**Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors**

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**SUMMARY**

The divergent homeobox gene Hex exhibits three notable expression patterns during early mouse development. Initially Hex is expressed in the primitive endoderm of the implanting blastocyst but by 5.5 dpc its transcripts are present only in a small patch of visceral endoderm at the distal tip of the egg cylinder. Lineage analysis shows that these cells move unilaterally to assume an anterior position while continuing to express Hex. The primitive streak forms on the opposite side of the egg cylinder from this anterior Hex expression domain approximately 24 hours after the initial anterior movement of the distal visceral endoderm. Thus, Hex expression marks the earliest unequivocal molecular anteroposterior asymmetry in the mouse embryo and indicates that the anteroposterior axis of the embryo develops from conversion of a proximodistal asymmetry established in the primitive endoderm lineage. Subsequently, Hex is expressed in the earliest definitive endoderm to emerge from the streak and its expression within the gut strongly suggests that the ventral foregut is derived from the most anterior definitive endoderm and that the liver is probably the most anterior gut derivative. Hex is also an early marker of the thyroid primordium. Within the mesoderm, Hex is transiently expressed in the nascent blood islands of the visceral yolk sac and later in embryonic angioblasts and endocardium. Comparison with flk-1 (T. P. Yamaguchi et al., *Development* 118, 489-498, 1993) expression indicates that Hex is also an early marker of endothelial precursors but its expression in this progenitor population is much more transient than that of flk-1, being downregulated once endothelial cell differentiation commences.

Key words: Hex, Axis formation, Endoderm, Gastrulation, Vasculogenesis, Angiogenesis, Mouse, Endothelial cell

**INTRODUCTION**

The divergent homeobox gene Hex (Bedford et al., 1993), also known as Prh (Compton et al., 1992; Hromas et al., 1993), is expressed in a range of multipotent haematopoietic progenitor cells and cell lines and is generally downregulated during terminal cell differentiation (Manioletti et al., 1995), suggesting a role for this gene in the early stages of haematopoietic cell differentiation. In a screen for homeobox genes represented in germ-layer-specific cDNA libraries prepared from gastrulating embryos (Harrison et al., 1995), we identified Hex and, from documenting its expression pattern during early mouse development, revealed precocious anterior pattern in the embryonic visceral endoderm of the peri-implantation embryo. In addition, Hex transcripts also appear to serve as one of the earliest markers of angioblast and early endothelial cell development during development of the extraembryonic and embryonic vasculature.

The first unequivocal morphological evidence of the anteroposterior axis in the mouse embryo is the formation of the primitive streak which defines the posterior pole of the embryo. For this reason, most experiments previously have concentrated on explaining why the streak forms where it does and how its derivatives, most notably the node or organiser, bestow anteroposterior pattern on the rest of the embryo. Histological analysis has revealed that the implanted blastocyst is a bilaterally symmetrical structure due to a slight distal extension of the inner cell mass on one side of the embryo (Smith, 1980, 1985). Retrospective interpretation of tissue sections has been used to argue that this asymmetry is preserved during implantation and that the longest side of the embryo with respect to the proximodistal axis corresponds to the future posterior pole of the embryo proper. However, although postimplantation embryos do exhibit consistent morphological asymmetry in the extraembryonic region, lineage labelling indicates that there is only a modest correlation between this asymmetry and the future positioning of the streak, and it does not accurately predict the polarity of the anteroposterior axis (Gardner et al., 1992). Recently, more elaborate tracing techniques have been used to show that the axis of bilateral symmetry in the blastocyst coincides with the animal-vegetal axis of fertilised egg (Gardner, 1997). This raises the possibility that information present in the oocyte could influence the subsequent definition of axes in the embryo (Gardner, 1997), but evidence for a causal link between bilateral symmetry in the...
blastocyst and the site of primitive streak formation is still missing.

