Development 124, 279-287 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV4798

Murine gastrulation requires HNF-4 regulated gene expression in the visceral endoderm: tetraploid rescue of *Hnf-4*$^{-/-}$ embryos

Stephen A. Duncan[1,*], Andras Nagy[2] and Wendy Chan[1]

[1]Laboratory of Molecular Cell Biology, The Rockefeller University, New York, New York 10021, USA
[2]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada

*Author for correspondence

SUMMARY

Immediately prior to gastrulation the murine embryo consists of an outer layer of visceral endoderm (VE) and an inner layer of ectoderm. Differentiation and migration of the ectoderm then occurs to produce the three germ layers (ectoderm, embryonic endoderm and mesoderm) from which the fetus is derived. An indication that the VE might have a critical role in this process emerged from studies of *Hnf-4*$^{-/-}$ mouse embryos which fail to undergo normal gastrulation. Since expression of the transcription factor HNF-4 is restricted to the VE during this phase of development, we proposed that HNF-4-regulated gene expression in the VE creates an environment capable of supporting gastrulation. To address this directly we have exploited the versatility of embryonic stem (ES) cells which are amenable to genetic manipulation and can be induced to form VE in vitro. Moreover, embryos derived solely from ES cells can be generated by aggregation with tetraploid morulae. Using *Hnf-4*$^{-/-}$ ES cells we demonstrate that HNF-4 is a key regulator of tissue-specific gene expression in the VE, required for normal expression of secreted factors including alphafetoprotein, apolipoproteins, transthyretin, retinol binding protein, and transferrin. Furthermore, specific complementation of *Hnf-4*$^{-/-}$ embryos with tetraploid-derived *Hnf-4*$^{+/+}$ VE rescues their early developmental arrest, showing conclusively that a functional VE is mandatory for gastrulation.

Key words: HNF-4, visceral endoderm, gastrulation, tetraploid, transcription, mouse, ES cells

INTRODUCTION

The visceral endoderm (VE) derives from the primitive endoderm, which itself differentiates from the inner cell mass cells of the blastocyst at around 4.0-4.5 days post coitum (E4.0) in the mouse (Gardner, 1983). The transcription factor HNF-4 is expressed in the primitive endoderm as soon as a morphologically distinct endoderm can be identified, suggesting that it could be important for differentiation of this tissue (Duncan et al., 1994). During gastrulation the VE joins with the extraembryonic mesoderm to form the visceral wall of the yolk sac. Its function as part of the yolk sac has been well characterized in postgastrulation rodent embryos where it has a critical role in the maternofetal exchange of nutrients prior to formation of a functioning placenta (Jollie, 1990). That a functioning yolk sac is critical for development of the postgastrulation mammalian embryo is illustrated by the observation that antisera which recognize proteins in the yolk sac are teratogenic (for review see Brent et al., 1990). During this period the VE is also responsible for the synthesis and secretion of several serum factors including α-fetoprotein (AFP), transthyretin (TTR), and several apolipoproteins (Apo) (Meehan et al., 1984) and, in addition, is the site of embryonic hematopoeisis. Although preceding and during gastrulation little is known about VE function, gene targeting studies have suggested that the VE may have important roles during early embryogenesis (Spyropoulos et al., 1994; Ang et al., 1994; Weinstein et al., 1994; Chen et al., 1994).

Hepatocyte nuclear factor 4 (HNF-4) is a transcription factor identified in liver extracts as a DNA binding protein which binds to the promoters of the transthyretin (TTR) and apolipoprotein CIII (ApoCIII) genes (Sladek et al., 1990). A member of the steroid hormone receptor family which lacks a known ligand (Sladek et al., 1990), HNF-4 is evolutionarily conserved, with homologues found in *Xenopus laevis*, *Drosophila melongaster*, and humans (Zhong et al., 1993; Drewes et al., 1996). Targeted disruption of the *Hnf-4* gene in mice revealed that it was critical for completion of gastrulation (Chen et al., 1994). Although *Hnf-4*$^{-/-}$ embryos initiate gastrulation, as evidenced by production of cells expressing markers of nascent mesoderm, eg. Brachyury, they fail to produce cells expressing late mesoderm markers such as mox-1, suggesting that HNF-4 has a critical role in supporting the progression of gastrulation (Chen et al., 1994). Analyses of *Hnf-4* expression by in situ hybridization revealed that during this stage of development *Hnf-4* mRNA was restricted to the extraembryonic visceral endoderm (VE), with no *Hnf-4* mRNA detected in embryonic tissues (Duncan et al., 1994). The first expression of *Hnf-4* in embryonic tissues was not detected until induction of the liver diverticulum at about E9.0 (Duncan et al., 1994; Taraviras et al., 1994). This implied that the VE has a critical function in maintaining gastrulation of the murine epiblast.

To determine whether HNF-4 is required for differentiation of the VE and, furthermore, to define the role of the VE during murine gastrulation we took advantage of the unique properties of embryonic stem (ES) cells to form VE in vitro and embryos in vivo. We show here that in the absence of HNF-4 differentiation of the VE is incomplete, resulting in a functionally defective tissue which fails to express several serum proteins including AFP, Apo-AI, Apo-AIV, Apo-B, TTR, retinol binding protein (RBP), and transferrin (TFN). Furthermore, *Hnf-4*$^{-/-}$ embryos are rendered gastrulation competent when they are complemented with *Hnf-4*$^{+/+}$ VE, confirming that *Hnf-4*$^{-/-}$ embryonic ectoderm cells can complete gastrulation and that the VE has a critical function in defining an environment supportive of gastrulation in the mouse.

MATERIALS AND METHODS

Growth and differentiation of ES cells in vitro and selection of HNF-4 null ES cells

Embryonic stem (ES) cells were maintained in ES cell medium supplemented with 1000 u/ml LIF on a primary embryonic fibroblast feeder layer as described by Robertson (1987). ES cells were induced to differentiate to form EBs in vitro according to the method of Robertson (1987). 3×10^6 ES cells were plated on gelatin-coated tissue culture dishes and grown for 3 days in the absence of feeder fibroblasts and LIF. After addition of trypsin/EDTA, ES cell clumps were collected, divided between bacterial grade Petri dishes (Fisher) containing ES cell medium and grown in suspension for the specified duration. To generate ES cells homozygous for a targeted mutation in the HNF-4 gene (*Hnf-4*$^{-/-}$) the method of Mortensen et al. (1992) was followed. Specifically, 5.0×10^6 HNF-4 heterozygote ES cells (*Hnf-4*$^{+/-}$) (clone 2-69; Chen et al., 1994) were plated in medium containing 1.25 mg/ml G418 and 1000 units/ml LIF. After 9 days in culture surviving colonies were expanded and their genotype ascertained by Southern blot.

Analysis of genotype by Southern blot or PCR

DNA was prepared from ES cells, mouse tail biopses and mouse embryos as described by Plump et al. (1992). In Southern blots of *Nco*I digested genomic DNA a wild-type *Hnf-4* allele generated a 4.5 kb restriction fragment which hybridized to an *Hnf-4* specific probe, while the targeted allele generated a 3.8 kb fragment, as previously reported (Chen et al., 1994). The genotype of ES cell/teraploid-derived mouse embryos was determined by polymerase chain reaction (PCR). Embryonic DNA served as templates for PCRs which contained all four deoxyribonucleotides (dNTPs), [α-^{32}P]dATP and *taq* DNA polymerase and utilized primers specific to either HPRT (agcgcaagttgaatctgc, agcgacaatctaccagag), the neomycin resistance gene (Neo^r) (gccaacgctatgtcctgatagcggt, agccggtcttgtcgatcaggatgat), or *Hnf-4* (ccccatctgaaggtgccaacctc, ggttcttcctcacgctcctcctgaa).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Conditions used for RT-PCR followed the method of Wilson and Melton (Wilson et al., 1994) with minimal modifications. Total RNA was extracted from ES cells or mouse embryos using TRIzol reagent and following the manufacturers instructions (Gibco-BRL) and contaminating genomic DNA was removed using 1 µl of RNase-free DNase-I (Boehringer)/10 µg RNA. cDNA was synthesized using MMLV-RT (Gibco-BRL) with dNTPs and random hexamer primers (Gibco-BRL). These cDNAs provided templates for PCRs using specific primers at an annealing temperature of 65ºC in the presence of dNTPs, [α-^{32}P]dATP and *taq* DNA polymerase. The following forward and reverse primers were used for specific amplification:

