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Postimplantation expression patterns indicate a role for the mouse *forkhead*/HNF-3 α, β and γ genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm

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SUMMARY

The HNF-3 α, β and γ genes constitute a family of transcription factors that are required for hepatocyte-specific gene expression of a number of genes, e.g. transthyretin, α-1 antitrypsin and tyrosine aminotransferase. These genes share a highly conserved DNA-binding domain first found in the *Drosophila* gene, *forkhead,* which is required for the normal patterning of the developing gut and central nervous system in *Drosophila.* In adult mouse tissues, transcripts from HNF-3 α and β have been localised to the liver, intestine and lung, whereas HNF-3 γ is found in the liver, intestine and testis. In light of the early developmental significance of *forkhead* in *Drosophila,* we have compared the patterns of expression of HNF-3 α, β and γ mRNAs during murine embryogenesis. We find that these genes are sequentially activated during development in the definitive endoderm. HNF-3 β mRNA is expressed in the node at the anterior end of the primitive streak in all three germ layers and is the first gene of this family to be activated. Subsequently, HNF-3 α is transcribed in the primitive endoderm in the region of the invaginating foregut and HNF-3 γ appears upon hindgut differentiation. These genes have different anterior boundaries of mRNA expression in the developing endoderm and transcripts are found in all endoderm-derived structures that differentiate posterior to this boundary. Therefore, we propose that these genes define regionalisation within the definitive endoderm. Furthermore, differential mRNA expression of HNF-3 α and β is detected in cells of the ventral neural epithelium, chordamesoderm and notochord. In the neural epithelium, expression of HNF-3 α and β mRNA becomes localised to cells of the floor plate. We propose that, in addition to their characterised requirement for liver-specific gene expression, HNF-3 α and β are required for mesoderm and neural axis formation. We also conclude that HNF-3 β is the true orthologue of the *Drosophila forkhead* gene.

Key words: liver-specific transcription, *forkhead*, definitive endoderm, notochord, floor plate

INTRODUCTION

Regional diversity during development is determined by the selective expression of genes in a cell- or tissue-specific manner. The identification of developmental control genes in organisms such as *Drosophila melanogaster* or *Caenorhabditis elegans,* has greatly expanded our knowledge of early events involved in regional specification. In addition, these genes have provided useful molecular probes for the isolation of the homologous vertebrate genes in order to investigate differentiation events during mammalian development. A number of conserved multigene families of transcription factor encoding genes have been identified that play a role in pattern formation. These families can be grouped according to their DNA-binding and/or protein dimerisation domains and include: (1) the paired box gene family (Kessel and Gruss, 1990); (2) families containing the helix turn helix motif (McGinnis and Krumlauf, 1992) (3) the zinc finger family (reviewed in Pabo and Sauer, 1992) (4) the basic helix-loop-helix and leucine zipper family of transcription factors (reviewed by Harrison, 1991) and (5) families containing the ankyrin dimerisation motif (Blank et al., 1992).

Genes containing the *forkhead* (*fkh*) domain constitute a family of recently described transcription regulators, with the *Drosophila* homeotic gene *forkhead* being the prototype. This conserved 110 amino acid domain is required for DNA binding and shares striking sequence similarity with members of the rat hepatic enriched transcription factors HNF-3 α, β and γ (Weigel and Jäckle, 1990; Lai et al., 1991). The *Drosophila fkh* gene is a region-specific homeotic gene, which promotes terminal, as opposed to segmental, development. *Fkh*-deficient embryos exhibit defects in the ectodermal and endodermal components of the gut, yolk nuclei, salivary glands and nervous system (Jürgens and Weigel, 1988; Weigel et al., 1989). Therefore,

it has been proposed that there is a primordium-specific requirement for the *fkh* gene product in the developing gut of *Drosophila.* A number of *fkh*-containing genes have been identified from a variety of species including *Saccharomyces cerevisiae* (Li et al., 1992), *Drosophila* (Grossniklaus et al., 1992; Häcker et al., 1992), *Xenopus* (Dirksen and Jamrich, 1992; Knöchel et al., 1992; Ruiz i Altaba and Jessel, 1992), rat (Tao and Lai, 1992; Clevidence et al., 1993), mouse (Kaestner et al., 1993; Sasaki and Hogan, 1993) and human (Li et al., 1991; Oliver et al., 1992). Initial characterisation of these genes has revealed that they encode proteins that exhibit unique DNA recognition specificities and are temporally and spatially regulated during embryogenesis. Furthermore, overexpression of *pintallavis*, a *Xenopus fkh* domain-containing gene, perturbs the development of the neural axis, suggesting that, in higher organisms, these genes may play a role in induction and pattern formation (Ruiz i Altaba and Jessel, 1992). The *fkh* genes therefore constitute a family of transcription factors, some of which are likely to be required for early differentiation events.

The *fkh* homologous proteins in rat, HNF-3 α, β and γ, were originally characterised as transcription factors that regulate the expression of liver-enriched genes (e.g. transthyretin and α-1 antitrypsin; Costa et al., 1988; Herbst et al., 1991; Pani et al., 1992). These proteins bind similar DNA sequences as monomers via the *fkh* domain, but exhibit different DNA-binding affinities for specific sites. In adult rat, all three genes are abundantly expressed in liver, and weakly expressed in small intestine and pancreas. In lung, only HNF-3 α and β can be detected, whereas HNF-3 γ is unique to the testis (Lai et al., 1990, 1991; Xanthopoulos et al., 1991). RNase protection experiments with probes for the mouse HNF-3 α, β and γ genes have shown that these genes are expressed in the developing embryo as early as day 9 post coitum (p.c.) (K. Kaestner et al., unpublished data). In addition, HNF-3 proteins have been shown to bind to the promoter of hepatic nuclear factor 1 (HNF-1) which is a sequence-specific DNA-binding protein also required for the transcription of a number of liver restricted genes. These data suggest that proteins encoded by the HNF-3 family act at an early position in the cascade which regulates hepatocyte differentiation (Kuo et al., 1992). In light of the pivotal role that these genes are therefore proposed to play in hepatic differentiation, we examined their spatial and temporal patterns of transcription during murine embryogenesis using in situ hybridisation. We find that these genes not only exhibit differential expression during early endoderm differentiation but that HNF-3 α and β are also highly expressed in the developing central nervous system. We conclude that these genes play a pivotal role in regionalisation within the embryonic endoderm and neuroectoderm during differentiation of the body axis.

MATERIALS AND METHODS

In situ hybridisation

Mouse embryos and fetuses were obtained from matings between NMRI F_1 mice. Day 0.5 was assumed to begin at the middle of the day of the vaginal plugging. Embryos were fixed in 4% paraformaldehyde (pH 7.2) overnight, dehydrated through an ethanol series, cleared in toluene and embedded in paraffin. 6 μm sections were cut for each stage. In situ prehybridisations and hybridisations were carried out as described in Wilkinson et al. (1987) except that the post ethanol fixation in paraformaldehyde was omitted. Hybridisations were carried out at 55°C with ^{35}S-labelled riboprobes at a concentration of 60 ng/ml. The first post-hybridisation wash was performed at 55°C and subsequent washes with 50% formamide were performed at 67°C. A final wash in 0.1× SSC at 55°C was performed before the sections were dehydrated. Slides were dipped in Kodak NTB2 emulsion diluted 1:1 with water and exposed at 4°C for 5 to 7 days and developed using Kodak D19 developing solution and Kodakfix at 15°C for 4 minutes. Sections were stained using eosin and haemotoxylin.

