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Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut, and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst

(visceral endoderm/organogenesis/murine development)

STEPHEN A. DUNCAN*, KATIA MANOVA†, WILLIAM S. CHEN*, PAMELA HOODLESS*, DANIEL C. WEINSTEIN*, ROSEMARY F. BACHVAROVA†, AND JAMES E. DARNELL, JR.*‡

*Department of Molecular Cell Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021; and †Department of Cell Biology and Anatomy, Cornell University Medical College, 1300 York Avenue, New York, NY 10021

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ABSTRACT **The expression of HNF-4 (hepatocyte nuclear factor 4) mRNA in postimplantation mouse embryos was analyzed by *in situ* hybridization. Expression was found in the primary endoderm at embryonic day 4.5 and was restricted to the columnar visceral endoderm cells of the yolk sac from day 5.5 to day 8.5. HNF-4 mRNA was first detected in embryonic tissues at day 8.5, in the liver diverticulum and the hindgut. At later times HNF-4 transcripts were observed in the mesonephric tubules, pancreas, stomach, and intestine and, still later, in the metanephric tubules of the developing kidney. This expression pattern suggests that HNF-4 has a role in the earliest stages of murine postimplantation development as well as in organogenesis.**

Specialized cell types in vertebrates are believed to depend for their development, at least in part, on the regulated expression of transcription factors (1, 2). Since cell-specific gene expression in adult mammalian cell types also relies on transcriptional control (1, 2), it has seemed plausible that such transcription factors might also be key elements in mammalian cell differentiation. Several approaches have been taken to identify key regulators in mammalian systems: identification of mammalian homologues of known regulators from invertebrates (3), gene trapping in embryonic stem cells (4), retroviral or transgenic mutagenesis *in vivo* (5), and analysis of differentiation *in vitro* using embryonal carcinoma cells (6). An alternative approach is to identify transcription factors that regulate adult tissue-specific gene expression and then determine whether they act during development. This approach is logical, based on the frequent occurrence in invertebrates of early-acting transcription factors which become quiescent and are then reactivated in a specific tissue at a later stage (7, 8).

Analyses of promoter elements from genes expressed in hepatocytes have identified a number of transcription factors with roles in tissue-specific gene regulation, such as HNF-1α and -1β, the C-EBP family, HNF-3α, -3β, and -3γ, and HNF-4 (reviewed in ref. 1). Several of these genes have expression patterns consistent with a function in developmental regulation. For example, HNF-3α and HNF-3β are expressed in late and early primitive-streak-stage embryos, respectively, prior to expression in the developing gut (9–12). In addition, ectopic expression of HNF-3β in the midbrain/hindbrain region resulted in the induction of a neural floor-plate-like tissue with the concomitant expression of HNF-3α, providing evidence for HNF-3β, and possibly HNF-3α, as regulators of early neural development (13).

Another hepatocyte transcription factor, HNF-4, is a member of the steroid hormone receptor superfamily (14); binding sites for this protein are found in many genes expressed in hepatocytes and are required for expression after acute transfection in hepatoma cells (15). The tissue distribution of HNF-4 mRNA and protein in the adult mouse is restricted, with expression in the liver, intestine, kidney, and pancreas but not in brain, spleen, skin, and muscle (reviewed in ref. 15). HNF-4 was considered a strong candidate for a developmental regulator after the isolation of a *Drosophila* homologue (dHNF-4) with strikingly similar sequence (8). The dHNF-4 gene is expressed in the egg as a maternal mRNA and, after fertilization, expression is briefly retained in the terminal segments of the developing embryo. Later, embryonic expression recurs in the midgut, fat bodies, and Malpighian tubules. These tissues are homologous to the intestine, liver, and kidney, where HNF-4 is prominently expressed in mammals, suggesting a conserved function during evolution.

To determine whether HNF-4 has a potential role as a mammalian developmental regulator, we have mapped its expression pattern during early mouse development by *in situ* hybridization. We found HNF-4 mRNA in the primary endoderm of implanting blastocysts at embryonic day 4.5 (e4.5) and in the extraembryonic visceral endoderm cells from e5.5 onward. The first appearance in embryonic tissue was detected around e8.5 in the liver and gut primordia. This expression pattern suggests a role for HNF-4 during formation of the extraembryonic endoderm lineage and in determining liver- and gut-like functions throughout embryogenesis.

