing the C509T and A541G mutations. These results demonstrate that some sporadic cases of the more common mild forms of pituitary hypoplasia have a genetic basis, resulting from heterozygous mutation of the HESX1 gene.

INTRODUCTION

The pituitary gland plays a central role in the hormonal control of many critical homeostatic processes, including growth, thyroid function, ability to cope with stress and sexual development. These processes are controlled via hormones secreted by five distinct cell types located within the anterior pituitary (AP), which include growth hormone (GH), adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), prolactin (PRL) and the gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Differentiated cell types that secrete these hormones (somatotrophs, corticotrophs, thyrotrophs, lactotrophs and gonadotrophs, respectively) arise during embryogenesis in an evolutionarily conserved sequence from a layer of oral ectoderm termed Rathke’s pouch (1,2). Failure of these trophic cells to differentiate and/or proliferate during embryogenesis results in congenital pituitary disorders that range in severity from panhypopituitarism (no pituitary function) to milder forms in which one or more of the hormone-secreting cells are absent (for a review see ref. 3). The most common form of these disorders is isolated GH deficiency, which has been reported to occur in 1 in 4000–10 000 live births (4). Combined pituitary hormone deficiencies (CPHDs), in which patients lack more than one of the AP hormones, are less common, but are associated with considerable morbidity, particularly if the diagnosis is delayed. Congenital pituitary hypoplasia also occurs in association with other developmental abnormalities. One such syndrome is septo-optic dysplasia (SOD) (also known as de Morsier’s syndrome), which is characterized by the triad of pituitary hypoplasia, optic nerve hypoplasia and agenesis of midline...
brain structures, including the corpus callosum and septum pellucidum (5–7). The phenotype is highly variable, with 62% of affected individuals having hypopituitarism and 30% having all three manifestations (8–10).

Although most cases of pituitary hypoplasia are sporadic and idiopathic (11), familial forms have been reported underlying the genetic contribution to the aetiology of these disorders (OMIM 262600, 312000). One of the most effective means of identifying the genes responsible for human pituitary disorders has been through the initial identification of genetic lesions in spontaneous mouse mutants with phenotypes closely resembling human pituitary disorders (for a review see ref. 12). Two pituitary disease genes that have been identified in this manner are POUF1 (13) and PROPI (14), both of which belong to the homeobox class of transcription factors. Mutations in the POUF1 murine orthologue (Pit-1) and Prop1 genes were originally identified in the Snell and Ames dwarf strains (15–17), respectively, and have subsequently been shown to be present in a variety of patients with isolated CPHD (4,18 and references therein).

An additional member of the homeobox gene family, which we have shown to be essential for pituitary and forebrain development in man and mouse, is HESX1/Hexx1 (19). During mouse embryogenesis, Hexx1 is expressed in the developing forebrain and Rathke’s pouch, and is subsequently downregulated during pituitary cell differentiation (20,21). Null mice generated using targeted mutagenesis display pituitary hypoplasia, micro- or anophthalmia, and agenesis of midline forebrain derivatives, including the corpus callosum and septum pellucidum (22). A small proportion of heterozygous mice are also affected, but have a considerably milder phenotype compared with homozygote pups. The similarity between the Hesx1 mutant mouse phenotype and SOD in humans prompted us to investigate whether HESX1 mutation in humans is associated with SOD. This was confirmed by the identification of a homozygous null mutation in two siblings with SOD from a consanguineous family (22).

The existence of HESX1 mutations in SOD patients provided the first compelling evidence that SOD, at least in familial cases, has a genetic basis. However, as most cases of hypopituitarism/SOD are sporadic, it is not clear whether the HESX1 mutation contributes to the aetiology of these disorders. Some support for an association between heterozygous HESX1 mutations and sporadic SOD/hypopituitarism is indicated by the mild, incompletely penetrant phenotype in heterozygous Hesx1 pups. Accordingly, we scanned for HESX1 mutations in 228 patients with a wide spectrum of congenital hypopituitarism/SOD phenotypes. Within this cohort we have identified three point mutations resulting in non-conservative amino acid substitutions, which display incomplete penetrance amongst heterozygous family members. These data indicate that despite the sporadic nature of most congenital pituitary hypoplasia/SOD cases, some of these cases have a genetic component to their aetiology, which includes mutation of the HESX1 gene.