Photography

Sections were visualised using a Zeiss confocal laser scanning microscope 310. The pictures shown are overlays of the dark-field images in the red image channel with the transmitted light images in the green channel.

RESULTS

Sagittal, transverse and frontal sections were prepared from mouse embryos collected between day 6.5 p.c. and day 16 p.c. and examined for the expression of HNF-3 α, β and γ mRNA using in situ hybridisation. Regions of the mouse HNF-3 α, β and γ genes corresponding to the 3′ end of each sequence (Fig. 1) were chosen as probes to avoid cross hybridisation between family members.

HNF-3 β mRNA is expressed in the node and subsequently in all three germ layers

HNF-3 β is the first gene of this family to be expressed. In prestreak embryos, weak mRNA expression can be detected in a few cells in the lateral part of the egg cylinder at the site of primitive streak formation in both the ectoderm and primitive endoderm cell layers (results not shown). Formation of the third germ layer, the mesoderm, is initiated on day 6.5 p.c. within the primitive streak. During early streak stages, transcription of the HNF-3 β gene is initially localized to the anterior tip of the primitive streak in all three germ layers, the ectoderm, endoderm and emerging mesoderm. This region later gives rise to the node that is the precursor of the head process, from which the notochord, head mesoderm and definitive endoderm differentiate (Fig. 2B) (Beddington et al., 1992; Lawson et al., 1987; Lawson and Pedersen, 1992; Stern and Canning, 1990; Tam and Beddington, 1987, 1992). As the primitive streak elongates, cells move forward from the node, to separate the neuroepithelium of the future head fold from the adjacent extraembryonic endoderm and the embryonic endoderm. Concomitantly, cells expressing HNF-3 β mRNA show a gradient of expression decreasing from the archenteron to the head fold, reflecting these patterns of cell movements (Fig. 2C). All axial endoderm cells stretching anteriorly from and including the node express HNF-3 β mRNA. In addition, transcripts are detected in the ectoderm and developing notochord (Fig. 2B,C). Expression is not detected throughout the neural ectoderm but is localised to cells in the midline adjacent to the endoderm and prechordal plate (Fig.

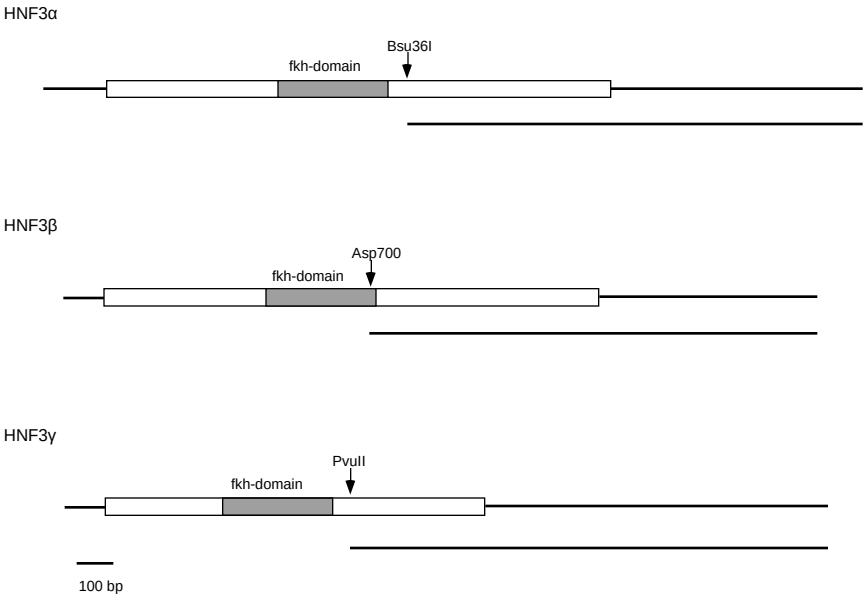

Fig. 1. Restriction map of HNF-3 α, β and γ. The bars under the maps show the regions cloned to generate riboprobes for the in situ hybridisation.

5B). HNF-3 β can not be detected at this stage in any germ layer stretching posterior to the archenteron (Fig. 2C) These results are in complete concordance with cell lineage tracing experiments that suggest that the node is the site of origin of the definitive endoderm which displaces the primitive endoderm anteriorly. It is the definitive endoderm that gives rise to all endoderm-derived structures of the embryo and HNF-3 β mRNA is expressed in all of these structures (see below). These experiments also suggest that the notochord and the neuroectoderm are also derived from cells emerging from the node (Beddington et al., 1982; Lawson et al., 1986, 1987; Lawson and Pedersen, 1992; Tam and Beddington, 1992). The patterns of expression of HNF-3 β mRNA therefore suggest that it is an early marker for the formation of the definitive endoderm and notochord, as they emerge from the primitive streak.

HNF-3 α, β and γ define domains in the developing embryonic endoderm

In mid to late primitive streak embryos (7.5 days to 8.5 days p.c.) weak expression of HNF-3 β mRNA in the endoderm and mesoderm extends to the allantois. HNF-3 α transcripts are detected in cells of the embryonic endoderm whereas HNF-3 γ mRNA is only weakly detected in the extraembryonic endoderm. On day 8 p.c., the foregut pocket becomes distinguished as a horseshoe invagination that extends rostrally into the head fold. Cells of the endodermal lining of the foregut are separated from the neural epithelium by cells of the notochord. Intense expression of HNF-3 β and α mRNA can be detected in the invaginating foregut (Fig. 2D,E). HNF-3 β mRNA is expressed throughout the invaginating gut endoderm, whereas a gradient of HNF-3 α transcripts extend from the more caudal parts of the invagination to the node (Fig. 2D,E, arrow). There is a specific anterior boundary of expression of HNF-3 β mRNA in cells of the underlying notochord and neuralepithelium at this stage (Fig. 2E, double arrow) whereas HNF-3 α mRNA is only weakly detected in the notochord. In transverse sections of neural fold at this stage, HNF-3 β transcripts in the neuroectoderm are localised to the base of the folding neural tube (Fig.6B), cells within this region will later give rise to the floor plate (Fig. 5B). Between days 8.75 p.c. and 9 p.c., expression of HNF-3 β mRNA in the notochord decreases to an undetectable level as the intensity of HNF-3 α transcripts continues to increase and expression of HNF-3 α mRNA in the base of the neural folds is observed (Fig. 2G).

The formation of the hindgut is initiated shortly after foregut invagination as cells of the posterior endoderm ingress into the surrounding mesenchyme. Expression of HNF-3 α and β mRNA is maintained in the endoderm in these ingressing cells (Fig. 2F,G). In addition, expression of HNF-3 γ is first detected in the embryonic endoderm in cells of the invaginating hindgut. A small patch of expression is also detected in the endoderm adjacent to the developing heart (Fig. 2H, arrow). Thus the extreme anterior and posterior portals of the gut develop first, and differential expression of HNF-3 α, β and γ mRNA is observed as this process is initiated.