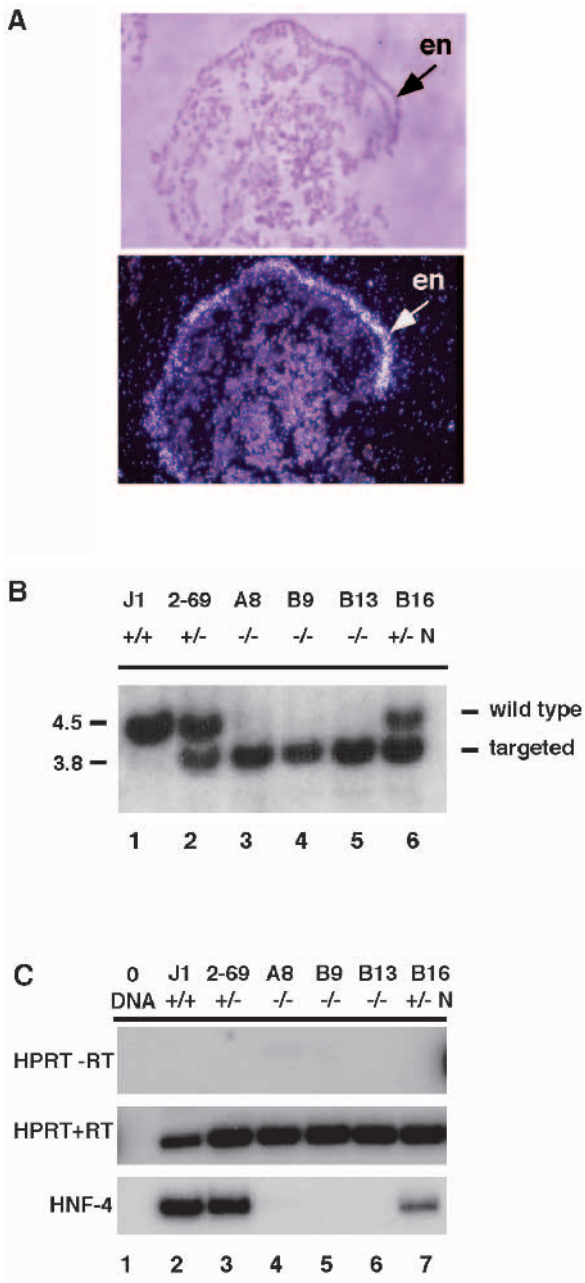

Fig. 1. Expression of HNF-4 in ES cell EBs and production of *Hnf-4*$^{-/-}$ ES cells. (A) In situ hybridization with an antisense RNA *Hnf-4* probe. Phase contrast micrograph of a 5 µm section through a d14 ES cell EB, stained with hematoxylin and eosin, is shown in the top panel with the corresponding dark-field image below. Visceral endoderm (en) is identified by an arrow. (B) Southern blot of *Hnf-4*$^{+/+}$ (J1; lane 1), *Hnf-4*$^{+/-}$ (2-69 and B16; lanes 2 and 6), and *Hnf-4*$^{-/-}$ (A8, B9, B13; lanes 3-5) ES cell genomic DNA. Wild-type and targeted *Nco*I fragments which hybridized to an *Hnf-4* specific probe (Chen et al., 1994) are indicated with sizes shown in kb. (C) RT-PCR showing lack of HNF-4 expression in *Hnf-4*$^{-/-}$ ES cell EBs. RT-PCR was performed on RNA from *Hnf-4*$^{+/+}$ (J1; lane 2), *Hnf-4*$^{+/-}$ (2-69, B16; lanes 3 and 7), and *Hnf-4*$^{-/-}$ (A8, B9, B13; lanes 4-6) EBs using HPRT- and *Hnf-4*-specific primers. No product was amplified by HPRT primers in the absence of reverse transcriptase (HPRT –RT) or cDNA (lane 1), confirming that all products were amplified from cDNA rather than contaminating genomic DNA.

HPRT; agcgcaagttgaatctgc, agcgacaatctaccagag, GATA-4; ctaagctgtccccacaaggctatgca, cagagctccacctggaaaggtgtttg, vHNF-1; gaaagcaacgggagatcctccgac, cctccactaaggcctccctctcttcc, Apo-E; aggatgcctagccgagggagagc, tagatcctccatgtcggctccgagt, *Hnf-4*; cttccttcttcatgccag, acacgtccccatctgaag, AFP; tcgtattccaacaggagg, aggcttttgcttcaccag, TFN; tggcacaggaacactttg, tcctgctgattccgaatg, Apo-AI; acacacgtagactctctg, ctgggctttggtcttaag, Apo-AIV; agccaaggaaactgagag, tctccttgatcgtggtct, Apo-B; cttcagggaacaaagcag, tcaagggtgagctgattg, TTR; ctcaccacagatgagaag, ggctgagtctctcaattc, RBP; atccagtggtcatcgtttcctcgct, gaacttcgacaaggctcgtttctctgg, HNF-1; ttctaagctgagccagctgcagacg, gctgaggttctccggctctttcaga.

In situ hybridization and lectin staining of ES cells aggregates

In situ hybridizations were performed as described previously (Duncan et al., 1994). Sense and anti-sense *Hnf-4*, [^{33}P]UTP labeled RNA probes were synthesized in vitro from the plasmid p4-is. The specificity of this probe for *Hnf-4* has previously been described (Chen et al., 1994). Probes were hybridized to paraffin sections of day 14 J1 ES cell aggregates (Hooper et al., 1987), exposed to photographic emulsion for 14 days, before developing and counterstaining with hematoxylin and eosin. Flourescein-isothiocyanate (FITC)-labeled *Sophora japonica* (SJA) (Sigma) was used to label the VE of either *Hnf-4*$^{+/+}$ or $^{-/-}$ day-11 ES cells aggregates in whole mount as described (Soudais et al., 1995; Wu et al., 1983).

Production of ES cell-derived embryos by tetraploid aggregation

ES cell-derived embryos were produced essentially following the method described by Nagy and Rossant (1993) with minor modifications. 2-cell stage embryos isolated from naturally mated CD-1 mice were collected in M2 medium (Specialty Media Inc.), equilibrated in fusion buffer (0.3 M mannitol, 0.1 mm $MgSO_4$, 50 µM $CaCl_2$, 3% BSA) (McLaughlin, 1993) and placed between the electrodes of a CF150 cell fusion instrument (Biochemical Laboratory Service LTD., Hungary). Embryos were aligned in a 0.7 volt AC field and fused with three 100 volt DC pulses of 45 µseconds each. Embryos were washed in M2 medium, transferred to KSOM medium (Specialty Media Inc.) and incubated for 1 hour at 37°C/5% CO_2 at which time successfully fused embryos were recovered and cultured overnight under the same conditions. ES cell-tetraploid chimaeric embryos were produced by aggregation as described elsewhere (Nagy and Rossant, 1993).