Recently, evidence has been obtained that the primitive endoderm lineage may be important in influencing anteroposterior pattern. Expression of the paired-like homeobox gene Hesx1 (Rpx) is restricted to a small group of cells within the anterior visceral endoderm (AVE) at the beginning of gastrulation (Hermesz et al., 1996; Thomas and Beddington, 1996). Although the onset of Hesx1 expression coincides with streak formation, it seems very unlikely that its anterior expression can depend on derivatives of the primitive streak because these will not have reached the anterior aspect of the embryo at the time that Hesx1 transcripts are first detected. Other molecules have also been detected in the AVE about 12 hours prior to gastrulation, including a novel cerberus-like molecule (Bouwmeester et al., 1996; Thomas et al., 1997) and VE-1, an antigen that is recognised by an antibody of unknown epitope specificity (Rosenquist and Martin, 1995). Furthermore, several genes shown by mutational analysis to be essential for normal anterior development, such as Otx2 (Acampora et al., 1995; Ang et al., 1994), Lim1 (Shawlot and Behringer, 1995), nodal (Varlet et al., 1997) and HNF-3β (Ang and Rossant, 1994; Weinstein et al., 1994; E. Robertson, personal communication), are expressed initially in the visceral endoderm. Removal of the AVE at the onset or during the early stages of gastrulation severely compromises development of the anterior CNS (Thomas and Beddington, 1996) and chimeric studies have shown that nodal is absolutely required in the visceral endoderm population but not in epiblast derivatives for normal forebrain development (Varlet et al., 1997). Finally, as in Xenopus (Miller and Moon, 1996), ectopic expression of Wnt8 in the mouse embryo is sufficient to cause duplications of the streak and organizer but, unlike the case in frog, this does not result in duplication of rostral structures such as forebrain or heart. This too would be consistent with the initial formation of anterior structures requiring cues from visceral endoderm rather than the organizer (Pöpperl et al., 1997; Thomas et al., 1997). The expression pattern of Hex, together with the cell lineage analysis reported here, strongly endorses the notion that visceral endoderm may be the first tissue to acquire anteroposterior polarity in the mouse conceptus and gives some indication of how this asymmetry is acquired.

The expression studies reported here also implicate Hex in the initiation of blood vessel formation. Formation of the vasculature during embryonic development occurs by two related mechanisms (reviewed in Risau, 1997). The first, termed vasculogenesis, is defined as the coalescence and concomitant differentiation of endothelial precursor cells (angioblasts) into vessels. This process prevails during the formation of the first major embryonic blood vessels, the paired dorsal aortae, at early somite stages. A secondary mechanism of blood vessel formation, defined as angiogenesis, describes the generation of additional vessels by sprouting from an existing vessel. The latter mechanism is used to generate the intersomitic vessels, (Coffin and Poole, 1988) and is also believed to be involved in the formation of the limb (Joterau and LeDouarin, 1978) and cerebral (Stewart and Wiley, 1981) vasculature. Several genes encoding receptor tyrosine kinases (RTKs) including flk-1 (Yamaguchi et al., 1993), fli-1 (Fong et al., 1995) tie-1 and tie-2 (Sato et al., 1995) function during endothelial cell differentiation. Expression and mutational analyses suggest that Flk-1, and its ligand Vascular Endothelial Growth Factor (VEGF), play a role at the earliest stages of endothelial cell differentiation (Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996). Flk-1 is the first of the endothelial RTKs to be expressed and is restricted to angioblasts and early endothelial cells. Moreover, flk-1 null mutant mice do not generate a vascular network and lack haematopoietic progenitor cells. This latter defect has led to the proposal that Flk-1 may be expressed by the haemangioblast, a hypothetical stem cell that gives rise to both angioblasts and haematopoietic lineages (Shalaby et al., 1995; Risau, 1997). However, the developmental potential of flk-1-positive angioblasts has yet to be determined and it is still not clear how angioblasts (or haemangioblasts) are selected from within the mesenchyme. Comparison of Hex and flk-1 expression reported here indicate that Hex is expressed at least as early as flk-1 in endothelial precursors but is a more transient marker of these progenitors.

MATERIALS AND METHODS

Whole-mount in situ hybridisation and histology

Embryos were recovered as described in Hogan et al. (1994). Prior to processing (Wilkinson, 1992), embryos were fixed in 4% paraformaldehyde in PBS for approximately 24 hours at 4°C and dehydrated through a methanol series. Early and pregastrulation embryos were processed in 12 μm tissue culture inserts (Costar) in a volume of 2 ml. Older embryos were processed in 74 μm mesh inserts (Costar). Digoxigenin-labelled antisense Hex probe was generated according to the manufacturer’s instructions (Boehringer Mannheim) and spanned positions 291-818 of the Hex cDNA sequence (EMBL accession no. Z21524). The Oct-4 antisense probe (a kind gift from Dr Hans Scholer) spanned positions 491-953 of the Oct-4 cDNA sequence (Scholer et al., 1990). The T probe was generated as previously described (Thomas and Beddington, 1996). flk-1 antisense probe was transcribed from a flk-1 cDNA clone (a kind gift from J. Rossant) as previously described (Yamaguchi et al., 1993). For histology, embryos were processed according to Hogan et al. (1994), sectioned serially at 7 μm, dewaxed and mounted in DPX (BDH, Ltd). Sections and intact embryos up to 6.5 dpc were photographed in an Axioskop (Zeiss) compound microscope and intact older embryos were photographed in a dissecting microscope using Tungsten corrected film (64T; Kodak).

