DEVELOPMENT AND DISEASE

Molecular effects of novel mutations in Hesx1/HESX1 associated with human pituitary disorders

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SUMMARY

The homeobox gene Hesx1/HESX1 has been implicated in the establishment of anterior pattern in the central nervous system (CNS) in a number of vertebrate species. Its role in pituitary development has been documented through loss-of-function studies in the mouse. A homozygous missense point mutation resulting in a single amino acid substitution, Arg160Cys (R160C), is associated with a heritable form of the human condition of septo-optic dysplasia (SOD). We have examined the phenotype of affected members in this pedigree in more detail and demonstrate for the first time a genetic basis for midline defects associated with an undescended or ectopic posterior pituitary. A similar structural pituitary abnormality was observed in a second patient heterozygous for another mutation in HESX1, Ser170Leu (S170L). Association of S170L with a pituitary phenotype may be a direct consequence of the HESX1 mutation since S170L is also associated with a dominant familial form of pituitary disease. However, a third mutation in HESX1, Asn125Ser (N125S), occurs at a high frequency in the Afro-Caribbean population and may therefore reflect a population-specific polymorphism.

To investigate the molecular basis for these clinical phenotypes, we have examined the impact of these mutations on the regulatory functions of HESX1. We show that Hesx1 is a promoter-specific transcriptional repressor with a minimal 36 amino acid repression domain which can mediate promoter-specific repression by suppressing the activity of homeodomain-containing activator proteins. Mutations in HESX1 associated with pituitary disease appear to modulate the DNA-binding affinity of HESX1 rather than its transcriptional activity. Wild-type HESX1 binds a dimeric homeodomain site with high affinity (Kd 31 nM) whilst HESX1(S170L) binds with a 5-fold lower activity (Kd 150 nM) and HESX1(R160C) does not bind at all. Although HESX1(R160C) has only been shown to be associated with the SOD phenotype in children homozygous for the mutation, HESX1(R160C) can inhibit DNA binding by wild-type HESX1 both in vitro and in vivo in cell culture. This dominant negative activity of HESX1(R160C) is mediated by the Hesx1 repression domain, supporting the idea that the repression domain is implicated in interactions between homeodomain proteins. Our data suggest a possible molecular paradigm for the dominant inheritance observed in some pituitary disorders.

Key words: Repression, Homeodomain, Pituitary, Hesx1/HESX1, Transcription

INTRODUCTION

The elaborate spatial and temporal patterns of gene expression generated during vertebrate embryogenesis are mediated by a large array of transcription factors. Homeodomain proteins constitute a major class of transcription factors responsible for this refined program of embryonic gene expression [reviewed by Duboule (Duboule 1994)]. Members of this family are characterised by the possession of a tripartite helical homeodomain, which recognises the core binding site TAAT and mediates the primary component of homeodomain protein functional specificity [reviewed by Gehring (Gehring et al., 1994)]. Further specificity in homeodomain-DNA interactions is generated by the ninth residue in the third helix of the homeodomain, which binds the two bases immediately 3’ to this core (Schier and Gehring, 1992; Treisman et al., 1989; Wilson et al., 1993). However, the broad classes of specificity generated by recognition of the TAAT core and its flanking sequences does little to define specific DNA sequences recognised by individual homeodomain proteins (Desplan et
The Paired (prd) class of homeodomain proteins binds to DNA cooperatively as dimers (Wilson et al., 1993). Some members of this family are characterised by the possession of an additional DNA-binding domain (the Prd domain), e.g. Drosophila prd and the Pax genes, while other members of this family contain only a prd class homeodomain, e.g. Hesx1, Goosecoid (Gsc). The prd homeodomain is known to bind cooperatively to adjacent TAAT cores and the spacing between cores preferred by specific prd domain proteins also depends on the ninth residue of helix three of the homeodomain.

