Hesx1 homeodomain protein represses transcription as a monomer and antagonises transactivation of specific sites as a homodimer

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Abstract

The homeobox repressor Hesx1, expressed throughout Rathke's pouch and required for normal pituitary development, has been implicated in anterior pituitary pathogenesis in man. Prolonged expression of *Hesx1* delays the appearance of anterior pituitary terminal differentiation markers in mice, particularly the gonadotroph hormones. We tested if Hesx1 could modulate gonadotrophin gene expression directly, and found that Hesx1 repressed both common alpha subunit (α GSU) and luteinising hormone β -subunit (LH β) gene promoters. Repression mapped to the Pitx1 homeodomain protein transactivation site in the proximal α GSU promoter, but did not map to the equivalent site on LH β . Hesx1 repression of the α GSU Pitx1 site was overridden by co-transfection of Pitx1. In contrast, Hesx1 antagonised Pitx1 transactivation of LH β in a dose-dependent manner. This was due to monomeric binding of Hesx1 on α GSU and homodimerisation on LH β . The homodimerisation site comprises the Pitx1 DNA binding site and a proximal binding site, and mutation of either inhibited homodimer formation. Conversion of the LH β Pitx1 DNA binding site to an α GSU-type did not promote homodimer formation, arguing that Hesx1 has pronounced site selectivity. Furthermore, mutation of the proximal half of the homodimerisation site blocked Hesx1 antagonisation of Pitx1 transactivation. We conclude that Hesx1 monomers repress gene expression, and homodimers block specific transactivation sites.

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Introduction

Homeodomain genes are crucial for controlling and specifying development of the pituitary gland (Watkins-Chow & Camper 1998, Dasen & Rosenfeld 1999). Correct spatial expression of homeodomain proteins, defined by morphogen signalling gradients, is necessary for stratifying the anterior pituitary (Treier *et al.* 1998) into the constituent cell-types: gonadotrophs, thyrotrophs, somatotrophs, lactotrophs and corticotrophs. A number of homeodomain genes have been implicated in commitment, cellular expansion and expression of terminal markers in these constituent cell-types (Bach *et al.* 1995, Gage *et al.* 1996, Hermes *et al.* 1996, Lamonerie *et al.* 1996, Sheng *et al.* 1996, Sornson *et al.* 1996, Acampora *et al.* 1998, Dattani *et al.* 1998, Tremblay *et al.* 1998, Gage *et al.*

1999, Szeto *et al.* 1999, Cushman *et al.* 2001, Thomas *et al.* 2001).

It is becoming increasingly evident that the combinatorial expression pattern of homeodomain factors is critical for regulation of pituitary gene expression. For instance, prophet of Pit-1 (Prop-1), which activates *Pit-1* gene expression in thyrotrophs, somatotrophs and lactotrophs (Sornson *et al.* 1996), is known to extinguish gene expression of another *paired*-like homeodomain factor Hesx1 (Gage *et al.* 1996, Sornson *et al.* 1996). Furthermore, *paired*-like pituitary factor-1 (Pitx1) is required for expression of all the differentiated cell markers in the anterior pituitary (Lamonerie *et al.* 1996, Tremblay *et al.* 1998, Szeto *et al.* 1999), but not for overall pituitary development, although the delayed expression of gonadotroph terminal differentiation markers clearly indicates a role for Pitx1 in this

cell-type (Szeto *et al.* 1999). The genetic targets of *Pitx1* are well defined (Tremblay *et al.* 1998, Quirk *et al.* 2001), with *Pitx1* modulating expression of pituitary terminal differentiation markers by interacting with non-homeodomain factors (Tremblay *et al.* 1999, Poulin *et al.* 2000, Lamolet *et al.* 2001).

The gene expression profiles of *Hesx1* and *Pitx1* overlap during pituitary development (Hermesz *et al.* 1996, Lanctot *et al.* 1999), but the gene targets for *Hesx1* have not been identified, and potential interaction between these factors has not been investigated. *Hesx1* is a homeodomain repressor protein *in vivo* (Ermakova *et al.* 1999) and both the N-terminal and C-terminal domains, when expressed as a GAL4 DNA-binding domain fusion protein, can recruit nuclear corepressor (N-CoR) and repress (Xu *et al.* 1998). It is known, however, that *Hesx1* gene expression is crucial for normal pituitary development in man and mouse (Dattani *et al.* 1998, Thomas *et al.* 2001), and continued expression prevents pituitary expansion prior to terminal differentiation (Gage *et al.* 1996, Wu *et al.* 1998). Furthermore, haploinsufficiency of *Hesx1*, associated with combined pituitary hormone deficiency (CPHD) in man, is as damaging as loss of function (Dattani *et al.* 1998, Thomas *et al.* 2001). Expression of *Hesx1* is initially dispersed throughout the presumptive pituitary; however this gradually regresses in a ventral to dorsal manner (Hermesz *et al.* 1996). The presumptive gonadotrophs form along the ventral axis (Treier *et al.* 1998), and continuous expression of *Hesx1* perturbs gonadotroph cell commitment (Gage *et al.* 1996, Wu *et al.* 1998). Thus, prolonged expression, or loss of expression, or changes in levels of expression of *Hesx1* homeodomain repressor are detrimental to anterior pituitary and gonadotroph function, as assessed by expression of the terminal differentiation markers, luteinising hormone β -subunit (LH β) and follicle-stimulating hormone β -subunit (FSH β). Although, on cognate palindromic binding sites, *Hesx1* can either heterodimerise with Prop-1 or homodimerise (Sornson *et al.* 1996, Dattani *et al.* 1998), the targets of this molecular interaction and the molecular mode of action of *Hesx1* are unknown.