RESULTS

Hnf-4 mRNA is expressed in the visceral endoderm of ES cell embryoid bodies

During the earliest stages of implantation, inner cell mass cells of the blastocyst which juxtapose the blastocoel cavity are specified to form the primitive endoderm lineage from which all extraembryonic endoderm is derived. The embryo at this time is relatively resistant to a molecular investigation and so, to determine whether HNF-4 has any role in differentiation of the extraembryonic endoderm lineages, we decided to use an ES cell in vitro differentiation assay. When ES cells are grown in suspension culture in the absence of LIF they differentiate to form embryoid bodies (EBs) (Evans et al., 1981; Martin, 1981). These EBs, which resemble mouse embryos at early developmental stages, contain differentiated VE (Doetschman et al., 1985). To ascertain whether HNF-4 expression was induced during EB formation in vitro, we assayed for the presence of *Hnf-4* mRNA by in situ hybridization (Fig. 1A). 5 µm paraffin sections of day-14 postaggregation EBs were hybridized to a [^{33}P]UTP labeled RNA probe which had previously been shown to recognize only *Hnf-4* (Duncan et al., 1994; Chen et al., 1994). While a sense-strand RNA probe showed no hybridization above background (data not shown), Fig. 1A shows that an anti-sense probe identified *Hnf-4* transcripts which were restricted to the VE layer of the EB. From

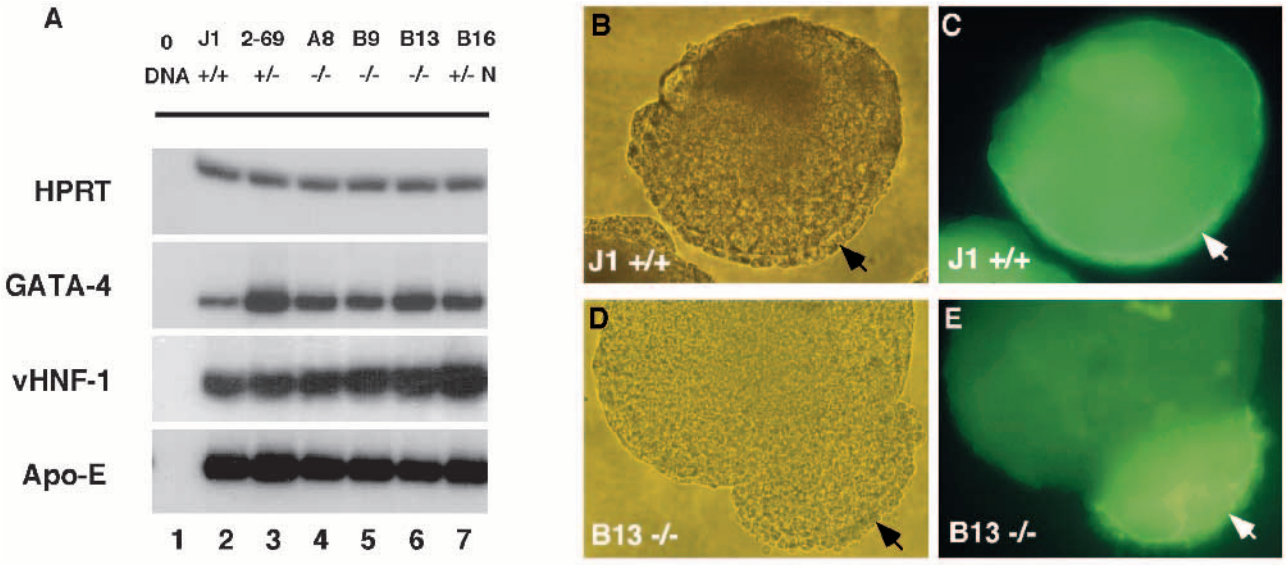

Fig. 2. HNF-4 is not required for specification of visceral endoderm. (A) Steady state levels of mRNAs expressed from the VE marker genes, *Gata-4*, *vHNF-1* and *Apo-E* were measured in d14 *Hnf-4*$^{+/+}$ (J1; lane 2), *Hnf-4*$^{+/-}$ (2-69, B16; lanes 3 and 7), and *Hnf-4*$^{-/-}$ (A8, B9, B13; lanes 4-6) EBs using HPRT, GATA-4, vHNF-1 and Apo-E specific primers. No product was amplified with HPRT primers in the absence of RT (not shown) or with any primers in the absence of cDNA (lane 1). (B) VE is produced in *Hnf-4*$^{-/-}$ EBs. *Hnf-4*$^{+/+}$ (J1) (B,C) and *Hnf-4*$^{-/-}$ (B13) (D,E) d14 EBs were examined for the morphological presence of endoderm (arrows) using phase contrast microscopy (B,D). The same EBs were processed for whole-mount staining with FITC-labeled *Sophora japonica* agglutinin (SJA) (C,E).

these data we conclude that *Hnf-4* mRNA is expressed in, and restricted to, the VE of ES cell EBs.

Production of *Hnf-4* homozygous mutant ES cells

The expression of HNF-4 in the VE of ES cell EBs allowed us to adopt an in vitro genetic approach to ask whether HNF-4 was central to VE differentiation and/or function. We have previously described the generation of HNF-4 heterozygote (*Hnf-4*$^{+/-}$) ES cells in which the DNA binding domain of one *Hnf-4* allele was deleted by homologous recombination (Chen et al., 1994). To construct ES cell lines which were homozygous for this targeted mutation (*Hnf-4*$^{-/-}$), *Hnf-4*$^{+/-}$ cells were passaged in high concentrations of G418 (high [G418]). This procedure has previously been shown to efficiently produce ES cell lines which are homozygous for a targeted allele (Mortensen et al., 1992). ES cell clones which survived 9 days in culture under 1.5 mg/ml G418 were assayed for loss of the wild-type *Hnf-4* allele by Southern blot analysis. An *Hnf-4* specific DNA probe identified a 4.5 kb *Nco*I fragment in wild-type genomic ES cell DNA (Fig. 1B; lane 1) while the targeted allele generated a 3.8 kb *Nco*I fragment (Fig. 1B; lanes 2-6) (Chen et al., 1994). Of 44 high [G418] resistant clones analyzed, 12 were *Hnf-4*$^{-/-}$ and the remainder *Hnf-4*$^{+/-}$. Three *Hnf-4*$^{-/-}$ lines (A8, B9 and B13) and one *Hnf-4*$^{+/-}$ line (B16, referred to as *Hnf-4*$^{+/-N}$), were selected for subsequent experiments.

To demonstrate that no functional HNF-4 could be expressed by the *Hnf-4*$^{-/-}$ ES lines, *Hnf-4*$^{+/+}$, *Hnf-4*$^{+/-}$ and *Hnf-4*$^{-/-}$ day-14 EBs were assayed for the presence of *Hnf-4* mRNA by RT-PCR (Fig. 1C). To control for the relative amounts of RNA used in each RT-PCR assay we included primers which identified HPRT mRNA since this gene is expressed ubiquitously at relatively constant levels. As shown in Fig. 1C, no product was amplified by HPRT primers in the absence of reverse transcriptase (HPRT −RT) or DNA (lane 1), showing that all products were amplified from cDNA rather than from contaminating genomic DNA. In the presence of reverse transcriptase (HPRT +RT) a specific 219 bp product was identified at comparative levels in each sample indicating that each reaction started with a similar amount of template. While HNF-4 primers generated a specific PCR product from *Hnf-4*$^{+/+}$ (lane 2), *Hnf-4*$^{+/-}$ (lane 3), and *Hnf-4*$^{+/-N}$ (lane 7) EB cDNAs, no product was detectable in any of the three *Hnf-4*$^{-/-}$ EB samples (lanes 4-6). These data verify that ES cell lines A8, B9 and B13 are homozygous *Hnf-4*$^{-/-}$ mutants.