The prd homeodomain protein HESX1 (also known as Rpx) was recently implicated in the human disease Septo-Optic Dysplasia (SOD) (Dattani et al., 1998). Hesx1 is expressed early in mouse development, beginning with a small patch of cells in the anterior midline visceral endoderm (Hermes et al., 1996; Thomas and Beddington, 1996). Expression later continues in the prospective forebrain and is then restricted to Rathke’s pouch, the primordium of the anterior pituitary. Mice homozygous for a null mutation in the Hesx1 locus display variable defects in the anterior CNS such as micro- or anophthalmia, and agenesis of midline forebrain derivatives, including the corpus callosum and septum pellucidum. They also exhibit pituitary dysplasia. The phenotype of the Hesx1 null mice is similar to human SOD, which consists of congenital panhypopituitarism, optic nerve hypoplasia and/or abnormalities of midline brain structures. A familial form of SOD has recently been described in association with a homozygous mutation in the HESX1 locus (Dattani et al., 1998).

Plasmid construction

Gal4-Hesx1 fusion proteins were constructed identically to the previously described Gal4-Hex fusions (Brickman et al., 2000). The sequence upstream of the Hesx1 ATG was modified by polymerase chain reaction (PCR) to ensure the presence of an optimal Kozak sequence flanked by an EcoRI site (5′-GGCGGACCCACTG-GTTGCTCCAGGCTTTC-3′). The stop codon was mutated and flanked by a BamHI site (5′-GGCGGACCCGAGCCCTTTCTGG-3′). A series of GAL4-VP vectors constructed in pBGX-1 (Emami and Carey, 1996) were used to construct Hesx1-VP2 and VP4 fusions using the same PCR product. pBME108 expresses the full length GAL4-Hesx1 fusion while pBME117/119 expresses GAL4-Hesx1-VP2/VP4. All deletions were generated by a PCR based strategy using an analogous set of oligonucleotides to those used to construct pBME108. The GAL4-Gsc constructs were made by amplifying the relevant fragments of Xenopus Gsc. Reporter constructs employing reiterated 17-mers upstream of the SV40 and E4 promoters were made as previously described (Brickman et al., 2000). The G5E4 reporter was a gift from Dr S Harrison. (P3)αE4 and CDNA3-Bix1 were gifts from Dr M. Tada.

Cell culture and transfections

Feeder-independent ES cells were maintained in gelatinised flasks in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal calf serum and leukaemia inhibiting factor (Smith et al., 1988). ES cell lines were E14.2 (Fisher et al., 1989) and CGR8 (Mountford et al., 1994). Transient transfections were performed as described previously (Brickman et al., 2000). COS-7 cells were cultured in DMEM containing sodium pyruvate supplemented with 10% fetal calf serum. Western blots on transfected COS cells were performed by transferring 1.2 μg of the indicated expression vector into a 1.0 cm dish by lipofection using Lipofectamine (Gibco BRL) according to the instructions of the manufacturer. Extracts were made in RIPA with gentle agitation at 4°C for 30 minutes. Lysates were clarified and diluted with 2X Laemmli sample buffer. Blots were probed with α-VP16 antibody (Santa Cruz) at a concentration of...
200 ng/ml and an &alpha;-mouse horseradish peroxidase antibody (Sigma) at a concentration of 100 ng/ml. They were visualised by chemiluminescence using an ECL kit (Amersham) according to the instructions of the manufacturer.

**Purification of recombinant proteins**

All HESX1 proteins were expressed and purified as previously described using derivatives of the plasmid pBE21, which contains ten histidine residues upstream of an amino-terminal poly-linker (Dattani et al., 1998). Briefly, insoluble material from lysates of *Escherichia coli* strain BL21(DE3) was solubilised in 20 mM Tris pH 8.0, 100 mM KCl, 20% (w/v) glycerol, 6 M urea, 1 mM phenylmethylsulfonyl fluoride and 1 mM imidazole and fractionated by nickel (Ni²⁺) affinity chromatography. HESX1-containing fractions were eluted in a stepwise manner using increasing concentrations of imidazole. The proteins were then renatured by dialysis against 50 mM Tris pH 8.0, 600 mM NaCl, 10 mM MgCl₂, 20% (v/v) glycerol, 0.1% Nonidet P40, 10 mM diithiothreitol and 1 mM phenylmethylsulfonylfluoride containing decreasing amounts of urea. Electrophoretic mobility shift assays (EMSA) were performed as previously described (Brickman et al., 1999). The oligonucleotides used in the EMSA assays were 5′AGCTTGGAGTCTAA TTGAA-3′ for the P3 site and 5′TCGACCAATTTAATTCGAGATTATTC-3′ and 5′GATCATGATAATCCCTGGGAAATTAAGCA-3′ for the monomeric site in the *Gsc* promoter. Anti-His antibody was pre-incubated with gel shift reactions as in Fig. 2C at a concentration of 12.0 ng/μl.

