Hex is a transcriptional repressor that contributes to anterior identity and suppresses Spemann organiser function

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SUMMARY

One of the earliest markers of anterior asymmetry in vertebrate embryos is the transcription factor Hex. We find that Hex is a transcriptional repressor that can be converted to an activator by fusing full length Hex to two copies of the minimal transcriptional activation domain of VP16 together with the flexible hinge region of the λ repressor (Hex-λVP2). Retention of the entire Hex open reading frame allows one to examine Hex function without disrupting potential protein-protein interactions. Expression of Hex-λVP2 in *Xenopus* inhibits expression of the anterior marker *Cerberus* and results in anterior truncations. Such embryos have multiple notochords and disorganised muscle tissue. These effects can occur in a cell non-autonomous manner, suggesting that one role of wild-type Hex is to specify anterior structures by suppressing

signals that promote dorsal mesoderm formation. In support of this idea, over-expression of wild-type Hex causes cell non-autonomous dorso-anteriorisation, as well as cell autonomous suppression of dorsal mesoderm. Suppression of dorsal mesoderm by Hex is accompanied by the down-regulation of *Goosecoid* and *Chordin*, while induction of dorsal mesoderm by Hex-λVP2 results in activation of these genes. Transient transfection experiments in ES cells suggest that *Goosecoid* is a direct target of Hex. Together, our results support a model in which *Hex* suppresses organiser activity and defines anterior identity.

Key words: Anterior pattern, Transcriptional repression, Homeobox, Endoderm, Organiser, *Xenopus laevis*

INTRODUCTION

Traditional models for anterior patterning in the vertebrate embryo view the anterior-posterior axis arising as a consequence of gastrulation. Anterior identity was thought to be determined by the position of involuting tissues relative to the embryonic signaling centre, or organiser (Beddington and Smith, 1993). The organiser, initially defined in Amphibia, comprises a group of cells capable of inducing a complete secondary embryonic axis in heterotopic grafting experiments (Spemann and Mangold, 1924). The ability of this cell population to induce a complete secondary axis suggests that the organiser contains all signals necessary to pattern the anterior-posterior axis. However, recent evidence, derived principally from the mouse, suggests that the classically defined organiser (or node in the mouse) is insufficient to pattern the anterior of the embryo. Instead, the signals required for the initiation of anterior patterning are localised to an extraembryonic tissue, the anterior visceral endoderm (AVE), a full day prior to the initiation of overt gastrulation and the formation of the organiser (reviewed by Beddington and Robertson 1998, 1999). Comparative expression analysis of a number of gene products implicated in anterior identity suggest

that other vertebrates have structures that may be functionally analogous to the AVE: the anterior hypoblast in chick (Yatskievych et al., 1999), the yolk syncytial layer in zebrafish (Yamanaka et al., 1998), and a deep endodermal population in the frog (Bouwmeester et al., 1996; Jones et al., 1999). The visceral endoderm of the rabbit (Knoetgen et al., 1999), mouse (Tam and Steiner, 1999) and the deep endoderm of the frog (Jones et al., 1999) have been shown to have anterior signaling capabilities.

One of the earliest markers of anterior/posterior asymmetry in a number of vertebrates is the homeobox gene *Hex* (Thomas et al., 1998). In the mouse, Hex RNA is initially expressed throughout the primitive endoderm at 4.5 dpc and later becomes restricted to a population of visceral endoderm cells at the distal tip, more than a day before the onset of gastrulation. These cells then undergo a directional migration before coming to rest on the presumptive anterior side of the embryo. This displacement precedes expression of *Brachyury*, a marker of the primitive streak, and the initiation of gastrulation, by at least half a day. In *Xenopus* a similar movement of an endodermal population expressing *XHex* is observed (Jones et al., 1999).

Hex is a homeodomain protein of the Antennapedia/Ftz class

(Crompton et al., 1992; Neidle and Goodwin, 1994). In both mouse and frog, *Hex* expression resembles that of *Cerberus*, a gene previously linked to anterior patterning (Belo et al., 1997; Biben et al., 1998; Bouwmeester et al., 1996; Jones et al., 1999; Pearce et al., 1999; Shawlot et al., 1998; Thomas et al., 1997; Zorn et al., 1999) and indeed in *Xenopus*, ectopic expression of *XHex* induces *Cerberus* (Jones et al., 1999; Zorn et al., 1999). In addition to its early endodermal expression in a number of vertebrates, *Hex*, like *Cerberus*, is expressed in the earliest anterior-most migrating mesendoderm (Jones et al., 1999; Thomas et al., 1998; Zorn et al., 1999). *Hex* expression subsequently continues in the gut, liver and thyroid primordia (Newman et al., 1997; Thomas et al., 1998).

Here we use a combination of in vitro cell culture experiments and over-expression in *Xenopus* to explore further the role of Hex in anterior patterning. We show that Hex is a transcriptional repressor with likely targets that include genes that respond to classical organiser inducing signals. By employing fusion proteins containing the *Hex* coding sequence and reiterated modular units of the transcriptional activation domain of VP16 (Hex-VP16), we demonstrate a correlation between ectopic expression of potential Hex target genes, a loss of *Cerberus* expression in deep endoderm, and anterior truncations. Anterior truncations induced by Hex-VP16 contain expanded dorsal mesoderm structures, whereas embryos injected with RNA encoding Hex are anteriorised and display a disruption of dorsal mesoderm at the site of injection.

Injection of RNAs encoding Hex or Hex-VP16 derivatives has allowed us to identify two potential targets of Hex-mediated transcriptional repression: *Goosecoid* (*Gsc*) and *Chordin* (*Chd*). Transient transfection in ES cells using the *Gsc* promoter suggests that the effects of Hex on *Gsc* are direct. Expression analyses show that *Hex* and *Gsc* are expressed in separate populations during gastrulation, and we suggest that the two genes mark distinct regions required for anterior patterning. Together, our results support a model in which *Gsc* and *Chd* mark a default state of trunk organiser, with Hex specifying anterior structures by suppressing the expression of these genes in anterior endoderm.

MATERIALS AND METHODS

Plasmid construction

All fusion proteins were constructed with PCR fragments generated to have an *Eco*RI site upstream of a perfect Kozak sequence, followed by an ATG and a second codon encoding Val. This was followed by the mHex sequence. These were cloned as *Eco*RI/*Bam*HI fragments into either pBGX-1, to make GAL4 fusions, or into the pBGX-1 based GAL4-VP derivatives (Emami and Carey, 1992). The Hex-λVP fusions were then removed from GAL4 and inserted into pCS2 (Rupp et al., 1994) as an *Eco*RI/*Xba*I fragment.

Reporter constructs were designed using pGL-3 (Promega). GAL4 sites were excised from the GxE4 series of reporters described previously (Carey et al., 1990). The *Gsc* promoter deletions were constructed by removing the promoter sequences (SV40) from pGL-3 and inserting upstream sequences from the *Gsc* promoter (Watabe et al., 1995), including the TATA box, and transcription start site. All deletion derivatives were synthesized in the identical context to the full length promoter using a PCR based strategy.

Point mutations were constructed using a PCR based mutagenesis strategy in which overlapping oligonucleotides containing the point mutation were used to generate a PCR product in which the desired bases had been modified.

Capped synthetic RNA was prepared according to Smith (1993). CS2 Hex derivatives were linearized with *Not*I and RNA encoding nucβ-gal was transcribed from pSP6nucβGAL (Smith and Harland, 1991).

Embryonic manipulations

Xenopus laevis embryos were obtained by in vitro fertilization (Smith and Slack, 1983). Embryos were cultured in 10% normal amphibian medium (NAM; Slack et al., 1984) and staged according to Nieuwkoop and Faber (1994). Embryos were injected at the 2-, 4-, 8-, 16- and 32-cell stages with 10 nl of RNA in water. RNA concentrations ranged from 10 pg/blastomere to 500 pg/blastomere and microinjections were performed according to Smith (1993). Nuclear β-galactosidase RNA was injected at concentrations of 100 pg/blastomere. For animal cap assays, embryos were dissected and cultured in 75% NAM or 75% NAM containing BSA when activin was included in the culture medium. A crude preparation of activin A was made from conditioned medium of COS cells transfected with a human inhibin β_A cDNA. Activin units are defined by Cooke et al. (1987).