MATERIALS AND METHODS

***In Situ* Hybridization.** Primers 5′-ACACGTCCCCATCTGAAGGTG-3′ and 3′-CTTCCTTCTTCATGCCAGCCC-5′ were used to amplify by polymerase chain reaction a fragment of the mouse HNF-4 cDNA (W. Zhong and J.E.D., unpublished work) which was subsequently cloned into the *Eco*RV site of pBluescript KS (Stratagene). From this plasmid, p4-is, ^{33}P-labeled sense and antisense RNA probes were transcribed *in vitro*, using T3 and T7 promoters, respectively, which contained nucleotides 147–416 [according to the published rat HNF-4 nomenclature (14)] of mouse HNF-4 sequence. Sections were hybridized with either labeled sense-strand RNA, which showed no signal above background (data not shown), or labeled antisense RNA. Hybridization of

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Abbreviation: e*n*, embryonic day *n*.

‡To whom reprint requests should be addressed.

the antisense probe to mouse embryos that lacked the HNF-4 gene gave only background labeling, confirming the specificity of the probe (data not shown).

Embryos were collected from crosses of CD-1 mice (Charles River Breeding Laboratories), with noon on the day of finding a vaginal plug designated e0.5, fixed overnight with 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated, and embedded in paraffin. Sections (5 μm) were mounted on Superfrost Plus slides (Fisher) and processed for *in situ* hybridization essentially as described (16), with the following modifications. After wax removal and rehydration in water slides were fixed with 4% paraformaldehyde in PBS before incubation with proteinase K (20 μg/ml) for 7.5 min at room temperature. Digestion was stopped by washing twice in PBS and then in 4% paraformaldehyde in PBS. Following a further wash in PBS, slides were treated in 0.1 M triethanolamine for 5 min followed by a 5-min incubation in 0.25% acetic anhydride 0.1 M triethanolamine. After two washes in 2× standard saline citrate (SSC) and one wash in water, slides were incubated overnight with 5×10^6 cpm of probe in 50 μl of hybridization buffer [40% formamide/0.5 M NaCl/8 mM Tris·HCl, pH 7.5/1.6 mM EDTA/0.8× Denhardt's solution/10% dextran sulfate with yeast tRNA at 1 mg/ml and poly(A) at 80 μg/ml] at 65°C in a moist chamber. Slides were washed as described (16), except that samples were digested with RNase A (50 μg/ml) in 0.3 M NaCl/10 mM Tris·HCl, pH 8.0/5 mM EDTA. Samples were dehydrated and exposed to emulsion for ≈14 days before staining with hematoxylin and eosin.

Histochemical Staining with 5-Bromo-4-chloro-3 indolyl β-D-Galactopyranoside (X-Gal). e7.5 embryos from either CD-1 or crosses of CD-1 × TM47/68 [transgenic lines containing 7.2 kb of upstream HNF-4 promoter sequence driving expression of a β-galactosidase reporter gene (17)] were dissected in PBS, fixed, and treated for X-Gal histochemical staining as described (17).

RESULTS

At approximately e4.5 uterine implantation of the blastocyst occurs and is accompanied by an early cell fate decision whereby the layer of cells of the inner cell mass which border the blastocoel differentiate to form primary endoderm. At this point, the inner cell mass consists of two cell lineages: the ectoderm (epiblast), which will contribute to all embryonic tissues, and the primary endoderm, which will form the parietal endoderm and the visceral endoderm of the yolk sac. *In situ* labeling of thin sections (Fig. 1 *A* and *B*) revealed that at e4.5 HNF-4 was expressed in the single layer of cells facing the blastocoel, establishing HNF-4 among the earliest known markers for primary endoderm.