**Determination of equilibrium constants**

Equilibrium constants were calculated for the binding of the various recombinant forms of the protein to target DNA sequences using standard methods. Briefly, single-stranded DNA was synthesised and purified using a Phenyl Sepharose FPLC column (Pharmacia-Biotech). Following initial purification the hyperchromicity of each single strand was determined using UV spectrophotometric methods and phospho-triesterase degradation. Following accurate determination of DNA concentration, complementary strands were annealed. Duplex DNA was again purified using a Phenyl Sepharose column, and concentrations determined as described above. Double-stranded oligonucleotides were labelled by T4 kinase with [γ-32P]ATP. Excess unincorporated label was removed using G25 spin columns (Pharmacia-Biotech). For a typical binding reaction, 0.1-1 ng of DNA was incubated with various concentrations of recombinant protein, and the reaction mixture loaded onto a pre-electrophoresed acrylamide gel (30 minutes at 90V in 1× TAE). Once loaded, the gels were run for a further 40-50 minutes at a constant current (110 mA). Once dried, gels were visualised by autoradiography, and the level of bound and unbound species was determined using phosho-imaging techniques.

**Patient recruitment and mutational screening**

A total of 461 patients (163 with classical septo-optic dysplasia, 223 with variable pituitary hormone deficiencies, 9 with optic nerve hypoplasia, 3 with midline neuroradiological abnormalities and 63 with a variety of midline disorders such as holoprosencephaly) were recruited from Great Ormond Street Hospital for Sick Children (GOSH), University College London Hospitals (UCLH) and a variety of other sources. The majority of cases were sporadic apart from the two pedigrees described below and eight other families with variable degrees of SOD and Combined Pituitary Hormone Deficiency (CPHD). Ethical Committee approval for the study was obtained at both GOSH and UCLH. Mutation screening was performed using PCR followed by single-stranded conformational polymorphism (SSCP) as described previously (Dattani et al., 1999; Thomas et al., 2001). Samples showing any band shifts on SSCP analysis were then sequenced. We originally described a homozygous missense mutation C478T (R160C substitution) in two siblings with SOD from a highly consanguineous pedigree (Dattani et al., 1999). More recently, we have described three non-conservative missense heterozygous mutations, namely C509T in pedigree 2 (S170L substitution), A541G (T181A substitution) and G18C (Q6H) (Thomas et al., 2001). The C509T and A541G mutations were not found in 140 control chromosomes, and the G18C mutation was not observed in 100 control chromosomes. The inheritance pattern was autosomal dominant with variable penetrance. Additionally, we have documented a heterozygous A374G (N125S) change in 5 children of Afro-Caribbean descent, all of whom have variable SOD phenotypes. We have identified this as a probable polymorphism in the Afro-Caribbean population (see below).

**RESULTS**

**Patient phenotypes**

**R160C**

The phenotype of two children with a homozygous R160C substitution, born to a highly consanguineous pedigree, with panhypopituitarism, absence of the septum pellucidum and agenesis of the corpus callosum, has previously been described (Wales and Quarrell, 1996). More recently, both children have had magnetic resonance imaging performed and representative views are shown in Fig. 1B and 1C (Fig. 1). Interestingly, the two affected siblings had an ectopic/undescended posterior pituitary associated with a hypoplastic anterior lobe in addition to abnormalities of the corpus callosum and septum pellucidum. The scans revealed a degree of optic nerve hypoplasia with small optic chiasma that had no obvious clinical impact. Surprisingly the appearance of the corpus callosum and the size of the pituitary fossa differed in the two siblings. Sibling 1 has partial agenesis of the corpus callosum with a shallow pituitary fossa, whereas the MRI scan performed on sibling 2 shows hypogenesis of the corpus callosum with a well-developed sella turcica.