RNA preparations and RT-PCR

RNA was extracted from animal caps by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Primers for RT-PCR were designed with Primer 3 (Whitehead Web Page); sequences are available upon request. RT-PCR was performed with Titan RT-PCR enzyme mix (Boehringer Mannheim) according to the manufacturer's instructions. *Gsc* PCR was done for 27 cycles with an annealing temperature of 58°C. *EF-1α* was carried out with the same number of cycles and an annealing temperature of 55°C. Primers for *EF-1α* were described previously (Jones et al., 1999).

In situ hybridization and lineage tracing

Whole-mount in situ hybridization was performed essentially as described by Harland (1991). *Cerberus* (Bouwmeester et al., 1996), *Gsc* (Cho et al., 1991), *Chd* (Sasai et al., 1994), *Noggin* (Smith et al., 1993) and *BMP4* (Jones et al., 1996) probes were as described. Fluorescein-lysine-dextran was used as a lineage tracer and was revealed using anti-fluorescein antibody (Jones and Smith, 1998). In situ hubridisation was carried out on 10 μm paraffin sections as described by Jones et al. (1999).

Histology, β-galactosidase staining and immunohistochemistry

For histological analysis, specimens were fixed, sectioned and stained as described by Smith (1993). Staining for β-galactosidase was carried out as described by Beddington et al. (1989). Embryos were fixed in 1% paraformaldehyde, 0.2 % glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA and 0.02% NP-40 (Tada et al., 1997) and washed in PBS with 0.02% NP-40. Staining was done overnight at room temperature. Whole-mount immunohistochemistry with the monoclonal antibody MZ15 (Smith and Watt, 1985) was performed as described by Smith (1993).

Transfections and cell culture

Feeder-independent ES cells were maintained on gelatinised flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum and leukemia 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 done by lipofection using Lipofectamine (Gibco BRL) essentially according to the directions of the manufacturer. Cells were seeded at a density of 1.5×10^5/ml and allowed to grow for approximately 24 hours prior to application of the transfection cocktail. Transfections were done

overnight and the following morning the transfection cocktail was replaced with fresh medium. Transfections contained 100 ng of reporter and 75 ng of internal reference plasmid, with a total of 1.2 µg DNA (see Brickman et al., 1999). Luciferase assays were conducted with Dual Luciferase reagent (Promega) according to the directions of the manufacturer. An internal reference plasmid consisting of the SV40 promoter driving Renilla luciferase (Promega) was used as an internal control for transfection efficiency. To control for variations in transfection efficiency all data are represented as relative activities. Units of relative activity are calculated by dividing firefly luciferase readings by the reference Renilla activities and multiplying by a constant. Fold induction was determined by normalizing the baselines of each experiment to unity. All transfections were carried out in duplicate.

RESULTS

Design and in vitro function of Hex fusions to reiterated modular activation domains

To test its ability to regulate transcription in an embryonic cell type, Hex was fused to the heterologous DNA binding domain of GAL4 (Ptashne, 1992) and the resulting GAL4-Hex fusion proteins were assayed using GAL4 site-containing reporters in ES cell culture. ES cells were chosen as a substrate cell line because they express Hex and because they represent an in vitro system that resembles conditions in the early embryo. Fig. 1A shows transcriptional repression by GAL4-Hex. GAL4-Hex and a reporter gene containing five consensus GAL4 sites upstream of the SV40 promoter driving luciferase were transfected into ES cells. Transfection of increasing amounts GAL4-Hex led to repression of the SV40 promoter by up to fivefold. These results extend the findings of Tanaka et al. (1999) who demonstrated that rat Hex is a repressor in HepG2 cells.

To convert Hex to a transcriptional activator without disrupting Hex protein structure we employed the strategy shown in Fig. 1B. Hex was fused to the flexible linker domain from λ repressor and the minimal activation domain from VP16. The linker domain gives λ the flexibility to bind cooperatively to non-adjacent DNA sites (Astromoff and Ptashne, 1995), and in the context of the VP16 fusions it enhances the ability of an activation domain to stimulate transcription without its own intrinsic activation function (Emami and Carey, 1992; Ohashi et al., 1994). Reiteration of a functional unit containing the λ repressor linker and minimal VP16 activation domain has been used previously to analyse the synergy between multiple activation domains bound to a single DNA binding domain (Emami and Carey, 1992; Ohashi et al., 1994). In principle these activation domain modules should allow the recapitulation of an enhancer bound to a single DNA site by allowing the simultaneous interaction of multiple activation domains with the transcription complex (see Fig. 1B). Hex-VP16 fusion proteins were constructed either with or without additional fusion to the DNA binding domain of GAL4 and with either two or four (Hex-λVP2 and Hex-λVP4) activation domain modules. The tripartite GAL4-Hex-λVP fusions were constructed to monitor DNA binding from the well-defined high affinity GAL4 site, in addition to native Hex binding sites.

DNA encoding either GAL4, GAL4-Hex-λVP2 or GAL4-Hex−λVP4 fusion proteins was co-transfected alongside the indicated reporters. Co-transfection of both GAL4-Hex-λVP2
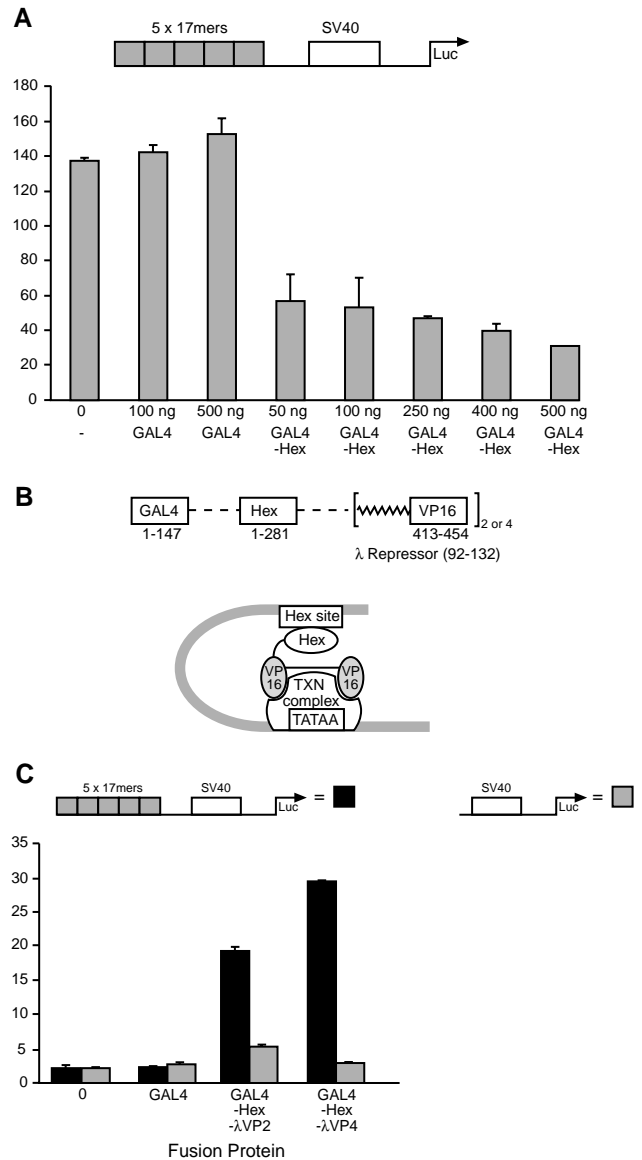

Fig. 1. Hex acts as a transcriptional repressor in ES cells. (A) GAL4-Hex represses transcription from upstream of the SV40 promoter. Increasing concentrations of DNA expressing either GAL4 or GAL4-Hex were co-transfected with the depicted reporter. (B) Cartoon illustrating the construction and principle behind the design of Hex-λVP fusions. (C) Activity of GAL4, GAL4-HexλVP2 and GAL-HexλVP4 in ES cells. DNA encoding the indicated fusion protein was co-transfected with the indicated reporters as in A. Bars represent mean activities in arbitrary units (see Materials and Methods); standard deviations are indicated. Amount of plasmids expressing the indicated GAL4 or Hex derivatives are indicated in A and is 100 ng for all transfections in C.

and GAL4-Hex-λVP4 led to approximately tenfold GAL4 site dependent induction of the SV40 promoter (Fig. 1C). There is also a small effect of these molecules on the SV40 promoter due to the presence of a DNA site for Hex within SV40 (data not shown). Both these fusion proteins, and versions in which GAL4 has been removed, are active on native Hex binding sites (see Fig. 7).