Gonadotrophin gene transcription is controlled by a complex combination of transcription factors (Brown & McNeilly 1999) including *Pitx1* (Tremblay *et al.* 1998, Quirk *et al.* 2001). Since the expression profiles of *Hesx1* and *Pitx1* overlap

(Hermesz *et al.* 1996, Lanctot *et al.* 1999), and persistent expression of *Hesx1* perturbs expression of the gonadotrophin genes in mice (Gage *et al.* 1996) and also presumably in man (Wu *et al.* 1998), we reasoned that *Hesx1* may modulate *Pitx1* transactivation of gene expression, and that *Hesx1* may repress gonadotrophin gene expression. Thus, we have used a model system of transient transfection assay of a *Hesx1* protein expression construct and the common α -subunit (α GSU) and LH β gonadotrophin gene promoters. *Hesx1* does indeed repress expression of both the α GSU and LH β gene promoters. Furthermore, *Hesx1* homodimerised on a novel paired-like homeodomain binding site in the LH β gene promoter, and antagonised *Pitx1* transactivation. In contrast, *Hesx1* bound the α GSU *Pitx1* transactivation site as a monomer and did not antagonise *Pitx1* activated gene expression, when co-transfected. This suggests that the molecular mode of action of *Hesx1* on the gonadotrophin gene promoters is to act as a short-range monomeric repressor and antagonise transactivation as a homodimer.

Materials and methods

Cell culture and transfection assays

HeLa cells were grown in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were seeded at 50 000 cells per well in 12-well tissue culture dishes 24 h prior to transfection. On the morning of transfection, cell media were replaced and cells were transfected using Fugene 6 transfection reagent (Roche Diagnostics), in a ratio of 3 volumes of Fugene to 1 weight of DNA, according to the manufacturer's protocols. For the promoter resection experiments, 25 ng of a control β -galactosidase construct, 1.5 μ g reporter construct, 250 ng of each effector plasmid and pBluescript II (Roche Diagnostics) carrier DNA, to a total of 2 μ g, were transfected per well. For the minimal promoter experiments, 25 ng of a control β -galactosidase construct, 0.5 μ g reporter construct, between 31–125 ng of each effector plasmid and pBluescript II carrier DNA to a total of 2 μ g were transfected per well. Cells were harvested 48 h post-transfection and luciferase/ β -galactosidase activity was determined using a chemiluminescent assay (Dual Light, Perkin Elmer) in an LB96 V luminometer (Bertold Technologies, Bad Wildbad,

Germany). All transfections were carried out in triplicate, repeated three times and corrected for β -galactosidase activity. Basal promoter expression levels were normalised to 1 and expression levels were calculated relative to this. All results are expressed as the mean $\pm$ S.E.M.

Murine L-cells were grown in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were seeded at 1 500 000 cells/75 cm³ tissue culture flask 24 h prior to transfection. On the morning of transfection, cell media were exchanged for Optimem (Invitrogen). Subsequently, 30 μ g effector plasmid were transfected using Gene Porter (Gene Therapies Inc. San Diego, CA, USA) and, after 4 h, transfection media were exchanged for DMEM/10% FCS. Cells were harvested for nuclear extract preparation after 48 h.

Electrophoretic mobility shift assay (EMSA) analysis

L-cell nuclear protein extracts were harvested using a modified protocol (Schreiber *et al.* 1989). Top and bottom strand oligonucleotides were synthesised (Sigma-Genosys Ltd) and annealed before end-labelling with 3000 Ci/mmol γ -ATP using T4 polynucleotide kinase (Roche Diagnostics). A 5 μ g aliquot of nuclear extract was pre-incubated on ice for 10 min in Pitx1 binding buffer (25 mM HEPES, pH 7.6, 84 mM KCl, 5 mM dithiothreitol, 10% glycerol) with 5 μ g polydI-dC (Sigma Ltd) and Complete protease inhibitors (Roche Diagnostics) before adding 30 000 c.p.m. oligonucleotide and incubating for 30 min on ice. Complexes were resolved on 6% PAGE gels in 0.5 $\times$ TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.3) at 10 v/cm.

Plasmid DNA constructs

Resections of the ovine LH β and murine α GSU gene promoters, generated using Exonuclease III (Amersham Pharmacia Biotech), were fused upstream of a luciferase reporter in the vector pA3 LUC. Hesx1 and Pitx1 expression constructs were generated in the eukaryotic expression vector pCDNA 3 (Invitrogen). Oligonucleotides containing 3 copies of a fragment spanning the homeodomain binding site from α GSU, LH β , the mutant M3 or a synthetic consensus sequence PIII respectively, were synthesised (Sigma Genosys) with

overhanging KpnI and NheI sites and ligated upstream of a minimal thymidine kinase promoter in the vector pTAL-Luc (BD Biosciences, UK).