**S170L**

This heterozygous substitution was recently described in two siblings with isolated GH deficiency as determined clinically and by the concentrations of insulin-like growth factor 1 (IGF-1) and IGF binding protein 3 (IGFBP3) (Thomas et al., 2001). The older sibling (sibling 1), who also had mild cranio-facial dysmorphism and optic nerve dysplasia, displayed an evolving growth hormone deficiency (GHD), with a reduction in the peak GH response to glucagon stimulation from 14.5 (5.6 μg/l) to 6.5 (2.5 μg/l) mU/l over a period of 3 years. In contrast, his brother (sibling 2) had clinical evidence of severe GH deficiency with undetectable concentrations of IGF-1 and IGFBP3, but with what would be considered to be a normal GH response to glucagon stimulation (43.9 mU/l; 16.7 μg/l) at the age of 14 months. Treatment with recombinant human growth hormone (hGH) led to a dramatic increase in the growth rate. Since the description of these phenotypes, sibling 2 has been re-tested at the age of 5 years, after discontinuing his GH treatment over a 6-week period. The peak GH on re-testing was 20.4 mU/l (7.8 μg/l) using the same GH radioimmunoassay as the first test. This borderline response reflected a considerable reduction in GH secretion as compared with the original test. The thyroid-stimulating hormone (TSH) response to thyrotrophin-releasing hormone (TRH) was normal (peak 15.7 mU/l) as was the prolactin response (peak 660 mU/l). The
pituitary glands in these two children were normal on MRI scanning. A third patient with the S170L substitution has recently been identified. The patient presented at the age of 6 years with short stature, having grown with a sub-optimal height velocity since the age of 2 years. His birth weight was 3.5 kg and the delivery was unremarkable. On presentation, the patient had a height well below the 0.4th height centile (99.7 cm at 6.4 years). His vision was reported as being normal and fundoscopy revealed no abnormal findings. An insulin tolerance test combined with a TRH test was performed to assess his pituitary function, and revealed a peak GH response to hypoglycaemia of 1.1 mU/l (0.4 ng/ml), confirming a diagnosis of severe GH deficiency. His cortisol secretion was normal (peak cortisol 772 nmol/l) as were his TSH (peak 8.2 mU/l) and prolactin (peak 799 mU/l) responses to TRH. He was treated with recombinant hGH until the age of 15 years, by which time he had achieved a height of 169.7 cm, which placed him between the 9th and 25th centiles (mid-parental centile 25th). He progressed through puberty normally. His hypothalamo-pituitary axis was re-tested following cessation of the GH treatment and he achieved a peak GH response of 0.9 mU/l (0.36 ng/ml) on re-testing, with normal thyroid function and cortisol concentrations. Magnetic resonance imaging of his brain and pituitary gland revealed an atrophic posterior lobe of the pituitary gland that had not descended into the fossa. The pituitary stalk was very thin with a poorly enhancing anterior pituitary gland that is hypoplastic and does not enhance well. The pituitary stalk is thin, but the optic chiasm (oc) is normal as is the corpus callosum (cc).

The heterozygous S170L mutation was clearly associated with highly variable phenotypes in terms of the presence of optic nerve hypoplasia and MRI scan appearances. However, it is noteworthy that all of the affected individuals demonstrated a defect in GH secretion with no other pituitary hormone deficit, suggesting that somatotropes may be particularly vulnerable to perturbations in HESXI.

N125S
Five of our 461 patients were heterozygous for the N125S substitution in HESX1. All of these patients were of an Afro-Caribbean background. This substitution is probably a frequent polymorphic variant within this particular ethnic group. Screening an unaffected Afro-Caribbean population for this polymorphism revealed 5 homozygotes for the wild-type allele, 17 homozygotes for the N125S substitution and 20 heterozygotes for the substitution. However, this mutation may not be entirely silent since the substitution of a serine residue by proline at the analogous position 18 in the homeodomain of Prop1 has previously been implicated in the phenotype of the Ames dwarf mouse (Sornson et al., 1996).