At e5.5 (Fig. 1 *C* and *D*) HNF-4 expression is seen in all visceral endoderm cells, which now form a low columnar epithelium surrounding the egg cylinder and extend up to the ectoplacental cone. Around e6.5 the primitive streak begins to form on the posterior side of the embryo. The visceral endoderm has become a tall columnar epithelium surrounding the upper part of the egg cylinder and extends past the junction between the embryonic and extraembryonic ectoderm, while the visceral endoderm around the lower half of the embryonic region is a squamous epithelium. At e6.5 HNF-4 mRNA continues to be expressed in the columnar visceral endoderm but is undetectable in the squamous endoderm cells surrounding the lower embryonic region (Fig. 1 *E* and *F*). At e7.5 extraembryonic mesoderm originating from the primitive streak has contributed to amnion, allantois, and visceral yolk sac, while embryonic mesoderm has spread throughout the embryo. Embryonic endoderm arising in the primitive streak covers much of the embryo at this time. HNF-4 expression is still restricted to the columnar

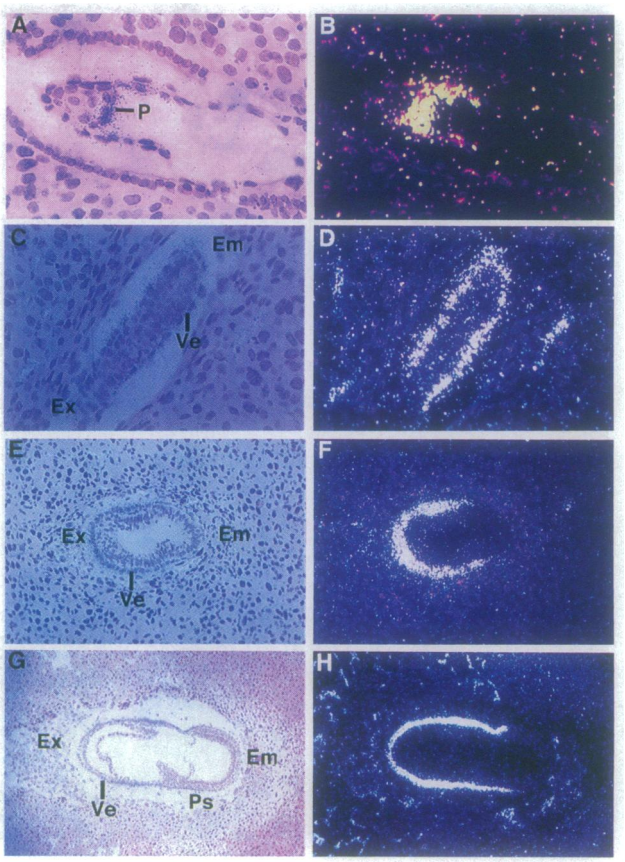

FIG. 1. Expression of HNF-4 mRNA in e4.5–e7.5 mouse embryos. *In situ* hybridizations were carried out on sagittal sections of e4.5 (*A* and *B*), e5.5 (*C* and *D*), e6.5 (*E* and *F*), and e7.5 (*G* and *H*) mouse embryos. *A*, *C*, *E*, and *G* show brightfield images of hematoxylin- and eosin-stained sections with corresponding darkfield images shown in *B*, *D*, *F*, and *H*. P, primary endoderm; Ex, extraembryonic pole; Em, embryonic pole; Ve, visceral endododerm; Ps, primitive streak. (Original objective used for *A* and *B* was ×40; for *C–F*, ×20; and for *G* and *H*, ×10.)

visceral endoderm (Fig. 1 *G* and *H*), often appearing to be more abundant on the posterior side; it is not detected in any embryonic cells or in the extraembryonic mesoderm or ectoderm.

Around e8.0 somite formation begins and the yolk sac has expanded to form a balloon-like structure. HNF-4 continues to be expressed in the yolk sac endoderm, and the border between expressing and nonexpressing cells corresponds approximately with the border between extraembryonic and embryonic endoderm (Fig. 2). Interaction of cardiac mesoderm with the endoderm of the ventral wall of the foregut (Fig. 2 *G* and *H*) induces formation of the liver primordium (18); however, in the four-somite embryos shown in Fig. 2 *A–H*, no labeling of foregut endoderm is identified. In a slightly later embryo (12 somites) a proliferation of liver cells can be seen growing ventrally from the ventral wall of the foregut/midgut junction, and these cells are strongly labeled (Fig. 3 *A–D*). In addition, the ventral wall of the hindgut is expressing HNF-4 mRNA (Fig. 3 *A* and *B*). The midgut is still open at this stage, and a transitional region of cells expressing HNF-4 at a low level can be seen between the dorsal embryonic endoderm and the yolk sac endoderm that will presumably form the lateral and ventral walls of the gut. At e9.5, the developing liver grows voraciously, interacting with the mesenchyme of the septum transversum, and strongly expresses HNF-4 (Fig. 3 *E–H*). The midgut, including the primordium of the pancreas, expresses HNF-4 at a moderate