HNF-4 is not essential for specification of the visceral endoderm lineage

Because HNF-4 is detected in the primitive endoderm at the earliest stages of its differentiation (Duncan et al., 1994) we wanted to determine whether HNF-4 was required for specification of the extraembryonic endoderm lineage. We therefore measured steady state mRNA levels from the VE marker genes *Gata-4* (Soudais et al., 1995), *vHnf-1* (Cereghini et al., 1992) and *Apo-E* (Basheeruddin et al., 1987; Harrison et al., 1995) by RT-PCR in *Hnf-4*$^{+/+}$, *Hnf-4*$^{+/-}$ and *Hnf-4*$^{-/-}$ EBs (Fig. 2A). As before, amplification with HPRT-specific primers demonstrated that each sample started with a comparable concentration of template. In several repetitions of this experiment no significant difference in levels of *Gata-4*, *Apo-E*, or *vHnf-1* mRNAs were detected between *Hnf-4*$^{+/+}$, *Hnf-4*$^{+/-}$ or *Hnf-4*$^{-/-}$ EBs, suggesting that VE tissue could be produced in the absence of HNF-4. To confirm this, day-11 *Hnf-4*$^{+/+}$ or *Hnf-4*$^{-/-}$ EBs were stained in whole mount with FITC-labeled *Sophora japonica* agglutinin (SJA) which specifically reacts with the VE (Sato et al., 1985). Fig 2B,D shows phase contrast micrographs in which the formation of endoderm, evident as a cuboidal epithelium, can readily be identified in both *Hnf-4*$^{+/+}$ and *Hnf-4*$^{-/-}$ EBs. The same tissue also stains with FITC-labeled SJA showing that this endoderm is VE (Fig. 2C,E). Control EBs did not label when N-acetylgalactosamine was pre-incubated with the lectin (data not shown), confirming the specificity of the staining (Sato et al., 1985). Cumulatively, these data demonstrate that *Hnf-4* is not required for early specification of the VE lineage.

HNF-4 is essential for the complete differentiation of visceral endoderm in vitro and in vivo

Differentiation of the VE requires the orderly formation of an epithelial layer upon a basement membrane with the subsequent expression of characteristic late marker genes (Grover et al., 1983a,b). Many of these genes encode secreted serum proteins that are also expressed in hepatocytes (Meehan et al., 1984), where HNF-4 is believed to be important in regulating their expression (Sladek, 1994). We therefore determined whether HNF-4 was required for late phase VE differentiation by using RT-PCR to measure the steady state levels of mRNAs expressed from such genes. Primers were designed to detect *AFP*, *TFN*, *Apo -AI*, *-AIV*, and *-B*, *TTR* and *RBP* mRNAs by RT-PCR in day-14 *Hnf-4*$^{+/+}$, *Hnf-4*$^{+/-}$ and *Hnf-4*$^{-/-}$ EBs. In addition, since HNF-4 has been implicated in the transcriptional regulation of the transcription factor HNF-1 (Tian et al., 1991; Kuo et al., 1992), primers were included which could detect *Hnf-1* mRNA. As before, no product was detected in the absence of DNA or reverse transcriptase showing that products were amplified from cDNAs (Fig. 3A; HPRT −RT, and lane 1). A similar amount of starting material was used in each reaction, as shown by an equivalent amount of product generated by HPRT primers (Fig. 3A; HPRT +RT). GATA-4 was expressed at comparable levels between samples indicating that similar amounts of VE had been formed by the different EBs and, as expected, while HNF-4 was expressed in *Hnf-4*$^{+/+}$ and *Hnf-4*$^{+/-}$ EBs none could be detected in the *Hnf-4*$^{-/-}$ EBs (Fig. 3A: GATA-4 and HNF-4). Analysis of serum protein gene expression gave the striking result presented in Fig. 3A. While expression of *AFP*, *TFN*, *Apo-AI*, *Apo-AIV*, and *Apo-B* was easily detected in *Hnf-4*$^{+/+}$ or *Hnf-4*$^{+/-}$ EBs, expression was virtually undetectable in *Hnf-4*$^{-/-}$ EBs. Expression of TTR and RBP mRNAs was also grossly reduced in the *Hnf-4*$^{-/-}$ EBs and, although less striking, levels of *Hnf-1* mRNA were also down. These data demonstrate that HNF-4 is a key regulator of VE gene expression and is essential for the complete differentiation of VE in vitro.

We next determined whether this disruption to the expression of serum protein genes in the absence of HNF-4 in EBs also held true in vivo. E8.5 embryos were collected from crosses of *Hnf-4*$^{-/-}$ embryos and pooled according to phenotype; E8.5 embryos which express HNF-4 have formed a distinct headfold and allantois, contain somites and exhibit clear organization of germ layers, while *Hnf-4*$^{-/-}$ embryos show no obvious morphological signs of gastrulation (Chen et al., 1994). The genotype of the pooled embryos was confirmed

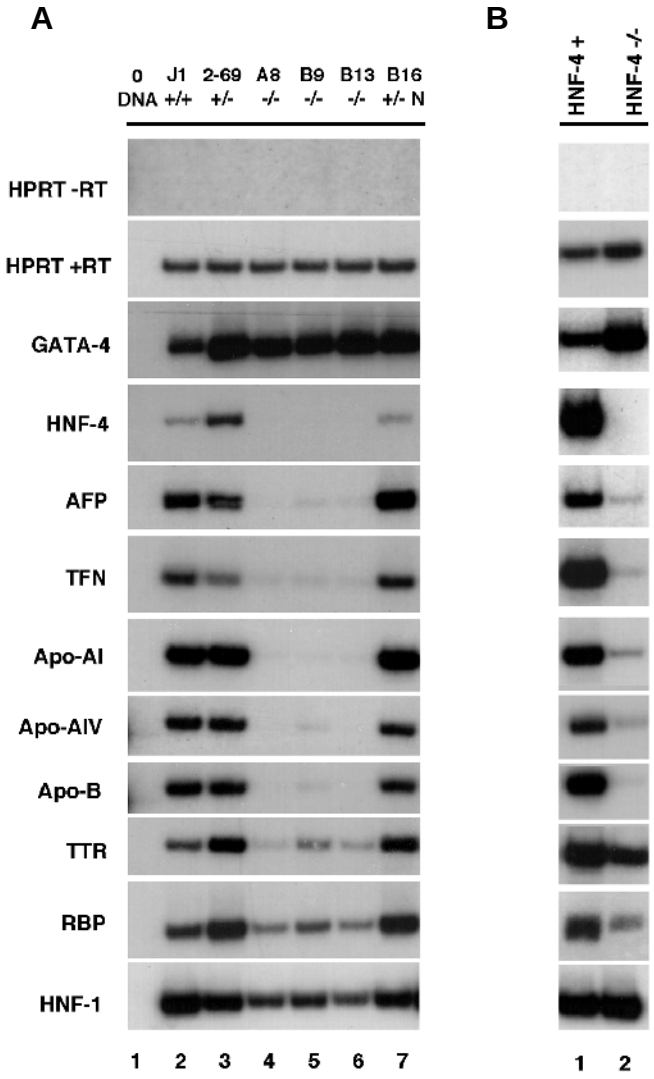

Fig. 3. HNF-4 is essential for late-phase differentiation of VE in vitro and in vivo. (A) *Hnf-4*$^{+/+}$ (J1; lane 2), *Hnf-4*$^{+/-}$ (2-69 and B16; lanes 3 and 7), and *Hnf-4*$^{-/-}$ (A8, B9, B13; lanes 4-6) ES cell EBs were assayed for the presence of mRNAs derived from genes encoding secreted serum factors. HPRT primers did not amplify product in the absence of RT (HPRT –RT) or cDNA (lane 1). Each sample started with a comparable amount of cDNA since HPRT primers +RT amplified similar amounts of product. Importantly, each EB sample contained similar amounts of VE as shown by the comparable levels of product amplified by GATA-4 primers. AFP, TFN, Apo-AI, Apo-AIV, Apo-B, TTR, RBP, and HNF-1 primers all amplified products of expected size in *Hnf-4*$^{+/+}$ and $^{+/-}$ EBs, but the levels of product generated in *Hnf-4*$^{-/-}$ EBs were greatly decreased. (B) Steady state mRNA levels of *HPRT, GATA-4, HNF-4, AFP, TFN, Apo-AI, Apo-AIV, Apo-B, TTR, RBP*, and *HNF-1* were measured in E8.5 HNF-4^{+} (lane 1) and HNF-4$^{-/-}$ (lane 2) embryos by RT-PCR. The HPRT +RT reaction shows that each sample started with a comparable amount of material; however, because HNF-4$^{-/-}$ embryos fail to complete gastrulation (Chen et al., 1994), a greater proportion of the starting material in these embryos is VE tissue, as confirmed by the greater levels of *Gata-4* mRNA. As in A, the expression of the genes encoding serum proteins was significantly decreased in *Hnf-4*$^{-/-}$ samples.