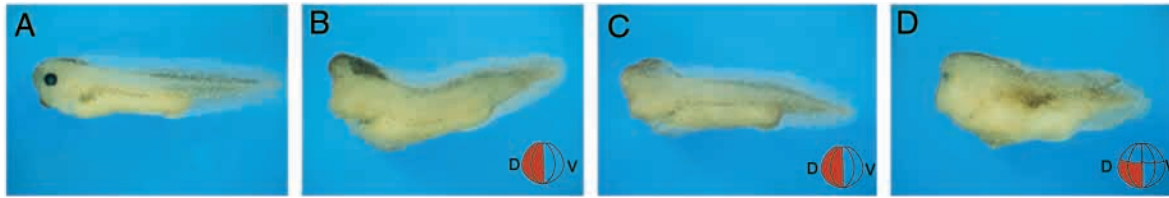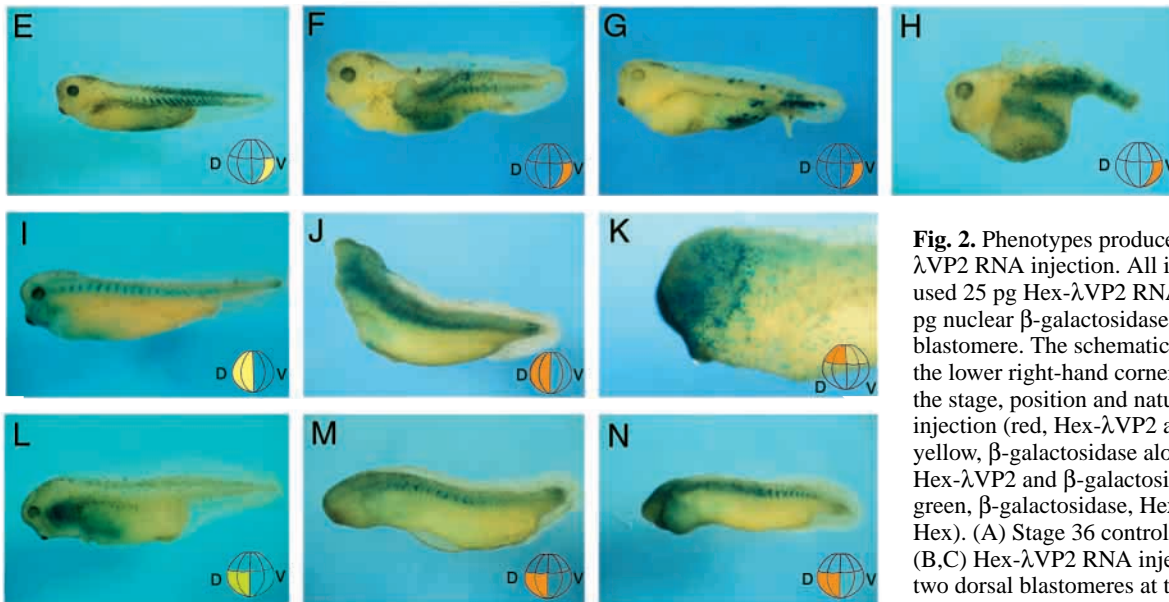Full length VP16 is one of the most potent transcriptional

Dorsal Hex-λ VP2 injection

Hex-λ VP2 + β-galactosidase

Fig. 2. Phenotypes produced by Hex-λVP2 RNA injection. All injections used 25 pg Hex-λVP2 RNA and 100 pg nuclear β-galactosidase per blastomere. The schematic diagram in the lower right-hand corner indicates the stage, position and nature of each injection (red, Hex-λVP2 alone; yellow, β-galactosidase alone; orange, Hex-λVP2 and β-galactosidase; lime green, β-galactosidase, Hex-λVP2 and Hex). (A) Stage 36 control tadpole. (B,C) Hex-λVP2 RNA injected into two dorsal blastomeres at the 4-cell stage. (D) Hex-λVP2 RNA injected into two dorsal-vegetal blastomeres at the 8-cell stage. (E) β-galactosidase RNA injected into a single ventral-vegetal blastomere at the 8-cell stage. (F-H) β-galactosidase and Hex-λVP2 RNA injected into a single ventral-vegetal blastomere at the 8-cell stage. (I-J) β-galactosidase and Hex-λVP2 RNA injected into two dorsal blastomeres at the 4-cell stage. (K) β-galactosidase and Hex-λVP2 RNA injected two dorsal animal blastomeres at the 8-cell stage. (L) β-galactosidase, Hex-λVP2 and Hex RNA injected into two dorsal blastomeres at the 8-cell stage. (M,N) β-galactosidase and Hex-λVP2 RNA injected into two dorsal-vegetal blastomeres at the 8-cell stage. Frequencies for the phenotypes described above are listed in Table 1. For dorsal animal injections, 62% of cases were normal, 10% showed anterior truncations, 10% were cyclopic and 20% had non-specific gastrulation defects (*n*=21).

Table 1. Phenotypic frequencies in response to injection of Hex-λVP2 derivatives

| | | Dorsal phenotypes (%) | | | | Marker expression in ventral-lateral injections | | | | | | Ventral vegetal phenotypes (%) | | | |
| | | | | | | *Gsc* | | *Chordin* | | *Noggin* | | | | | |
RNA	*n*	Anterior truncation	Cyclopic	Gastrulation defect	Normal	*n*	Ectopic (%)	*n*	Ectopic (%)	*n*	Ectopic (%)	*n*	Normal	Mild	Severe
HexλVP2	160	44	41	9	6	52	71	36	78	39	0	38	5	37	63
Hex(N186P)λVP2	42	5	0	19	76	20	10**	nd	-	nd	-	nd	-	-	-
Hex(N186K)λVP2	22	18	23	59	0	16	6**	nd	-	nd	-	nd	-	-	-
HexλVP2:Hex 1 : 1	18	17	56	0	27	nd	-	nd	-	nd	-	nd	-	-	-
HexλVP2:Hex 1 : 2	33	6	18	76*	0	nd	-	nd	-	nd	-	nd	-	-	-
HexλVP2:Hex 1 : 4	nd	-	-	-	-	nd	-	nd	-	nd	-	20	50	45	5
HexλVP2 Δ56	nd	-	-	-	-	10	0	11	18**	7	0	nd	-	-	-

Summary of phenotypes obtained in injection experiments. Frequency of the phenotypes are given as percentages. *n* represents the number of embryos scored from several independent experiments and in cases where appropriate, only those embryos in which the correct domain of β-galactosidase expression was observed at the tadpole stage were scored. The phenotypes scored in response to ventral vegetal injection are scored as mild (ectopic muscle) or severe (induction of a partial axis duplication and/or dorsalisation).

*Hex over-expression phenotype.
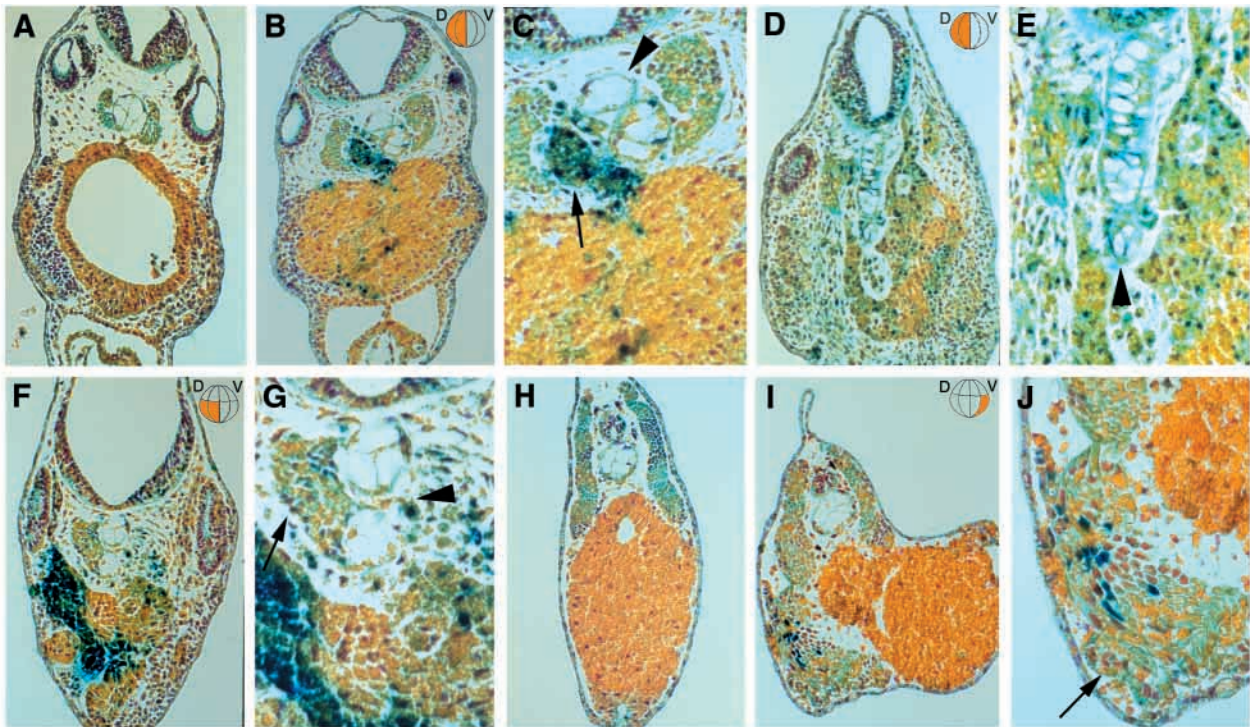
**Faint, diffuse. Potential ectopic expression.