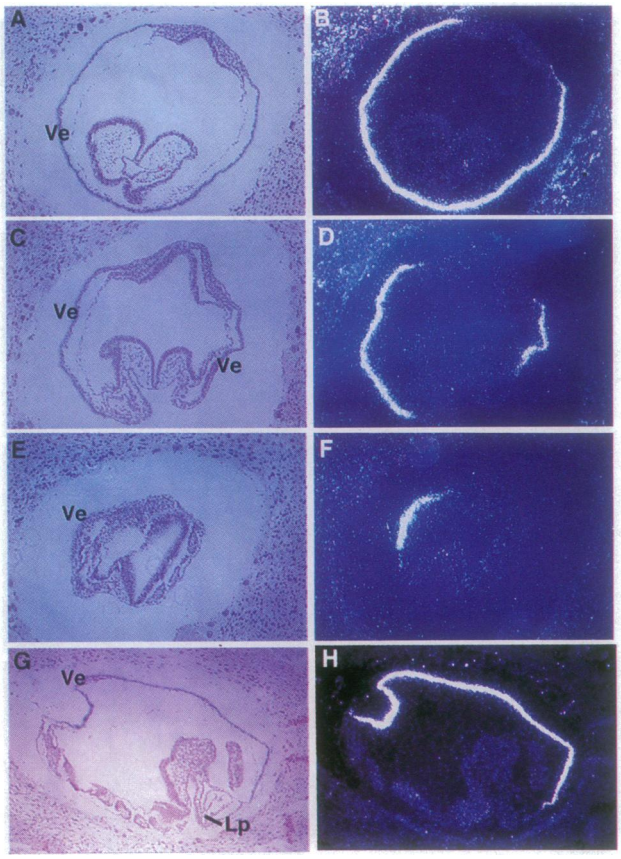

FIG. 2. Expression of HNF-4 mRNA at e8.0. *In situ* hybridizations were performed on 5-μm transverse (*A–F*) and sagittal (*G* and *H*) sections of e8.0 (about four somites) mouse embryos. Representative transverse sections through an e8.0 embryo are presented proximal to distal, in *A–F*, demonstrating that HNF-4 expression is restricted to the extraembryonic yolk sac endoderm. *G* and *H* show a mid-sagittal section through another e8.0 embryo with the site of the liver primordium indicated by Lp. *A*, *C*, *E*, and *G* show brightfield images of hematoxylin- and eosin-stained sections with corresponding darkfield images shown in *B*, *D*, *F*, and *H*. Ve, visceral endoderm; Lp, liver primordium. (Original objective, ×10.)