Bioinformatics and statistics

All DNA sequence comparisons were carried out using Wisconsin Package version 10.2, Genetics Computer Group (GCG), Madison, WI, USA, housed on the Medical Research Council's Human Genome Mapping Project server (MRC HGMP, Hinxton, UK). Statistical significance was established as $P < 0.05$ using ANOVA one-way analysis of variance on Statview 4.02 (SAS, Cary, NC, USA).

Results

Hesx1 represses transcription of the α GSU and LH β gene promoters

A -480 bp fragment of α GSU and -320 bp of LH β gene promoter were fused upstream of a luciferase reporter and transfected into HeLa cells to determine basal reporter gene expression levels, which were expressed as 1 (Fig. 1a). A Hesx1 expression vector was co-transfected with these promoters and the change in luciferase gene expression calculated relative to basal levels (Fig. 1a). Since Hesx1 repressed expression of both α GSU and LH β gene promoters, a series of resections were used to map the site of repression (Fig. 1b and c). The site of α GSU promoter repression was mapped between α GSU resections -120 and -109 bp (Fig. 1b), while co-transfection of Hesx1 did not affect basal promoter activity of either -136 or -80 bp LH β gene promoters. This paradoxically suggests that the homeodomain DNA binding site contained within the -120 bp α GSU resection could be a DNA target for Hesx1 repression, but the site encoded within the -136 bp LH β promoter was not. These two homeodomain DNA binding sites are targets for the *paired*-like homeodomain transcriptional activator Pitx1 (Tremblay *et al.* 1998). The DNA sequence of these sites differ, the α GSU Pitx1 DNA binding site 5'-CTTA-3' varies from the consensus homeodomain binding site 5'-ATTA-3' present in LH β . Therefore, this difference in repression of gene expression by Hesx1 of the two promoters, via these two homeodomain DNA binding sites, was further investigated.

Figure 1 Hesx1 homeodomain represses α GSU and LH β gene promoter activity. Hesx1 was co-transfected with either α GSU or LH β promoters into HeLa cells and luciferase activity was measured. (a) -480 bp α GSU and -320 bp LH β gene promoter activity was repressed by Hesx1. *** $P<0.001$, -480 bp α GSU vs -480 bp α GSU + Hesx1; * $P<0.05$, -320 bp LH β vs -320 bp LH β + Hesx1. (b) Hesx1 repression mapped to between -120 and -109 bp of the α GSU promoter. *** $P<0.001$, -480 bp α GSU + Hesx1 vs -480 bp α GSU and -109 bp α GSU + Hesx1; * $P<0.05$, -120 bp α GSU + Hesx1 vs -120 bp α GSU and -109 bp α GSU + Hesx1. (c) Only the -320 bp LH β promoter was repressed by Hesx1, and not the -136 or -80 bp LH β promoter resections. ** $P<0.01$, -320 bp LH β + Hesx1 vs -136 bp LH β + Hesx1; * $P<0.05$, -320 bp LH β + Hesx1 vs -80 bp LH β + Hesx1. Basal promoter expression in the absence of Hesx1 (-) was expressed as 1, and determined by dividing luciferase activity by β -galactosidase activity. Co-transfection of Hesx1 is denoted by +. The known Pitx1 homeodomain binding sites are depicted by open boxes.

Hesx1 can modulate Pitx1 transactivation of gene expression

A Pitx1 expression construct was transfected with -480, -120 and -109 bp resections of the α GSU gene promoter into HeLa cells. As expected, Pitx1 transactivated the -480 and -120 bp constructs (Fig. 2a). However, when Hesx1 was co-transfected with Pitx1, Hesx1 attenuated Pitx1 transactivation of the -480 bp α GSU construct, but did not repress, and had a variable effect on expression of the -120 α GSU resection (Fig. 2a). This experiment was repeated for the LH β gene promoter (Fig. 2b). In this instance, Pitx1 transactivated both the -320 and -136 bp gene promoters, again as expected, but this was modulated by co-transfection of Hesx1. Although Hesx1 did not repress the -136 bp LH β gene promoter *per se* (Fig. 1c), when Hesx1 was co-transfected with Pitx1, gene expression was reduced (although not significantly, a value of $P<0.07$ was obtained for -136 bp LH β + Pitx1 vs -136 bp LH β + Pitx1 +

Hesx1) but again, as for α GSU, gene expression was not repressed below basal. The insensitivity of the -80 bp LH β promoter to either Pitx1 or Hesx1 suggests that the effect is mediated via the Pitx1 DNA binding site contained within the -136 bp LH β promoter.