Fig. 3. Hex-λVP2 induces dorsal mesoderm. Transverse sections through embryos described in Fig. 2. Dark blue stain is β-galactosidase lineage tracer and sections are stained with Feulgen, Light Green and Orange G. Muscle tissue and somite are denoted by an arrow, and notochord by an arrowhead. The type of injection is depicted in cartoon form in the upper right hand corner of each panel as in Fig. 2. (A) Control embryo at the hindbrain level, (B-E) dorsal injections, (F-G) dorsal-vegetal injection, (H) control embryo, (I-J) ventral-vegetal injection. The images in C, E, G and J are higher magnification views of sections in B, D, F and I.

activators known, but as a result it can be lethal in yeast and mammalian cells as a result of the phenomenon known as squelching (reviewed by Ptashne, 1992). The modular forms of the minimal amino terminal domain of VP16 described in this study appear not to have toxic side effects as overexpression of a point mutation in Hex fused to λVP2 has no phenotype in *Xenopus* (see below) and over-expression of Hex-λVP2 at levels tenfold higher than those used in this study did not kill animal cap explants (data not shown). However, to avoid potential problems as a result of squelching, all in vivo experiments in this paper use Hex-λVP2 rather than Hex-λVP4.

Mis-expression phenotypes of Hex and Hex-λVP2

To assess the role of repression by Hex on anterior patterning, RNA encoding Hex-λVP2 was injected into *Xenopus* embryos at the 4- and 8-cell stages (Table 1). Fig. 2 shows the results of these experiments. Embryos shown in the top panel of Fig. 2 were injected on the dorsal side at the 4-cell stage (B,C; also see I,J for co-injection with β-galactosidase lineage tracer) and 8-cell stage (D; also see L,M,N for co-injection with a β-galactosidase lineage tracer). Complete anterior truncation occurred in 44% of injected embryos while the cyclopic phenotype shown in Fig. 2D occurred with a frequency of 41%. Truncations occurred just anterior of the mid-hind brain boundary as demonstrated by in situ hybridization with *Krox 20* and *Engrailed* (data not shown). These embryos represent the range of phenotypes described in Table 1. Transverse sections at the level of the hindbrain of injected embryos (Fig.

3A-G) show expanded somites and duplication of the notochord. Use of a β-galactosidase lineage tracer indicates that this expansion of dorsal structures occurs in both a cell-autonomous and a cell-non-autonomous manner (Fig. 3C,E,G).

The basis for the expansion of dorsal structures and loss of anterior tissues became apparent when RNA encoding Hex-λVP2 was injected on the ventral side at the 8-cell stage (Fig. 2E-H). In these embryos the range of phenotypes includes ectopic induction of disorganized muscle (sections through embryos such as that shown in Fig. 2G, see for example Fig. 3I), partial secondary axis formation (Fig. 2F), or dorsalisation (Fig. 2H). Sections through ventral-vegetally injected embryos show cell-non-autonomous induction of muscle (Fig. 3H-J). Fig. 2 also shows that when similar concentrations of RNA encoding Hex-λVP2 were injected into dorsal-animal blastomeres at the 8-cell stage, there was relatively little anterior phenotype (Fig. 2K), suggesting that at these RNA concentrations, Hex-λVP2 has little effect when expressed in blastomeres that are fated to become anterior ectoderm. This is consistent with molecular marker analysis which shows that induction of markers in response to Hex-λVP2 in the ectoderm is not as dramatic as in other tissues (data not shown). When wild-type Hex was co-injected with Hex-λVP2 in ventral vegetal blastomeres, rescue of the Hex-λVP2 phenotype was observed (Table 1). When wild-type Hex was co-injected with Hex-λVP2 into dorsal blastomeres at a 1:1 ratio we observed a low frequency of rescue (for example, Fig. 2L) and at a 2:1 ratio we observed a defect in gastrulation as a result of Hex over-expression (Table 1, see below). The pronounced defect
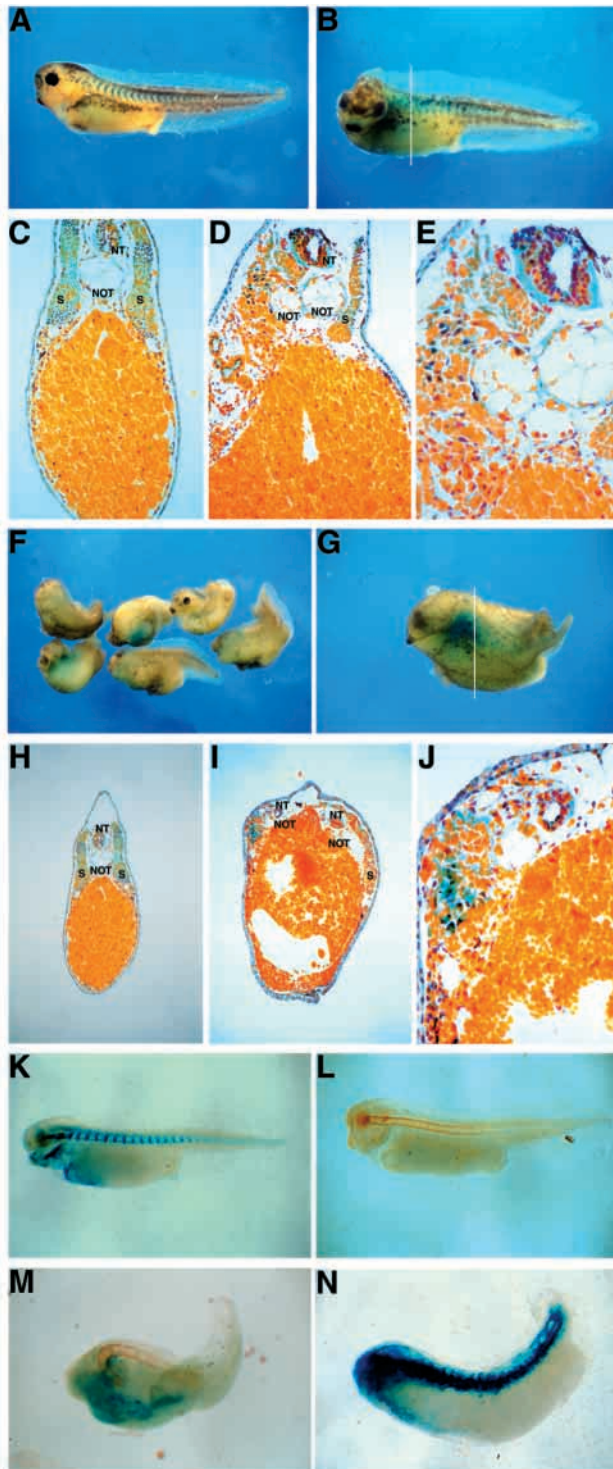
Fig. 4. Phenotypes produced by Hex RNA injection. Hex RNA (250 pg) was co-injected with a β-galactosidase lineage tracer into a single dorsal or a single dorsal-vegetal blastomere. (A) Control embryo injected with β-galactosidase lineage tracer. (B) Embryo injected with RNA encoding both Hex and β-galactosidase. (C,H) Sections through control embryos at comparable levels to D,E and I,J, respectively. Injection of Hex RNA caused either bulges around the mid-line (B) or loss of anterior and posterior axial structures (F,G). (D,E) Sections through the embryo in B showing disruption of the somite on the injected side (indicated by blue labelled cells), a yolky endoderm-like extension and induction/expansion of notochord. (I,J) Sections through the embryo in G showing a second neural tube in addition to disruption of the somite and induction of notochord on the injected side. Identification of notochord is confirmed by examination of serial sections. Antibody staining with the notochord-specific MZ15 shows these effects to be non-cell autonomous. (K) Cleared embryo injected with RNA encoding β-galactosidase, and stained with MZ15. (I) Embryo stained solely with MZ15 (orange); comparison with K reveals co-localisation of β-galactosidase and MZ15 staining. (M) Embryo injected with RNA encoding Hex has an enlarged notochord (orange) distinct from the population of Hex-injected cells (blue). (N) Embryo injected with Hex-λVP2 and stained with MZ15. The enlarged notochord shown in Fig. 3 is obscured by labeled cells which occupy axial positions. S, somite; NT, neural tube; NOT, notochord. After 4-cell dorsal injections (n=32), 33% showed a severe phenotype (Fig. 4F) and 66% a mild phenotype (Fig. 4B). After 8-cell dorsal injections (n=32), 19% showed a severe phenotype (Fig. 4F) and 81% a mild phenotype (Fig. 4B).

