Expression patterns of vHNF1 and HNF1 homeoproteins in early postimplantation embryos suggest distinct and sequential developmental roles

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Summary

The homeoproteins HNF1 (LFB1/HNF1-A) and vHNF1 (LFB3/HNF1β) interact with an essential control element of a group of liver-specific genes. During development, these putative target genes are initially expressed in the visceral endoderm of the yolk sac and subsequently in fetal liver. To assess the possible involvement of HNF1 and/or vHNF1 as transcriptional regulators in the early steps of visceral endoderm differentiation, we have analyzed the expression pattern of both factors both in vitro during differentiation of murine F9 embryonal carcinoma cells and in vivo during early postimplantation mouse development. We show here that differentiation of F9 cells into either visceral or parietal endoderm is accompanied by a sharp induction in vHNF1 mRNA and protein. By contrast, only low levels of aberrantly sized HNF1 transcripts, but not DNA-binding protein, are found in F9 cells and its differentiated derivatives. At 6-7.5 days of gestation, high levels of vHNF1 mRNA are present in the visceral extraembryonic endoderm, which co-localize with transcripts of the transthyretin gene. HNF1 transcripts are first detected in the yolk sac roughly two embryonic days later, after the developmental onset of transcription of target genes. As development proceeds, discrepancies are observed between the level of transcripts of both vHNF1 and HNF1 and their respective nuclear binding proteins, notably in the yolk sac and embryonic kidney. In addition, we show that two alternative spliced isoforms of vHNF1 mRNA, vHNF1-A and vHNF1-B, are expressed in both embryonic and adult tissues. Taken together, these data suggest that vHNF1 participates as a regulatory factor in the initial transcriptional activation of the target genes in the visceral endoderm of the yolk sac, whereas the later appearance of HNF1 could be required for maintenance of their expression. Our results also provide evidence of a posttranscriptional level of control of vHNF1 and HNF1 gene expression during development, in addition to the spatial restriction in transcription.

Key words: HNF1 and vHNF1, homeoproteins, transcription factors, visceral endoderm, mouse embryo, embryonal carcinoma, posttranscriptional control.
In situ hybridization analyses of mid to late embryonic stages have indicated that HNF1 and vHNF1 mRNAs are present, albeit at different levels, in polarized epithelia of organs of endodermal and mesodermal origin - kidney, liver and the digestive tract. In both the developing liver and kidney, vHNF1 expression precedes that of HNF1 (De Simone et al., 1991; Ott et al., 1991; Lazzaro et al., 1992). Moreover, HNF1 and vHNF1 transcripts are not always located in the same cell types within these organs (Ott et al., 1991; Lazzaro et al., 1992). At present, however, the relationship between the temporal expression of HNF1 and vHNF1 and developmental activation of putative target genes has not been precisely defined.

Visceral and parietal endoderm derive from the primitive endoderm and constitute one of the first cell lineages to differentiate in the mouse embryo. In rodents, visceral endoderm cells form a layer of polarized epithelial cells at the periphery of the egg cylinder. Organized as an absorptive/secretory epithelium, it is the first structure to provide nutritional and hematopoietic function, before the hemorrhoidal placenta is formed (Rugh, 1990). In the early postimplantation stage, visceral endoderm, but not parietal endoderm, synthesizes and secretes a number of serum proteins, shown to be regulated by HNF1 in cultured cells, including alpha-fetoprotein (AFP), albumin, transthyretin (TTR), alpha-1-antitrypsin (A1-AT) (Dziadek and Andrews, 1983; Meehan et al., 1984; Makover et al., 1989).

The murine F9 embryonal carcinoma (EC) cells are able to differentiate in vitro toward either visceral or parietal endoderm and therefore mimic features of the endoderm differentiation in the mouse embryo shortly before and after implantation (Hogan et al., 1981; Scott et al., 1984; Soprano et al., 1988; Grover and Adamson, 1986). In the present study, we examine whether vHNF1 and/or HNF1 could have a role in the commitment and differentiation of the visceral endoderm, by performing a detailed analysis of the expression profiles of these homeoproteins both in vitro during differentiation of embryonal carcinoma F9 cells and in vivo in the early postimplantation mouse embryo.

We show here that the most significant change upon differentiation of F9 cells to either visceral or parietal endoderm is a sharp induction of vHNF1 mRNA and protein. In contrast, low levels of aberrantly sized HNF1 message are found in both non-differentiated and differentiated F9 cells. Moreover, no HNF1-binding activity is detected in either F9 or its differentiated derivatives.

In early postimplantation embryos, high levels of vHNF1 transcripts are detected by in situ hybridization in the visceral extraembryonic endoderm at 6 to 7.5 days of gestation. Active vHNF1 binding protein is also detected in 7.5-day embryos. At these stages no significant levels of either HNF1 mRNA or protein are observed.

The results of these analyses support the hypothesis that vHNF1 may participate as a regulatory factor in the early transcriptional activation of the presumed target genes in the visceral endoderm of the yolk sac, while the later appearance of HNF1 could be required for maintenance of their expression. These results are discussed in relation to other transcriptional regulators implicated in hepatospecific gene expression.