RESULTS

HESX1 mutation detection

We scanned for HESX1 mutations in 228 patients, including 85 with isolated pituitary hypoplasia (including isolated and combined pituitary hormone deficiency), 105 with SOD and 38 with holoprosencephaly or related phenotypes. All of these cases were sporadic apart from pedigree 1 and four other families in whom no band shifts/mutations were observed. Three different missense mutations were identified in this cohort, all of which were heterozygous in affected individuals. The C509T mutation (Fig. 1a), which results in the non-conservative S170L substitution, was identified in two affected brothers in pedigree 1 (II.1 and II.2 in Fig. 2a). The S170 residue is located immediately C-terminal to the homeodomain within an RESQFL motif, which is completely conserved in all HESX1/ANF class homeoproteins (23). Both brothers exhibit GH deficiency and individual II.1 also has optic nerve hypoplasia. In pedigree 2, an A541G mutation, which results in a non-conservative T181A substitution, was identified in individual II.1 who has isolated GH deficiency (Figs 1b and 2a). Individual II.1 in pedigree 3, who has multiple AP hormone deficiencies, has a G18C mutation, which causes a Q6H non-conservative substitution in exon I (Figs 1c and 2a). Mutations and patient phenotypes are summarized in Table 1 and Figure 3a.

Apart from the mutations described above, all of the HESX1 alleles that were sequenced from our cohort were wild-type, with the exception of one Afro-Caribbean male who was heterozygous for an A374G substitution. As only a small proportion
of our cohort was of Afro-Caribbean heritage, we investigated the possibility that this allele was a polymorphic variant prevalent in the Afro-Caribbean population. Sequence analysis of 42 unaffected control Afro-Caribbean individuals demonstrated that five were homozygous for the A374G allele, 20 were heterozygous and 17 did not have the change. The relatively high proportion of normal individuals who were homozygous for the A374G allele indicates that this change represents a benign polymorphism that is relatively common in the Afro-Caribbean population.

Genotype analysis of family members in pedigrees 1, 2 and 3 showed that the affected individuals had inherited the mutation from one of their parents, who was not affected in each case (Fig. 2a). Furthermore, in pedigree 2 an unaffected heterozygote sibling (II.2) of an affected individual (II.1) was also present. The incomplete penetrance of heterozygous HESX1 mutations in individuals with relatively mild pituitary hypoplasia is consistent with our previous finding that Hesx1<sup>+/−</sup> mice display low penetrance of a mild phenotype (22). However, an alternative explanation for the presence of the mutated allele in unaffected individuals is that these sequence changes represent polymorphisms that do not compromise HESX1 function. To address this possibility, we sequenced exons I and IV, amplified from 100 control chromosomes. All control sequences for these exons were identical to our previously published sequence (22), indicating that all three mutations are not common sequence polymorphisms.

**HESX1 containing haplotype segregation in pedigrees 1 and 2**

In contrast to our previous report, in which two siblings with severe SOD were homozygous for a null HESX1 mutation (22), all affected individuals in this study were heterozygotes and were more mildly affected. The apparent lack of a mutation in one HESX1 allele in affected individuals indicates that mild pituitary hypoplasia/SOD is associated with heterozygous HESX1 mutations. However, an alternative explanation is that the affected individuals are compound heterozygotes due to the inheritance of a mutation from their mother (in whom no mutation has been identified). We investigated the possibility that the affected individuals in pedigrees 1 and 2 are compound heterozygotes by examining the inheritance of a maternal haplotype that contains the HESX1 locus. This analysis was not performed for pedigree 3 due to the lack of unaffected heterozygous siblings.