by RT-PCR using HNF-4 specific primers (Fig. 3B; HNF-4). Expression of mRNAs for the same genes described above were, once again, assayed by RT-PCR (Fig. 3B). HPRT –RT primers did not generate a product confirming the absence of contaminating genomic DNA, while the HPRT +RT reaction shows that equivalent amounts of starting material were used in each sample (Fig. 3B; HPRT –RT, HPRT +RT). Fig 3B also shows that higher levels of *Gata-4* mRNA are found in *Hnf-4*$^{-/-}$ embryos than in embryos expressing HNF-4, reflecting the fact that the ratio of VE cells to cells of embryonic lineage is greater in *Hnf-4*$^{-/-}$ embryos because they fail to undergo normal gastrulation (Chen et al., 1994). Fig. 3B shows that the transcripts of all genes assayed were detected in normal embryos; however, as is the case in vitro, expression of *AFP, TFN, Apo-AI, Apo-AIV*, and *Apo-B* mRNAs was almost undetectable in *Hnf-4*$^{-/-}$ embryos and, as before, expression of *TTR, RBP* and *Hnf-1* was reduced. In sum, from both in vitro and in vivo data, we conclude that ablation of HNF-4 results in a striking dysregulation of gene expression in the VE that severely compromises its paracrine activity.

Hnf-4$^{-/-}$ ES cell-derived embryos complete gastrulation when complemented with tetraploid *Hnf-4*$^{+/+}$ VE

In situ hybridization anlayses demonstrated that expression of *Hnf-4* mRNA was restricted to the VE prior to formation of the hepatic diverticulum at around E9.0. Since no expression could be detected in the embryonic tissues before this stage, we postulated that the disruption to gastrulation was due to dysfunction of the VE (Duncan et al., 1994; Chen et al., 1994). If this model is correct, specific complementation of *Hnf-4*$^{-/-}$ embryos with *Hnf-4*$^{+/+}$ VE should allow *Hnf-4*$^{-/-}$ embryos to complete gastrulation. As illustrated in Fig 4A, when chimaeras are formed between tetraploid (4n) morulae and diploid (2n) ES cells, the resulting fetuses are derived entirely from ES cells, whereas tetraploid cells contribute to the extraembryonic tissues including the VE (Nagy et al., 1990). This procedure has previously been used to rescue *Mash-2*$^{-/-}$ embryos, which die due to deficiencies in development of extraembryonic tissues (Guillemot et al., 1994). Using this system we produced *Hnf-4*$^{-/-}$ fetuses containing *Hnf-4*$^{+/+}$ VE (Fig. 4).

Hnf-4$^{-/-}$ ES cells were aggregated with *Hnf-4*$^{+/+}$ tetraploid morulas, cultured to blastocyst stage and transferred to surrogate mothers. Post-gastrulation ES cell-derived embryos were collected after 9.5 days of gestation (E9.5) and their genotype ascertained by PCR. As shown in Fig. 4A, primers were designed which could detect either ES cell-derived DNA alone (Neo^{r}), tetraploid morula-derived DNA alone (HNF-4), or both (HPRT). Embryos derived from all three *Hnf-4*$^{-/-}$ ES cell lines (A8, B9 and B13) were recovered (n=60) and shown to be devoid of *Hnf-4*$^{+/+}$ cells (Fig. 4B;E), whereas the yolk sacs from these embryos were strongly HNF-4 positive (Fig. 4B;Y). The phenotypes of tetraploid-A8 and -B13 (*Hnf-4*$^{-/-}$) embryos as well as wild-type and *Hnf-4*$^{-/-}$ embryos are shown in Fig. 4C-F. As described previously, *Hnf-4*$^{-/-}$ embryos are grossly abnormal by E8.5 (Fig. 4D), and by E9.5 the majority are being resorbed (Chen et al., 1994). As with wild-type control embryos (Fig. 4C), tetraploid-*Hnf-4*$^{-/-}$ ES cell-derived embryos (Fig. 4E,F) had undergone gastrulation, as illustrated by distinct postgastrula features including defined anterior-posterior and dorsal-ventral axes, segmental patterning (somites), neural tube formation and onset of oganogenesis. All