HNF-3 α, β and γ mRNAs are expressed in all endoderm-derived structures that differentiate posterior to their anterior boundary

The spatial and temporal patterns of expression of the HNF-3 α, β and γ genes in the differentiating endoderm and its derivatives suggest an involvement in anterior-to-posterior regionalisation. On the ninth day of development, these genes exhibit different anterior expression boundaries reflecting their earlier patterns of expression (Fig. 3). HNF-3 α and β mRNAs are similar and are expressed along the entire endodermal lining of the embryo except that HNF-3 β has an anterior boundary in front of the oral plate in the ectodermally derived stomatodaeum (Fig. 3B arrow) whereas HNF-3 α mRNA is restricted anteriorly by the oral plate and at this stage is not detected in the stomatodaeum (Fig. 3A, arrow). In contrast, the anterior boundary of HNF-

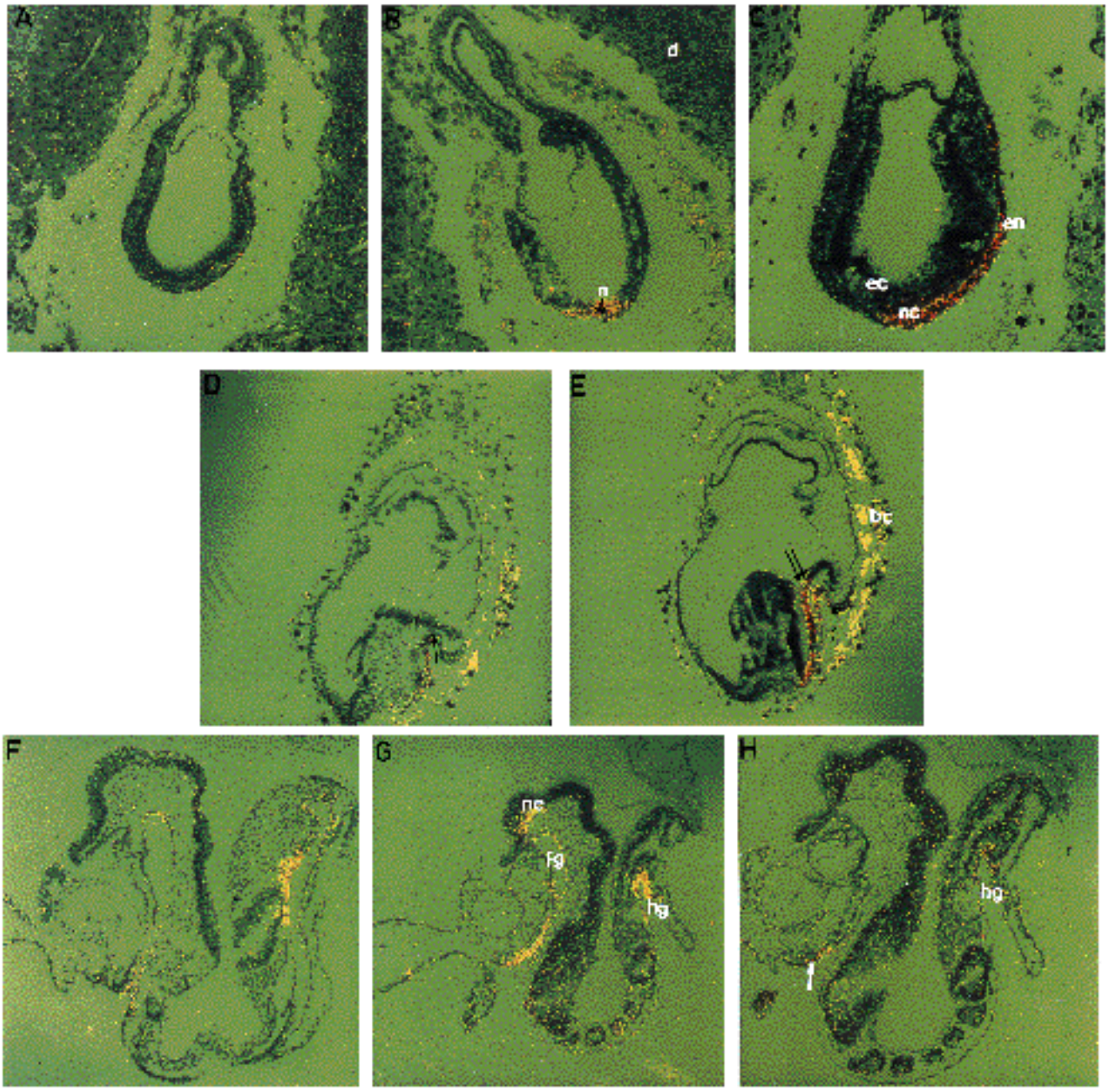

Fig. 2. Expression of HNF-3 α, β and γ mRNA during primitive streak formation. Superimposition of the transmitted light (green) and reflected light (red/yellow). (A-C) Sagittal section of a 7.5 day p.c. embryo hybridised with (A) HNF-3 α mRNA, which is not expressed, and (B,C) HNF-3 β mRNA which is expressed in the node, notochord and definitive endoderm. (D,E) Late 7 day p.c. to early 8 day p.c. embryo showing the foregut indentation. (D) HNF-3 α transcripts extend from the dorsal endoderm (arrow) to the node. (E) HNF-3 β mRNA is expressed in more anterior foregut endoderm (arrow). There is a strict anterior boundary of HNF-3 β mRNA in the neuroepithelium (double arrow). (F-H) Slightly older embryo showing both foregut and hindgut indentation. (G) HNF-3 α and (F) HNF-3 β mRNAs are expressed in the endoderm of the foregut and hindgut pocket. (H) HNF-3 γ mRNA is observed in the hindgut invagination and in endoderm in the region of the cardiac mesoderm (arrow). Blood cells which are in yellow strongly reflect light but are not positive. Key: bc, blood cells; ec, ectoderm; d, decidua; en, endoderm; fg, foregut; hg, hindgut; nc, notochord; ne, neural epithelium; s, somites. (A,B) ×25; (C) ×20; (D,E) ×20; (F) ×18; (G,H) ×16.

3 γ mRNA (Fig. 3C) is found at the junction of the liver diverticulum. The posterior boundary of expression of all three genes is similar, extending to the posterior hindgut. Subsequently expression of HNF-3 α, β and γ mRNAs are maintained in all endoderm-derived structures that differentiate posterior to these boundaries. For example, HNF-3 α and β transcripts can be detected in all of these structures as soon as they begin to differentiate, but HNF-3 γ transcripts are only detected in structures that are located posterior to the liver.