**Material and methods**

**Cell lines and culture conditions**

The rat hepatoma cells used, H4II and its dedifferentiated derivative H5 (Deschatrette and Weiss, 1974), the somatic hybrid HF1, which causes extinction of the expression of liver-specific functions, and HF1-5, a revertant of HF1 reexpressing liver functions (Deschatrette et al., 1979), were cultured as described (Cereghini et al., 1988). Mouse F9 EC, P19 EC, the trophoblastoma 3TDM-1 and the parietal-like PYS-2 cell-lines were provided by J. F. Nicolas and cultured as described by Nicolas et al. (1976). For differentiation into either ‘parietal’ or ‘primitive endoderm’-like cells, 2.5×10⁵ F9 cells were plated on gelatinized 10 cm tissue culture Petri dishes containing 5×10⁻⁷ M RA (Sigma Chemical Co) (primitive endoderm) or 5×10⁻⁷ M RA + 0.1 mM dCAMP (parietal endoderm). Differentiation of F9 aggregates to visceral endoderm was carried out as described by Scott et al. (1984).

**Isolation of cDNA clones encoding mouse HNF1 and vHNF1**

Oligo(dT)-primed mouse liver and F9 differentiated visceral cDNA libraries were constructed using the kits ‘cDNA synthesis’ and ‘Lambda-gt-10 cloning system’ from Amersham, as described by the manufacturers. The nonamplified libraries (5×10⁶ plaques) were screened by hybridization in low-stringency conditions with a 511 bp NcoI fragment that contains the entire homeodomain of rat HNF1 (334 to 845 nt residues) essentially as described (Bach et al., 1991). Isolation and sequencing of positive clones was performed as described by Bach et al. (1991). Three full-length mouse vHNF1 clones were obtained, while the longest mouse HNF1 clone lacks 45 nt from the ATG initiator codon. The sequence of this clone was identical to that of mouse HNF1 reported by Kuo et al. (1990b). As in previous screening of a H5 rat hepatoma cDNA library (Rey-Campos et al., 1991), two types of mouse vHNF1 clones were obtained. Most of them contained a 78 nt insertion between the B domain and the homeodomain, while others lack this sequence. The mouse vHNF1 sequence reported by Mendel et al. (1991a) corresponds to the type of clones lacking such insertion, whereas that reported by De Simone et al. (1991) corresponds to those containing this insertion. The coding sequences of our mouse (± insertion) vHNF1 clones were identical to that shown by Mendel et al. (1991a) and differ in two regions with that reported by De Simone et al. (1991). These differences are probably due to sequencing errors, since a human vHNF1 clone (Bach et al., 1991) displays identical amino acid residues in these regions to either our or Mendel et al. (1991a) mouse clones.

**Nuclear extracts and gel mobility-shift assays**

Nuclear extracts from cell-lines and mouse adult tissues were prepared as described (Cereghini et al., 1988). Mouse embryo extracts were prepared at the indicated times after fertilization, by microsurgery and pooled. Nuclear extracts from whole embryo or microdissected organs were prepared in the presence of 2.5 µg/ml of BSA, at a microscale level, essentially as described for adult tissues, except that the ammonium sulfate precipitation and dialysis steps were omitted. The amount of nuclear protein from embryo extracts used in the DNA-binding assays ranged from 0.1 to 0.5 µg. Normalization of embryo extracts was performed by assaying the ubiquitous NF-Y binding factor. Gel mobility shift assays were as described (Cereghini et al., 1988). When mentioned, specific polyclonal antibodies at the indicated dilution in
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PBS + 1 µg/ml of BSA, were preincubated with nuclear extracts at room temperature for 10 minutes, before adding the labeled probe. Double-stranded oligonucleotide probes encompassing the HNF1 (PE) and the NF-Y binding sites of the rat albumin promoter (Cereghini et al., 1988) and the HNF4 site of the apoCIII promoter (Sladek et al., 1990), kindly provided by M.Zakin, were 32P-labeled with T4 polynucleotide kinase. Total protein extracts from embryonic organs were prepared by tissue homogenization as described for the isolation of nuclei, followed by addition of 1/10 volume of 4 M ammonium sulfate, sonication and centrifugation. Western blots with either total or nuclear protein were carried out essentially as described by Blumenfeld et al. (1991), using 40-60 µg and 25 µg of total and nuclear protein, respectively.

Antiserum preparation

HNF1 and vHNF1-specific antisera were raised, respectively against residues 306 to 391 of mouse HNF1 and residues 293 to 424 of mouse vHNF1, fused to glutathione in the pGEX3 bacterial expression vector (Smith and Johnson, 1988). The cDNA fragments were generated using specific primers, following amplification from Bluescribe plasmids containing either mouse HNF1 or mouse vHNF1 cDNAs. The two polyclonal antibodies were raised into white Bouscat rabbits. Boost injections were performed each 21-28 days with 100 µg of fusion protein purified on glutathione agarose columns (Smith and Johnson, 1988). Sera used in this study were obtained after three boosts.