The novel LHβ paired homeodomain binding site is conserved across species

It was possible that the differences between Hesx1 repression of the two promoters, -120 bp α GSU and -136 bp LH β , were due to differences in the cognate DNA binding sites on the α GSU and LH β promoters. Examination of the -136 bp LH β gene promoter fragment identified a potential homeodomain DNA binding site 8 bp proximal to the Pitx1 DNA binding site 5'-ag**ATT**Agtgtccag**GTT**A-3'; both sites are shown in bold, with the putative site underlined. The DNA sequence corresponding to this novel paired

Figure 2 Modulation of Pitx1 transactivation of the α GSU and LH β promoters by Hesx1. Pitx1 or both Pitx1 and Hesx1 were co-transfected with α GSU or LH β promoters. (a) Pitx1 transactivated the -480 bp and -120 bp, but not the -109 bp α GSU promoters. Co-transfection of Pitx1 and Hesx1 down-regulated Pitx1 transactivation of the -480 bp α GSU promoter. ** $P < 0.01$, -480 bp α GSU + Pitx1 vs -480 bp α GSU and -480 bp α GSU + Pitx1 + Hesx1. * $P < 0.05$, -120 bp α GSU + Pitx1 vs -120 bp α GSU. (b) The -320 and -136 bp LH β promoters were transactivated by Pitx1. This transactivation was diminished by co-transfection of Pitx1 and Hesx1. The -80 bp LH β promoter was insensitive to both Pitx1 and Hesx1. *** $P < 0.001$, -320 bp LH β vs -320 bp LH β + Pitx1; *** $P < 0.001$, -136 bp LH β vs -136 bp LH β + Pitx1 and -136 bp LH β + Pitx1 + Hesx1; ** $P < 0.01$, -320 bp LH β + Hesx1 vs -320 bp LH β ; * $P < 0.05$, -320 bp LH β + Pitx1 vs -320 bp LH β + Pitx1 + Hesx1 and -136 bp LH β + Pitx1 + Hesx1. Results are expressed relative to basal promoter levels determined by the ratio of luciferase to β -galactosidase activity and expressed as 1, and after transfection of either Pitx1 (+) or Hesx1 (+). The Pitx1 DNA binding sites are depicted by open boxes.

Mouse LH β -115	AAAGAGATTAGTGTCTAGGTTACCCAAGCCTGTAGCCACTACTTAGTGGCCTTGCCA
Rat LH β -106	AAAGAGATTAGTGTCTAGGTTACCCAGGCCTGTAGCCTCTGCTTAGTGGCCTTGCCA
Cow LH β -97	GGGGAGATTAGTGTCCAGGTTACCCACCATGCTGCCACCC. . GGCCTTGCCG
Sheep LH β -102	GGGGAGATTAGTGTCCAGGTTACCCACCATGCTGCACCCCTCG. . GCGCTTGCCG
Human LH β -106	GGGGGATTAGTGTCCAGGTTACCCAGCATCCTATCACCTCCTGGTGGCCTTGCCG
Mouse LH β	CCCCACAACCCGCAGGTATAAAGCCAGGTGCCAAGGTAGGGAAGGTATCAAAGAA
Rat LH β	CCCCACAACCCGCAGGTATAAAGCCAAGTGCCAAGGTAGGGAAGGTA
Cow LH β	CCCCACAGCCTGCAGGTATAAGACCAGGTAAACACAGCAGGGGAGGCA
Sheep LH β	CCCCACAGCCTGCAGGTATAAGACTAGGTGAACACAGCAGGGGAGGCA
Human LH β	CCCCACAACCCGCAGGTATATAGCCAGATACACGAGGCAGGGGATGCA

Figure 3 Conservation of a second potential homeodomain binding site in the LH β promoter. LH β promoter sequences from mouse, rat, cow, sheep and human were aligned using GCG version 10-2 (Genetics Computer Group). A 19 bp sequence containing a homeodomain consensus binding element (ATTA), and a second potential homeodomain binding site (GTTA) is highly conserved across species. Transcription start sites are shown (bold).

homeodomain binding site was used in a database sequence similarity search across the LH β gene promoters of other species and the DNA sequence line-up is shown in Fig. 3. There was complete conservation of the sequence across these species.

Hesx1 can antagonise Pitx1 transactivation

To investigate the cognate DNA binding sites for Pitx1 from the α GSU and LH β gene promoters,

and the putative homeodomain DNA binding site identified in LH β , a series of constructs were built. Three copies of the DNA sequence from α GSU (5'-agCTTAgtgtgctgatccctg-3') or LH β (5'-agATTAggtgtccagGTTAc-3'), or a PIII consensus site (5'-tgagtcTAATtgaATTActgtacagc-3') known to bind Hesx1 as a homodimer (Dattani *et al.* 1998), were fused upstream of a minimal thymidine kinase promoter. Transfection of each of these DNA

Figure 4 Hesx1 can antagonize Pitx1 transactivation of the LHβ Pitx1 DNA binding site, but not αGSU. DNA constructs containing three copies of the Pitx1 DNA binding site from αGSU, LHβ and a consensus paired DNA binding site (PIII) were transfected into HeLa cells with varying amounts of Hesx1 or Pitx1 or both. (a) Hesx1 repressed the αGSU trimer. Pitx1 increased luciferase activity, and this was not affected by co-transfection of Hesx1. ** $P<0.01$, αGSU trimer vs αGSU trimer and ++ or ++++ Hesx1; * $P<0.05$, αGSU trimer vs αGSU trimer and + Hesx1. (b) Hesx1 marginally repressed the LHβ trimer. Luciferase activity was increased by co-transfection of Pitx1, and activity decreased in a dose-dependent manner when Hesx1 was co-transfected. * $P<0.05$, LHβ trimer vs LHβ trimer and ++++ Hesx1 and LHβ trimer and ++++ Pitx1. (c) PIII was insensitive to Hesx1. Pitx1 transactivated the PIII trimer, and this was not affected by co-transfection of Hesx1. * $P<0.05$, PIII trimer vs PIII trimer and ++++ Pitx1 or ++++ Pitx1 and ++ Hesx1. Relative activity was standardised to basal promoter activity, expressed as 1, and was calculated as the ratio of luciferase to a control β-galactosidase construct. Hesx1 and Pitx1 were either omitted (–) or transfected in amounts of 125 ng (++++), 63 ng (++) or 31 ng (+).