To identify closely linked HESX1 flanking markers, three overlapping yeast artificial chromosomes (YACs) containing the HESX1 gene, 810-E-3, 927-F-2 and 956-E-7, were identified (Fig. 2b). These YACs form part of a contig (WC3.10) located on 3p21.1 within the region previously defined by fluorescence in situ hybridization (FISH) to contain the HESX1 gene (22). Haplotypic segregation analysis of four HESX1 flanking (CA)<sub>n</sub> markers within the WC3.10 contig, spanning a 6.1 cM region, was performed (Fig. 2a). In pedigree 1, the heterozygous affected siblings II.1 and II.2 have inherited different maternal haplotypes (22 + 21 versus 65 + 27, respectively), and therefore different HESX1 alleles. As their mother is not affected, and must therefore carry at least one wild-type HESX1 allele, at least one of the affected brothers does not harbour a second unidentified HESX1 mutation. Therefore, our analysis of this pedigree demonstrates that heterozygous HESX1 mutations are associated with mild pituitary hypoplasia/SOD. In pedigree 2, the same haplotype has been passed on from the mother (I.2) to both the affected (II.1) and unaffected (II.2) daughters. If this haplotype harboured an HESX1 mutation, then we would expect both II.1 and II.2 to be affected, as homozygous HESX1 mutations are completely penetrant in both humans and mice (22). Therefore, haplotype analysis of pedigree 2 indicated that individual II.2 is not a compound heterozygote, and provides additional support for the association between heterozygous mutations in HESX1 and mild pituitary phenotypes.

**DNA binding activity of the S170L HESX1 protein**

Since the S170L missense mutation lies in a conserved sequence close to the homeodomain, we compared its DNA binding affinity with that of the wild-type protein (Fig. 3b). To test the relative DNA binding affinity of HESX1 and S170L HESX1, we performed equilibrium competition experiments (24,25) in which DNA binding affinity is measured by the concentration of unlabelled probe required to compete for the formation of a protein–DNA complex on trace amounts of the same radioactively labelled oligonucleotide. The occupancy...
of the labelled oligonucleotide is monitored from a starting point of ~80% in the absence of cold competitor. The probe for this analysis was the palindromic PIII sequence, which has been previously shown to be bound by HESX1 with high affinity (22). For the wild-type HESX1 protein, occupancy remained at 80% for concentrations of unlabelled competitor probe between 1 and 16 nM (Fig. 3b, lanes 3–7). A reduction in occupancy was observed using 32 nM competitor (Fig. 3b, lane 8), and at 128 nM competitor occupancy had reduced to 0% (lane 10). In contrast, the mutant protein maintained 80% occupancy at much higher concentrations of competitor probe (up to 307 nM) (Fig. 3b, lane 22) and required 4.9 µM competitor probe to reduce the occupancy to 0% (lane 26). These data suggest that S170L HESX1 binds significantly less well, in the order of 10-fold, than the wild-type protein. The reduced binding activity of the mutant protein supports a functional role

**Table 1. Summary of patient phenotypes and mutations**

<table>
<thead>
<tr>
<th>Patientsa</th>
<th>Mutation</th>
<th>Protein</th>
<th>GH</th>
<th>TSH</th>
<th>ACTH</th>
<th>LH/FSH</th>
<th>ON</th>
<th>CC/SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/II.1</td>
<td>C509T</td>
<td>S170L</td>
<td>Deficient</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Hypoplastic</td>
<td>+</td>
</tr>
<tr>
<td>1/II.2</td>
<td>C509T</td>
<td>S170L</td>
<td>Deficient</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2/II.1</td>
<td>A541G</td>
<td>T181A</td>
<td>Deficient</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3/II.1</td>
<td>G18C</td>
<td>Q6H</td>
<td>Deficient</td>
<td>Deficient</td>
<td>+</td>
<td>Deficient</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+, normal; GH, growth hormone; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotropic hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; ON, optic nerve; CC, corpus callosum; SP, septum pellucidum.

*Pedigree/ID.

bFrom age 7 years.