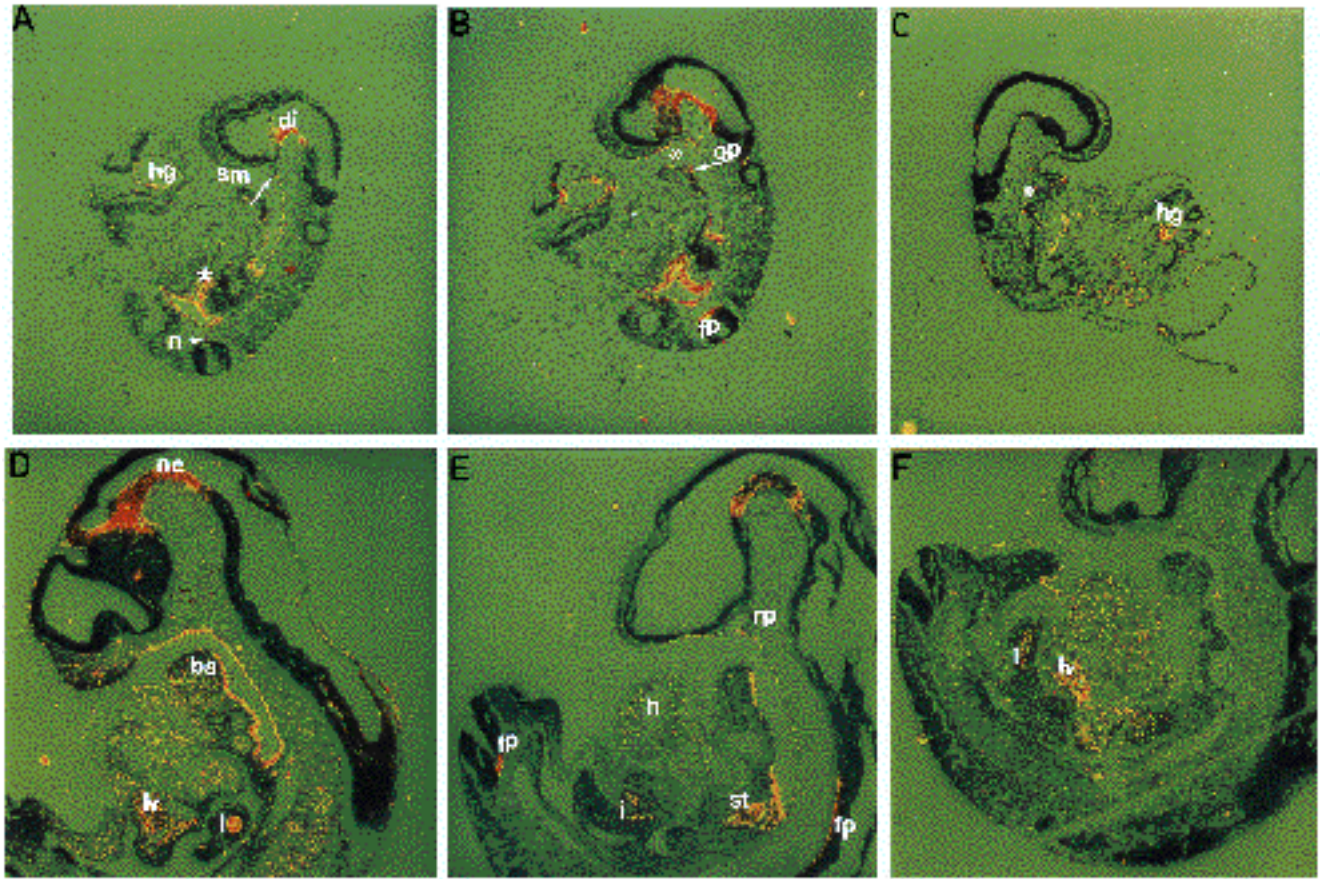

Fig. 3. HNF-3 α, β and γ mRNA in sagittal sections of 9 day p.c. (A,B,C) and 10 day p.c. embryos (D,E,F). HNF-3 α mRNA (A) is restricted to cells of the digestive track located posterior to the oral plate (arrow). In the neural epithelium, HNF-3 α is observed in the diencephalon (di), notochord (n) and weakly in the floorplate (fp). (B) HNF-3 β is observed in all endoderm-derived structures extending from the stomadaeum posterior (star). Remnants of the oral plate can be seen (op). HNF-3 β mRNA is also observed in the diencephalon and floorplate. HNF-3 γ mRNA is only detected in the mid/hindgut region but can not be detected in the foregut (dot) or neural epithelium. On day 10, HNF-3 α mRNA (D) and HNF-3 β mRNA (E) have identical patterns of expression in the endoderm and neuroepithelium. Transcripts are detected in the differentiating lung epithelium (l), stomach (st) and in the developing liver (lv). Rathkes pouch (rp) does not express HNF-3 α or HNF-3 β (E). HNF-3 γ (F) is also detected in the developing liver and weakly in the intestine (i). Expression of HNF-3 α and β mRNA is maintained in the floorplate. Key: ba, branchial arches; hg, hindgut; h, heart; ne, neuroepithelium; op, oral plate; sm, stomadaeum. ×120.

On day 9.5 p.c., all three genes are highly expressed in the ventral floor of the foregut at the site of the primary hepatic diverticulum (Fig. 3A star, B). Subsequently these endodermal cells proliferate and give rise to epithelial cords, which invade the surrounding mesenchyme and differentiate into parenchymal cells. As this process occurs, transcripts of HNF-3 α, β and γ are detected in these cells, reflecting their involvement in parenchymal cell differentiation (Fig. 3D,F). At day 9.5 p.c., expression of HNF-3 α and β mRNA is quite high but decreases to an almost undetectable level between day 12.5 and day 15.5 p.c. (Figs 4E, 5C). Later, their expression increases as they are strongly transcribed in adult liver (Kaestner et al., unpublished data). In contrast, HNF-3 γ mRNA is initially weakly detected but increases on day 10.5 p.c. and remains high throughout liver development (Figs 4C,E, 5C,D).

Anterior to the liver diverticulum, the oesophagus and laryngotracheal groove differentiate. Intense expression of HNF-3 α and β mRNA is detected in this region as cells become committed to differentiate into these structures. On day 10.5 p.c., both HNF-3 α and HNF-3 β mRNA have the same anterior boundary of expression in the oral cavity (Fig. 3D,E) Their restriction to pharyngeal derivatives is reflected by their lack of expression in Rathke's pouch, a structure that buds off from the ectodermal portion of the foregut and migrates cranially to form part of the developing anterior pituitary (Fig. 3E). As the branchial arches and lungs develop, HNF-3 α and β transcripts are localised only to their epithelial layers (Figs 3D, 4A,E and 5C). Expression of both genes is maintained throughout development and is detected in the fully differentiated adult bronchial epithelium. Initially both genes are strongly expressed in the epithelium of the future tongue (Fig. 4A,B) but, on day 13 p.c., the anterior limit of HNF-3 β mRNA begins to retract (Fig. 4D). Subsequently, on day 14 p.c., expression of HNF-3 β mRNA is no longer detected in the trachea and oesophagus. In contrast, expression of HNF-3 α mRNA is maintained in the trachea and oesophagus but retracts from the tongue on day 14 p.c.

Adjacent and posterior to the developing liver, the

stomach, gall bladder, spleen, adrenal glands and pancreas bud from the endodermal tube. HNF-3 α and β mRNAs are expressed in all these structures as they differentiate (Figs 4A,B,D,E, 5C). Initially HNF-3 γ mRNA is only weakly detected in the intestine and has an anterior boundary at the junction of the liver diverticulum and extends to the cloaca (Figs 4C, 5D). HNF-3 α and β mRNAs are strongly expressed in these structures as they differentiate, but on day 14.5 p.c., HNF-3 β mRNA is transiently decreased as it can not be detected in the stomach or intestine. Table 1 summarises these results.

HNF-3 β and γ mRNAs are expressed in developing bone tissue

In the developing cervical, thoracic and lumbar vertebrae between day 13.5 p.c. and day 15.5 p.c, weak expression of HNF-3 β mRNA is observed. HNF-3 γ mRNA is also expressed in the developing vertebrae from day 13 p.c. In addition, HNF-3 γ mRNA is detected in all developing bone tissue in the embryo including bones of the forelimb and hindlimb, ribs and bones of the jaw and skull (Fig. 4F). Expression of HNF-3 γ mRNA in developing bone is first weakly detected in the blastemal condensations of the scapula, humerus and ulna on day 12 p.c. As chondrification occurs, expression of HNF-3 γ mRNA increases between days 13 and 14.5 p.c. By day 16 p.c. expression of HNF-3 β and γ mRNAs can no longer be detected in developing bone tissue.