constructs with Pitx1 increased reporter gene expression, including PIII (Fig. 4a, b and c). However, Hesx1 repression of expression appeared to be graded in the three constructs. Hesx1 strongly repressed basal gene expression of the Pitx1 DNA binding site from the αGSU promoter (Fig. 4a); in contrast there was little to moderate repression of the LHβ trimer, and repression increased with increasing amounts of Hesx1 (Fig. 4b). There was minimal to no repression of PIII when Hesx1 was co-transfected (Fig. 4c). In agreement with the promoter resection data, Hesx1 did not repress or antagonise Pitx1 transactivation of the αGSU

trimer (Fig. 4a). There was variation in the amount of Pitx1 mediated transactivation when Hesx1 was co-transfected, possibly implying weak interference of Hesx1 for the same DNA binding site, or simply variation in the levels of Pitx1 stimulation. However, Pitx1 transactivation of the LHβ paired DNA binding site was clearly antagonised by Hesx1 in a dose-dependent manner (Fig. 4b). Indeed, when Pitx1 and Hesx1 were transfected in equivalent amounts, Hesx1 nearly abolished Pitx1 transactivation via the LHβ homeodomain DNA binding site. Interestingly, Hesx1 did not antagonise Pitx1 transactivation of the PIII construct (Fig.

Figure 5 Hesx1 protein binds to α GSU and LH β homeodomain DNA elements. Nuclear extracts from untransfected L-cells, or from L-cells transfected with Hesx1 or Pitx1 were used in EMSA analysis of homeodomain binding sites in α GSU and LH β . Specific bound complexes and free probe are indicated. Hesx1 bound α GSU as a monomer, and LH β as a homodimer. Pitx1 competed off Hesx1 bound to α GSU. Presence of Pitx1 did not interfere with Hesx1 homodimer formation on LH β and no Hesx1/Pitx1 heterodimer was observed on LH β . A 4% PAGE gel was used to resolve complexes bound by LH β .

4c), even though Hesx1 has been shown to homodimerise on this DNA element (Dattani *et al.* 1998).

EMSA analysis of a novel paired DNA binding site from LH β

Hesx1 differentially represses the α GSU and LH β gene promoters via the Pitx1 DNA binding site. Furthermore, Hesx1 can only antagonise Pitx1 on the paired binding site contained within the LH β promoter. Therefore, EMSA was used to analyse potential binding of Hesx1 protein to α GSU and LH β dsoligonucleotides. Hesx1 protein was isolated as part of a mouse L-cell nuclear extract and incubated with α GSU and LH β DNA elements (Fig. 5). Specific Hesx1 shifted complexes were observed for each oligonucleotide, that were distinct from endogenous L-cell complexes. The specific Hesx1 complex, shifted by the α GSU dsoligonucleotide, was smaller than that of LH β . The binding of Hesx1 to the α GSU and LH β elements was also compared and contrasted to

binding in the absence and presence of nuclear extract containing Pitx1 (Fig. 5). Although Pitx1 binding to the α GSU dsoligonucleotide was weak, there was clearly a shifted Pitx1 complex on the LH β dsoligonucleotide. There was no evidence of a larger Hesx1 complex forming on the α GSU dsoligonucleotide, confirming that a Hesx1 homodimer did not form on this site, and this monomeric Hesx1 shifted complex was competed when Pitx1-containing extract was added, as evidenced by the decrease in Hesx1 complex formation. In contrast, the Hesx1 specific homodimer complex shifted by LH β persisted when nuclear extract containing Pitx1 was added. Furthermore, although there was some weak residual Pitx1 binding to LH β , there was no evidence of a Hesx1 and Pitx1 protein heterodimer forming on this element, even on prolonged exposure of the gel.