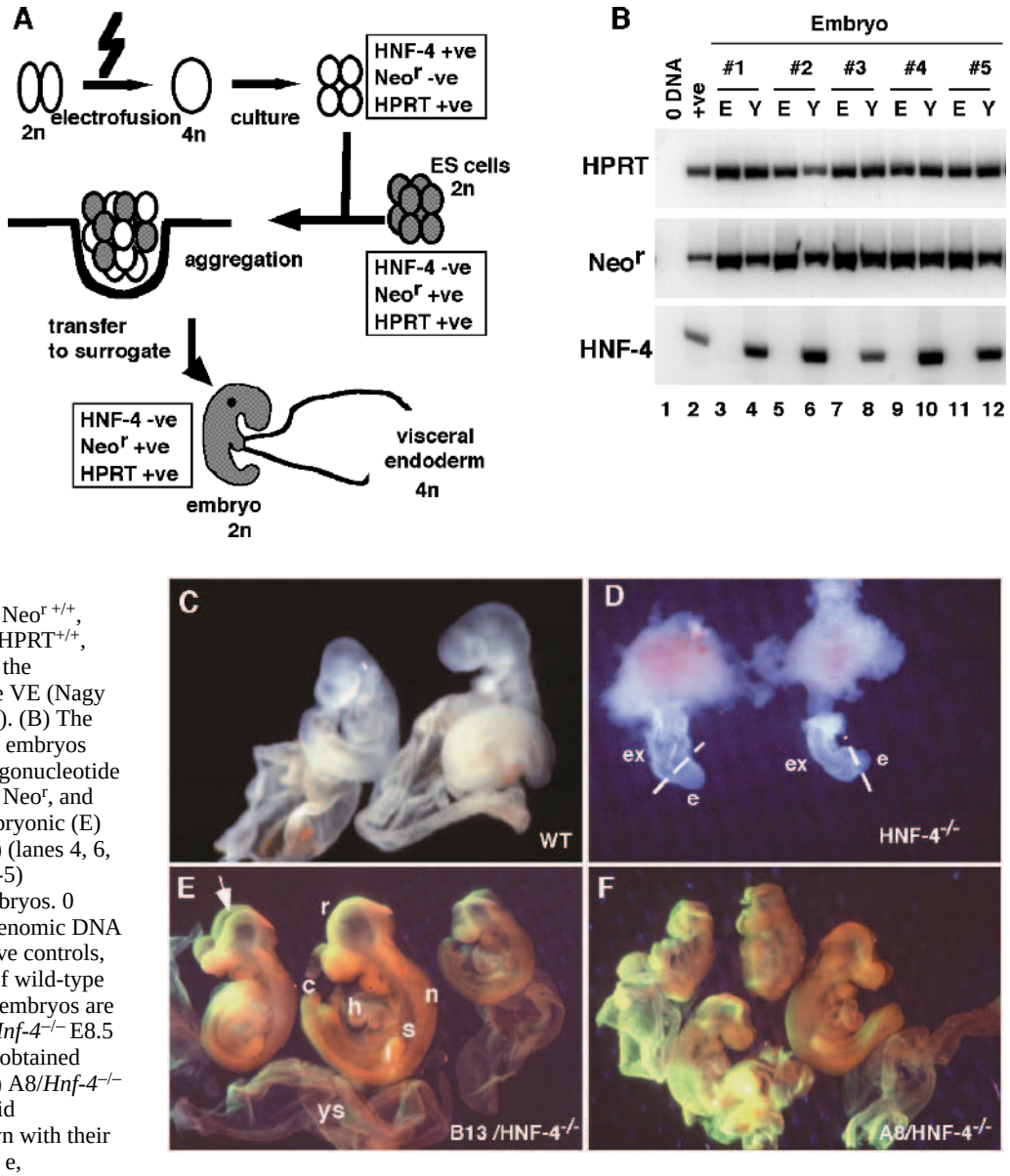

Fig. 4. Complementation of *Hnf-4*$^{-/-}$ embryos with *Hnf-4*$^{+/+}$ VE by tetraploid aggregation. (A) 2-cell diploid (2n) blastomeres can be induced to form a 1-cell tetraploid embryo (4n) by electrofusion (Kubiak et al., 1985). Such embryos can be cultured to 4 cell stage morulae overnight and have a HPRT$^{+/+}$, Neo$^{r\ -/-}$, *Hnf-4*$^{+/+}$ genotype. Aggregation of these tetraploid morulae with 2n ES cells (HPRT$^{+/+}$, Neo$^{r\ +/+}$, *Hnf-4*$^{-/-}$) produces chimaeric blastocysts which can be transferred to surrogate mothers where they will develop into fetuses (Nagy et al., 1990). Lineage analysis of the embryonic versus extraembryonic tissues in these fetuses shows that embryos can be recovered which are derived entirely from the ES cells (HPRT$^{+/+}$, Neo$^{r\ +/+}$, *Hnf-4*$^{-/-}$), while the tetraploid cells (HPRT$^{+/+}$, Neo$^{r\ -/-}$, *Hnf-4*$^{+/+}$) contribute toward the extraembryonic tissues, including the VE (Nagy et al., 1990; Nagy and Rossant, 1993). (B) The genotype of *Hnf-4*$^{-/-}$ ES cell-derived embryos was determined by PCR. Specific oligonucleotide primers were used to amplify HPRT, Neo^r, and HNF-4 genomic sequences from embryonic (E) (lanes 3, 5, 7, 9, 11) and yolk sac (Y) (lanes 4, 6, 8, 10, 12) DNA isolated from five (1-5) representative tetraploid:*Hnf-4*$^{-/-}$ embryos. 0 DNA (lane1), and *Hnf-4*$^{+/-}$ ES cell genomic DNA (lane 2) provided negative and positive controls, respectively. (C-F) The phenotypes of wild-type (wt), mutant (*Hnf-4*$^{-/-}$), and rescued embryos are shown. (C) WT E9.5 embryos; (D) *Hnf-4*$^{-/-}$ E8.5 embryos; (E) B13/*Hnf-4*$^{-/-}$ embryos obtained after tetraploid complementation; (F) A8/*Hnf-4*$^{-/-}$ E9.5 embryos obtained after tetraploid complementation. Embryos are shown with their yolks sacs (YS). ex, extraembryonic; e, embryonic; r, rostral; c, caudal; h, heart; l, limb bud; s, somites; n, neural tube. The arrow in E indicates an exencephaly which was seen in all ES-cell-derived embryos, regardless of their HNF-4 genotype, and is therefore likely to be a defect inherent to the starting ES cell line.

ES-cell-derived embryos, including those from wild-type (*Hnf-4*$^{+/+}$) ES cells (not shown), exhibited an exencephaly that was presumably inherent to the parental ES cell line and not a reflection of a specific defect attributed to loss of HNF-4. These data demonstrate that *Hnf-4*$^{-/-}$ embryos are capable of completing gastrulation in the presence of *Hnf-4*$^{+/+}$ VE and, futhermore, demonstrate that a fully differentiated VE is required to support murine gastrulation.

DISCUSSION

Mouse embryos lacking a functional *Hnf-4* gene are unable to support gastrulation (Chen et al., 1994). The *Hnf-4*$^{-/-}$ embryos first show evidence of an abnormal phenotype as early as E6.5, around the onset of gastrulation, at which time the *Hnf-4*$^{-/-}$ embryonic ectoderm exhibits an increase in apoptotic cell death relative to normal littermates (Chen et al., 1994). By E7.5, when normal embryos are at the late primitive streak stage, *Hnf-4*$^{-/-}$ embryos show no morphological evidence of gastrulation and by E8.5 they are grossly abnormal. Further investigation of the *Hnf-4*$^{-/-}$ embryos found that although gastrulation did initiate it was delayed and failed to progress beyond the expression of early primitive streak stage marker genes (Chen et al., 1994). The tissue distribution of *Hnf-4* mRNA during early development was shown by in situ hybridization to be restricted to the visceral endoderm with no expression found in the fetus prior to E9.0 (Duncan et al., 1994; Taraviras et al., 1994). From these data we postulated that HNF-4 regulated the expression of VE secretory proteins which were required to support gastrulation (Duncan et al., 1994; Chen et al., 1994). For this model to be correct we

predicted that two criteria should be satisfied: (i) that expression of secreted protein genes should be dysregulated in *Hnf-4*$^{-/-}$ VE, and (ii) that specific complementation of *Hnf-4*$^{-/-}$ embryos with *Hnf-4*$^{+/+}$ VE should allow *Hnf-4*$^{-/-}$ embryos to complete gastrulation.

Expression of VE secreted proteins could be blocked if either the VE failed to differentiate or if gene expression was directly affected by the absence of HNF-4. To test this we made *Hnf-4*$^{-/-}$ ES cells and asked whether they were capable of producing fully differentiated VE in vitro. We found that both *Hnf-4*$^{+/+}$ and *Hnf-4*$^{-/-}$ EBs could form a morphological endoderm whose identity was confirmed as VE by positive staining with the diagnostic lectin SJA. In addition, both *Hnf-4*$^{+/+}$ and *Hnf-4*$^{-/-}$ EBs expressed similar levels of the VE marker genes *Gata-4*, *Apo-E*, and *vHnf-1*, confirming that the initial stages of VE differentiation do not require HNF-4. It has been proposed that one of the VE markers tested, the transcription factor GATA-4, is essential for the earliest stages of VE differentiation in ES cell EBs (Soudais et al., 1995). This would suggest that HNF-4 acts downstream of GATA-4 and is consistent with the proposal that differentiation of the VE is a multistep process (Grover et al., 1983a,b). Whether there is any direct regulation of HNF-4 by GATA-4, resulting in a transcriptional cascade during VE differentiation, is currently under investigation.

Having found that HNF-4 is non-essential for early differentiation of the VE we were able to ask whether HNF-4 was required for expression of secreted protein genes in the VE. Since HNF-4 is believed to be important for the regulation of many genes expressed in hepatocytes it seemed reasonable to suggest that the most likely candidates for serum protein genes regulated by HNF-4 would be those expressed in both liver and VE (Meehan et al., 1984; Sladek, 1994). We therefore analysed the expression of *AFP, TTR, Apo-AI, Apo-AIV, Apo-B, RBP,* and *TFN* in *Hnf-4*$^{-/-}$ ES cell EBs by RT-PCR. We found that while all genes were expressed in *Hnf-4*$^{+/+}$ and *Hnf-4*$^{+/-}$ EBs their expression was grossly reduced in all *Hnf-4*$^{-/-}$ EBs. Furthermore, we also found that expression of these genes was extremely reduced in *Hnf-4*$^{-/-}$ embryos, demonstrating that HNF-4 is required for expression of several proteins secreted from the VE both in vitro and in vivo. Many of the genes analyzed above contain HNF-4 binding sites within their promoters/enhancers, and some of these sites are important for their expression, at least in tissue culture cells (reviewed by Sladek, 1994). This would suggest that these genes are direct targets of HNF-4 and that their expression is directly dependent upon HNF-4 action. It is generally believed that tissue specific transcriptional regulation is the result of the coordinated interplay of several *trans*-acting factors. Redundancy at the promoter/enhancer level is suggested by the observation that mutation of specific *cis*-acting elements does not usually abolish but, more frequently, subtly modulates the level of transcription of a given gene. Correspondingly, targeted disruption of transcription factor genes often has surprisingly little effect on target gene expression (see for example, Pontoglio et al., 1996). However, in striking contrast to this, our data show that HNF-4 has a central role in establishing the expression of many VE genes and supports the proposal that HNF-4 is a key regulator of complete differentiation of the VE. Whether this is due to the direct action of HNF-4 on target gene expression or as the result of an HNF-4 regulated transcriptional cascade, possibly involving HNF-1, is unknown. However, it is interesting to note that HNF-1 expression appears to be only moderately reduced in *Hnf-4*$^{-/-}$ VE. This would suggest that while HNF-4 modulates, it is not essential for HNF-1 expression in the VE.