Table 1. Summary of expression patterns of HNF-3 α, β and γ mRNA during mouse development. Not determined (ND)

Developmental Expression of the murine HNF-3 α, β and γ genes during embryogenesis

Tissue	6.5d	7.5d	8.5d	9.5d	10.5d	11.5d	12.5d	13.5d	14.5d	15.5d	Adult
HNF-3 α											
HNF-3 β											
HNF-3 γ											
Node											
Ectoderm:											
Neural fold											
Forebrain											
Floorplate											
Mesoderm:											
Notochord											
Vertebrae											
Long Bones											
Endoderm:											
Stomodaeum											
Tongue											
Pharynx											
Oesophagus											ND
Thyroid											ND
Submandibular											ND
Lung											
Foregut											
Midgut											
Liver											
Stomach											
Pancreas											
Adrenal											ND
Hindgut											ND

HNF-3 α and β mRNAs are expressed in the floor plate and in the developing central nervous system

Sagittal, transverse and frontal sections of the developing mouse brain between day 8 p.c. and day 15.5 p.c. were hybridised with HNF-3 α and β probes. HNF-3 β mRNA is expressed in the developing neuroectoderm as soon as it emerges from the primitive streak from day 7 p.c. HNF-3 α mRNA cannot be detected in the neuroectoderm at this time (Figs 2A, 6A). From day 8.5 p.c., expression of HNF3 α and β mRNA in the neuroectoderm is very similar. In the folding neural tube, expression of HNF-3 α and β mRNA is initially detected in ventral regions in an area slightly larger than the prospective floor plate (Fig. 6B). In addition, there is an anterior-to-posterior sequence of activation of HNF-3 α and β mRNA in the neural epithelium. In posterior regions, transcripts are only detected in the notochord while, in anterior regions, both the notochord and floorplate show transcripts. Thereafter, transcripts for both genes become localised to the developing floor plate cells as soon as the floor plate becomes morphologically distinct (Fig. 5A,B). In the spinal cord, expression becomes restricted to cells of the floor plate but this domain expands covering adjacent lateral regions anterior to the hindbrain (Fig. 6C,D). In transverse sections, at this stage, extended expression of HNF-3 α and β mRNA is observed in the ventricular zone in the midbrain and diencephalon (Fig. 6C,D). In frontal sections on day 12.5 p.c., expression of both genes is observed in the hypothalamus and ventral thalamus with an anterior border located in the ventral/dorsal thalamic boundary region. HNF-3 α mRNA has a slightly broader region of expression in the mesencephalon and is expressed in more anterior regions of the diencephalon than HNF-3 β mRNA (Fig. 6C,D). Expression is excluded from the nuclei of Cajal in the mesencephalon (Fig. 6C,D arrowheads). On day 15.5 p.c., transcripts for HNF-3 α and β become localised to defined regions in the brain, including cells in the ventricular zone of the IV ventrical, the ventral tegmental nucleus, the red nuclei and dorsal thalamic regions.

HNF-3 β mRNA is detected in the notochord only for a short time (day 7-8 p.c.) but transcription of HNF-3 α is maintained in the notochord until it degenerates. HNF-3 α transcripts are also detected in cartilagenous cells which condense around the notochord and differentiate into structures of the vertebral column. A broad band of expression extends dorsally to the floorplate and a narrow band extends ventrally toward the developing gut (Fig. 5A,C).

DISCUSSION

We have compared the temporal and spatial patterns of mRNA expression of the transcription factors HNF-3 α, β and γ during development in the mouse. RNase protection experiments have shown that, in the adult, transcription of all three genes can be detected in the gut, liver and pancreas (K. Kaestner et al., unpublished data). In addition, HNF-3 α and β are transcribed in the lung whereas HNF-3 γ is tran-

scribed in the testis. These restricted patterns of expression for the HNF-3 genes in addition to their requirement for liver-specific gene expression, led to the proposal that they are endoderm-specific transcription factors (Costa et al., 1989). We have shown that the expression patterns of these genes in the developing endoderm suggest that they are involved in regional specification within the developing endoderm from the very first steps of differentiation and that HNF-3 α and β are required for the differentiation of the notochord and floorplate.

HNF-3 β is expressed in the node

Gastrulation occurs in mouse on the sixth day of development with the appearance of the primitive streak. The formation of the third germ layer, the mesoderm, which occurs within the primitive streak, is a crucial event in the establishment of the basic body plan. Cell lineage tracing experiments have shown that the definitive endoderm and mesoderm are derived from ectodermal cells and that this recruitment is most active at the anterior end of the primitive streak: the node. Mesoderm that originates from the node gives rise to prechordal and chordal plate mesoderm. Thus cells of the notochord and endoderm are derived from the same population of ectodermal cells in the node. (Beddington, 1981, 1982; Lawson et al., 1986, 1987, 1991; Lawson and Pedersen, 1992; Tam, 1989; Tam and Beddington, 1987, 1992).

During primitive streak formation, HNF-3 β transcripts are observed in all three germ layers in the node and in cells of the definitive endoderm, prechordal and chordal plate mesoderm as they extend toward the future head fold. Other genes have been identified that are expressed in the node at this time and have been shown to play key roles in axis formation: goosecoid, a homeobox-containing gene, which marks the site of primitive streak formation and subsequently becomes localised to the mesodermal cells at the node (Blum et al., 1992); nodal, a TGF-β-like molecule, which is transiently expressed in the endodermal cells of the node, and embryos homozygous for this mutation do not produce mesoderm (Zhou et al., 1993); and *Brachyury*, a putative transcription factor, which is initially expressed in nascent mesoderm within the primitive streak and is required for notochord and posterior axis formation (Herrmann, 1990; Wilkinson et al., 1990). Although a comparative expression analysis of these genes has not been performed in mouse, it can be concluded that they are expressed in overlapping populations of nascent mesoderm in the node during primitive streak formation. Similar conclusions have been reached by Sasaki and Hogan (1993). HNF-3 β mRNA is additionally expressed in adjacent ectoderm and endoderm cells. Furthermore, expression of HNF-3 β mRNA is detected in more anterior mesoderm than brachyury and does not extend into the posterior mesoderm where brachyury mRNA is still expressed. These findings, in addition to its known role in liver-specific gene activation, suggest that HNF-3 β plays a key role in mesoderm induction, in particular in notochord differentiation and subsequently in axis formation in the developing mouse.

Expression of HNF-3 α, β and γ and regionalisation of the definitive endoderm

The formation of the definitive endoderm in the developing mouse has been extensively studied histologically (Snell and Stevens, 1966; Poelmann, 1981; Lamers et al., 1987), using both orthotopic and heterotopic transplants (Beddington, 1981, 1982; Levak-Svajger and Svajger, 1971) and in situ using single-cell lineage-tracing experiments. These experiments all indicate that the definitive endoderm is derived from a population of ectodermal cells that are inserted into the primitive endoderm at the anterior end of the primitive streak. It has been shown that there is a continuous recruitment of new cells at the node into the definitive endoderm during the very early stages of gastrulation which gradually displace cells of the primitive endoderm anteriorly. Cell lineage tracing experiments have further demonstrated that the visceral endoderm primarily gives rise to the extraembryonic endoderm but that it is cells from the definitive endoderm that colonise the anterior and posterior intestinal track (Beddington et al., 1992; Lawson and Pedersen, 1992). The expression of HNF-3 β mRNA in the endoderm during these stages is coincident with the emergence of the primitive endoderm. Expression is first detected on day 6.5 p.c. in a small population of endoderm and ectoderm cells. Subsequently HNF-3 β mRNA is localised to ectodermal cells adjacent to the node as soon as it becomes morphologically distinguished. In the endoderm, expression of HNF-3 β mRNA is localised only to cells extending from the node toward the head process suggesting that it is specifically labelling the emerging definitive endoderm cells (Fig. 2C).