RNA isolation, analysis and quantitative PCR amplification of cDNA

Total RNA, poly (A)+ RNA preparation and northern blot analysis were carried out as previously described (Cereghini et al., 1990). For PCR analysis of RNA, 1 µg of poly (A)+ RNA prepared from cell lines and mouse tissues was reverse transcribed in a final volume of 80 µl using random hexamers, as described by Montarras et al. (1989). When indicated, 4 µg of total RNA was used to prepare the corresponding cDNAs. The conditions of cDNA synthesis and PCR amplification were essentially as described by Blumenfeld et al. (1991). The PCR reactions were performed in a final volume of 50 µl using either 2.5 µl or 10 µl of the cDNA mixture prepared respectively from poly (A)+ RNA or total RNA, for 15 cycles of 94°C denaturation (30 seconds), 55°C annealing (30 seconds) and 72°C extension (1 minute) in an automated thermal cycler. Due to the preferential amplification of some products, independently of their size, the results of RT-PCR experiments were confirmed with at least two different sets of primers. The primers chosen were located in different exons and control experiments showed that the generation of PCR products were strictly dependent on the synthesis of cDNA and on the presence of the selected 5′- and 3′-primers (not shown). The quality and amount of the RNA samples were previously examined by northern blot, using GAPDH as probe. In addition, specific primers for hypoxanthine phosphoribosyl transferase (HPRT) transcripts were also used as positive control of the corresponding cDNA. PCR primer sequences used in the experiments shown here were: vHNF1 - 5′: AACGGGAGATCCCTCCGACAG; vHNF1 - 3′: CATTGCAACTGTACATTTGTT; HNF1 - 5′: TGGTTTTCTAAGCTGAGCAG; HNF1 - 3′: GGGCTTCCAATCGATCCGC; HPRT - 5′: GATATTTAGCATGATAACTATT (exon 6); HPRT - 3′: TCAAGGGGCTACACAGATCCAAAC (exon 8) (see Melton et al., 1984). Unless otherwise indicated, the PCR conditions were such that amplification of cDNA was linear and dependent on the concentration of the respective RNA : low number of cycles (no more than 15) and transfer of the amplified products to a membrane, followed by hybridization. 18 µl of the PCR reactions (final volume 50 µl) were loaded on agarose gels, transferred to Hybond N (Amersham) membranes and hybridized as described for northern blot analysis. Full-length mouse vHNF1 cDNA and the mouse HNF1 cDNA (see above) were used as hybridization probes. The hp2 probe was generated by RT-PCR from mouse liver RNA.

Molecular probes and in situ hybridization procedures

The HNF1 and vHNF1 probes used for in situ hybridization encompassed the same DNA sequences from the carboxy terminal part of the mouse vHNF1 and HNF1 cDNAs that were used to generate the fusion proteins in pGEX3 vectors. The PCR amplified fragments (see above) were cloned into Bluescribe plasmid (Stratagene). AFP (Tyner et al., 1990) and TTR probes (Makover et al., 1989) were kindly provided by S. Thilghman and W. Blanes, respectively. C3H mice embryos were used. Noon of the day when the vaginal plug was detected is considered gestational day 0.5. Preparation of 25S riboprobes, tissue sections and hybridization conditions were as described (Ott et al., 1991). For each hybridization, antisense and sense riboprobes were used: only background levels of hybridization were detected with sense probes and are not shown.

Results

Induction of vHNF1 mRNA, but not of HNF1 RNA, in differentiated F9 cells

In the presence of RA, F9 EC cells differentiate into endoderm-like cells which, depending on the culture conditions, form either parietal or visceral endoderm. When grown as monolayers in the presence of RA, or RA + dibutyryl cyclic AMP (dbcAMP), they differentiate into parietal endoderm cells (Strickland et al., 1980). However, if F9 cells are treated with RA and allowed to aggregate, most of the cells of the outer surface of the aggregate differentiate into visceral endoderm, characterized by the synthesis of AFP, albumin and transthyretin (Hogan et al., 1981; Grover et al., 1983; Soprano et al., 1988).

We examined the levels of HNF1 and vHNF1 mRNA by northern blot analysis using poly(A)+ RNA from F9 cells differentiated into either parietal or visceral endoderm at various times after differentiation (Fig. 1). As markers of differentiation, we followed both the keratin Endo A and AFP mRNA levels. As expected, Endo A is expressed at very low levels in F9 EC cells and is highly induced in parietal endoderm derivatives (Oshima, 1982; Fig. 1A), while AFP mRNA is induced exclusively in visceral cells (Fig. 1B).

As shown in Fig. 1A, a strong increase in vHNF1 mRNA levels is observed in both visceral and parietal endoderm-type cells. Low levels of HNF1 transcripts are present in both nondifferentiated F9 cells and after differentiation into these two pathways. Moreover, these transcripts display different sizes ranging from 3 to 3.5 kb instead of a unique HNF1 transcript of 3.5 kb, as is observed in liver (Fig. 1A), kidney or intestine (Rey-Campos et al., 1991). The different sizes of HNF1 transcripts are not due to nonspecific mRNA degradation as indicated by the sequential hybridization of the same northern blot with the vHNF1, Endo A and GAPDH probes (Fig. 1A). The origin of these aberrantly sized HNF1 transcripts is presently under investigation. The presence of HNF1 transcripts in nondifferentiated...
Fig. 1. Induction of vHNF1 mRNA in RA-induced differentiation of F9 cells along parietal and visceral endoderm pathways. F9 EC cells were grown either as monolayer in the presence of 5x10^{-7} M RA + 0.1 M cAMP (parietal endoderm pathway : Par) or in suspension in the presence of 7.5x10^{-8} M (visceral endoderm pathway : VIS). Cells were harvested for RNA and nuclear protein (Fig. 2) analyses at the times indicated in days above the respective lanes. F9EC corresponds to undifferentiated stem cells. Mouse liver RNA was used as a control for HNF1 mRNA; RNA from the parietal endoderm-like cell line PYS-2 and the trophoblastoma cell line 3TDM-1 were also included. Northern blot analyses using 3 µg of poly (A)^+ RNA per lane were performed as described in Materials and methods. Similar results were obtained in several independent differentiation experiments. The sizes in kb of the mRNAs hybridized are shown on the left with pointed arrows. (A) The same filter was consecutively hybridized with the probes indicated on the left of the respective autoradiograms and comprise from top: the full-length mouse cDNA fragment (kindly provided by P. Duprey). The origin of the extra band observed with the vHNF1 probe in the 3TDM RNA sample is under investigation. Note that less RNA was loaded in lane VIS 10d. (B) Specific induction of AFP mRNA in visceral F9 derivatives. Filter hybridized with a mouse AFP cDNA probe (Tyner et al., 1990).