### DNA-binding properties of Hesx1/HESX1 and HESX1 mutants
We have previously shown that the R160C substitution in HESX1 resulted in a loss of DNA binding, even at micromolar concentrations, as compared with the wild-type protein which had a high DNA-binding affinity for the consensus P3 DNA sequence (Wilson et al., 1993), a synthetic palindrome shown to bind prd homeodomain proteins (Table 1). To further

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_d$ at P3 (nM)</th>
<th>$K_d$ at GBS (nM)</th>
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<tr>
<td>HESX1</td>
<td>31</td>
<td>180</td>
</tr>
<tr>
<td>HESX1 (N125S)</td>
<td>20</td>
<td>78</td>
</tr>
<tr>
<td>HESX1 (S170L)</td>
<td>150</td>
<td>1000</td>
</tr>
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The sequences of these sites and sample EMSA experiments can be found in Fig. 2.
examine the molecular consequences of point mutations associated with both heritable and sporadic forms of SOD, we over-expressed and purified from bacteria wild-type HESX1, HESX1(S170L), HESX1(R160C) and HESX1(N125S). Fig. 2 shows the binding of the HESX1(S170L) and HESX1(N125S) proteins to two classes of homeodomain site, the dimeric P3 site and a naturally occurring site, known as GBS, from the Brachyury promoter, which is thought to bind paired class homeodomains as monomers (Latinkic and Smith, 1999). Table 1 shows the dissociation constants for the binding of the various proteins to these two sites. Fig. 2C shows a ternary complex formed by HESX1 and an antibody to the amino-terminal His tag. The presence of this antibody appears to stabilize the DNA-protein complex formed by both the wild-type HESX1 and HESX1(S170L).

HESX1(R160C) did not bind DNA even in the presence of the α-His antibody, confirming our previous observation (Dattani et al., 1998). Moreover, HESX1(N125S) did not appear to interact with the α-His antibody under native conditions. This is not a result of the removal of the His tag since a western blot using the same antibody confirmed the presence of the His-tag in a protein preparation that is 80% homogeneous (data not shown). Interestingly, HESX1(N125S) migrated faster in EMSA than the wild-type protein (Fig. 2B), suggesting that the substitution of asparagine by serine resulted in a difference in either the conformation or the charge of the protein. HESX1(N125S) also appeared to bind DNA with a slightly higher affinity than wild-type HESX1 (Fig. 2B; Table 1).

**HESX1/Hesx1 is a transcriptional repressor and contains a 36 amino acid repression domain**

In order to further characterise mutations in HESX1 associated with SOD we extended our functional studies from DNA binding in vitro to transcriptional regulation and DNA binding in vivo as assayed by transient transfection assays. Fig. 3A shows that Hesx1 is a transcriptional repressor when fused to the DNA-binding domain of GAL4. DNA encoding GAL4-Hesx1 was co-transfected into ES cells together with a reporter containing GAL4 DNA-binding sites upstream of the SV40 promoter. Identical results were obtained in COS cells (data not shown). Fig. 3A shows that the repression activity mapped to 36 amino acids (aas) at the amino terminus of Hesx1 in a region that is highly conserved across species boundaries. Deletion or removal of this region from Hesx1 resulted in a dramatic reduction in repression by GAL4-Hesx1, but had no...
noticeable effect on the levels of Hesx1 expressed in transfected COS cells as determined by western blot analysis (Fig. 4). This region contains a stretch of seven amino acids, FSIESIL, previously implicated in transcriptional repression by Engrailed and Goosecoid, the eh-1 or Engrailed homology domain. A point mutation in eh-1 resulting in the substitution of the conserved Phe by Glu significantly compromises its activity (Smith and Jaynes, 1996; Tolkunova et al., 1998). Fig. 3A shows that mutation of the analogous position in Hesx1, Hesx1F21A, resulted in a loss of repression activity by GAL4-Hesx1.

Consistent with the localisation of repression activity to this conserved amino terminal domain, we find that GAL4-HESX1 (human as opposed to mouse), GAL4-HESX1(R160C), GAL4-HESX1(N125S) and GAL4-HESX1(S170L) were equally able to repress transcription stimulated by the SV40 promoter (Fig. 3B). Moreover, another eh-1 containing protein, Gsc also contains a 40 aa peptide capable of repressing SV40 (Fig. 3C).

Fig. 3D shows that a completely different result was obtained when these GAL4 fusion proteins were assayed from the minimal Adenovirus E4 promoter element. When a minimal 49 amino acid Hesx1 peptide fused to GAL4 was assayed off this promoter, it had no effect on transcription, whereas the same fragment repressed SV40 transcription 6-fold. Repression by HESX1 therefore appears to be a promoter-specific phenomenon, and presumably involves interaction with partner proteins since the SV40 promoters contains several consensus homeodomain sites while the E4 promoter does not.