**Figure 3.** (a) Schematic representation of the HESX1 gene showing the position of the three mutations identified in this study. Boxes denote exons that are numbered underneath. The coding region is lightly shaded and the homeobox is represented by dark shading. (b) Gel shift analysis of the wild-type HESX1 and S170L HESX1 proteins showing the reduced binding affinity of the mutant protein. Plus and minus symbols indicate the presence and absence of protein and unlabelled competitor probe ([P3]), respectively. The diagonal line indicates increasing concentrations of unlabelled probe which were 2-fold increments from 1 nM (lane 3) to 128 nM (lane 10) for the wild-type protein and 0.6 nM (lane 13) to 19.7 µM (lane 28) for the mutant protein.
for S170 in DNA binding of wild-type HESX1, and indicates that the S170L mutation in heterozygous individuals is functionally compromised.

DISCUSSION

It is notable that only one of the affected heterozygous individuals in this study has a mild form of SOD. The three remaining affected individuals have variable degrees of hypopituitarism which, in its mildest form, is recognized as isolated GH deficiency. The relatively mild phenotypic expression in the heterozygote patients identified in this study contrasts with the severe form of SOD previously described in homozygote individuals (22). We believe that the most likely explanation for this difference in phenotype is that heterozygous HESX1 mutations in humans result in mild forms of pituitary disease with or without recognizable midline defect in forebrain structures. This is supported by the observation of a mild phenotype in heterozygous Hesx1 neonate mice (22). Furthermore, as in humans, the mouse heterozygous phenotype is incompletely penetrant, with obvious symptoms occurring in only 1% of the neonates. This phenomenon of semidominance (i.e. milder phenotype expression in heterozygotes compared with homozygotes), with incomplete penetrance of a heterozygous phenotype, has also been described for at least three other congenital disorders resulting from homeobox gene mutation, namely symplectydaactyly (HOXD13) (26), Leber congenital amaurosis (CRX) (27) and sacral agenesis (H9B) (28). We therefore suggest that gene dosage-related phenotypic expression may be a general feature of congenital disorders resulting from mutations in developmental homeobox genes, at least in individuals that survive to term.

How could this semidominant mode of inheritance and incomplete penetrance be explained? One model is that heterozygous mutations reduce the wild-type HESX1/Hesx1 levels in embryonic cells to concentrations around the threshold required for normal embryogenesis. Due to the stochastic nature of gene transcription, HESX1/Hesx1 protein levels below this threshold may occur at critical stages during early development, resulting in a lack of cellular proliferation and/or inappropriate differentiation of forebrain and pituitary primordia. The early inception of such embryonic defects as demonstrated in the Hesx1 null mice (22) could then become amplified during embryogenesis, so that mild deficiencies are manifest at birth as significant hypoplasia. Since Hesx1/HESX1 is implicated in the original allocation of tissue in the prospective forebrain, followed by the later restriction of lateral anterior structures both within and between individual Hesx1 mutant embryos and pups (22). Our conclusion based on human and mouse data is that all individuals who are heterozygous for HESX1 mutations are at risk of developing a phenotype of pituitary hypoplasia due to the reduced cellular concentration of functional Hesx1/HESX1 protein in the embryonic pituitary. However, it remains possible that either modifier genes or as yet unidentified environmental factors also exert an influence.

All three of the HESX1 mutations that we have identified result in non-conservative amino acid changes, consistent with the possibility that these mutant proteins are functionally compromised. To investigate the functional consequence of one of these mutations (C509T), we performed gel shift analysis of the S170L HESX1 protein, which revealed a significant reduction in DNA binding activity compared with wild-type HESX1. The S170L substitution is located immediately C-terminal to the homeodomain in an RESQFL motif, which is completely conserved in, and unique to, HESX1/ANF homeoproteins. It is interesting that despite the location of this mutation outside the homeodomain, DNA binding activity of the S170L protein is compromised, indicating that this motif is important for homeoprotein–DNA interactions. Recent structural studies of Pbx1 class homeodomains (29, 30) highlight the importance of homeodomain flanking residues/motifs and may provide insight into the functional role of the RESQFL motif. Pbx class homeodomains, which bind as heterodimers with Hox class proteins, also contain a conserved stretch of residues C-terminal to the homeodomain. In Pbx1 these residues fold into a fourth α-helix that forms an integral part of the homeodomain binding complex by making specific contacts with homeodomain residues. Interestingly, replacement of the F298 residue within the Pbx homeodomain C-terminal tail, which occupies the same relative position to the homeodomain as the HESX1 S170 residue, results in reduced homodimeric and cooperative DNA binding activity (31). This suggests that, in addition to the reduction in DNA binding activity of the S170L homodimer, interaction with partner proteins such as PROP1 (17) may also be compromised by this mutation, and raises the possibility that this protein may have a dominant negative effect. On this point it is worth noting that two of the three individuals in pedigree 1 who are heterozygous for the C509T mutation (S170L) are affected. In contrast, none of the nine heterozygous individuals who carry the C478T substitution, which generates a protein that is incapable of binding to the PIII sequence, display a pituitary phenotype (22). It is therefore possible that the greater penetrance of the C509T mutation is due not only to the reduced DNA binding activity of the S170L HESX1 protein but also to the inappropriate interaction of the mutant protein with binding partners (which may include other homeoproteins) and pituitary gene regulatory sequences.