F9 cells is not a particularity of this cell-line as they are also detected in embryonic stem cells (ES), albeit at lower levels than in F9 (see below, Fig. 3).

In addition, two stably differentiated teratocarcinoma cell lines, the parietal endoderm-like cell line PYS-2 and 3TDM-1, a trophoblastoma cell-line, characterized by the presence of endoderm-like and giant trophoblastic-like cells (Nicolas et al., 1976), behave similarly to differentiated F9 cells. Cells of both lines express constitutively high levels of vHNF1 mRNA, but low levels of aberrantly sized HNF1 mRNAs. In contrast, no vHNF1 or HNF1 mRNA is observed in P19 teratocarcinoma cells that undergo neuronal differentiation upon aggregation and treatment with RA (not shown). Thus, the induction of vHNF1 mRNA appears to be associated with the endodermal differentiation character of the cells.

The HNF1 binding activity induced in differentiated F9 cells is composed essentially of vHNF1 homodimers

vHNF1 and HNF1 proteins display an identical DNA-binding sequence specificity, but vHNF1 has a lower molecular weight and exhibits a faster mobility than HNF1 in gel retardation assays (Cereghini et al., 1988; Baumhueter et al., 1988). To assay for the presence of HNF1/vHNF1 DNA-binding activities, gel shift assays were performed using as probe the HNF1 site of the albumin proximal promoter (PE oligonucleotide). Nuclear extracts from H5 cells, which lack HNF1 (Cereghini et al., 1988), were used as a control for the mobility of vHNF1 homodimers.

As shown in Fig. 2A, no complexes were detected in nondifferentiated F9 cells. After RA addition, a complex, which comigrates with that formed by vHNF1 homodimers of H5 nuclear extracts is observed in both parietal and visceral derivatives. As a control, we monitored the ubiquitous NF-Y (CCAAT) binding activity in the same extracts and found roughly the same activity (Fig. 2B).

Cross-competition experiments with wild-type and mutant PE oligonucleotides clearly show that the complex formed in differentiated F9 cells exhibits an identical behavior to that which we previously described for both HNF1 and vHNF1 (Fig. 2C; Cereghini et al., 1988). UV cross-linking experiments showed that the complex detected in both parietal and visceral F9 extracts is formed by a polypeptide of apparent relative molecular mass identical to that of vHNF1 from H5 extracts (i.e. ~70-75x10^3; data not shown).

To confirm further the presence of only vHNF1 homodimers in F9 differentiated cells, we generated specific antibodies against the most highly divergent region in the C-terminal part of the mouse HNF1 and vHNF1 proteins (see Material and methods). In no case was the complex generated by either visceral or parietal differentiated F9 cells affected by HNF1 antibodies, while it was totally competed by vHNF1 antibodies (Fig. 2D).

These results show that the PE-binding activity induced upon differentiation of F9 cells is primarily composed of vHNF1 homodimers. Consequently, the HNF1 transcripts present in both undifferentiated and differentiated F9 cells are either not translated into active HNF1 binding protein or the protein is extremely unstable. We did not detect HNF1 protein in either nuclear or total cellular extracts from these cells by either western blot or gel shift assays (data not shown), a result that argues against the presence of either inactive DNA-binding protein(s) or cytoplasmic
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protein not transported to the nucleus. However, we cannot exclude the presence of trace levels of HNF1 protein that could escape detection by these assays.

We also investigated whether HNF1 and vHNF1 might exhibit different sensitivity to RA. No evidence of HNF1 binding activity was observed in extracts from F9 cells grown in suspension and exposed to RA concentrations ranging from $7.5 \times 10^{-8}$ M to $1 \times 10^{-6}$ M, for either 5, 10 or 15 days, while vHNF1 activity was similarly induced at all of these concentrations and time points (data not shown).

**Developmental expression of HNF1 and vHNF1 mRNAs**

To verify the developmental relevance of the results obtained in differentiated F9 cells, we examined the time
course of transcript accumulation of vHNF1/HNF1 during mouse development by a quantitative reverse transcription-polymerase chain reaction (RT/PCR) assay (see Material and Methods).