Cell lineage tracing experiments have shown that there is a strict craniocaudal recruitment of definitive endoderm cells into the developing gastrointestinal track; cells that arise first colonise regions of the ventral foregut and anterior intestinal track whereas cells that colonise the dorsal foregut arise later (Beddington et al., 1992). Consistent with these observations, HNF-3 β mRNA is intensely expressed in the anterior foregut invagination. Upon invagination, intense expression of HNF-3 α mRNA is observed only in the caudal regions of the foregut pocket. Hindgut invagination is initiated shortly thereafter and expression of HNF-3 α and β mRNA extends into this pocket. The first transcripts of HNF-3 γ are now also detected in the invaginating posterior intestinal portal. In addition, HNF-3 α, β and γ mRNA expression is detected in endoderm adjacent to the heart mesoderm. This region of the endoderm has not yet been accurately fate mapped but the subsequent anterior boundary of expression of HNF-3 γ mRNA indicate that this region may be involved in liver differentiation. Therefore the temporal and spatial patterns of the initiation of expression of HNF-3 α, β and γ mRNA in the definitive endoderm and differentiating foregut and hindgut, reflect the craniocaudal sequence of commitment of endoderm cells to intestinal track differentiation.

The expression of HNF-3 α, β and γ mRNAs in endoderm-derived structures is dictated by the anterior boundary of expression of each gene. HNF-3 β is initially transcribed in more anterior regions than HNF-3 α but their expression patterns subsequently become indistinguishable. Upon differentiation of the endoderm, transcripts of HNF-3 α and β are detected in all endoderm-derived structures. HNF-3 γ mRNA, which has an anterior boundary in the midgut, is only expressed in organs that differentiate

posterior to this boundary. Expression extends from the junction of the liver diverticulum to the colon. This suggests that the anterior boundaries of expression of these genes in the endoderm may be reflecting regionalisation in developing digestive track.

The HNF-3 gene family and liver development

The HNF-3 genes were originally characterised as transcription factors that are required for the expression of liver-specific genes (Costa et al., 1988; Herbst et al., 1991; Lai et al., 1991; Pani et al., 1992). Additional liver-enriched tran-

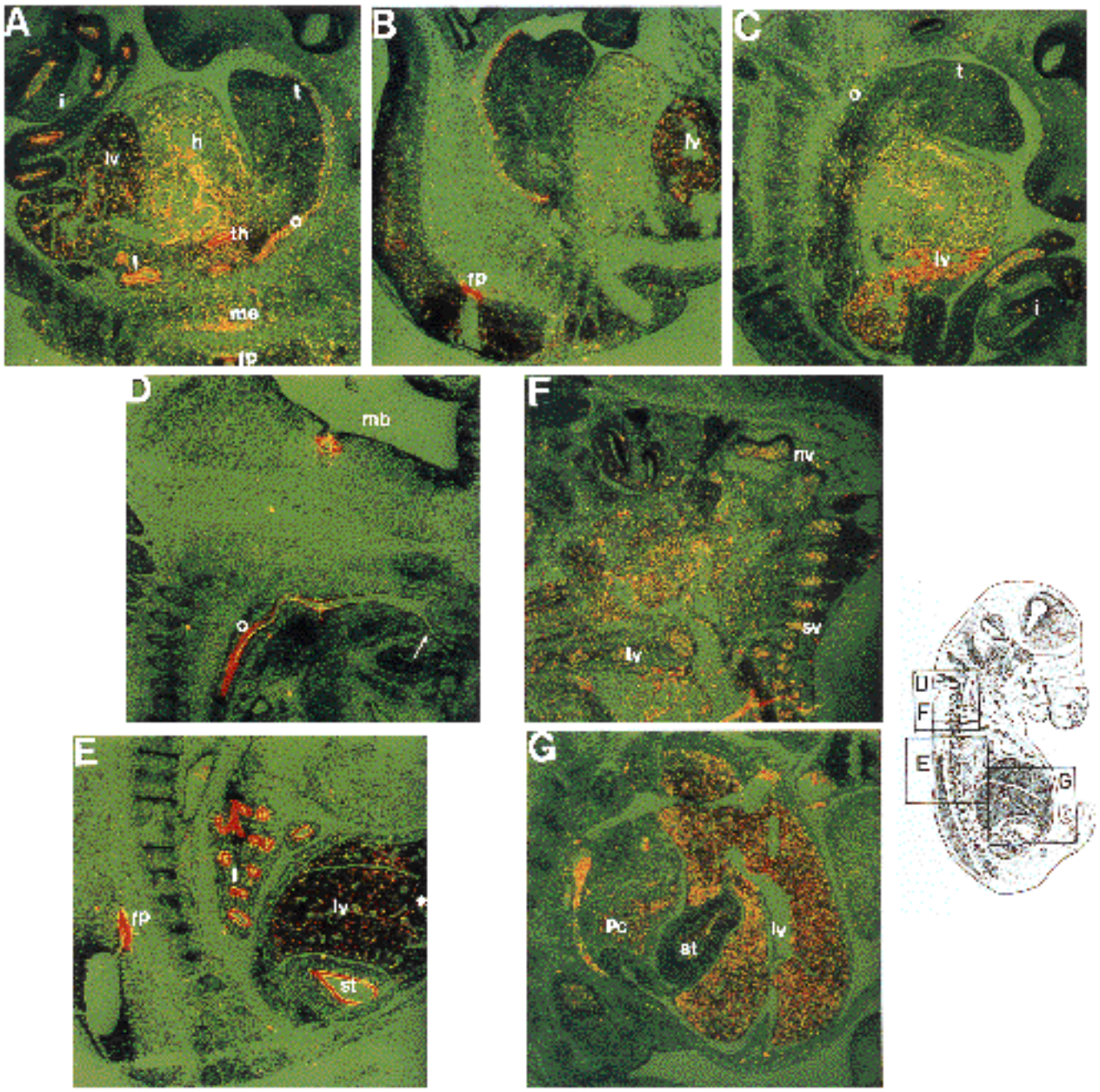

Fig. 4. Sagittal sections of 11.5 day (A,B,C), 13.4 day (D,E,F) and 14.5 day (G) embryos. (A) Expression of HNF-3 α mRNA is maintained on day 11.5 in the tongue (t), oesophagus (o), thymus (th), lung (l), intestine (i), floorplate (fp) and mesenchyme (me). The expression in the liver is decreasing (lv). The heart, which contains blood cells is not positive (h). (B) Expression of HNF-3 β mRNA in more lateral regions showing expression in the developing tongue, floorplate and absence of expression in the mesenchyme. Expression in the liver is also decreasing. (C) HNF-3 γ mRNA is highly expressed in the liver and weakly expressed in the intestine. (D,E) Composite reconstruction of the dorsal regions of 13 day intestinal track. Transcripts of HNF-3 β are retracted from the anterior regions of the tongue (arrow) but maintained in the oesophagus, lung, floorplate and stomach (st). The liver is very weakly positive (lv) with nonexpressing regions (star). (G) In contrast, HNF-3 γ mRNA is highly expressed in the liver on day 13.5. The stomach is also positive. The pancreas (pc) which is also positive for HNF-3 α and β transcripts at this stage is shown. (F) Sagittal section through the neck of a 14.5 day embryo hybridised with HNF-3 γ mRNA. Note the expression in the spinal vertebrae (sv), neck vertebrae (nv) and ribs (tv). Midbrain (mb). ×4.6.