gastrulation can occur, we targeted Hex RNA either into a single dorsal blastomere at the 4-cell stage or a single dorsal vegetal blastomere at the 8-cell stage. Fig. 4 shows that over-expression of Hex under these conditions leads either to mild distortions of the embryonic axis (Fig. 4B) or to a more extreme phenotype, which displays defects in both anterior and posterior structures (Fig. 4F,G). Despite the anterior defects displayed in the more extreme phenotypes all embryos appear to have an enlarged head with a dramatically shortened A-P axis (Fig. 4F). Histological analysis of injected embryos shows that anterior neural defects notwithstanding, a large proportion of injected embryos have enlarged cement glands (data not shown). Consistent with the results obtained with RNA encoding Hex-λVP2, injection of Hex RNA leads to disruption of somitic tissue on the injected side and replacement of somite with a yolky, endoderm-like tissue, extending up from the endodermal yolk mass (Fig. 4D,I). Surprisingly, immediately adjacent to the cells injected with Hex, expanded or ectopic dorsal structures such as notochord are formed (Fig. 4E,J). Double staining using the β-galactosidase lineage tracer and the notochord-specific MZ15 antibody (Smith and Watt, 1985) (orange in Fig. 4K-N) shows how this result contrasts with the ectopic induction of dorsal mesoderm observed in response to injection of Hex-λVP2. The effects of Hex are cell non-autonomous and injected cells appear to segregate from the expanded notochord (Fig. 4M) whereas the expanded notochord induced by Hex-λVP2 is populated by injected cells; the induction of axial mesoderm in response to Hex-λVP2 is therefore cell autonomous (Fig. 4N). Thus Hex-λVP2-injected cells appear to form trunk organiser derivatives whereas Hex-injected cells have the capacity to induce dorsoanterior structures.
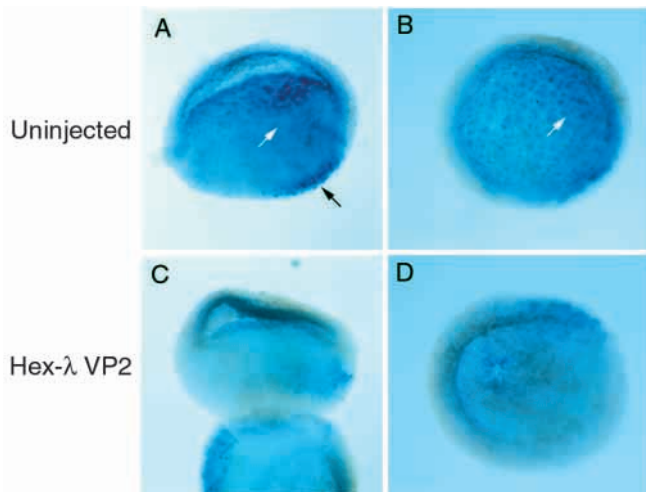
in gastrulation (see below), which is a consequence of Hex over-expression on the dorsal side, makes it difficult to determine a ratio of Hex:Hex-λVP2 at which there are no phenotypic consequences.

The induction of dorsal mesoderm by Hex-λVP2 suggests that wild-type Hex defines an anterior state by suppressing the induction and/or propagation of dorsal mesoderm (or trunk organiser). To determine the consequences of Hex over-expression on the dorsal side under conditions in which

Fig. 5. Injection of Hex-λVP2 RNA inhibits *Cerberus* expression. (A) Lateral view and (B) animal view of early gastrula stage 10-10.5 uninjected embryo. (C) Lateral view and (D) animal view of embryo injected with Hex-λVP2 RNA. White arrows indicates the deep expression domain of *Cerberus* and the black arrow the suprablastoporal domain. Embryos were injected with 25 pg of Hex-λVP2 RNA into both dorsal blastomeres at the 4-cell stage. All embryos were cleared in Murray's agent to visualize deep tissues.

Hex is a suppressor of dorsal mesoderm that is necessary for the maintenance of anterior endoderm

Correlating with the anterior truncations caused by injection of Hex-λVP2 is a reduction in the anterior endoderm domain marked by expression of *Cerberus*. At the early gastrula stage, *Cerberus* is expressed in two domains, an involuting suprablastoporal population of anterior mesendoderm (Fig. 5A, black arrow) and a population of deep, non-involuting endoderm cells, extending from the floor of the blastocoel into the vegetal hemisphere (Fig. 5A,B, white arrows). Injection of RNA encoding Hex-λVP2 on the dorsal side of the embryo causes a reduction in *Cerberus* expression (Fig. 5C,D). The effect is particularly pronounced in the deep, non-involuting endoderm population, which has been compared to the AVE of the mouse. Since Hex is a transcriptional repressor in vitro, and Hex-λVP2 functions as an activator, any inductive relationship between Hex and *Cerberus* must be indirect.

The ability of Hex-λVP2 to induce trunk organiser or dorsal mesoderm is also reflected in induction or expansion of certain dorsal mesodermal markers early in development. At gastrulation stages (10-11), *Chd* (Sasai et al., 1994) and *Noggin* (Smith et al., 1993) are expressed in dorsal mesoderm and function to antagonize BMP4 signaling (McMahon et al., 1998; Piccolo et al., 1996). *BMP4* at this stage is expressed in the ventral marginal zone and the ectoderm (Hemmati-Brivanlou and Thomsen, 1995). Fig. 6A shows that injection of RNA encoding Hex-λVP2 induced *Chd* and inhibited *BMP4* expression. However, in contrast to *Chd*, expression of *Noggin* was not affected by Hex-λVP2 (Fig. 6A). Consistent with these results, injection of RNA encoding Hex into dorsal blastomeres inhibited *Chd* expression, but not *Noggin* (Fig. 6B).

Together, our results suggest that Hex suppresses trunk organiser formation. These observations are supported by axis duplication experiments with a dominant negative form of the
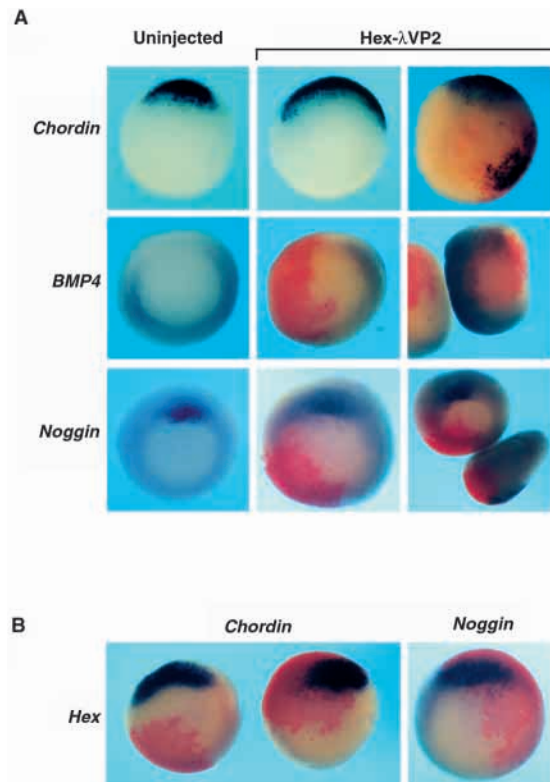

Fig. 6. Induction of dorsal mesoderm by Hex-λVP2 and suppression by Hex. (A) Injections of RNA encoding Hex-λVP2 induces ectopic *Chd*, causes down-regulation of *BMP4* and has no effect on *Noggin*. (B) Injections of RNA encoding Hex inhibit expression of *Chd* and not *Noggin*. Embryos were co-injected with synthetic RNA and a fluorescent dextran lineage trace. In situ hybridization for the indicated marker is in blue and the lineage tracer is stained in red. All embryos were fixed at stage 10-10.5 and are orientated with dorsal upwards. The majority of injections were done at the 4- or 8-cell stage into ventral lateral blastomeres or in one case, at the top of (A), throughout the dorsal side (second panel from left). In B all embryos were injected at the 4-cell stage into either a single dorsal or ventral blastomere. Embryos were injected with either 25 pg of Hex-λVP2 RNA or 250 pg Hex RNA.