We therefore tested the ability of Hesx1 to repress transcription from the minimal Adenovirus E4 promoter containing an upstream P3 site when it was activated by a
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Co-transfection of DNA encoding full-length Hesx1, together with expression vectors for paired class activators of the Mix family (Germain et al., 2000), Bix (Tada et al., 1998), Mix-1 (Mead et al., 1996) and Mixer (Henry and Melton, 1998) led to a dramatic repression of these transcriptional activators (Fig. 5A). Hesx1 was able to repress transcription activated by Bix 91-fold. However, when DNA encoding Bix was co-transfected with a plasmid expressing a Hesx1 deletion derivative lacking the repression domain, Hesx1(50-185), Bix-mediated activation was repressed by only up to 4.2-fold, even when DNA encoding Hesx1(50-185) was present in equimolar amounts to that encoding the Bix activator (Fig. 5B). When DNA expressing full-length Hesx1 was present at 10-fold lower levels than those of the vector expressing the Bix activator, activation by Bix was still significantly repressed (greater than 10-fold) whereas DNA expressing Hesx1(50-185) had no significant effect on Bix activity at these levels (Fig. 5B). Since the quantity of Hesx1 required for these effects appears well below that required to saturate for DNA binding, we refer to this as cooperative repression.

One simple interpretation of these results is that Hesx1(50-185) binds DNA less well than the full-length protein and that the loss of repression by Hesx1(50-185) is the result of reduced DNA-binding affinity in vivo. To control for this possibility, we have employed a tripartite fusion protein in which Hesx1 was fused to both the DNA-binding domain of GAL4 and the transcriptional activation domain of VP16. Hesx1, Hesx1(50-185) and HESX1 mutant proteins were fused to a modular activation domain derived from the Herpes Simplex Virus transactivator, VP16 (Emami and Carey, 1992). This fusion contains homeodomain-containing activator.
reiteration of a modular activation unit that has been used to convert homeodomain-containing transcriptional repressors to activators without removing their endogenous repression domain (Brickman et al., 2000). The construction of these fusion proteins is illustrated in Fig. 6A. Fig. 6A shows that the tripartite fusion protein GAL4-Hesx1-\(\lambda\)VP4 was a potent activator of transcription when bound to GAL4 binding sites positioned upstream of the SV40 promoter. The activity of the tripartite fusion GAL4-Hesx1-\(\lambda\)VP4 was the same as that of GAL4-Hesx1(50-185)-\(\lambda\)VP4. This is consistent with the expression level of these proteins as determined by western blots on transfected COS cells (Fig. 4). Moreover, when these tripartite proteins were tested from P3 site containing reporters (Fig. 6B) similar results were obtained, at least when the tripartite proteins were present at limiting concentrations. Thus when lower levels of either Hesx1 or Hesx1(50-185) expression vectors were transfected alongside the P3 site containing reporter, they activate transcription to similar degrees, suggesting that the removal of the N terminus in the context of this fusion protein does not impair binding to the P3 DNA element.

**Dominant negative activity of HESX1(R160C) requires the presence of the Hesx1 repression domain**

Our previous data have shown that HESX1(R160C) cannot bind DNA in its own right. However, we tested its binding in the context of a heterodimer, fully anticipating that it would not affect the ability of the wild-type protein to bind target DNA since all of the nine human heterozygotes for the R160C substitution did not manifest a phenotype (Dattani et al., 1998). Fig. 7A shows that addition of stochiometric quantities of HESX1(R160C)-\(\lambda\)VP4 completely blocks the activity of HESX1-\(\lambda\)VP4, but when HESX1(R160C)-\(\lambda\)VP4 is present at stochiometric or substochiometric levels (last two points in the curve), HESX1-\(\lambda\)VP4 regains partial activity on the P3 site reporter suggesting a possible 1:1 relationship between the wild-type and mutant proteins. Fig. 7B also shows that the eh-1-containing amino-terminal repression domain is required for the inhibitory effect of HESX1(R160C) as co-transfection of DNA expressing HESX1(50-185)-\(\lambda\)VP4 has no effect on the activity of HESX1-\(\lambda\)VP4.