Dil labelling and lineage analysis of preimplantation embryos

5.5 dpc embryos were dissected from the uterus and Reichert’s membrane reflected. Embryos were transferred individually to hanging drops of M2 medium (Hogan et al., 1994) in a micromanipulation chamber (Gardner, 1978) and all labelling was carried out using a Leitz micromanipulator. Micropipettes were prepared as described previously (Beddington, 1987) and approximately 10 nl of Dil solution (Serbedzija et al., 1989) drawn into the injection pipette (internal diameter 10 μm), using a DeFonbrune suction and force pump, from a drop of Dil solution on the floor of the manipulation chamber. Each embryo was immobilised using a holding pipette (internal diameter 100 μm) applied to the ectoplacental cone, and the embryo orientated by rotating the ectoplacental cone using the injection pipette so that the distalmost tip of the egg cylinder lay in the same focal plane as the holding pipette. The injection pipette was apposed to the endodermal epithelium at the distal tip and Dil solution slowly expelled for approximately 30 seconds. Embryos were washed once in M2.
medium and then either immediately fixed in 4% paraformaldehyde at 4°C for verification of labelling or transferred to siliconised (Replicote) embryological dishes containing DMEM + 10% rat serum. Embryos were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air and after 24 hours fixed in 4% paraformaldehyde at 4°C. Labelled embryos were inspected in an Axiohot (Zeiss) compound microscope as described previously (Wilson and Beddington, 1996) using rhodamine optics and photographed using P1600 film (Kodak).

RESULTS

Hex transcripts reveal pregastrulation anteroposterior asymmetry in the visceral endoderm and later mark anterior definitive gut endoderm

The earliest stage of development at which in situ analysis was carried out was hatched blastocysts (4.5 dpc) and, at this stage, Hex transcripts are restricted to the primitive endoderm cells at the interface between the epiblast (embryonic ectoderm) and the blastocoel cavity. No expression was observed in nascent parietal endoderm cells, nor in the epiblast or trophectoderm lineage (Fig. 1B,C). In contrast, transcripts of Oct-4 are found throughout the inner cell mass (ICM) at this stage (Fig. 1A). A day later (5.5 dpc), Hex expression remained restricted to the visceral endoderm and was detected in a small group of cells at the distal tip of the egg cylinder (Fig. 1D) or a unilateral patch of visceral endoderm immediately proximal to the distal tip (Fig. 1E). At 6.0 dpc, double in situ analysis of Hex and T, a primitive streak marker that has been shown to be expressed prior to gastrulation in a symmetrical ring of epiblast cells immediately distal to the embryonic/extraembryonic junction (Thomas and Beddington, 1996), confirmed that asymmetrical Hex expression in the primitive endoderm preceded primitive streak formation (Fig. 1F). The Hex expression domain was clearly restricted to visceral endoderm on one side of the egg cylinder and extended from the distal tip to about half way up the embryonic region (Fig. 1F). At the onset of gastrulation (6.5 dpc; Fig. 1G), T mRNA is localised to the nascent primitive streak which defines the posterior end of the embryo, while Hex transcripts remain localised to the visceral endoderm diametrically opposite the streak, which therefore corresponds to the anterior aspect of the embryo. The Hex expression domain extended from the embryonic-extraembryonic junction to within about 30 μm of the distal tip of the cylinder (Fig. 1G). These expression data provide direct evidence that anterior identity within the embryo may be established up to 24 hours prior to the formation of the primitive streak.