BMP receptor. Ventral-vegetal injection of this construct induces partial secondary axis formation in injected embryos (Ishikawa et al., 1995), and co-injection of Hex suppressed the axis duplication activity of the dominant negative BMP receptor (data not shown).

Another marker of dorsal mesoderm, and one of the earliest markers of organiser tissue, is the homeobox gene *Gsc*. In ectopic expression experiments Gsc apparently has the opposite effect to that of Hex, as Gsc will induce *Chd* and not *Noggin* (Sasai et al., 1994). Like *Chd* and *Noggin*, *Gsc* is expressed on the dorsal side of the *Xenopus* gastrula (Cho et al., 1991). Expression of RNA encoding Hex-λVP2 throughout the dorsal region of the embryo led to an expansion of the endogenous *Gsc* domain (Fig. 7A). Similarly, injection of RNA encoding Hex-λVP2 into ventrolateral locations led to ectopic *Gsc* expression in a cell-autonomous fashion (Fig. 7A). Hex-λVP2 expression also led to induction of *Gsc* in animal cap explants (Fig. 7C). The reciprocal experiment of targeting wild-type *Hex* to either the entire dorsal side or to half of the

endogenous domain of *Gsc* (to allow gastrulation to proceed) caused down-regulation of *Gsc* (Fig. 7B), and injection of wild-type Hex RNA inhibited induction of *Gsc* in animal cap explants treated with activin (Fig. 7C). Animal cap explants treated with activin undergo convergent extension or gastrulation-like movements (Symes and Smith, 1987). Injection of Hex inhibited these movements, while injection of Hex-λVP2 induced them (data not shown).

Direct interaction of Hex with the Goosecoid promoter

The effect of Hex on *Cerberus* expression appears indirect. However, Hex is a transcriptional repressor and in mis-expression experiments it suppresses *Gsc* in a cell-autonomous manner, suggesting that Hex might regulate *Gsc* directly. Fig. 8 shows that Hex-λVP2 recognizes the *Gsc* promoter in cell culture. Examination of the *Gsc* activin and Wnt response elements (Watabe et al., 1995) revealed the presence of a potential Hex binding site between nucleotides −122 and −115 (CATTAAAT); this is based on identity (at 7 out of 8 positions) to the site defined previously by Crompton et al. (1992) in binding site selection experiments. Fig. 8A shows an experiment in which Hex-λVP2 was co-transfected with different Luciferase reporter genes containing fragments of the *Gsc* promoter positioned upstream of the *Gsc* minimal TATA box. Co-transfection of a plasmid expressing Hex-λVP2 over a range of concentrations led to a fourfold induction of a 300 bp fragment of the *Gsc* promoter. This region is sufficient to mediate responses to both Wnt and activin signalling in animal cap explants (Watabe et al., 1995). A 5′ deletion of this promoter element containing sequences from −142 nucleotides upstream of the start of transcription also responded to Hex-λVP2, but a smaller fragment comprising nucleotides from position −37 did not. Full induction of the *Gsc* promoter in response to Hex-λVP2 appears to require the putative Hex binding site at position −122.

Specificity of Hex-λVP2 activity in vivo and in vitro

Hex is a member of the Antennapedia /Ftz homeodomain sub-class, having an asparagine at position 9 in the recognition helix (Crompton et al., 1992). Mutation of this position to a lysine has been shown in two cases to alter the class of DNA site recognized by the homeodomain to that of Bicoid (Schier and Gehring, 1992; Treisman et al., 1989; Wilson et al., 1993). Alteration of this position in Hex (homeodomain position 50) to either lysine (HexN186K), which alters the specificity of the Hex homeodomain, or proline (HexN186P), which should break the recognition helix, yields fusion proteins that are unable to induce transcription of the *Gsc* promoter in transient transfections (Fig. 8B). Similar results are obtained when these proteins are expressed fused to GAL4 (also see Fig. 8B), creating the tripartite proteins GAL4-Hex(N186P)-λVP2 and GAL4-Hex(N186K)-λVP2. Thus, co-transfection of DNA encoding GAL4-Hex-λVP2 with the *Gsc* promoter resulted in a tenfold induction of transcription, whereas co-transfection of DNA encoding either GAL4-Hex(N186P)-λVP2 or GAL4-Hex(N186K)-λVP2 with the same reporters over a tenfold range of concentrations resulted in no detectable induction. However, when DNA encoding GAL4-Hex(N186P)-λVP2 or GAL4-Hex(N186K)-λVP2 is co-transfected along with reporter genes containing GAL4 sites (Fig. 8C), they are at
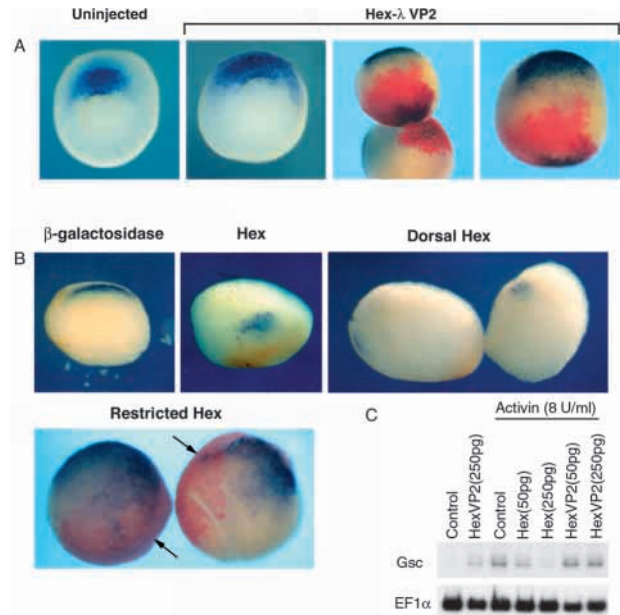

Fig. 7. Regulation of *Goosecoid* by Hex. (A) Injection of Hex-λVP2 RNA induces ectopic *Gsc* expression. *Gsc* expression is in blue and the fluorescent dextran lineage tracer is in red. (B) Injection of Hex RNA leads to a reduction in *Gsc* expression. Equivalent concentrations of *Hex* or β-galactosidase RNA (1 ng), as a negative control, were injected into embryos at the 2-cell stage, on the dorsal side at 4-cell stage or, in the bottom panel, 500 pg of *Hex* RNA was injected into either a single dorsal or a single ventral blastomere at the 4-cell stage (the arrow indicates the location of the injection relative to the endogenous *Gsc* domain). As in (A), in situ hybridization for *Gsc* is in blue and the lineage tracer is in red. The stage and orientation of embryos is as Fig. 6. (C) Hex and Hex-λVP2 activity in animal cap explants. Hex RNA and *EF-1α*, as an internal control, were detected by RT-PCR. The bar over the second set of lanes indicates that the animal cap explants were cultured in the presence of 8 units/ml of activin, to induce *Gsc*. Embryos were injected with the same amount of RNA as those in Fig. 5 unless otherwise indicated.

least as active as GAL4-HexλVP2, demonstrating that these mutations specifically interfere with the ability of the Hex homeodomain to recognize its natural site, rather than by disrupting the overall activity or stability of these fusion proteins.

When assayed by electrophoretic mobility shift assays, in vitro translated Hex, Hex-λVP2 and Hex(N186K)-λVP2, but not Hex(N186P)-λVP2, bound to the putative Hex site CATTAAAT (data not shown). The ability of Hex(N186K) to bind this site was surprising because ES cell transfection assays show a tenfold reduction in specific recognition of the *Gsc* promoter by the N186K mutation. However, the transient transfection experiments were done in ES cells and these cells may contain additional components that affect Hex-DNA binding. The *Gsc* reporter constructs may also contain additional DNA sequences involved in Hex-DNA binding.