The homodimerisation of Hesx1 on the LH β dsoligonucleotide was studied using a series of oligonucleotides with mutations in one or both sites. Either the Pitx1 site or the second site was mutated, and compared with binding of Hesx1 to α GSU (Fig. 6). EMSA experiments were undertaken with these oligonucleotides using either L-cell nuclear extracts or L-cell nuclear extracts containing Hesx1 (Fig. 6). Hesx1 bound the wild-type sequence as a homodimer, mutation of the 5'-ATTA-3' DNA binding site abolished complex formation, with weak monomer binding (Fig. 6, M1). Mutation of 5'-ATTA-3' to the Pitx1 DNA binding site in the α GSU promoter 5'-CTTA-3' also prevented homodimer complex formation (Fig. 6, M2); however, again a weak monomer complex was formed. In contrast, mutation of 5'-GTTA-3' to 5'-GGTA-3' (Fig. 6, M3) markedly reduced both homodimer and monomer complex formation, when compared with wild-type. Although prolonged exposure of the gel indicated that some homodimer did form, the complex was very diffuse and weak (data not shown). Mutation of both sites completely abrogated homodimer and monomer complex formation (Fig. 6, M4). As a comparison, there was clear formation of a monomer on the α GSU dsoligonucleotide (Fig. 6, α GSU). These results suggest that Hesx1 must bind both sites to form a homodimer, but that 5'-ATTA-3' is obligate, followed by co-operative binding to 5'-GTTA-3'. Furthermore, the lack of homodimerisation, but some monomer binding to

Figure 6 Mutational analysis of the LHβ homeodomain DNA binding site. Hesx1 homodimerisation on LHβ was investigated using dsoligonucleotides containing mutations in the homeodomain binding sites and nuclear extracts from untransfected L-cells or L-cells transfected with Hesx1. Free probe and specific bound monomer or homodimer complexes are indicated. The oligonucleotide sequences are shown above the autoradiograms. Hesx1 binds wild-type αGSU and LHβ oligonucleotides as a monomer and homodimer respectively. Mutation of either half of the LHβ homeodomain binding site reduced homodimer complex formation (M1, M2, M3 and M4). Hesx1 bound M1, M2, and αGSU as a monomer.

oligonucleotide M2, indicates that there is a hierarchy of Hesx1 binding sites with monomer formation favouring 5'-CTTA-3'.

Transfection analysis of a novel paired DNA binding site from LHβ

Therefore, Hesx1 antagonises Pitx1 transactivation of the novel paired homeodomain DNA binding site in the LHβ promoter by homodimerising. If this paired homeodomain binding site is mutated, then Hesx1 will no longer homodimerise. Three copies of the M3 oligonucleotide (5'-agATTAggtgtccagGTTAc-3') were ligated upstream of a minimal thymidine kinase promoter as already described. This was co-transfected with Hesx1, Pitx1 or both and luciferase activity was assayed (Fig. 7). Substituting one base-pair markedly altered the luciferase expression of the construct, in response to co-transfection of either Hesx1 or Pitx1 or both. Hesx1 only repressed activity when transfected at high concentrations and this may be a feature of occupancy of the 5'-ATTA-3' site, since lower concentrations resulted in a weak transactivation. Pitx1 still transactivated the construct, although weakly when compared with wild-type, and when Hesx1 and Pitx1 were co-transfected there was no dose-dependent antagonism of Pitx1 transactivation.

Discussion

We have analysed Hesx1 modulation of gene expression *in vitro*, in an effort to understand how this transcriptional repressor operates. To study this, we used the gonadotrophin gene promoters, and found that both αGSU and LHβ gene promoters were repressed by Hesx1. Resections mapped this repression to the *paired*-like Pitx1 DNA binding site contained within -120 bp of αGSU, but outside the Pitx1 DNA binding site contained within the LHβ gene promoter. Since the DNA sequence of these Pitx1 binding sites only differs by one base-pair, this difference in repression of gene expression was further investigated and compared with Pitx1 transactivation of the same promoters. In this instance, Hesx1 did not repress Pitx1 transactivation of -120 bp αGSU, but did modulate Pitx1 transactivation of -136 bp LHβ promoter. This difference was due to the presence of a novel paired DNA binding site 5'-agATTAggtgtccagGTTAc-3' contained within the -136 bp LHβ promoter, that is conserved across species. Indeed, when these DNA binding sites were cloned upstream of a minimal promoter,

Figure 7 Hesx1 homodimer formation and subsequent antagonism of Pitx1 transactivation requires an intact 5'-GTTA-3'. Trimeric DNA constructs of the mutant M3 oligonucleotide were transfected into HeLa cells with increasing amounts of Hesx1 or a constant amount of Pitx1 and increasing amounts of Hesx1. Hesx1 only repressed the M3 trimer at high concentrations. Pitx1 transactivated expression from the M3 trimer, but this was insensitive to co-transfection of increasing amounts of Hesx1. Relative activity was standardized to basal promoter activity, and was calculated as the ratio of luciferase to a control β -galactosidase construct. Hesx1 and Pitx1 were either omitted (-) or transfected in amounts of 125 ng (++++), 63 ng (++) or 31 ng (+). * $P < 0.05$, M3 trimer vs M3 trimer and ++ Hesx1 or ++++ Pitx1 or ++++ Pitx1 and + Hesx1.

Hesx1 strongly repressed basal α GSU activity, but minimally repressed LH β , and had no effect on a PIII paired DNA binding site. EMSA analysis confirmed that Hesx1 binds as a monomer to the Pitx1 DNA binding site in α GSU (5'-agCTTAgtgctgatccctg-3'), but binds as a homodimer to the novel paired DNA binding site in LH β , described above. Furthermore, this homodimer persists, even in the presence of Pitx1 DNA binding. Mutational analysis of the paired DNA site indicated that Hesx1 preferentially bound the 5'-ATTA-3' DNA sequence, and once tethered at this site, cooperatively bound the 5'-GTTA-3' sequence forming a homodimer. This was further supported by mutation of the 5'-GTTA-3' site.