We have previously shown by in situ hybridization that before 9.0 days of gestation *Hnf-4* mRNA is restricted to the VE (Duncan et al., 1994). The demonstration that VE lacking HNF-4 expressed greatly reduced levels of secreted serum factors further supported our hypothesis that, in the absence of HNF-4, disruption of VE paracrine activity could block normal gastrulation. If this was indeed the case, specific complementation of *Hnf-4*$^{-/-}$ embryos with *Hnf-4*$^{+/+}$ VE should rescue the *Hnf-4*$^{-/-}$ block to gastrulation. To address this we used the tetraploid aggregation technique to produce *Hnf-4*$^{-/-}$ ES cell-derived fetuses which contained *Hnf-4*$^{+/+}$ tetraploid-derived extraembryonic tissues, as described by Nagy et al. (1990) and Nagy and Rossant (1993). This procedure has been used successfully to rescue *Mash-2*$^{-/-}$ embryos and, more recently, to produce *VEGF*$^{-/-}$ fetuses directly from ES cells (Guillemot et al., 1994; Carmeliet et al., 1996). We found that in the presence of *Hnf-4*$^{+/+}$ VE, *Hnf-4*$^{-/-}$ embryos would complete gastrulation, exhibiting anterior-posterior and dorsal-ventral structures which were essentially indistinguishable from wild-type embryos. Cumulatively, these data show that *Hnf-4*$^{-/-}$ embryonic ectoderm is competent to complete gastrulation and that the VE has a critical role in supporting gastrulation of the epiblast.

Following gastrulation, the extraembryonic VE forms a bilayer structure with extraembryonic mesoderm to produce the visceral wall of the yolk sac. From this time until maturation of the placenta, the yolk sac is responsible for maternofetal transport of nutrients for the growing embryo (reviewed by Jollie, 1990). Although yolk sac function has been well studied much less is known about the role of the VE before formation of the yolk sac. Recently, however, the VE has been shown to provide a signal for apoptosis required during cavitation of the egg cylinder which occurs prior to gastrulation (Coucouvanis et al., 1995). Furthermore, other pre-gastrulation functions of the VE have been suggested by mutations in the *Hβ58* and *even-skipped* (*evx-1*) genes. Disruption of *Hβ58* by a transgene insertion causes defects in growth of the embryonic ectoderm during a time when the highest level of Hβ58 expression is found in the extraembryonic VE (Lee et al., 1992). Mouse embryos lacking evx-1, whose expression at early times is found in the VE, fail to form extraembryonic endoderm and are resorbed before gastrulation begins (Spyropoulos et al., 1994). Further insight into the putative roles of the VE during early development has been provided by analyses of parthenogenetic embryos. Early reports showed that embryos derived entirely from maternal genomes could develop to mid-gestation stages although yolk sac development was severely impaired (Kaufman et al., 1977; Barton et al., 1984; McGrath et al., 1984; Surani et al., 1984). However, a recent detailed morphological analysis of parthenogenetic embryos during early developmental stages found that they fell into four categories exhibiting increasingly severe abnormalities (Sturm et al., 1994). While the least affected parthegenones developed to mid-gestation periods, as described previously, the remaining embryos all exhibited gross defects in the production of mesoderm and in axial patterning (Sturm et al.,

1994). Furthermore, in the most severely affected parthegenones, VE was grossly abnormal or completely absent while in the less affected the VE, although not normal, was present and capable of forming a yolk sac (Sturm et al., 1994). These observations are therefore consistant with a critical role for the VE during murine gastrulation.

In sum, our data establish that *Hnf-4*$^{-/-}$ ectodermal cells are competent to form postgastrulation embryos and that gastrulation requires an HNF-4^{+} VE. Since many of the genes whose expression is down-regulated in the absence of HNF-4 are serum factors, we propose that, during early stages of postimplantation development in the mouse, VE paracrine activity is critical for defining and maintaining an embryonic environment which will support gastrulation of the epiblast. Whether any of the serum factors we have shown to be down-regulated in the absence of HNF-4 are the cause of the *Hnf-4*$^{-/-}$ phenotype is under investigation. However, since gene targeting studies have shown that many are dispensable for gastrulation (Williamson et al., 1992; Episkopou et al., 1993; Farese et al., 1995; Huang et al., 1995), we believe it more likely that the effect is cumulative and the result of a gross serum deficiency. In this regard it is of interest to note that growth of PC13 cells, a murine embryonal carcinoma cell line, can be maintained in serum-free media if the media are supplemented with transferrin (TFN), high density-lipoprotein (HDL), and low-density lipoprotein (LDL) (Heath et al., 1983). We have shown that expression of TFN, as well as that of Apo-AI and Apo-B which are major components of HDLs and LDLs respectively, are almost undetectable in the absence of HNF-4.

Recent years have seen rapid advances in the application of targeted genetics to the study of mouse development and gene function. Application of the tetraploid aggregation technique has enabled us to bring a new dimension to our investigation of the early embryonic lethality seen in *Hnf-4*$^{-/-}$ mice. By complementing the lethal VE defect, the competence of *Hnf-4*$^{-/-}$ ES cell-derived embryos to progress beyond gastrulation was uncovered. This complementation opens the way for dissection of roles that HNF-4 may play in later stages of organogenesis.

We gratefully thank P. Traktman, R. F. Bachvarova, C. Horvath, and M. Stoffel for help and discussions; J. E. Darnell Jr for continued support and encouragement; S. Cereghini for advice on vHNF-1 and HNF-1 probes and the Rockefeller University transgenic facility for helping S. A. D. set up the tetraploid aggregation system. S.A.D. is a Naomi Judd American Liver Scholar and a recipient of an Alexandrine and Alexander Sinsheimer Scholar Award.

REFERENCES

Ang, S. L. and Rossant, J. (1994). HNF-3β is essential for node and notochord formation in mouse development. *Cell* **78**, 561-574.

Barton, S. C., Surani, M. A. H. and Norris, M. L. (1984). Role of paternal and maternal genomes in mouse development. *Nature* **311**, 374-376.

Basheeruddin, K., Stein, P., Strickland, S. and Williams, D. L. (1987). Expression of the murine apolipoprotein E gene is coupled to the differentiated state of F9 embryonal carcinoma cells. *Proc. Natl. Acad. Sci. USA* **84**, 709-713.

Brent, R. L., Beckman, D. A., Jensen, M. and Koszalka, T. R. (1990). Experimental yolk sac dysfunction as a model for studying nutritional disturbances in the embryo during organogenesis. *Teratology* **41**, 405-413.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Erberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W. and Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-439.

Cereghini, S., Ott, M.-O., Power, S. and Maury, M. (1992). Expression patterns of vHNF1 and HNF1 homeoproteins in early postimplantation embryos suggest distinct and sequential developmental roles. *Development* **116**, 783-797.

Chen, W. S., Manova, K., Weinstein, D. C., Duncan, S. A., Plump, A. S., Prezioso, V. R., Bachvarova, R. F. and Darnell, J. E., Jr. (1994). Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. *Genes Dev.* **8**, 2466-2477.

Coucouvanis, E. and Martin, G. R. (1995). Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. *Cell* **83**, 279-287.

Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* **87**, 27-45.

Drewes, T., Senkel, S., Holewa, B. and Ryffel, G. U. (1996). Human hepatocyte nuclear factor 4 isoforms are encoded by distinct and differentially expressed genes. *Mol. Cell Biol.* **16**, 925-931.

Duncan, S. A., Manova, K., Chen, W. S., Hoodless, P., Weinstein, D. C., Bachvarova, R. F. and Darnell, J. E., Jr. (1994). 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. *Proc. Natl. Acad. Sci. USA* **91**, 7598-7602.

Episkopou, V., Maeda, S., Nishiguchi, S., Kazunori, S., Gaitanaris, G. A., Gottesman, M. E. and Robertson, E. J. (1993). Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone. *Proc. Nat. Acad. Sci. USA* **90**, 2375-2379.