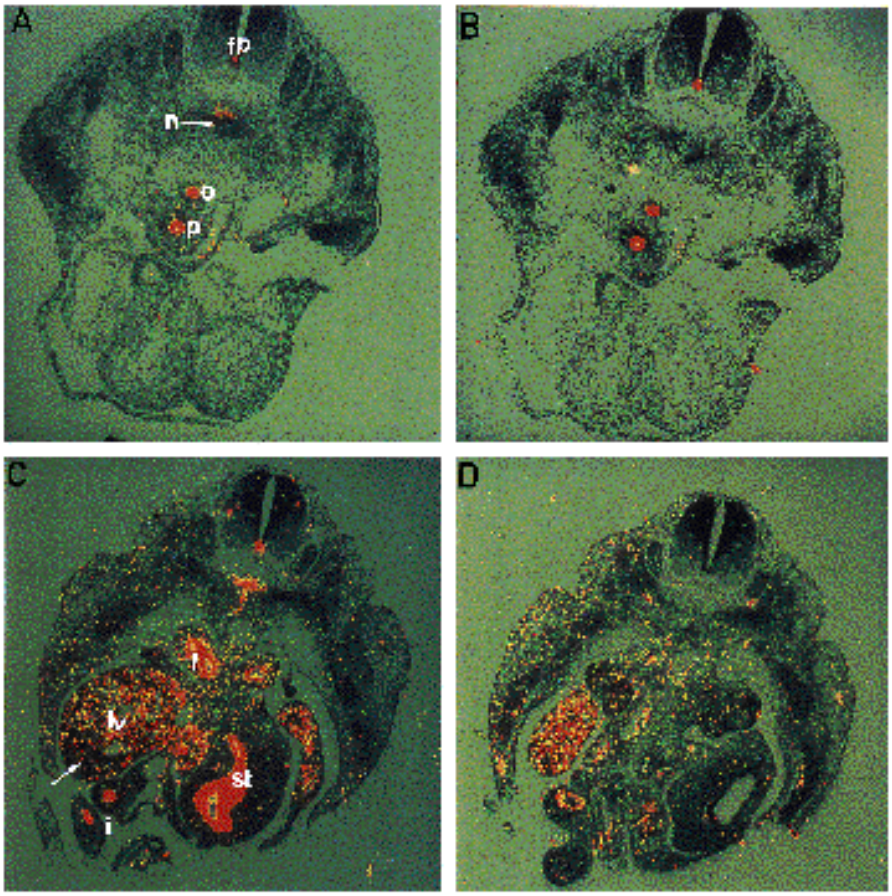

Fig. 5. Transverse sections of 12.5 day p.c. embryos. (A) From dorsal to ventral expression of HNF-3 α mRNA is localised to cells of the floorplate (fp), notochord (n), oesophagus (o) and trachea (p). Note the additional expression of HNF-3 α mRNA in cells of the mesenchyme surrounding the notochord. A thin band extends from the notochord toward the oesophagus and a broader triangle of expressing cells extends toward the floorplate (A,C). (B) HNF-3 β mRNA is also observed in the floorplate, oesophagus and trachea but is absent from the notochord and surrounding mesenchyme cells. (C,D) Transverse sections in more posterior regions demonstrate differences between HNF-3 α and γ mRNAs. In the developing floorplate, notochord and lung epithelium (l), HNF-3 α mRNA (C) can be detected. Both HNF-3 γ (D) and α mRNA (C) are observed in the intestine (i). Expression of HNF-3 α mRNA in the liver (lv) is continuing to decrease (arrow) (the observed signal is mainly reflection from differentiating haematopoeitic cells within the liver) but expression in the stomach is high (st). In contrast, HNF-3 γ mRNA is highly expressed in the liver but is only weakly detected in the stomach. ×5.

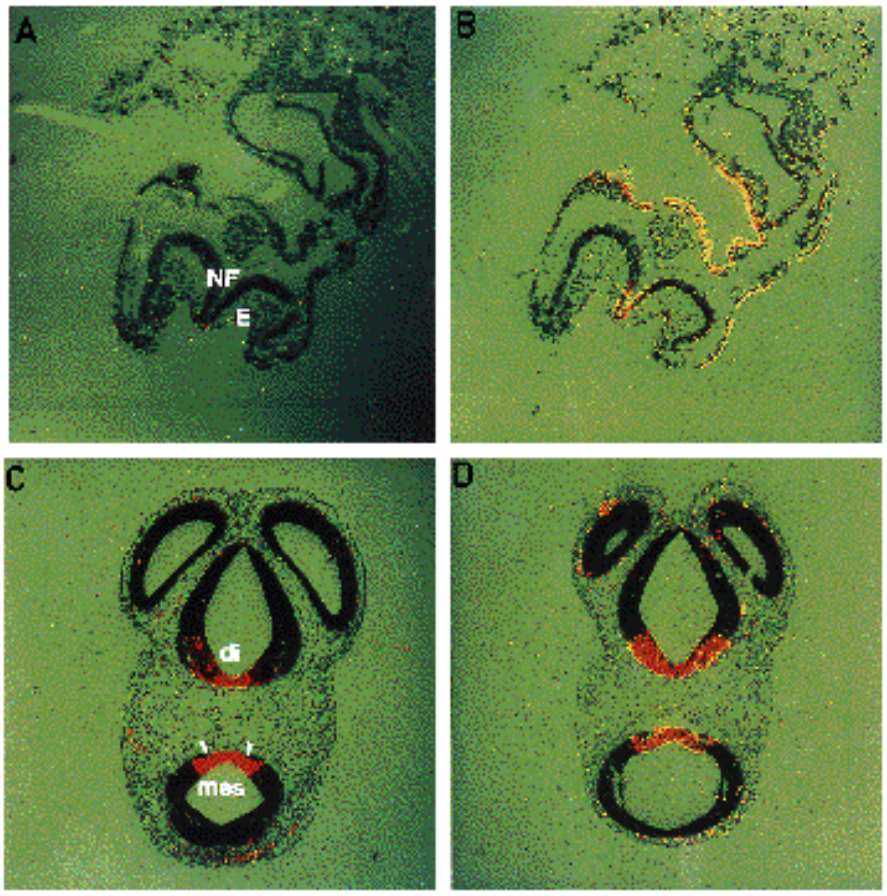

Fig. 6. Expression in the developing neural epithelium of HNF-3 α and β mRNA. (A,B) Transverse section through the folding neural epithelium of a 7.75 day p.c. embryo showing expression of HNF-3 β mRNA (B) in the floor of the neural tube (nf) and endoderm (e). HNF-3 α mRNA (A) can not be detected. (C,D) Transverse section through the developing brain of a 12.5 day p.c. embryo showing expression of HNF-3 α mRNA (C) and HNF-3 β mRNA (D) in the diencephalon (di) and mesencephalon (mes). Note the two small excluded areas of expression (arrowheads) in the mesencephalon. (A,B) ×22; (C,D) ×8.