When this unique site was mutated and the construct subsequently tested for antagonism, Hesx1 could no longer transcriptionally antagonise Pitx1.

Wild-type and chimaeric Hesx1 proteins can act as transcriptional repressors *in vivo* (Ermakova *et al.* 1999) and *in vitro* by recruitment of the nuclear co-repressor N-CoR (Xu *et al.* 1998). This investigation expands these observations to include the α GSU and LH β gene promoters, and identifies specific DNA targets within them. Hesx1 repression was mediated over the entire length of -480 bp α GSU and -320 bp LH β DNA promoters so, once bound, Hesx1 interfered with the basal transcriptional apparatus. This indicates that Hesx1 can act

α GSU -480bp

AAATCCAGATGCCTGTT**TAAT**TTAAGAA**ATTG**GAG**CAAT**TGTTTTATTTTCTGTTCTCTGTTGAAAT**TAATG**
TAATCCTGAAAAATGTTTTTTTTTATCCTGCCTTATGAAATATCAGGTA**CTTAGCTAAT**TAAATGTGCTAC
TCTTAGAGTCCTGTTTATTTTAAAAAGTGCAACTTTCAGGATGTTTGTG**TAAGGGTCAATAATATTACAT**
AGAAAATGGCCAAATGCTCTCTCTTCA**TAAG**CTGTCCTTGAGGTCACCACTACCTCAAATGTCTAAAAAC
AAAAGTATCTGAGGGTTG**CAAT**GTGATATGAT**CAAT**TGATGTCATGG**TAAT**TATACCAAGTGCCAT**CCAA**
TCACTGGGTAG**CAAT**TTACCCCTGATCCCTGAG**CTTA**ATGTCAGCTGGGAACATGCAATTTGT**ATTACT**CTG
CTGGTATAAAAGACAGTGGGAGACTTTATTAACTGCAGGCACTGAAAAATCCAGA

LH β -320bp

CGCGCTCACAAGGCAAGCGGCACAGCTGAGGGTCTC**CAAT**ATCTGATTTCTGTACTCCACCACGACCTC
CCTAGCACCCGGGG**ATTG**AGTCTGTGAAGTCACCTTCTCCTTGGGTGTCTTCTGCTTCGTGGTGCGGGAGC
CGCTCAGGGAGTTGGGGGAGGGATGCGCCGTTGAGCCGCTCTTGCCTCTCCCTGACCTTCGCCCGGGGA
GATTAGTGTCCAG**GTTA**CCCCACCATGCTGCACCCTCGGCGCTTGCCGCCCCACAGCCTGCAGGTATAAG
ACTAGGTGAACACAGCAGGGGAGGCA

Figure 8 α GSU and LH β gene promoters contain putative paired homeodomain DNA binding sites. Putative half-sites are shown in bold and those matching the Hesx1 binding sites identified in this study are underlined. Actual Hesx1 binding sites are highlighted (solid stars) and TATAA sequences are boxed.

as a short-range transcriptional repressor, since repression was mapped to within -120 bp on α GSU and between -320 and -136 bp on the LH β promoter. Short- and long-range repression of transcription is used to define the category of repressor: long-range repression is non-specific, controlling global locus silencing, while short-range repressors act at the promoter level and also specifically block transactivation (Courey & Songtao 2001). There were no canonical paired DNA binding sites, defined by 5'-TAAT-3' palindromes separated by a 3 bp spacer, in either promoter (Fig. 8); instead, each distal promoter fragment contained numerous copies of possible half-binding sites. Consistent with this, the half-site contained within the -120 bp α GSU promoter was bound as a monomer and expression from this promoter was strongly repressed by Hesx1. In addition, repression was mapped upstream of -136 bp in LH β , so again Hesx1 repressed presumably via half-sites.

Hesx1 only antagonised Pitx1 transactivation of LH β and not α GSU gene promoters. The lack of Hesx1 antagonisation of Pitx1 activated α GSU gene expression was initially surprising, until the hitherto equivalent Pitx1 binding sites in the promoters were actually found to be different. The Hesx1 DNA binding site in the LH β promoter was comprised of an imperfect inverted palindrome separated by an elongated 8 bp spacer. This DNA sequence was entirely novel, conserved across species and, importantly, was not repressed by Hesx1. There is known to be a high degree of redundancy in DNA binding site selection by homeodomain proteins, that is mediated by their context (Catron *et al.* 1993), and by the spacing of the core binding site (Tucker & Wisdom 1999). However, conversion of the paired site in the LH β promoter to the site present in the α GSU promoter did not promote Hesx1 homodimerisation, and instead Hesx1 bound as a monomer to this site.