By the midstage stage (7.0 dpc), an additional site of Hex expression was detected at the anterior end of the primitive streak (Fig. 2A). Transverse sections through midgut embryos showed Hex-positive cells in the ectoderm and endoderm layers immediately adjacent to the prospective node region mapped by Lawson et al. (1991), indicating that Hex is expressed by the presumptive definitive endoderm cells as they exit from the epiblast to intercalate into the visceral endoderm cell layer (Fig. 2H; Lawson et al., 1987). At the anterior aspect of the embryo Hex expression was restricted to a strip of medial visceral endoderm approximately 10 cells wide. During extension of the streak towards the distal tip of the egg cylinder, Hex-positive definitive endoderm cells extended rostrally from the node to merge with the Hex-expressing cells in the AVE (Fig. 2B,C). By the late streak stage (7.5 dpc), Hex expression in the anterior endoderm extended from just rostral to the node (at the distal tip) to the embryonic/extraembryonic junction (Fig. 2D), but once the neural groove was evident transcripts were restricted to a group of anteromedial endoderm cells immediately distal to the embryonic/extraembryonic junction (Fig. 2E). These probably correspond to the most anterior definitive endoderm (Lawson et al., 1987). During headfold formation and subsequent involution of the foregut pocket, the domain of anterior endoderm Hex expression had spread laterally to generate a crescent that extended to the lateral edges of the involuting foregut endoderm (Fig. 2F). By late head-fold stage, this expression domain had expanded to encompass the entire ventral foregut endoderm (Fig. 2G). At early somite stages, Hex expression continued within the ventral gut endoderm, which extended anterolaterally to include endoderm adjacent to the forming heart tube, but the blind ending ‘anterior’ extremity of the foregut pocket was devoid of transcripts (Fig. 3A,B,E), presumably because this region of the gut is actually derived from more caudal definitive endoderm. At the 10-somite stage, Hex ventral foregut
expression had resolved into two discrete domains (Fig. 3C) which by the 18-somite stage could be unequivocally identified as the thyroid primordium and the hepatic diverticulum (Fig. 3D).

Unidirectional cell movement within the embryonic visceral endoderm

The time course of peri-implantation Hex expression (Fig. 1) strongly suggested that coherent and directional cell movement may occur within the 5.5 dpc visceral endoderm layer such that cells initially located at the distal tip of the cylinder, and their immediate descendants, become distributed along the anterior midline of the future embryonic axis. This possibility was explored using DiI lineage tracing to follow the movement of distal tip visceral endoderm cells (Fig. 4A). All but two of the embryos selected for labelling had an incipient proamniotic cavity and corresponded to the developmental stages shown in Fig. 1D and E. The two exceptions were slightly larger and more advanced having an expanded proamniotic cavity and corresponded to the developmental stage shown in Fig. 1F. The distal endoderm of 38 embryos was labelled with DiI and 9 of these embryos were fixed immediately after labelling to assess the accuracy and efficiency of labelling. Five of these embryos showed bright fluorescent labelling of approximately 3-6 distal endoderm cells (Fig. 4B) while the remaining 4 embryos showed either no sign of label or only one or two minute particulate spots of fluorescence at their distal extremity. In all cases, the ectoplacental cone showed signs of labelling, due to some DiI leakage during the orientation of the embryo before labelling, but ectopic label was not observed in any other region. Although the efficiency of distal endoderm labelling is low (55.6%), aspiration of more DiI solution during labelling, which generates a higher percentage of embryos with distal endoderm labelled, also frequently caused labelling of endoderm elsewhere in the embryonic region.

Of the 29 embryos that continued development in culture, 4

<table>
<thead>
<tr>
<th>No. of embryos labelled initially</th>
<th>No. normal development after 24h in culture (% labelled)</th>
<th>No. labelled after 24h in culture (% normal embryos)</th>
<th>No. low level punctate label also in epiblast (% labelled embryos)</th>
<th>No. uniform cell labelling (% labelled embryos)</th>
<th>No. unilateral label (% uniform labelled)</th>
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<tr>
<td>29</td>
<td>25 (86.2%)</td>
<td>17 (68%)</td>
<td>3 (17.6%)</td>
<td>14 (82.4%)</td>
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*4 embryos had a discernible primitive streak and in all 4 the labelled endoderm was located on the opposite side of the embryo to the streak.
Hex gene expression