**DISCUSSION**

We have shown that Hesx1 is a high affinity DNA-binding protein that behaves as a promoter-specific repressor. The repression domain maps to the N terminus and contains a sequence known as eh-1 that was initially identified in Engrailed and is necessary for repression. One mechanism by which eh-1 may function is via the recruitment of the Drosophila co-repressor, Groucho (Jimenez et al., 1997). In fact, a nine amino acid sequence including the eh-1 sequence from Gsc can interact with Groucho in vitro (Jimenez et al., 1999). In the case of Drosophila Gsc, eh-1 has also been shown
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mediated repression by facilitating the formation of heterodimers with other homeodomain proteins. Eh-1 is required for Gsc-mediated repression of Otd (now known as ocelliless; Oc), but not for direct repression of transcriptional activation induced by the glucocorticoid receptor (Mailhos et al., 1998).

The eh-1-containing domain from Hesx1 repressed transcription from the SV40 promoter, or transcriptional activation stimulated by prd homeodomain-containing activators, but not from the minimal Adenovirus E4 promoter. Mutations in HESX1 associated with SOD affect DNA binding, rather than transcriptional repression. One of these mutations, HESX1(R160C), has a dominant negative effect on HESX1, both in vivo and in vitro. This activity is dependent on the eh-1-containing N-terminal repression domain.

Promoter-specific/cooperative repression by Hesx1

Hesx1-mediated repression does not affect the minimal Adenovirus E4 promoter, which contains a consensus TATA box and initiator but no additional regulatory sequences (Lin et al., 1988). Hesx1 is therefore unable to mediate repression through a direct interaction with the basal transcription complex bound at the E4 promoter. However, when Prd class homeodomain-binding sites are placed upstream of the E4 promoter and a prd class homeodomain activator is added, Hesx1 can now repress transcription stimulated by the homeodomain activator. There are two possible mechanisms by which this might occur: direct competition between the two homeodomain proteins for the P3 DNA site, or direct inhibition by Hesx1 of the activity of the homeodomain-containing activator through a physical interaction. Direct competition would not explain the magnitude of repression (up to 100-fold) observed in these experiments unless there were major differences in DNA binding affinity. However, the activities of Bix and Hesx1-VP4 over a range of concentrations suggest similar DNA binding affinities for the P3 site. Assuming that these proteins do have similar DNA binding affinities, direct competition could explain the magnitude of repression observed by Hesx1(50-185) (4-fold) when Hesx1(50-185) and Bix are present at a 1:1 ratio. Hesx1 can also repress transcription when it is present at 10-fold lower levels than the Mix family activator protein suggesting that formation of a heteromeric complex with the Mix family protein on DNA is cooperative and that repression occurs via an active quenching mechanism.

The ability of Gal4-Hesx1 fusions to repress the SV40 promoter is probably due to the presence of homeodomain activators. In addition to the multiple Sp1 sites and AT rich sequences immediately adjacent to the transcription start site [reviewed by McKnight and Tjian (McKnight and Tjian, 1986)], the SV40 promoter contains two perfect consensus sites for binding homeodomain proteins of the Caudal-like and Deformed families.

Several recent studies have implicated the eh-1 sequence within engrailed (Jimenez et al., 1997; Tollkunova et al., 1998), UNC-4 (Winnier et al., 1999), and Gsc (Jimenez et al., 1999) in the recruitment of the co-repressor Groucho. General recruitment of a global co-repressor such as Groucho would imply a more direct mechanism for transcriptional repression by eh-1 and suppression of basal transcription. However, the implication of our data is that eh-1 in Hesx1 mediates an interaction with other homeodomain-containing proteins to form a complex that can serve as a promoter-specific binding surface for Groucho. The interaction between a homeodomain...
or DNA binding motif with a repression domain may be a general feature of co-repressor recruitment. For example, Dorsal, which functions as both an activator and a repressor, interacts with co-factors that modulate both its promoter-specific regulatory activity (Brickman et al., 1999) and its capacity to recruit Groucho (Dubnicoff et al., 1997; Valentine et al., 1998) via its conserved DNA binding motif, the Rel domain.