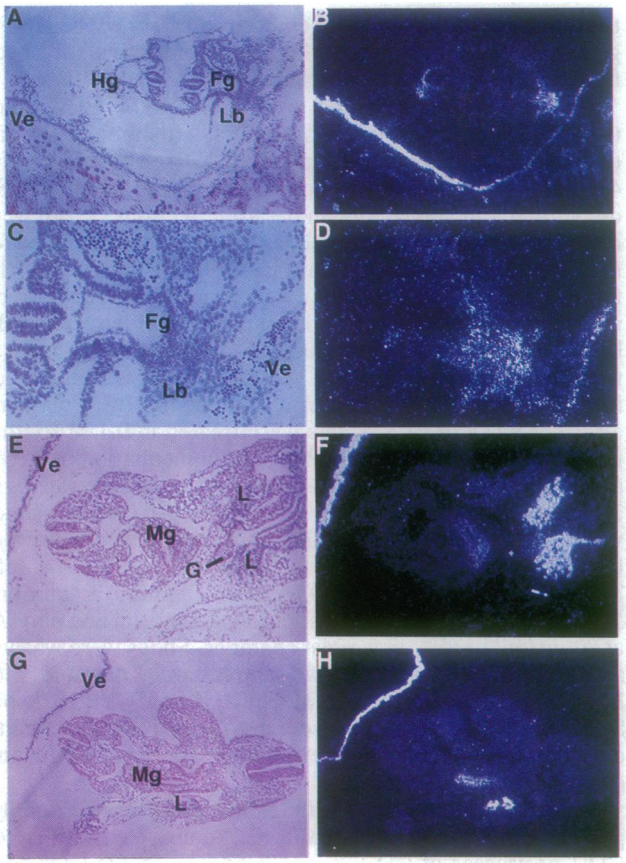

FIG. 3. HNF-4 expression at e8.5 and e9.5. *In situ* hybridizations were performed on 5-μm transverse sections of e8.5 (*A–D*) and e9.5, (*E–H*) embryos. *A*, *C*, *E*, and *G* show brightfield images of hematoxylin- and eosin-stained sections with corresponding darkfield images shown in *B*, *D*, *F*, and *H*. Ve, visceral endoderm; Hg, hindgut; Mg, midgut; Fg, foregut; Lb, liver bud; L, liver; G, gallbladder. (Original objective used for *A*, *B*, *G*, and *H* was ×6; for *C* and *D*, ×20; and for *E* and *F*, ×10.)

level, while the gall bladder (Fig. 3 *E* and *F*) and hindgut (not shown) display low expression.

At e10.5, this pattern is maintained, with the dorsal pancreas observed as a distinct outgrowth from the gut (Fig. 4 *A–D*). Expression is now also detected in the stomach in a gradient increasing toward the caudal and ventral region, and in the mesonephric tubules. High magnification of the e10.5 liver shows that sinusoids and blood cells are readily apparent and that HNF-4 expression is apparently restricted to the hepatocytes (Fig. 4 *E* and *F*). At e11.5 (Fig. 5 *A* and *B*), the expression pattern is similar to the previous stage. At e13.5 (Fig. 5 *C–H*), moderate to high expression extends from the midgut caudally through the hindgut, ending apparently within the endoderm approximately at the junction between the rectum and the anus. At this stage the mesonephric tubules begin to degenerate and are replaced by the metanephric tubules of the maturing kidney; high HNF-4 expression was detected in these tubules (Fig. 5 *C* and *D*), compatible with the reported expression of HNF-4 in the cortex of the adult kidney (19).

Our laboratory has recently developed a transgenic mouse strain that has sufficient upstream sequences derived from the HNF-4 gene to direct expression of a reporter gene in the liver and intestines of adult mice and additional expression in the pancreas and mesonephros of e11.5 mouse embryos (17). We wished to test whether these transgenic strains would also express β-galactosidase in the visceral endoderm of early mouse embryos. Fig. 6 shows β-galactosidase expression at e7.5 in the transgenic strain TM47 which was absent from CD-1 control embryos, indicating that 7.2 kb of sequence lying upstream of the HNF-4 gene is adequate to direct cell-specific expression throughout embryogenesis and in adults.

DISCUSSION

How do we interpret these findings that a gene encoding a transcription factor present at high levels in adult cells (hepatocytes, intestinal epithelium, and kidney tubules) is also expressed in the primary and visceral endoderm of the developing embryo? First, it is clear from studies in *Drosophila* that transcription factors acting early in embryogenesis are often expressed later during organogenesis or in adults to serve a function in the differentiated cell, dHNF-4 being one such protein (8). Second, we note that there are many parallels in function between the visceral endoderm in the early embryo and the adult organs where HNF-4 is expressed, particularly the liver. By e5.5 the visceral endoderm surrounds the egg cylinder, forming a tight epithelial sheet that provides a barrier between the embryo and the surrounding maternal environment. The environment within the developing embryo is therefore regulated by transport across and secretion from the visceral endoderm (20–23). In this respect, the visceral endoderm of the early embryo has a function analogous to that of the liver and intestine combined, interposed between the external and internal environ-