Fig. 3. Whole-mount in situ hybridisation analysis of Hex and flk-1 expression during somitogenesis. All embryos were hybridised with Hex antisense probe except for G. (A) Ventral view of a 4-somite-stage embryo. Hex expression was detected in the involuting ventral foregut endoderm (black arrow), clusters of angioblasts (white arrow) and in the allantois (white arrowhead). (B) Same embryo as in A shown in lateral view. The white arrowhead depicts isolated or small groups of Hex-positive cells in the posterior lateral mesoderm. (C) Lateral view of 10-somite embryo. Increased numbers of Hex-positive angioblasts were detected throughout the lateral mesoderm (white arrowhead) and cranial mesenchyme. Expression in the gut has resolved into two distinct region (black arrowheads). (D) 18-somite embryo. Hex expression in the endoderm is restricted to the thyroid (black arrowhead) and liver (white arrowhead) primordia. Expression in the intersomitic vessels (white arrows) and roof of the dorsal aorta (black arrow) was also apparent. (E) Sagittal section of a 4- to 6-somite-stage embryo in similar orientation to the embryo shown in B. Hex expression is evident in the ventral foregut endoderm (black arrows) adjacent to the forming heart (but not in the anterior extremity of the foregut pocket) and in the endocardium (black arrowhead). Isolated positive cells (angioblasts) are evident in the cranial mesenchyme (white arrow). (F) Transverse section through the heart region of 10-somite-stage embryo. Hex transcripts were present in the thyroid primordium (white arrowhead) and endocardium (black arrowhead). Hex expression was no longer evident in the endothelial cells lining the paired dorsal aortae (black arrows). (G) Posterior view of an 8-somite-stage embryo with VYS intact. Many more flk-1-positive cells were detected throughout the length of the dorsal aorta (black arrows) and within the VYS vascular plexus (white arrow) than Hex-positive cells. (H) Transverse section through posterior region of 10-somite embryo. Hex-positive cells were detected lining the umbilical (black arrow) and vitelline (white arrow) veins, and in the posterior lateral mesoderm (black arrowhead). Restricted expression within the ventral endoderm was also detected (white arrowhead). (I) Transverse section of 10-somite embryo showing Hex expression in angiogenic cell clusters. Note the absence of staining in the endothelium of the dorsal aorta (DA). (J) Hex expression in the same stage embryo as that shown in G. Far fewer Hex-positive cells were detected in the dorsal aorta (black arrows) and VYS (white arrow) compared with flk-1. Bar, 100 µm (A,C,G,H); 110 µm (B); 150 µm (D); 75 µm (E-H); 40 µm (I).

were clearly retarded and abnormal and were excluded from further analysis (Table 1). Only 17 of the remaining embryos contained fluorescent cells in the embryonic region, but 3 of these exhibited anomalous labelling in that, instead of uniform labelling of individual cells, only particulate fluorescence was evident, occurring throughout the epiblast as well as the endoderm population (Fig 4C), which is indicative of autofluorescing necrotic tissue. All 14 embryos that contained uniformly labelled cells exhibited a similar distribution of label (Table 1): labelled cells occurred in a single coherent patch, which extended up one side of the egg cylinder (Fig. 4D-G). A radial or bilateral distribution of labelled endoderm cells was never observed. 10 of these embryos had yet to form a primitive streak indicating that the unilateral movement of endoderm preceded mesoderm formation and overt gastrulation movements. A primitive streak could be identified in 4 of these labelled embryos, including the 2 which were slightly more advanced at the time of labelling (Fig. 4F,G) and, in all 4 embryos, the DiI stripe of labelled cells occurred on the opposite side of the embryo to the streak (Fig. 4E-G). The consistency of these results strongly suggests that the pattern of movement in the primitive endoderm is such that cells initially located at the most distal extremity of the visceral endoderm, and thus those that express Hex, are always displaced to one side of the cylinder before primitive streak formation and that their destination corresponds to the future anterior aspect of the embryo.

Hex expression in the mesoderm marks endothelial cell precursors

Hex expression within the mesoderm was first detected at midstage stages (7.0 dpc) in a narrow band of cells within the nascent blood islands of the visceral yolk sac (VYS; Fig. 2C) at what appears to be the same stage that flk-1 is first expressed in this population (Yamaguchi et al., 1993). By the late streak stage (7.5 dpc), increased numbers of Hex-positive cells were detected in the expanding blood islands and these cells formed a discrete band around the circumference of the VYS (Fig. 2D). High levels of Hex transcripts persisted within the blood islands until neural plate (Fig. 2E) and early headfold (Fig. 2F) stages. However, by the late headfold stage relatively few Hex-positive cells were present in the blood islands: only isolated cells expressed Hex and the level of transcripts per cell appeared to be reduced (Fig. 2G). Hex expression in the VYS remained low during early somite stages when the blood islands coalesce to form intact vasculature (Fig. 3J). Therefore,
**Hex** expression during development of the VYS vasculature appeared to be restricted to the endothelial cell precursors (or angioblasts), and was downregulated during differentiation into endothelial cells. This is supported by comparison of **Hex** expression with **flk-1**, a marker of angioblast and early endothelial cells. In 8-somite-stage embryos, **flk-1** was strongly expressed in the nascent vascular endothelium of the VYS (Fig. 3G) whereas by this stage **Hex** expression was barely detectable (Fig. 3I). **Hex** transcripts were also evident in the developing allantois, initially at the neural plate stage being present only in the tip of the allantoic bud but by the late headfold stage being present at high levels throughout the entire allantois (Fig. 2G). The allantois remained a site of intense **Hex** expression at least up to 9.5 dpc (Fig. 3; data not shown).