Evans, M. J. and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154-156.

Farese, R. V., Ruland, S. L., Flynn, L. M., Stokowski, R. P. and Young, S. G. (1995). Knockout of the mouse apolipoprotein B gene results in embryonic lethality in homozygotes and protection against diet-induced hypercholesterolemia in heterozygotes. *Proc. Natl. Acad. Sci. USA* **92**, 1774-1778.

Gardner, R. L. (1983). Origin and differentiation of extraembryonic endoderm in the mouse. *J. Embryol. Exp. Morphol.* **80**, 251-288.

Grover, A., Andrews, G. and Adamson, E. D. (1983a). Role of laminin in epithelium formation by F9 aggregates. *J. Cell Biol.* **97**, 137-144.

Grover, A., Oshima, R. G. and Adamson, E. D. (1983b). Epithelial layer formation in differentiating aggregates of F9 embryonal carcinoma cells. *J. Cell Biol.* **96**, 1690-1696.

Guillemot, F., Nagy, A., Auerbach, A. and Joyner, A. L. (1994). Essential role of *Mash-2* in extraembryonic development. *Nature* **371**, 333-336.

Harrison, S. M., Dunwoodie, S. L., Arkell, R. M., Lerach, H. and Beddington, R. S. P. (1995). Isolation of novel tissue-specific genes from cDNA libraries representing the individual constituents of the gastrulating mouse embryo. *Development* **121**, 2479-2489.

Heath, J. K. and Deller, M. J. (1983). Serum-free culture of PC13 murine embryonal carcinoma cells. *J. Cell. Physiol.* **115**, 225-230.

Hooper, M., Hardy, K., Handyside, A., Hunter, S. and Monk, M. (1987). HPRT-deficient (Lesch-Nyan) mouse embryos derived from germline colonization by cultured cells. *Nature* **326**, 292-295.

Huang, L. S., Voyiaziakis, E., Markenson, D. F., Sokol, K. A., Hayek, T. and Breslow, J. L. (1995). Apo B gene knockout in mice results in embryonic lethality in homozygotes and neural tube defects, male infertility, and reduced HDL cholesterol ester and Apo A-I transport rates in heterozygotes. *J. Clin. Invest.* **96**, 2152-2161.

Jollie, W. P. (1990). Development, morphology, and function of the yolk-sac placenta of laboratory rodents. *Terat.* **41**, 361-381.

Kaufman, M. H., Barton, S. C. and Surani, A. A. H. (1977). Normal postimplantation development of mouse parthenogenetic embryos to the forelimb bud stage. *Nature* **265**, 53-55.

Kubiak, J. C. and Tarkowski, A. K. (1985). Electrofusion of mouse blastomeres. *Exp. Cell. Res.* **157**, 561-566.

Kuo, C. J., Conley, P. B., Chen, L., Sladek, F. M., Darnell, J. E., Jr. and Crabtree, G. R. (1992). A transcriptional hierarchy involved in mammalian cell-type specification. *Nature* **355**, 458-460.

Lee, J. J., Radice, G., Perkins, C. P. and Constantini, F. (1992). Identification and characterization of a novel, evolutionarily conserved gene disrupted by

the murine Hß58 embryonic lethal transgene insertion. *Development* **115**, 277-288.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78**, 7634-7638.

McGrath, J. and Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* **37**, 179-183.

McLaughlin, K. L. (1993). Production of tetraploid embryos by electrofusion. In *Guide to techniques in mouse development*, (P. M. Wasserman and DePamphilis, M. L.), pp. 919-929. Academic Press, Inc., San Diego.

Meehan, R. R., Barlow, D. P., Hill, R. E., Hogan, B. L. M. and Hastie, N. D. (1984). Pattern of serum protein gene expression in the mouse visceral yolk sac and foetal liver. *EMBO J.* **3**, 1881-1885.

Mortensen, R. M., Conner, D. A., Chao, S., Geisterfer-Lowrance, A. A. T. and Seidman, J. G. (1992). Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell. Biol.* **12**, 2391-2395.

Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., Ivanyi, E., Markkula, M. and Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. *Development* **110**, 815-821.

Nagy, A. and Rossant, J. (1993). Production of completely ES cell-derived fetuses. In *Gene Targeting: A Practical Approach*, (A. Joyner), pp. 147-179. Oxford Unviersity Press, Oxford, UK.

Plump, A. S., Smith, j. D., Hayek, T., Aalto-Setaa, K., Walsh, A., Verstuyft, J. G., Rubin, E. M. and Breslow, J. L. (1992). Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* **71**, 343-353.

Pontoglio, M., Barra, J., Hadchouei, M., Doyen, A., Kress, C., Bach, J. P., Babinet, C. and Yaniv, M. (1996). Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria and renal Fanconi syndrome. *Cell* **84**, 575-585.

Robertson, E. J. (1987). Embryo-derived stem cell lines. In *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, (E. J. Robertson), pp. 71-112. IRL press, Oxford, UK.

Sato, M. and Muramatsu, T. (1985). Reactivity of five N-acetylgalactosamine-recognizing lectins with preimplantation embryos, early postimplantation embryos, and teratocarcinoma cells of the mouse. *Diff.* **29**, 29-38.

Sladek, F. M. (1994). Hepatocyte nuclear factor 4 (HNF-4). In *Transcriptional Regulation of Liver Specific Genes* (ed. F. Tronch and Yanish, M.), pp. R.G. Landes Company, Austin, TX, USA.

Sladek, F. M., Zhong, W., Lai, E. and Darnell, J. E., Jr. (1990). Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.* **4**, 2353-2365.

Soudais, C., Bielinska, M., Heikinheimo, M., MacArthur, C. A., Narita, N., Saffitz, J. E., Simon, M. C., Leiden, J. M. and Wilson, D. B. (1995). Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro. *Development* **121**, 3877-3888.

Spyropoulos, D. D. and Capecchi, M. R. (1994). Targeted disruption of the *even-skipped* gene, *evx1*, causes early postimplantation lethality of the mouse conceptus. *Genes Dev.* **8**, 1949-1961.

Sturm, K., Flannery, M. L. and Pederson, R. A. (1994). Abnormal development of embryonic and extraembryonic cell lineages in parthenogenetic mouse embryos. *Dev. Dyn.* **201**, 11-28.

Surani, M. A. H., Barton, S. C. and Norris, M. L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* **308**, 548-308.

Taraviras, S., Monaghan, A. P., Schutz, G. and Kelsey, G. (1994). Characterization of the mouse HNF-4 gene and its expression during mouse embryogenesis. *Mech. Dev.* **48**, 67-79.

Tian, J. M. and Schibler, U. (1991). Tissue-specific expression of the gene encoding hepatocyte nuclear factor 1 may involve hepatocyte nuclear factor 4. *Genes Dev.* **5**, 2225-2234.

Weinstein, D. C., Altaba, A. R. i., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M. and Darnell, J. E., Jr. (1994). The winged-helix transcription factor HNF-3β is required for notochord development in the mouse embryo. *Cell* **78**, 575-588.

Williamson, R., Lee, D., Hagaman, J. and Maeda, N. (1992). Marked reduction in high density lipoprotein cholesterol in mice genetically modified to lack apolipoprotein A-I. *Proc. Natl. Acad. Sci. USA* **89**, 7134-7138.

Wilson, P. A. and Melton, D. A. (1994). Mesodermal patterning by an inducer gradient depends on secondary cell-cell communication. *Curr. Biol.* **4**, 676-686.

Wu, T.-C., Wan, Y.-J. and Damjanov, I. (1983). Flourescein-conjugated *Badeira simplicifolia* lectin as a marker of endodermal, yolk sac and trophoblastic differentiaition in the mouse embryo. *Diff.* **24**, 55-59.

Zhong, W., Sladek, F. M. and Darnell, J. E., Jr. (1993). The expression pattern of a *Drosophila* homolog to the mouse transcription factor HNF-4 suggests a determinative role in gut formation. *EMBO J.* **12**, 537-544.

(Accepted 28 October 1996)