**Repression and dimerisation**

The repression domain of Hesx1 is required for the dominant negative activity of HESX1(R160C). These findings are consistent with two observations with respect to Gsc eh-1; Gsc eh-1 mediates its physical interaction with the homeodomain of Otd and heteromeric repression by Gsc eh-1 is disrupted by mutations in the Otd homeodomain that interfere with dimerisation (Mailhos et al., 1998). In contrast to the isolated prd homeodomain, thought to be a monomer in solution (Wilson et al., 1993), our experiments with the HESX1(R160C) in vivo and in vitro suggest that full-length Hesx1 forms dimers in solution, and that this is dependent upon eh-1. The requirement of eh-1 for dimerisation and repression would be consistent with a model in which eh-1 interacts with homeodomain containing proteins to form a complex that can then recruit co-repressors such as Groucho.

This is one of the first reports of binding constants for intact recombinant proteins. We have determined the $K_D$ for the binding of all proteins to both monomeric and dimeric sites. Our binding studies on the dimeric P3 site show a significant change in the dissociation constant, suggesting that the difference in $K_D$ (6-fold) between the monomeric and dimeric sites was based upon the affinity of a dimer formed in solution for the P3 target sequence.

**Implications for clinical phenotypes associated with HESX1 mutations**

Our studies have suggested a rare role for HESX1 in milder pituitary phenotypes. It is noteworthy that all of our patients who have been documented as having mutations in HESX1 have impaired GH secretion with clinical evidence of GH deficiency. Additionally, HESX1 is the only gene to date associated with an ectopic/undescended posterior pituitary gland. Mutations in PIT1, PROP1 and LHX3 are associated with a posterior pituitary in the normal position in the sella turcica (Fofanova et al., 2000; Netchine et al., 2000; Parks et al., 1999). We screened 93 individuals with an ectopic/undescended posterior pituitary and found HESX1 mutations in only 5 individuals. The descent of the posterior pituitary may therefore be dependent upon a number of developmental genes that are expressed at an early stage of pituitary organogenesis. The association of an ectopic/undescended posterior pituitary with isolated GH deficiency suggests that somatotrope differentiation/proliferation and consequent GH secretion may be particularly vulnerable to dissociation between the anterior (derived from Rathke’s pouch) and posterior (derived from neuroectoderm) lobes.

Based on our in vitro studies, we would predict that, in the absence of allelic exclusion, a patient who is heterozygous for HESX1(R160C) would have much less than 50% of active HESX1 protein. Based on dominant negative experiments with wild-type HESX1 we would predict that these patients may have as little as 10% of the normal concentrations of active wild-type HESX1 dimers. The implication of these findings is that there may be sufficient active protein to escape a phenotype in a statistically significant number of patients. These observations may be consistent with the variable penetrance of the Hesx1 targeted mutation in mice (Dattani et al., 1998). By analogy, it seems unlikely that patients heterozygous for HESX1(S170L) would display a phenotype, if the only consequence of this mutation was to reduce DNA binding of one allele by 5-fold (based on $K_D$ determined for both dimer and monomer sites). The S170L substitution is located immediately C-terminal to the homeodomain in an RESQLF motif, which is completely conserved in, and unique to, HESX1 homeoproteins. Recent structural studies of Pbx class homeodomains highlight the importance of homeodomain-flanking residues/motifs and may provide insight into the functional role of the RESQLF motif (Piper et al., 1999). Pbx class homeodomains, which bind as heterodimers with Hox class proteins, also contain a conserved stretch of residues C-terminal to the homeodomain. In Pbx 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. Replacement of the F298 residue within the Pbx homeodomain C-terminal tail (F298 is the homologous position to S170 in Hesx1) results in a reduction in both monomeric and cooperative DNA binding (Lu and Kamps, 1996). This analogy suggests that the S170L mutation is positioned such that it alters interactions between Hesx1 and its partners, suggesting a possible mode for dominant inheritance.

Modulation of the interactions between Hesx1 and partner proteins involved in normal forebrain and pituitary development may explain the variable penetrance and expressivity of the SOD phenotype (Arslanian et al., 1984). The expression patterns of members of the Mix family suggest that they may be true physiological partners of Hesx1 and thus candidates for additional mutations associated with the SOD phenotypic spectrum.

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