Within the embryonic region at late streak and neural plate stages, isolated **Hex**-positive cells were detected in the proximalateral mesoderm immediately distal to the embryonic/extraembryonic junction (Fig. 2E). Rostrally, the **Hex** expression domain included tissue fated to form the heart (Tam and Beddington, 1992). During headfold formation, **Hex**-positive cells in the anterior mesoderm formed a horseshoe-shaped domain corresponding to the developing paired endocardial tubes (Fig. 2G). This aspect of **Hex** expression is remarkably similar to **flk-1** (see Yamaguchi et al., 1993), which is one of the earliest known endocardial markers. In early-somite-stage embryos, high levels of **Hex** transcripts were detected within the endocardial cells (and in the ventral gut endoderm adjacent to the heart), but not in the myocardial cell layer (Fig. 3E). **Hex** expression in the endocardium (Fig. 3F) persisted until at least 9.5 dpc.

The first major blood vessels to form within the embryonic vasculature are the paired dorsal aortae which, by the 8-somite stage, extend posteriorly from the heart to the forming vitelline artery at the base of the allantois (Kaufman, 1992). In 4-somite-stage embryos, small groups of **Hex**-positive cells were detected in the mesoderm lateral to the neural tube in the region where the dorsal aortae will form (Fig. 3A,B). However, by the 8-somite stage few of the endothelial cells lining the dorsal aorta contained **Hex** transcripts (Fig. 3J). Transverse sections through the anterior region of a 10-somite embryo confirmed that the endothelial cells of the dorsal aorta were almost exclusively **Hex** negative (Fig. 3F, I) and therefore **Hex** expression in these major vessels differs significantly from that of **flk-1**, whose transcripts remain abundant throughout the dorsal aortic endothelium at this stage (compare Fig. 3G and 3I). Thus, **Hex** appears to be expressed by angioblast cells as they coalesce to form the dorsal aorta, but (unlike **flk-1**) not once did endothelial cells differentiate.

In 10-somite-stage embryos, a significant increase in the number of **Hex**-positive cells was detected in the cephalic mesenchyme and in the posterior lateral mesoderm (Fig. 3C). Transverse sections through the anterior region showed **Hex** expression in small groups of angioblasts which were Anastomosing to form the cranial vasculature (Fig. 3I). Generally, larger vessels contained fewer **Hex**-positive cells than small vessels and no **Hex** expression was detected in primitive erythrocytes. By the 18-somite stage, **Hex** expression in the head mesenchyme appeared more organised presumably reflecting the formation of the cranial vessels and associated capillary plexi (Fig. 3D). Caudally, **Hex** transcripts were present in the developing vitelline artery and umbilical veins (Fig. 3H). In addition, isolated cells or groups of cells expressing **Hex** were present in the posterior splanchnopleure and somatopleure which is also consistent with **Hex** being involved in angioblast formation since this region is thought to be an intraembryonic source of endothelial cells (Shalaby et al., 1995). **Hex** was also expressed in a restricted domain within the caudal ventral gut endoderm, a region whose developmental history and fate have not been charted. Trails of **Hex**-positive cells were also present between the somites where the intersomitic arteries form (Fig. 3D). These are generated...
by sprouting of endothelial cells from the roof of the dorsal aorta (Coffin and Poole, 1988), demonstrating that \textit{Hex} is also expressed by cells undergoing angiogenesis.