scription factors that have been identified include: the homeoprotein HNF-1 (Baumhueter et al., 1990); HNF-4, a member of the steroid hormone receptor superfamily (Sladek et al., 1990); DBP, the albumin D-box-binding protein (Mueller et al., 1990) and members of the bZIP C/EBP protein family (Cao et al., 1991; Landschulz et al., 1988). These genes are required for the coordinate expression of a number of liver-enriched genes, e.g. TAT, TTR, α-1AT, AFP, albumin and PEPCK (reviewed in De Simone and Cortese, 1991). Furthermore, it has been shown that the expression of C/EBP, HNF-4, HNF-3 and, to a lesser extent, HNF-1 are controlled transcriptionally, suggesting that a transcriptional hierarchy exists in hepatic-specific gene expression (Xanthopoulos et al., 1991; Kuo et al., 1992). So far two genes seem to be related by a transcriptional hierarchy: HNF-4 regulates the expression of HNF-1 (Kuo et al., 1992; Tian and Schibler, 1991). Our results suggest that the members of the HNF-3 gene family may be the first genes in this cascade which leads to liver-specific gene expression. No other genes encoding liver-enriched transcription factors have been found that are expressed as early as HNF-3 β in the embryonic endoderm. vHNF-1 (Cereghini et al., 1992; Ott et al., 1991) and HNF-4 (A. P. M. unpublished observation) are detected in the hepatic diverticulum on day 9 p.c., whereas C/EBP, DBP and HNF-1 are activated after the initiation of hepatic differentiation (Ott et al., 1991; Kuo et al., 1990). Furthermore, HNF-1, HNF-4 and C/EBP do not bind to the proximal promoter of HNF-3 β gene, but HNF-3 β is positively autoactivated, putting it in a primary position in the hierarchy (Pani et al., 1992).

The early expression of HNF-3 α and β in the developing gut suggests that they may be required for the activation of genes crucial for the initiation of hepatogenesis. HNF-3 γ may also contribute to initiation, but its continued expression in the developing liver in the absence of detectable HNF-3 α and β transcripts (between day 11 and day 15) suggests that it is further required for the activation of genes in later development. Tissue culture and transplantation experiments have shown that endodermal cells require two inductive signals to induce hepatic differentiation. First, in avian and mouse embryos, cardiac mesoderm can induce the development of hepatic epithelium in anterior endoderm. Second, proliferating endodermal cells must interact with mesoderm of the septum transversum to differentiate into hepatocytes (LeDouarin, 1968, 1975; Houssaint, 1980; Fukuda-Taira, 1981; Cascio and Zaret, 1991). Intense expression of all three genes HNF-3 α, β and γ in the endoderm adjacent to the differentiating cardiac mesoderm on day 8 suggests that they may reflect induction events that initiate liver development. In addition, these genes are also expressed in the mesenchyme surrounding the septum transversum where hepatocyte differentiation is induced. These genes therefore provide useful molecular markers for the initiation events in hepatic differentiation.

The recent identification of the *Drosophila* C/EBP and HNF-4 genes and the demonstration that they are structurally and functionally similar to their mouse homologues has been reported (Rorth and Montell, 1992; Zhong et al., 1993). These findings in addition to the demonstration that, in *Drosophila*, there is a strict requirement for C/EBP, HNF-4 and *forkhead* for normal development suggests that this transcriptional hierarchy may be conserved between vertebrates and invertebrates. Furthermore the patterns of HNF-3 β mRNA expression in the developing mouse embryo is strongly reminiscent of the expression of the *Drosophila fkh* gene, which has been shown to be critical for normal *Drosophila* development. The HNF-3 α and β genes and the *fkh* gene are expressed in the invaginating anterior and posterior gut primordium, salivary glands and developing central nervous system In addition, the HNF-3 β gene and the *fkh* gene are expressed in the stomadaeum, suggesting that HNF-3 β is the murine forkhead orthologue.

HNF-3 α and β in central nervous system development

HNF-3 β mRNA expression in the neuroepithelium is coincident with the onset of mesoderm formation in the mouse embryo. Cells in this region contribute to formation of the head process, which is the progenitor of all cephalic structures in the adult. Upon formation of the head process on day 7.5 p.c., expression of HNF-3 β mRNA is observed only in the ventral regions of the neuroepithelium and in the underlying chorda mesoderm. If the expression of HNF-3 β mRNA is examined in the folding neural groove, it can be seen that only cells in the base of the folding neural plate express HNF-3 β mRNA. At early stages of development (between day 7 and day 9 p.c.), expression is also detected in the notochord, which is in close proximity to the neural plate during these stages. Subsequently, this expression becomes localised to cells of the floor plate. HNF-3 α transcripts are first detected in the notochord and neural epithelium on day 8 p.c. Expression of both HNF-3 α and β mRNAs in the notochord precedes expression in the floor plate and this occurs with an anterior-to-posterior progression. Subsequently, HNF-3 α mRNA expression is also detected in a restricted population of mesenchyme cells surrounding the notochord. In contrast to the transient HNF-3 β mRNA expression, HNF-3 α transcripts persist in the notochord until it becomes integrated into the spinal cord.

These observations are interesting in light of the role that these regions play in neural induction and dorsoventral patterning in the neural tube. During neurulation, one of the first cell types to differentiate is the ventral midline cells which give rise to the floor plate. Two inductive signals are necessary for the formation of the floor plate. The first is a contact-dependent signal, emanating from the notochord to cells of the neural plate. The second signal is generated by the floor plate cells themselves. The importance of the notochord in dorsoventral patterning has been highlighted in experiments in which removal during early embryonic stages results in loss of the floor plate and adjacent motor neurons. Furthermore, implantation of an additional notochord adjacent to the neural plate resulted in the induction of a second floor plate and the generation of additional motor neurons. The floor plate itself has also been implicated in the patterning of commissural neurons in the developing central nervous system (van Straaten et al., 1985; van Straaten and Hekking, 1991; Yamada et al., 1991; Placzek et al., 1991 and references therein). The expression of HNF-3 β mRNA in the mesoderm and neural epithelium during these formative stages of neural devel-

opment suggest that its activation may be a primary response to these inductive events. This proposal is supported by the observation that expression of HNF-3 α and β mRNAs is detected in the notochord first and then in the floor plate. Expression in ventral neural cells before neural tube closure and overt floor plate differentiation may reflect the potential of these cells to form floor plate. In addition HNF-3 β mRNA is only transiently expressed in the notochord possibly reflecting the transient capacity of the notochord to induce floor plate differentiation. It is interesting that as expression of HNF-3 β mRNA in the notochord is being reduced, expression of HNF-3 α mRNA is increasing, suggesting that HNF-3 α mRNA is activated as a secondary response to induction and may be required primarily for maintenance of the notochord.

The suggestion that expression of HNF-3 β mRNA in the floor plate may be a response to primary induction is supported by the observation that Axial (the zebrafish HNF-3 β orthologue) is not expressed in the floor plate of cyclops homozygous embryos although the expression in the notochord and mes-endodermal lineages is unaffected (Strähle et al., 1993). These embryos lack a recognizable floor plate even though the notochord is present (Hatta et al., 1991). The HNF-3 α and β genes therefore provide useful molecular markers to address the mechanisms involved in floor plate differentiation.

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