This study broadens the role of HESX1 from severe forms of familial SOD to much milder and more common forms of pituitary hypoplasia. Future studies focusing on patients with sporadic and familial pituitary hypoplasia, in particular isolated GH deficiency, will be required to assess the relative contribution of heterozygous HESX1 mutations to the manifestation of this disorder. Since Hesx1/HESX1 is expressed initially in the prospective forebrain, followed by the later restriction of
expression to Rathke’s pouch, it is conceivable that alterations in the expression pattern of these two phases accounts at least partly for the variability of the SOD phenotype. Additionally, misregulation of the gene may arise as a result of changes in pituitary and forebrain-specific promoters/enhancers, which would not have been identified in this study.

MATERIALS AND METHODS

Patient phenotypes

Pedigree 1. Individual II.1 was born at 39 weeks gestation with a birth weight of 2.61 kg. He had an uneventful neonatal course apart from mild jaundice. During the first year, slow growth and a poor sleeping pattern were noted, with frequent waking for feeds at night. His development was noted to be slow with mildly autistic social behaviour and dysmorphic features were noted. These included very small hands and feet, simian creases, a low nasal bridge with a down-turned mouth, frontal bossing and mild syndactyly of the second and third toes. He presented with a left convergent strabismus at the age of 3 years, when bilateral dysplastic optic discs were also noted. His vision was, however, reported to be normal, as was a magnetic resonance imaging (MRI) scan of his brain. His height was well below the third centile (85.5 cm at the age of 3.8 years). Glucagon stimulation revealed a suboptimal peak GH response (peak 14.5 mU/l) with normal cortisol, TSH and prolactin responses. GH therapy was instituted at 15 IU/m²/week with an excellent response (increase in height velocity from a basal of 5.3 to 10.6 cm/year during the first year). He continued to grow well, and at the age of 7 years his height was 113.2 cm (10th centile). In view of fatigue, the glucagon test was repeated at the age of 6 years with a peak GH of 6.5 mU/l and with normal cortisol and TSH responses. He requires educational assistance at present.

Individual II.2 was born at 39 weeks gestation with a birth weight of 3.4 kg. He developed mild neonatal jaundice, but had an otherwise unremarkable neonatal course. He woke frequently at night for feeds. He was referred at the age of 12 months with poor growth and a disproportionately large head. His length was below the 3rd centile with a head circumference in the 90th, and with clinical features of severe GH deficiency. Investigations revealed a normal MRI scan of the brain, undetectable levels of insulin-like growth factor 1 (IGF-1), low levels of insulin-like growth factor binding protein 3 (IGFBP3) (0.8 mg/l), a peak GH response to glucagon provocation of 43.9 mU/l and normal cortisol and thyroid hormone secretion. An IGF-1 generation test showed an appropriate increase in IGF-1 and IGFBP3. In spite of the normal GH response to provocation, the diagnosis of GH deficiency was felt to account for his suboptimal growth velocity and the low values of IGF-1 and IGFBP3. He was therefore commenced on 15 IU m²/week of human growth hormone (hGH) and mounted an excellent response to hGH in terms of growth and an increase in IGF-1 and IGFBP-3. He had no dysmorphic features and his optic discs and vision have been reported to be normal.