\textbf{DISCUSSION}

\textbf{Anteroposterior patterning of the embryo}

Historically, the generation of embryonic anteroposterior pattern was thought to be mediated exclusively by mesodermal or mesendodermal derivatives generated by the primitive streak (and in particular the organiser) during gastrulation. In part this view stemmed from the extensive analyses of axis formation in Amphibia which has primarily revealed cell biological and molecular mechanisms responsible for establishing an organiser, and characterised some of the signalling interactions responsible for the organiser’s unique patterning properties including those that influence anteroposterior pattern (Sharpe and Gurdon, 1990; Hemmati-Brivanlou et al., 1990, Saha and Grainger, 1992; and reviewed in Kessler and Melton, 1994; Sive, 1993; Miller and Moon, 1996). The asymmetrical expression of \textit{Hex} in the AVE, a derivative of primitive endoderm that does not contribute to the embryo itself (Gardner and Rossant, 1979), precedes any molecular markers or functional attributes associated with an organiser by at least 24 hours. As such it represents the earliest known example of asymmetrical gene expression related specifically to anteroposterior patterning in a vertebrate embryo and, moreover, highlights extraembryonic tissues as a possible source of early patterning influences. Initially \textit{Hex} appears to be symmetrically expressed in the primitive endoderm of the hatched blastocyst (Figs 1B,C. 5) although an Expressed Sequence Tag corresponding to \textit{Hex} is present in a 2-cell cDNA library (Rothstein et al., 1992). Following implantation, \textit{Hex} transcripts are still symmetrically distributed but now restricted to a small subset of primitive endoderm descendants located at the distal tip of the egg cylinder (Figs 1D, 5), thus providing a unique distal marker. How \textit{Hex} expression is restricted to this discrete population of primitive endoderm cells is unclear, although these cells have a unique morphology in that they are more columnar than other visceral endoderm cells (R. S. P. Beddington, unpublished observation). It is possible that they represent the original putative primitive endoderm stem cells, which when the primitive endoderm becomes multilayered in the blastocyst are thought to remain in contact with the epiblast (Gardner, 1983). Remarkably, when these cells are labelled with DiI, they invariably move proximally as a coherent population to the future anterior side of the embryo (Fig. 4) apparently maintaining \textit{Hex} expression as they do so (Figs 1E, 5). The cue for such polarised cell movement within an epithelium is not obvious but, since the embryonic visceral endoderm comprises less than 100 cells at 5.5 dpc (Snow, 1977), it is possible that differential growth could be responsible for such non-random movement. This might explain the correlation between implantation orientation and the future site of streak formation (Smith, 1980, 1985; Gardner et al., 1992) if closer proximity to maternal blood sinuses were sufficient to give the visceral endoderm on one side of the conceptus a slight growth advantage. Alternatively, the inherent bilateral asymmetry documented in the blastocyst, which may derive from asymmetries in the zygote (Gardner, 1997), could be responsible for generating differential growth or differences in the orientation of cell division in the visceral endoderm. Whatever the mechanism, this vectorial movement transforms a proximodistal axis into an anteroposterior one (Fig. 5).

That \textit{Hex} is expressed on the future anterior side of the embryo while \textit{T} transcripts are symmetrically distributed proximally (Fig. 1F) suggests that anterior influences could be responsible for dictating the site of streak formation. Such an anterior signal(s) could either direct the movement of proximal epiblast caudally or actively repress expression of genes such as \textit{T} associated with streak formation. Fate mapping of prestreak epiblast indicates that there is a net caudal movement of proximal epiblast cells (Lawson et al., 1991) and, therefore, establishment of the streak may also require vectorial movement but in this case within the epiblast (Fig. 5). Although \textit{nodal} is expressed in the visceral endoderm prior to streak formation and its expression in this tissue is required for normal anterior development (Varlet et al., 1997), it does not appear to be asymmetrically expressed (Conlon et al., 1991). The only signalling molecule to date that is asymmetrically expressed in AVE prior to gastrulation and might influence the behaviour of proximal epiblast is the putative homologue of...
cerberus, a gene originally identified in *Xenopus*, which is capable of inducing ectopic heads (Bouwmeester et al., 1996; Thomas et al., 1997). It is, of course, also possible that streak formation is dependent on a separate posterior signal for which there is as yet no marker, but which might be established either independently or in concert with the AVE.

**Definitive gut pattern**

At midstream stages, *Hex*-expressing cells are apparent in the definitive endoderm derived from the prospective node region and, by the late streak stage, these *Hex*-expressing cells have moved rostrally and in so doing displaced the AVE (Lawson et al., 1987; Thomas and Beddington, 1996). Therefore, as with *Lilm* (Shawlot and Behringer, 1995), HNF3ß (Ang et al., 1993; Sasaki and Hogan, 1993) and *Otx2* (Acampora et al., 1995; Ang et al., 1994), the rostral expression of *Hex* in the AVE is recapitulated later by node derivatives (axial mesoderm and definitive endoderm). The difference in this later expression domain of *Hex* (being restricted only to the anterior definitive endoderm) compared with those of *Lilm* and *Otx2*, indicates that different anterior derivatives of the node may have distinct, but perhaps related, roles in embellishing rostral pattern. The fact that the same genes are expressed in the AVE as the rostral derivatives of the node also argues that the AVE is likely to have a direct role in initiating anterior pattern and that the node derivatives are more likely required to maintain and refine this pattern.