Fig. 8 also shows that progressive truncation of the amino terminus of Hex prevents Hex from recognizing the *Gsc* promoter in ES cell transient transfection. Removal of either the amino terminal Engrailed homology domain (a sequence conserved in a large number of transcription factors and

necessary for repression by Engrailed; Smith and Jaynes 1996) or the entire amino terminus up to the homeodomain, leads to a loss of Hex DNA binding activity in transient transfection assays. Interestingly the binding site selection experiments performed with Hex were done with a protein fragment missing all coding sequence amino terminal of the homeodomain (Crompton et al., 1992) and this may explain why this isolated sequence appears insufficient for us to detect sequence specific DNA binding in vitro.

The phenotypes obtained when RNA encoding these mutant forms of Hex-λVP2 was injected into *Xenopus* embryos are summarized in Table 1. Mutation K186P, in the context of Hex-λVP2, led to a complete loss of phenotype in response to RNA injection, while mutation Q186K resulted in defects in gastrulation movements which were distinct from the phenotype induced by wild-type Hex-λVP2. Neither fusion protein activated ectopic *Gsc* (Fig. 9 and Table 1) and neither *Gsc* nor *Chd* was significantly upregulated in response to ectopic expression of Δ56Hex-λVP2 (Table 1).

The expression patterns of *Gsc* and *Hex* reveal independent domains established prior to gastrulation

At first sight, the observation that induction of *Gsc* by Hex-λVP2 is associated with loss of anterior structures is surprising, because *Gsc* is expressed in anterior mesendoderm and anterior organiser derivatives (Cho et al., 1991). We note, however, that *Hex* and *Gsc* occupy distinct expression domains from late blastula to mid gastrula stages, with *Hex* being expressed more anteriorly by late gastrula stages (Fig. 10). Inspection of published data and our own in situ analyses (not shown) suggest the same is true in the mouse.

Fig. 8. Hex recognises the *Goosecoid* promoter in ES cells. (A) Deletion analysis of the activin response element in the *Gsc* promoter by transient transfection with DNA encoding Hex-λVP2. (B) Activity of Hex-λVP2, GAL4-Hex-λVP2 and different point mutations in Hex from the *Gsc* promoter. (C) Activity of GAL4-Hex-λVP2 and point mutations from 5 GAL4 binding sites. The level of activation of Gsc is fold induction. The data represented here is based on multiple experiments in which the basal levels have all been normalized to 1. Error bars represent standard deviations. The overall activity in C is the total level of transcription from this promoter. Each number represents an average of multiple independent transfections with the error bar representing the standard deviation. Arrows indicate transfection of increasing amounts of DNA encoding the protein indicated. In A and B 200, 400 or 800 ng of a plasmid expressing the indicated Hex derivative were co-transfected with 100 ng of the indicated reporter. In C a range of concentrations of GAL4-Hex-λVP was transfected (100-600 ng), while subsets of these values (one low point and one high point) were used for all the other indicated Hex derivatives.

DISCUSSION

In this paper we show that Hex is a transcriptional repressor required for the establishment of anterior identity and that it has the capacity to suppress markers associated with the organiser. Conversion of Hex to an activator by fusion to modules of the VP16 activation domain results in a chimeric
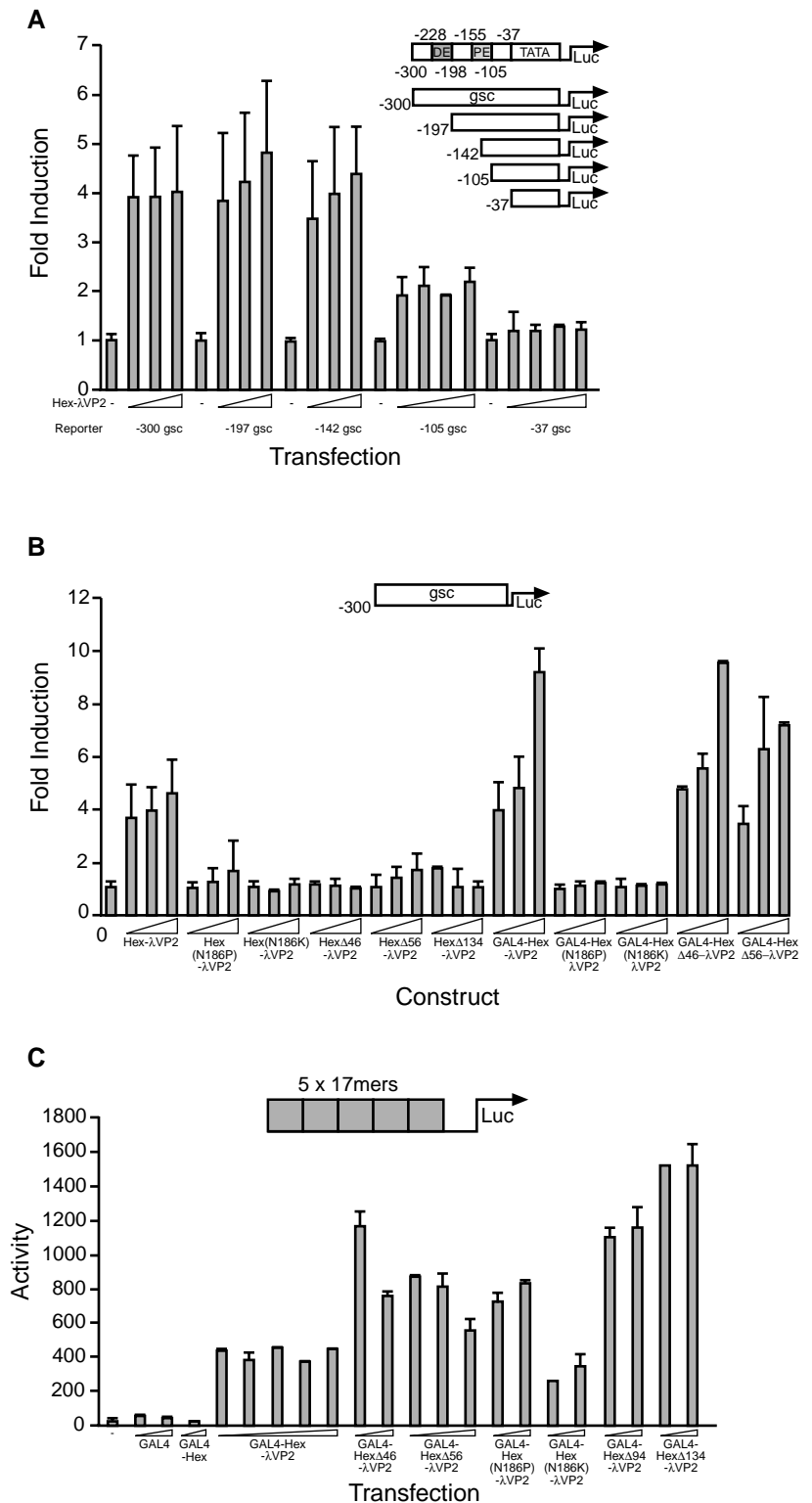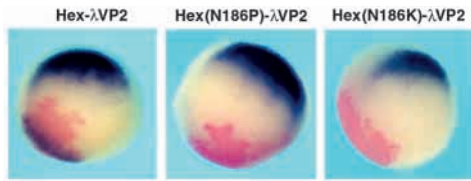
Fig. 9. Hex-λVP2 but not Hex(N186P)-λVP2, or Hex(N186K)-λVP2 induces *Gsc*. All injections were 25 pg of RNA in a ventral position at the 16-cell stage.

protein that in mis-expression experiments produces anterior truncations, induction of trunk dorsal mesoderm and induction of early mesodermal markers such as *Gsc* and *Chd*, while inhibiting the anterior endoderm marker *Cerberus*. Transient transfection experiments in ES cells indicate that this chimeric protein can recognize sequences in the activin response element of the *Gsc* promoter and that mutations in Hex-λVP2 which affect recognition of *Gsc* in ES cells either alter or eliminate the phenotype of Hex-λVP2 RNA injection in *Xenopus* embryos.