Pitx1 regulates gene expression by interacting with non-homeodomain binding proteins (Tremblay *et al.* 1999, Poulin *et al.* 2000, Lamolet *et al.* 2001). Hesx1 did not heterodimerise with Pitx1 on the LH β paired DNA binding site. Although Pitx1 can compete with Hesx1 for the Pitx1 site on the LH β promoter, since Pitx1 does not appear to dimerise, homodimerisation of Hesx1 stabilizes DNA binding and antagonises Pitx1. This was supported by co-transfection of Hesx1 and Pitx1, where transfecting increasing amounts of Hesx1 progressively decreased Pitx1 transactivation. In contrast, the α GSU trimer, PIII trimer and M3 trimer were not antagonised by Hesx1, but there was variability in Pitx1 transactivation. In these instances our data indicate that Hesx1 is competing with Pitx1 for monomeric DNA binding sites, but in every case co-transfection of increasing amounts of Hesx1 did not antagonise transactivation in a dose-dependent manner. This suggests that Hesx1 and Pitx1 factors probably have differing affinities for monomer binding sites, and this was reflected by the absence of antagonisation of Pitx1 transactivation. In addition, these results also imply that although the PIII trimer can bind Hesx1 as a homodimer (Dattani *et al.* 1998), the spacing is crucial for antagonisation of Pitx1 transactivation. The PIII trimer contains a 3 bp linker, while the LH β paired sites are separated by 8 bp. However, there may be an encrypted α GSU-like binding site for Pitx1 contained within the PIII DNA element (5'-ttgagtcTAATtgaATTA ctgtacagc-3') that could also account for these differences.

Our observation that Hesx1 can repress α GSU gene expression *in vitro* could have significant consequences for expression during embryonic development. Studies from a number of groups indicate that the expression patterns of α GSU and Hesx1 overlap in the developing anterior pituitary, and that as Hesx1 expression regresses in a ventral to dorsal manner, coincidentally α GSU expression increases (Burrows *et al.* 1996, Hermesz *et al.* 1996). Pitx1 and α GSU co-localise in embryonic cells (Lanctot *et al.* 1999), and Pitx1 is required for efficient α GSU gene expression (Tremblay *et al.* 1998, Szeto *et al.* 1999). Furthermore, Pitx1 protein levels decline (Lanctot *et al.* 1999) coincident with restriction of Hesx1 to Rathke's pouch (Hermesz *et al.* 1996), but significantly recover as Hesx1

expression declines. However, there is no evidence that Hesx1 can regulate pituitary expression of Pitx1 or *vice versa*.

Pitx1 is also required for LH β gene expression in mice (Quirk *et al.* 2001 and references therein), and affects lineage specification and maintenance of gonadotrophs (Szeto *et al.* 1999). There are numerous incidences of persistent expression of *Hesx1* (Gage *et al.* 1996, Wu *et al.* 1998), and in these instances, gonadotroph terminal differentiation markers fail to express. Our observations of Hesx1 repression and antagonism of Pitx1 transactivation of LH β gene expression could explain the delay in expression of gonadotroph terminal differentiation markers in *Prop-1* defective mice and patients (Gage *et al.* 1996, Wu *et al.* 1998) and also support a role for Hesx1 in perturbation of terminal differentiation markers. Furthermore, it has not been established how α GSU gene expression is activated, while LH β gene expression remains quiescent during pituitary development. It is likely that, although Hesx1 can act as a short-range repressor of LH β , another factor which is expressed over a longer developmental time-frame mediates long-range repression and gene silencing. Control of the LH β gene locus is undoubtedly complex, since studies in transgenic mice with a distal LH β promoter documented premature promoter activation during embryogenesis (P Brown and A S McNeilly, personal communication), although there was normal targeting and regulation of expression in gonadotrophs in adults (Brown *et al.* 1993, McNeilly *et al.* 1996). Therefore, control elements needed to silence and target LH β gene expression during embryogenesis are probably located at greater distances than that reported for α GSU (Brinkmeier *et al.* 1998).

A role for Hesx1 in suppression of expression of gonadotroph cell markers as a monomer, and specific antagonism of gene expression as a homodimer also indicates that *Hesx1* gene expression levels are critical. Haploinsufficiency in mice, and the occurrence of septo-optic dysplasia and CPHD in man are both linked to *Hesx1* expression levels and the ability of Hesx1 to homodimerise (Dattani *et al.* 1998, Thomas *et al.* 2001). The homodimerisation of Hesx1 on LH β and the repression of α GSU, which was efficiently repressed by low concentrations of Hesx1, imply that Hesx1 could have a two-tier mode of action.

Repression of target gene expression could be relatively insensitive to levels of *Hesx1*, since this only requires *Hesx1* to bind as a monomer, whereas antagonism either by homo- or heterodimerisation could be critically dependent on the levels of *Hesx1* gene expression. This model is also consistent with the proposed threshold response gradient action of the *paired*-like homeodomain factor, bicoid, in *Drosophila* (Burz *et al.* 1998).

In conclusion, our results show that *Hesx1* can suppress expression of both the α GSU and LH β gene promoters. *Hesx1* repression of α GSU is overridden by *Pitx1*, and was mediated via the *Pitx1* DNA binding site contained within the proximal α GSU promoter. In contrast, *Hesx1* repression of LH β mapped out with the equivalent proximal *Pitx1* DNA binding site. Instead, *Hesx1* antagonised *Pitx1* transactivation of the promoter in a dose-dependent manner, by binding as a homodimer to a novel DNA homeodomain binding site that encompassed the LH β *Pitx1* transactivation site.

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