With regard to vHNF1, specific primers were designed to verify whether the two distinct vHNF1 cDNA clones previously identified (Rey-Campos et al., 1991) are two spliced forms of vHNF1. As reported for rat vHNF1, two different mouse vHNF1 cDNA clones were also obtained upon screening a cDNA library from F9 differentiated cells; most of them contained a 78-nucleotide (nt) insertion between the POU domain and the homeodomain (referred as vHNF1-A), while roughly one out of four clones lacked this sequence (named vHNF1-B). Otherwise, mouse vHNF1-A and vHNF1-B display identical coding and 3′ non-coding sequences (see Materials and Methods). Since restriction analyses of mouse genomic DNA indicated that vHNF1 is a single copy gene (data not shown), these two classes of clones are likely generated by alternative splicing. To examine this more directly, specific primers, located at both sides of the 78 nt insertion, were used to amplify cDNAs transcribed from RNAs from various cell lines and tissues at different developmental stages.

The results of these analyses clearly indicate that both isoforms are present, but vHNF1-A represents the major mRNA species expressed in the different cell lines as well as in the different embryo stages and adult tissues (Fig. 3B,C). However, the ratio of vHNF1-A/vHNF1-B transcripts is reproducibly higher in adult liver and intestine than in other embryo or adult tissues.

vHNF1 and HNF1 mRNA levels were then examined in whole embryos at 7.5 days post coitum (d.p.c.), the stage when visceral and parietal extraembryonic endoderm are clearly differentiated, as well as at later developmental stages both in the embryo proper and the yolk sac. High levels of vHNF1 transcripts are observed in 7.5 d.p.c. embryos, while at stage HNF1 transcripts are barely detectable. In the yolk sac, the levels of vHNF1 mRNA remains high throughout gestation from 8.5 to 14.5 days. At 8.5 days of gestation HNF1 mRNA is indeed present in the significantly low levels of hprt amplified product observed in yolk sac samples at 9.5 and 14.5 days are presently unknown and are not due to low levels of RNA because similar levels of GAPDH mRNA were found in these samples (not shown). Numbers 1 to 8 below lanes of hprt amplifications correspond to the same RNA samples ES to YS 9.5 d, as indicated above lanes of vHNF1 amplifications.
the yolk sac and its levels increase at later developmental stages (Fig. 3C and results not shown). Thus, both HNF1 and vHNF1 mRNAs are expressed in the yolk sac from 8.5 days on, but vHNF1 precedes HNF1 expression.

vHNF1 and TTR transcripts co-localize in the visceral extraembryonic endoderm during early postimplantation mouse development

To localize transcripts in early postimplantation embryos, we performed RNA in situ hybridization analysis of embryo sections. At 6.5 days of gestation, the endoderm cells that remain in contact with the egg cylinder are organized into visceral extraembryonic and visceral embryonic endoderm, surrounding the embryonic and extraembryonic ectoderm, respectively. The primitive endoderm cells, which have migrated onto the mural trophoblast, form the parietal endoderm that consists of a flat layer lining the decidua.

As seen in Fig. 4, high levels of vHNF1 transcripts are detected in the visceral endoderm and in the uterine epithelium. Serial sections showed that vHNF1 transcripts are predominant in the extraembryonic endoderm. The decidua and trophoblast appear negative, indicating that parietal endoderm cells do not express vHNF1.

At 7.5 days of gestation, the egg cylinder continues to develop, the proamniotic cavity enlarges and the mesoderm forms between the posterior embryonic ectoderm and the endoderm. At this stage, vHNF1 transcripts are present in the columnar visceral endoderm, both in the extraembryonic part and in the middle region surrounding the mesoderm. The expression pattern of vHNF1 mRNA correlates spatially and temporally with that of transthyretin (Makover et al., 1989; Fig. 5C). However, it partially overlaps with that of AFP, which is confined to the visceral endoderm cells surrounding the embryonic part of the egg cylinder (Dziadek and Andrews, 1983; see also Fig. 5D,E). The synthesis of AFP in the visceral extraembryonic endoderm appears to be suppressed as a result of an inhibitory influence of the underlying extraembryonic ectoderm (Dziadek, 1978). At these two stages, no hybridization signal for HNF1 mRNA was detected in any embryo or extraembryonic structure, even after longer exposure (40 days; data not shown).

Thus the initial induction of transthyretin and AFP mRNAs is preceded by or concomitant with the expression of vHNF1 and occurs in the absence of detectable HNF1 expression.

Discrepancy between HNF1 and vHNF1 transcript and protein levels in the yolk sac and embryonic kidney

Since in several instances the presence of HNF1 transcripts is not correlated with the presence of DNA-binding protein (i.e. F9 and ES cells), we decided to examine whether similar non correlations are observed in embryo extracts. To this end, nuclear extracts from early embryos and microdissected developing organs were prepared and examined by gel mobility shift assays with specific antisera.

As shown in Fig. 6A, vHNF1 homodimers are detected in the nuclei of 7.5 d.p.c. whole embryos to 10.5 d.p.c. trunk embryos. The highest vHNF1 DNA-binding activity is present at 7.5 to 8.5 days of gestation, after which a decrease occurs until it becomes barely detectable at 10.5 days. In part, this decrease may reflect a reduction in a restricted number of positive cells relative to total embryo cells. Conversely, HNF1 is not detected in 7.5 to 9.5 d.p.c. embryos and becomes weakly detectable at 10.5 days of gestation. Thus, as expected, through 7.5 to 9.5 days of gestation, the DNA-binding complex detected in embryo nuclear extracts is composed essentially of vHNF1 homodimers.