Pedigree 2. Individual II.1 was born at full term by forceps delivery. After an uncomplicated neonatal period, she presented at 3 years 11 months with short stature (height 89.7 cm, <1st centile), poor appetite and high energy. A glucagon stimulation test showed severe GH deficiency with a basal GH level of <1 mU/l rising to a maximum of 3 mU/l. TSH and ACTH levels were within the normal range. MRI imaging revealed hypoplasia of the anterior pituitary and absence of the posterior pituitary bright spot. The optic nerves, optic chiasm and midline brain were normal in appearance. She commenced GH therapy at 5 years 4 months with a height of 95 cm (<<1st centile). She has responded well and at her last appointment (May 1999) was 140.5 cm (25th centile). She has also recently entered puberty.

Pedigree 3. Individual II.1 was born by vaginal delivery (birth weight 3.68 kg) after an uncomplicated pregnancy. He presented to the endocrinology clinic aged 6 years and 3 months with short stature (height 98.7 cm, <1st centile) and a small penis. An arginine-insulin stimulation test revealed profound GH deficiency and normal levels of LH, FSH, ACTH and TSH. GH replacement therapy commenced at this stage, to which he has responded well. Secondary hypothyroidism was evident at age 7 years (low TSH and T4) and replacement therapy was commenced at this time. A computed tomography scan at 7 years and 4 months revealed normal optic nerve structures and no visual problems are evident. An MRI scan at 9 years and 2 months showed anterior pituitary hypoplasia and an ectopic posterior pituitary. No midline brain defects were apparent.

Patient recruitment and mutation scanning

A total of 33 patients (13 with SOD, 17 with isolated/combined AP hormone deficiency, two with GH deficiency with single central incisor and one with holoprosencephaly) were recruited via the Endocrinology Clinic, Royal Children’s Hospital, Melbourne. Sixteen patients with SOD were recruited via the SOD/ONH Internet Support Group (http://members.tripod.com/~roses8/index.html). These 49 patients were scanned for mutations by direct cycle sequencing using exon flanking primers (22) of a 1.8 kb template spanning the entire coding region amplified from genomic DNA extracted from either venous blood or buccal cell samples. The HESX1 template was generated using the 1F and 4R primers (22) using the following cycle: 95°C for 3.5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 50 s with a final extension at 72°C for 5 min. This analysis identified the A541G and G18C mutations. All remaining patients (76 with SOD, 68 with isolated/combined AP hormone deficiency and 35 with holoprosencephaly or related disorders) were recruited via the Great Ormond Street Hospital for Sick Children, Middlesex Hospital Endocrine Clinics and other sources. Mutation scanning of these patients was performed using single-strand conformation polymorphism analysis and subsequent cloning and sequencing of PCR products as described previously (22). The C509T mutation was identified using this approach.

YAC screen

HESX1 3F and 4R primers (22) were used to PCR screen the 23 CEPH megaYAC “B” block pools (Research Genetics) identifying three positive pools (nos 25, 40 and 43). Subsequent amplification of block number plate DNA corresponding to...
these pools identified three HESX1-positive YACs. PCR was performed using standard conditions (including 1.5 mM MgCl2) with the following profile: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s with a final extension at 72°C for 5 min.

**Gel shift**

Preparation of recombinant proteins and electrophoretic mobility shift assays were performed as described previously (32).

**Haplotype analysis**

Primer sequences and amplification conditions were obtained from the genome database (http://gdbwww.org:80/). Haplotype analysis was performed using standard conditions (including 1.5 mM MgCl2) with the following profile: 95°C for 30 s, 60°C for 30 s and 72°C for 30 s with a final extension at 72°C for 5 min.

**Conclusions**

We would like to thank Sherry Cook and Elaine Doherty for excellent technical assistance. We also wish to extend our thanks for all the families participating in this study. P.T. is a Ramaciotti Foundation. National Health and Medical Research Council (NH and MRC) Howard Florey Centenary Research Fellow (Reg. Key No. 987209). This work was also supported by a grant from the Ramaciotti Foundation.

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