Although gene expression cannot serve as a cell lineage marker, the time course of *Hex* expression in the definitive endoderm strongly suggests that the ventral foregut represents the most anterior definitive gut derivative and this conclusion is supported by available clonal lineage analysis (Lawson et al., 1987). Therefore, despite its final abdominal location, it is supported by available clonal lineage analysis (Lawson et al., 1987; Thomas and Beddington, 1996). Therefore, as with *Lilm* (Shawlot and Behringer, 1995), HNF3ß (Ang et al., 1993; Sasaki and Hogan, 1993) and *Otx2* (Acampora et al., 1995; Ang et al., 1994), the rostral expression of *Hex* in the AVE is recapitulated later by node derivatives (axial mesoderm and definitive endoderm). The difference in this later expression domain of *Hex* (being restricted only to the anterior definitive endoderm) compared with those of *Lilm* and *Otx2*, indicates that different anterior derivatives of the node may have distinct, but perhaps related, roles in embellishing rostral pattern. The fact that the same genes are expressed in the AVE as the rostral derivatives of the node also argues that the AVE is likely to have a direct role in initiating anterior pattern and that the node derivatives are more likely required to maintain and refine this pattern.

**Vasculogenesis and angiogenesis**

Mesodermal *Hex* expression was first detected in the nascent VYS blood islands where primitive erythrocyte and blood vessel formation is initiated. Within the embryo proper, where the location of the endothelial cell precursors is less rigorously defined, *Hex* transcripts at the neural plate stage were associated with the proximolateral mesoderm where angioblasts are thought to arise (Coffin et al., 1991). Later, *Hex* is expressed in regions where definitive vessels are known to form and in a pattern commensurate with coalescing endothelial progenitors (e.g. dorsal aorta; Fig 3) or the sprouting of new vessels (e.g. intersomitic vessels; Fig. 3D). In this respect, *Hex* appears to participate in the initial phases of both angiogenesis and vasculogenesis, supporting the notion of a common underlying molecular mechanism for these related processes (Yamaguchi et al., 1993). *Hex* expression strongly resembles that of *flk-1* which is the earliest known marker of endothelial cell precursors (Yamaguchi et al., 1993) and has been shown to be essential for endothelial cell differentiation (Shalaby et al., 1995) in a cell autonomous fashion (Shalaby et al., 1997). The striking similarity in expression of *Hex* and *flk-1* implicates *Hex* in the initial stages of endothelial cell selection and/or differentiation and make it a likely candidate to be expressed in the putative haemangioblast stem cell (Sabin, 1920; Wagner, 1980). Unfortunately, attempts to determine whether *Hex* and *flk-1* are coexpressed in individual angioblasts using double in situ hybridisation produced equivocal results, both for technical reasons and because of the transient nature of *Hex* expression in endothelial progenitors. Unlike *flk-1*, *Hex* transcripts do not persist in differentiated endothelial cells making it the most specific marker for endothelial progenitors so far identified. This rapid extinction of *Hex* expression once differentiation is underway is reminiscent of its downregulation in the differentiated progeny of haematopoietic progenitors and cell lines (Manfioletti et al., 1995; Bedford et al., 1993).

In late streak/neural plate stage embryos, the cardiac progenitor cells are located in the anterior proximal mesoderm, the presumptive endocardium expression *flk-1* (Yamaguchi et al., 1993) and the myocardium *Nkx2.5* (Lints et al., 1993). Isolated *Hex*-positive cells are evident in the cardiogenic field at this stage and later, like *flk-1*, expression is restricted to the endocardium and maintained until at least 9.5 dpc. Therefore, unlike its transient expression in endothelial precursors *Hex* expression persists in the endocardium suggesting either that *Hex* serves additional functions in heart development (possibly during trabeculation which is mediated, at least in part, by the endocardium (Fishman and Chien, 1997)), or that endocardial cells mature more slowly than endothelial cells.

In summary, *Hex* constitutes the earliest known example of anteroposterior asymmetric gene expression in the conceptus, and points to a mechanism whereby the proximodistal axis of the conceptus is translated into an anteroposterior one by vectorial cell movement in the embryonic visceral endoderm shortly after implantation. Within the mesoderm, restriction of *Hex* transcripts to angioblasts and early endothelial cells indicates that it may be involved in the selection and/or initial differentiation of the angioblast, and possibly haemangioblast, lineages. These two aspects of *Hex* gene expression may have little in common although, if *Hex* is a general marker of tissue immaturity (being expressed only in the least differentiated progenitors of blood vessels and haematopoietic cells), then this would endorse the notion that anteroposterior patterning of the embryo may rely on the behaviour of the primitive endoderm stem cell population initially formed in the blastocyst.

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