Analysis of nuclear extracts from yolk sac show that at 8.5 d.p.c., although vHNF1 homodimers represent the major activity, HNF1 protein is also present essentially as HNF1:vHNF1 heterodimers (Fig. 6A). As development proceeds, however, there is a significant decrease of vHNF1 homodimers and a parallel increase of HNF1 homodimers. At 14.5 days of gestation, yolk sac nuclear extracts contain almost exclusively HNF1 homodimers and are qualitatively and quantitatively similar to embryo liver of the same developmental stage. In contrast, no drop in vHNF1 mRNA levels occurs between 8.5 days and 14.5 days, while only a 2-fold increase in the levels of HNF1 transcripts is observed over the same period (compare Fig. 3C with Fig. 6A).

Likewise, in the developing mouse kidney essentially vHNF1 activity is detected at 14.5 to 15.5 days of gestation, while HNF1 transcripts are readily detected in this organ from 14.5 days on, not only by the highly sensitive RT-PCR assays (Fig. 3C), but also by in situ hybridization (Ott et al., 1991). It is also relevant to note that, although similar levels of HNF1 transcripts are present in 14.5 and 15.5 d.p.c. embryonic liver, intestine and kidney, HNF1 binding activity is only detected in liver and intestine. Indeed, the binding activities detected in either embryo liver, intestine or lung are in good agreement with the levels of the respective mRNAs: mainly HNF1 homodimers are present in embryo liver, HNF1 and vHNF1 homodimers and heterodimers in the expected proportions in the developing intestine and exclusively vHNF1 homodimers in embryo lung (compare Fig. 3C with Fig. 6B). It is possible that the observed discrepancy between the HNF1 and vHNF1 binding activities and their transcript levels is due to changes in the affinity and/or in their translocation to the nucleus. To examine this, we performed western blot analysis on total protein extracts from embryo kidney and yolk sac. The results indicate that the levels of protein detected by this assay (Fig. 6C) are roughly in agreement with the levels of the respective binding activities detected by gel shift assays (Fig. 6A,B). Thus, neither the affinity of either HNF1 or vHNF1 nor their translocation to the nucleus appear to be severely perturbed in embryo kidney and yolk sac, respectively. Table 1 summarizes the mRNA levels and binding activities of HNF1 and vHNF1 at different embryonic stages and in developing organs.

Taken together, these results suggest that both vHNF1 and HNF1 may be posttranscriptionally regulated.

Developmental expression of HNF4 protein

At present, there is little information about the earliest appearance of other hepatocyte transcription factors during development (Lai and Darnell, 1991; see also Discussion).

Recent studies indicated that HNF1 and HNF4, a member
Fig. 4. For legend see p. 792
Expression patterns of HNF1 homeoproteins in mouse embryo of the steroid hormone receptor superfamily (Sladek et al., 1991), are regulated in a hierarchy by a higher-order locus (Kuo et al., 1992). HNF4 is a crucial transactivator of HNF1 (Kuo et al., 1992; Tian and Schibler, 1991) and, more importantly, stable transfection of HNF4 cDNA in dedifferentiated hepatoma cells rescues the expression of endogenous HNF1 mRNA and DNA-binding activity (Kuo et al., 1992). HNF1 and HNF4 appear also to cooperate in

Fig. 5. For legend see p. 792
Fig. 4. Pattern of vHNF1 mRNA expression at embryonic day 6.5. Sections through the implantation site containing the embryo were hybridized with 35S-labeled antisense vHNF1 RNA probe, whereas the section shown in C was hybridized with HNF1 riboprobe. (A-F) Dark-field views and (a-f) the respective bright-field micrographs. Horizontal bars in A and B indicate the regions enlarged and shown in E, F and G. The embryo sections are sagittal in E and oblique transverse through the visceral endoderm in F. Only one section is shown for the HNF1 probe, but other serial sections were also negative. Significant labeling is observed in the visceral extraembryonic endoderm (vee) and the uterine epithelium (ue). Abbreviations in this and Fig. 5 are: em, embryo; ee, embryonic ectoderm; exe, extraembryonic ectoderm; epc, ectoplacental cone; pac, proamniotic cavity; uc, uterine cavity; ue, uterine epithelium; vee, visceral extraembryonic endoderm.

Fig. 5. Coexpression of vHNF1 and TTR mRNAs at 7.5 days of gestation. Serial sections through a 7.5-day embryo were hybridized with 35S-labeled antisense vHNF1, TTR and AFP riboprobes, as indicated. (A-C) Dark-field views and (a-c) the respective bright-field views. For AFP hybridizations, only the dark-field images are shown (D,E). Coexpression of vHNF1 and TTR is observed in the columnar visceral endoderm (vee) overlying the extraembryonic ectoderm as well as in visceral endoderm cells overlaying the nascent mesoderm (m), whereas expression of AFP is observed in the visceral embryonic endoderm (ve) and, as vHNF1 and TTR, in the visceral endoderm surrounding the mesoderm. Parietal endoderm (pe), which is partially detached from the trophoblast in these sections, does not show significant labelling for either vHNF1, TTR or AFP. Serial sections hybridized with HNF1 probe did not give a positive signal in either embryo or extraembryonic structures (not shown). Additional abbreviations in this figure are: ac, amniotic cavity; ec, exocoelom; m, mesoderm; ve, visceral embryonic endoderm; pe, parietal endoderm; yc, yolk cavity.