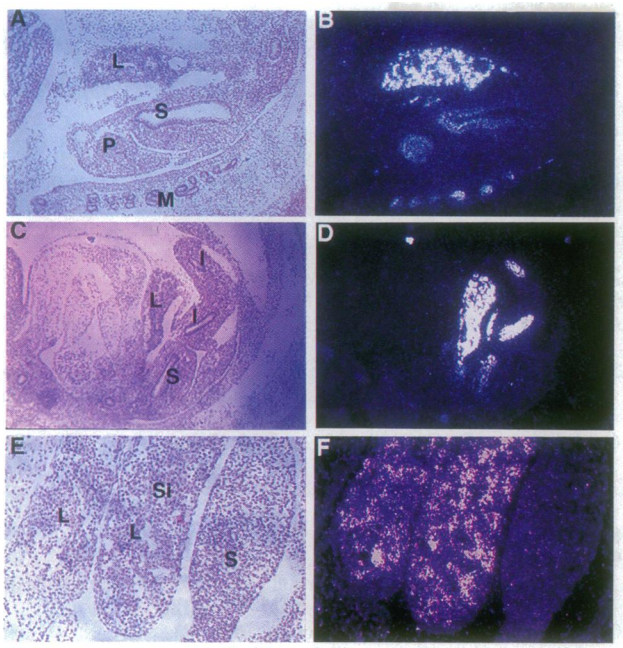

FIG. 4. HNF-4 expression pattern at e10.5. *In situ* hybridizations were carried out on 5-μm sagittal sections of e10.5 embryos. *A*, *C*, and *E* show brightfield images of hematoxylin- and eosin-stained sections with corresponding darkfield images shown in *B*, *D*, and *F*. L, liver; S, stomach; I, intestine; P, pancreas; M, mesonephric tubules; Si, sinusoids. (Original objective used for A–D was ×10 and for *E* and *F*, ×20.)

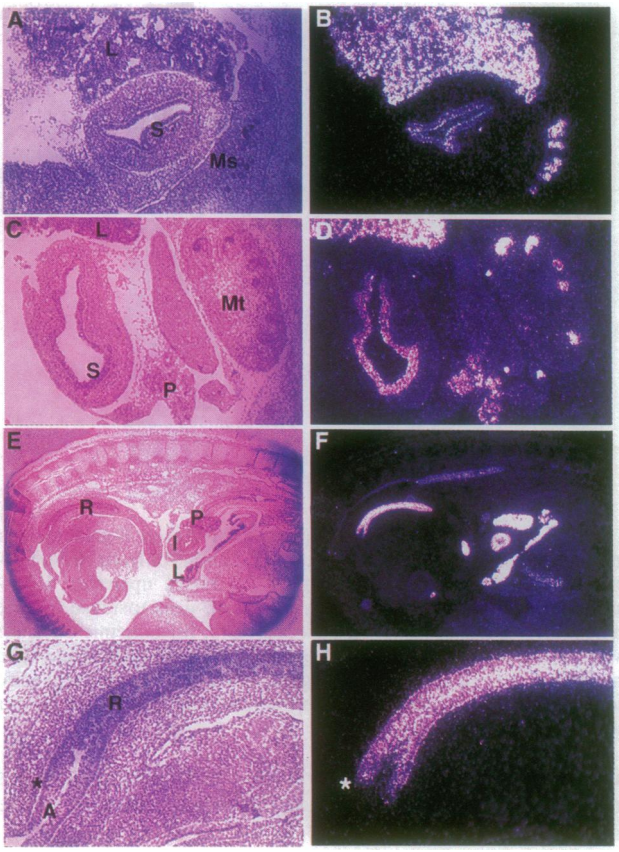

FIG. 5. Expression of HNF-4 at e11.5 and e13.5. *In situ* hybridizations were carried out on 5-μm sagittal sections of e11.5 (*A* and *B*) and e13.5 (*C–H*) embryos. *A*, *C*, *E*, and *G* show brightfield images of hematoxylin- and eosin-stained sections with corresponding darkfield images shown in *B*, *D*, *F*, and *H*. The caudal limit of HNF-4 expression at the anal–rectal boundary is indicated by a star in *G* and *H*. L, liver; S, stomach; I, intestine; P, pancreas; Ms, mesonephric tubules; Mt, metanephric tubules; A, anus; R, rectum. (Original objective used for *A–D* was ×10; for *E* and *F*, ×4; and for *G* and *H*, ×20.)

ments; later the placenta takes over some aspects of this function. Many proteins are secreted by both visceral endoderm and hepatocytes, including albumin, transferrin, several apolipoproteins, α_1-antitrypsin, α-fetoprotein, transthyretin, and retinol-binding protein (21, 24–26). In addition, the visceral yolk sac is the site of blood island formation and the fetal liver is the site of embryonic hematopoeisis.