Structure-function analysis of Hex and the generation of dominant negative proteins

The Hex-λVP2 molecule is a potent inducer of target gene expression. When the reiterated VP16 modules described in this study were tested as GAL4 fusions by both transient transfection and in yeast nuclear extracts in vitro, they were shown to behave synergistically (Emami and Carey, 1992; Ohashi et al., 1994). Moreover, in both yeast and mammalian cells, the λ repressor linker was shown to potentiate the activity of these modules without having intrinsic transcriptional activation function (Emami and Carey, 1992; Ohashi et al., 1994). In the case of Hex, the positioning of a flexible linker between the *Hex* coding sequence and transcriptional activation domains may avoid steric constraints generated when an activation domain is fused to a transcriptional repressor. As a result, we have been able to modify the activity of Hex without removing the endogenous repression domain. This has the advantage that there is little risk of perturbing additional structural motifs involved in promoter recognition that may lie outside the homeodomain. For example, in both transient transfections and in vivo ectopic expression experiments, we observed a defect in DNA binding by Hex-λVP2 derivatives lacking portions of the Hex amino terminus. Our smallest 46 amino acid deletion removes the conserved Engrailed homology domain 1 which, in *Drosophila* Gsc, mediates interactions between Gsc and the homeodomain of Otd (Mailhos et al., 1998). In the context of Hex this domain might therefore mediate homo-dimerisation or interaction with other DNA binding factors that mediate promoter specificity in the embryo.

Negative regulation and anterior induction

Recent models of anterior patterning suggest that the induction of head structures involves the simultaneous inhibition of TGFβ and Wnt signals (Piccolo et al., 1999; Glinka et al., 1997). For example, induction of heads by *Cerberus* is due to its capacity to inhibit signalling by BMP4, Xnr1 and XWnt8 (Piccolo et al., 1999), and indeed head formation can also be observed following simultaneous and independent inhibition of
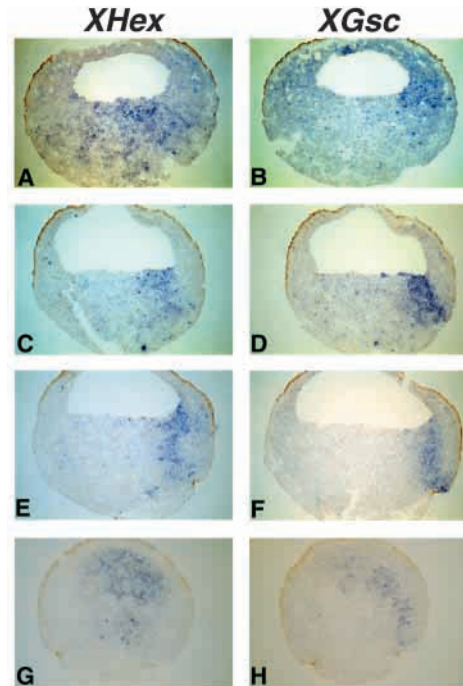

Fig. 10. Spatial expression patterns of *Gsc* and *Hex* during gastrulation. In situ hybridization in near-adjacent sections of *Xenopus* embryos with probes to *Hex* and *Gsc*. A,C,E and G are stained for *Hex* and B,D,F,H are stained for *Gsc*. Embryo in A and B is stage 9, embryo in C and D is stage 10-10.5, embryo in E and F is stage 10.5-11, embryo in G and H is stage 11-11.5. The sections in G and H go through the dorsal side at an angle that is imperfectly transverse. Thus anterior is at the top and dorsal extends down to the right.

these three signalling pathways (Glinka et al., 1997). These results suggest that the role of *Cerberus* and, more generally, of anterior endoderm, is to suppress formation of the trunk tissue that is normally induced by TGFβ and Wnt signals. Our experiments indicate that Hex, a transcriptional repressor, plays a role in this process.

Comparison of the phenotypes observed in our ectopic expression experiments with those following *Cerberus* over-expression suggests that *Hex* and *Cerberus* may act in similar pathways. For example, ventral marginal zone explants injected with the inhibitory molecule *Cerberus* develop head-like structures lacking any evidence of somites and notochord (Bouwmeester et al., 1996; Piccolo et al., 1999). In what may be the reciprocal experiment we observe induction of additional somites and notochord in response to Hex-λVP2.

Our findings that Hex is a transcriptional repressor, and that Hex-λVP2 inhibits *Cerberus* expression, suggest that Hex-mediated induction of *Cerberus* occurs indirectly through the suppression of factors that normally repress *Cerberus* in non-anterior endoderm. This indirect induction may explain why Hex induction of *Cerberus* is highly context dependent, occurring only when *Hex* is expressed in the endoderm (Jones et al., 1999). It contrasts with the more promiscuous induction of *Cerberus* by other factors such as *Sox17* and *Mixer* (Henry and Melton, 1998; Hudson et al., 1997).

Ectopic expression of *Hex* RNA in zebrafish has been recently shown to expand the expression of dorsal markers

such as *Chordin* (Ho et al., 1999), whereas our data show that Hex suppresses both *Chordin* and *Gsc*. We can best explain this discrepancy by suggesting that the expansion of chordin in zebrafish reflects the non cell-autonomous effects of Hex, which include, in *Xenopus*, the induction of dorsal tissues (Fig. 4). In *Xenopus* it is easier to distinguish between cell autonomous and non cell-autonomous effects, because it is possible to target RNA expression to particular regions of the embryo. Consistent with the idea that Hex plays similar roles in *Xenopus* and zebrafish, injection of RNA encoding Hex-λVP2 into zebrafish embryos causes dorsalisation at high concentrations and anterior truncation at lower concentrations (C. Houart and S. Wilson, personal communication).

Finally, we note that the phenotypes observed in response to mis-expression of Hex resemble those obtained following expression of dominant negative Wnt molecules (Deardorff et al., 1998; Hoppler et al., 1996; Itoh and Sokol, 1999) or of Wnt antagonists (Glinka et al., 1998; Leyns et al., 1997; Salic et al., 1997; Wang et al., 1997; Xu et al., 1998). The cell non-autonomous effects of Hex might therefore be due, at least in part, to the induction of secreted Wnt antagonists, including, perhaps, Cerberus (reviewed by Niehrs, 1999). The simultaneous induction of a secreted molecule such as Cerberus and the cell-autonomous suppression of dorsal mesoderm fated to become prechordal plate could explain the anterior defects we observe in the phenotype depicted in Fig. 4F.

Goosecoid as a direct target of Hex

Several observations support a direct role for Hex in the regulation of *Gsc*. First, ectopic expression of Hex inhibits *Gsc* whereas Hex-λVP2 result in cell-autonomous induction of *Gsc*. Second, mutations in Hex-λVP2 that do not activate the *Gsc* promoter in ES cell culture do not induce *Gsc* in vivo. Third, both Hex and Hex-λVP2 can regulate *Gsc* in animal caps. Finally, during gastrulation stages, *Gsc* and *Hex* RNA are expressed in adjacent, but distinct, domains.

The finding that there is an antagonistic relationship between Hex and Gsc, two factors required for anterior patterning, suggests that head induction requires two distinct cell types. Cells expressing organiser markers such as *Gsc* and *Chd* would be distinct from anterior endodermal cells expressing *Hex* and *Cerberus*. In the absence of anterior endoderm expressing *Hex* and *Cerberus*, tissue expressing organiser markers may only form trunk derivatives. In support of this idea are recent findings showing that when chicken axial mesoderm expressing *Chd* and *Gsc* is cultured in vitro it forms notochord, but when recombined with anterior endoderm it forms prechordal plate mesoderm (C. Vesque, S. Ellis, P. Thomas, R. S. P. B. and M. Placzek, unpublished observations).

Inspection of published data on the mouse suggests that *Hex* and *Gsc* are initially co-expressed and that their domains, in both in visceral and definitive endoderm, then segregate (Belo et al., 1997; Blum et al., 1992; Shawlot and Behringer 1995; Wakamiya et al., 1997; Thomas et al., 1998; Wakamiya et al., 1998). Interestingly, marker analysis in mice with a targeted mutation in the *Cripto* locus suggests that the visceral endoderm domains of *Gsc* and *Hex* are distinct, even during the period in which the two genes are co-expressed. Thus, in null *Cripto* mutants, *Hex*-expressing cells remain at the distal tip of the egg cylinder (Ding et al., 1998). Consequently,

Cerberus is expressed at the distal tip whereas *Gsc* is only expressed at the embryonic-extraembryonic junction. In *Xenopus*, *Chd* and *Gsc* are expressed in a more superficial domain than *Hex* and *Cerberus* (Zorn et al., 1999).

Conclusions

Our experiments suggest that Hex regulates the formation of anterior structures by acting through both cell autonomous and cell non-autonomous routes. First, it suppresses expression of *Gsc* and *Chd* in a cell-autonomous (and, at least in the case of *Gsc*, a direct) fashion, and in doing so it prevents the formation of organiser derivatives such as axial mesoderm. Secondly, and in an indirect manner, it activates the production of secreted factors which promote the formation of anterior structures. Such factors may include Cerberus or other Wnt inhibitors. In the absence of these factors, progeny of the classical Spemann organiser can form only trunk derivatives.

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