Table 1. Expression of HNF and vHNF1 mRNA and DNA-binding activities in embryonic cells

<table>
<thead>
<tr>
<th>Stage</th>
<th>HNF1 mRNA</th>
<th>vHNF1 mRNA</th>
<th>HNF1 protein</th>
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</tr>
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<tr>
<td>8.5 d</td>
<td>–/−</td>
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<tr>
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<td>–/−</td>
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<tr>
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<tr>
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Summary of HNF1 and vHNF1 mRNA levels and the respective DNA-binding activities in whole/trunk embryo and developing organs embryo. –/+, barely detectable or not clearly positive; +/−, very weak; +, weak, ++, Medium; ++++, high; n.d., not determined. Data were taken from both results shown in Figs 3, 6 and 7 and data not shown.

(*) HNF1 mRNAs of different sizes ranged from 3 to 3.5 kb.

Discussion

The present study was undertaken to examine whether HNF1 and/or vHNF1 are expressed in a temporal and spatial pattern in early postimplantation embryos that is consistent with their potential role as transcriptional regulators of several cell-specific markers. The fact that both HNF1 and vHNF1 possess the same DNA sequence specificity and heterodimerize raised the intriguing question whether each of these homeoproteins display distinct or similar developmental function. We focused our attention on the expression pattern of HNF1 and vHNF1 during the formation of visceral endoderm of the yolk sac.

Our results show that induction of the putative HNF1 target genes in the visceral endoderm is preceded by and/or concomitant with the expression of vHNF1 but not HNF1.
similar sequence of events seems to occur in vitro after differentiation of F9 cells: vHNF1 (mRNA and protein), but not HNF1, is highly induced in visceral derivatives. However, no vHNF1 transcripts were detected in the parietal yolk sac by in situ hybridization, while vHNF1 is also present in F9 parietal derivatives. We do not know yet whether vHNF1 is expressed transiently in the parietal endoderm at stages earlier than those examined here.

While this work was in progress, another study reporting the regulation of vHNF1 and HNF1 after differentiation...
of F9 cells into visceral endoderm was published (Kuo et al., 1991). That study and ours agree with regard to the earlier induction of both vHNF1 mRNA and protein relative to HNF1. However, in contrast to our results, Kuo et al. (1991) showed an increase in HNF1 mRNA of ~4-fold after 5 days of RA treatment of F9 cells in suspension, which correlates with a concomitant elevation of HNF1 binding activity, as measured by a supershift generated by polyclonal antibodies. At present, we have no explanation for this discrepancy. It is plausible that the F9 cell lines used in each study partially differ in the differentiation state induced.

Whatever the case, in vivo no HNF1 transcripts were detected by in situ hybridization in 6.5 to 7.5 days embryos; a weak signal was only observed by RT-PCR at 7.5 days of gestation. In contrast, high levels of vHNF1 mRNA are already present at 6.5 days of gestation in the visceral extraembryonic endoderm, which co-localizes with TTR and RBP transcripts, first detected at 7-7.5 days of gestation (Makover et al., 1988, present study : Fig. 5). Altogether the results suggest that vHNF1, but not HNF1, may participate in the initial transcriptional activation of these genes. Maintenance of their expression may require additional expression of HNF1, which appears in the visceral yolk sac roughly 2 embryonic days later (Ott et al., 1991; Blumenfeld et al., 1991). Interestingly, at least with regard to the yolk sac, vHNF1 and HNF1 proteins appear to display a reciprocal regulation during development (decrease of vHNF1 and concomitant increase of HNF1 binding activity), whereas the levels of both mRNAs remain similarly high throughout development.

The sequential expression of vHNF1 followed by HNF1 during kidney organogenesis led to the suggestion that vHNF1 may be critical in the early inductive phase characterized by a heterotypic inductive interaction between the epithelium of the ureter bud and the condensed mesenchymal cells, whereas HNF1 is activated later, during the postinductive phase, in already committed cells (Lazzaro et al., 1992). Since inductive interactions with mesenchyme are also required for endodermal cells to differentiate in hepatocytes (LeDouarin, 1975; Houssaint, 1980), vHNF1 and HNF1 may also execute distinct roles in the inductive and postinductive phases during liver organogenesis. Indeed, vHNF1, but not HNF1 mRNA, is detected in the hepatic precursor cells (Ott et al., 1991), which already express the cell-specific markers AFP and albumin (Schmid and Schulz, 1990; Cascio and Zaret, 1991). HNF1 mRNA is first detected in the liver primordium at a later developmental stage (10.5 days), after the initiation of the hepatic cell lineage (Ott et al., 1991).