Many of the proteins secreted by liver and visceral endoderm are thought to be involved in embryonic growth. For example, transthyretin acts as a carrier of thyroid hormone in rodent plasma (27), and the retinol-binding protein is involved in retinol transport (28). Since vitamin A is known to be essential for normal embryonic growth as is thyroid hormone, these proteins produced by visceral endoderm may contribute to transport of maternal retinol and thyroid hormone to the developing embryo. Consistent with such a role was the reported disruption of the transthyretin gene, which did not prevent development of viable animals but did lead to reduced serum levels of retinol and thyroid hormone (29). The finding that both high density lipoproteins and low density lipoproteins were required to support the growth of PC-13 murine embryonal carcinoma cells prompted the hypothesis that they might act as growth factors by transporting lipids to the growing embryo (30). Such an idea gains support by the observation that apolipoproteins A-I, A-IV, B, and E, all components of high density lipoproteins or low density lipoproteins, are secreted from the visceral yolk sac (21).

Analyses of the promoters of many of these genes reveal HNF-4 binding sites which are crucial for their expression in the adult liver (15), suggesting the possibility of a similar regulation in the visceral endoderm. Moreover, the role of HNF-4 may not be restricted to direct targets, since expression of HNF-1α, another liver-enriched transcription factor expressed in the visceral endoderm, is positively regulated by HNF-4 (31). Perhaps HNF-4 triggers a cascade of positively acting transcription factors and therefore mediates a global regulation of yolk-sac gene expression. In this regard it is important to address whether HNF-4 also regulates the expression of other transcription factors which are present in the visceral endoderm, such as HNF-1β (32) and HNF-3α and -3β (refs. 9–12; and S.A.D., W.S.C., and R.F.B., unpublished work).

Liver development is thought to occur in a three-step process: interaction of cardiac mesoderm with foregut endoderm, inductive interaction with the mesenchyme of the septum transversum, and further differentiation (18, 33, 34). We have shown that HNF-4 expression is first detected as the foregut endoderm interacts with mesenchyme of the septum transversum (e8.5–9.0) and is expressed at higher levels during later differentiation phases. This result agrees with previous studies which have found that the serum albumin gene, which serves as a marker for hepatocyte differentiation, is expressed in a similar pattern (34). The pattern of HNF-4 expression we have seen during organogenesis presages that described for the adult, although the expression reported here in the stomach between e11.5 and e13.5 has not been previously described for adult mice. It is interesting that HNF-4 is expressed in functionally related structures as development progresses; i.e., both the visceral endoderm and liver express HNF-4 mRNA, as do the mesonephric tubules, metanephric tubules, and cortex of the kidney. This pattern appears to have been conserved throughout evolution since a similar situation occurs with HNF-4 expression at the tips of the Malpighian tubules in *Drosophila* (8).

These results provide a clear basis for a role for HNF-4 during early development and may provide insight into the

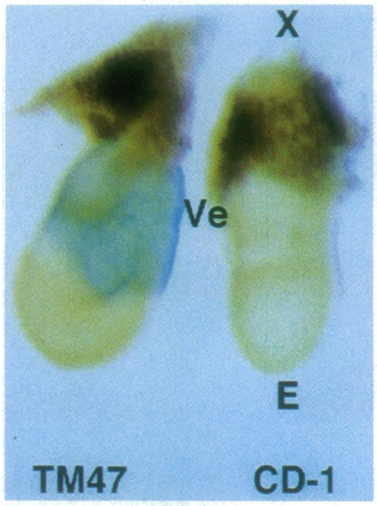

FIG. 6. Expression of HNF-4-promoter-driven β-galactosidase in transgenic e7.5 embryos. Dissected e7.5 embryos from either CD-1 or TM47 [transgenic line containing 7.2 kb of upstream HNF-4 promoter sequence driving expression of a β-galactosidase reporter gene (17)] × CD-1 crosses were processed for histochemical staining with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. Blue staining identifies the expression of β-galactosidase in the visceral endoderm. Ve, visceral endoderm; X, extraembryonic pole; E, embryonic pole.

function of the primary/visceral endoderm during early gastrulation. HNF-4 is obviously an important target for further manipulation in the mouse.

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