It is likely that the transcriptional activation of the various HNF1-dependant functions induced either in the yolk

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**Fig. 7.** HNF4 and COUP-TF/Arp-1 binding activities in nuclear extracts of early embryos, developing organs and differentiated F9 cells. (A) ‘HNF4’ binding activities in embryonic nuclear extracts. Gel shift assays were carried out using as probe a ds oligonucleotide encompassing the HNF4 recognition sequence present in the apo CIII promoter and the embryonic nuclear extracts shown in Fig. 6. For each extract, binding assays were done in the absence and in the presence of 40-fold molar cold oligonucleotide as competitor. (B) Identification of HNF4 and COUP-TF+Arp-1 binding activities in embryonic and F9 parietal and visceral nuclear extracts. When indicated, nuclear extracts were incubated with 1 µl of HNF4 antiserum (Ab-HNF4) diluted 1:6 and 1 µl of COUP-TF antiserum (Ab-COUP) diluted 1:5, before the addition of the labeled HNF4-apoCIII probe. Thin and thick arrows indicate the supershifts generated by HNF4 and COUP-TF antibodies, respectively. Only extracts in which both antibodies were used are shown; the complex observed in 7.5-day embryo extracts in A is not affected by HNF4 antibody (not shown).
est expression of C/EBP mRNA was observed in fetal liver at 13 days of gestation (Kuo et al., 1990a), which is around four days after the formation of the liver primordium. Members of the extended HNF3/fork head family are expressed not only in the gut-endoderm-derived tissues, but also in the developing rat brain (Tao and Lai, 1992 and references therein). Although no data are still available about the earliest expression of the different HNF3 genes in the mouse, the emerging picture is that HNF3 is a large family of transcription factors, with distinct subfamilies each implicated in differentiation of different cell lineages (Dirksen and Jamrich, 1992; Grossniklaus et al., 1992).

Finally, our results suggest that HNF4, as HNF1, may appear after initiation of visceral endoderm and hepatic cell lineage differentiation. HNF4 and HNF1 seem to be related by a transcriptional hierarchy in which HNF4 regulates the expression of HNF1 and, ultimately, both cooperate or synergize to activate transcription of downstream genes (Tian and Schibler, 1991; Kuo et al., 1992 and references therein). The developmental role of HNF4 was further suggested by mutagenesis studies in transgenic mice showing that disruption of either HNF1 or HNF4 binding sites present in the promoter-enhancer region of the A1-antitrypsin gene drastically affect its expression in fetal liver and yolk sac, whereas only the HNF1 mutation has marked effect in adult liver (Tripodi et al., 1991). Since HNF4 is not regulated by HNF1 (Tian and Schibler, 1991; Kuo et al., 1992), we predicted that its expression was an early event during development. In contrast, no HNF4, but COUP-TF/ARP-1 binding activities were detected in embryo extracts from 7.5 to 9.5 days of gestation. It remains to be established whether COUP-TF and/or ARP-1, although they antagonize the HNF4-transactivation of the ApoCIII promoter in liver and intestinal cells (Mietus-Snyder et al., 1992), may function differently with other promoters or in other cell types.

Two alternative spliced vHNF1 RNA isoforms are present in embryonic and adult tissues

We have shown that vHNF1 is expressed as two alternative spliced isoforms (vHNF1-A and vHNF1-B), which differ by the utilisation of an alternative exon of 78 nt present between the POU-like domain and the homeodomain. vHNF1-A, the transcript containing this extra exon, is predominant both in embryonic and adult tissues. However, the ratio of vHNF1-A/vHNF1-B mRNAs in adult liver and intestine is roughly 3- to 6-fold higher than in other tissues, suggesting that a particular ratio of these two transcripts may be functionally relevant. This pattern of alternative splicing of vHNF1 has been conserved throughout evolution, since both transcripts are also present in adult human liver (Bach et al., 1991 and personal communication).

Alternative splicing appears to be a general mechanism of generating multiple isoforms of transcriptional regulators, which in some cases results in dramatic changes in their function (i.e. a transactivator switched to a repressor) and can modulate the developmental function of a transcription factor in a tissue-specific manner (see Foulkes and Sassone-Corsi, 1992, for a recent review). Transient cotransfection experiments indicate that vHNF1-A and vHNF1-B exhibit similar transactivation potential, suggesting that these two isoforms likely do not possess antagonistic or distinct function (S. Power and P. M. Shaw unpublished observations). Further studies are required to establish whether these two transcripts are equally translated during development and whether in vivo functional differences do exist between vHNF-A and B.

Non-correlation between the developmental levels of HNF1 and vHNF1 transcripts and the respective DNA-binding proteins

In previous studies, we have shown that HNF1 expression in differentiated hepatoma cells is controlled primarily at the transcriptional level (Cereghini et al., 1990). Similarly, the high level of induction of vHNF1 mRNA in F9 visceral or parietal derivatives is due to a similar increase in the rate of transcription (S. Cereghini, unpublished data). Thus, it is likely that the appearance of HNF1 and vHNF1 transcripts in a restricted manner during development, is due to transcriptional activation.

The present results suggest a posttranscriptional level of control of HNF1 and vHNF1 gene expression, in addition to spatially regulated transcription. The first evidence of such additional level of control came from our studies in the F9 system, where we have been unable to detect HNF1 DNA-binding activity or protein in non-differentiated F9 cells and its differentiated derivatives, although HNF1 transcripts were present.

Similarly, during mouse development, we observe quite high levels of HNF1 mRNA in the developing kidney, but little or no active binding protein. Reciprocally, vHNF1 binding activity, while easily detected in the yolk sac at 8.5 d.p.c., becomes undetectable at 14.5 days of gestation, whereas the levels of vHNF1 mRNA are similarly high at both embryonic stages (see Fig. 6 and Table 1). Western blot analyses suggest that the DNA-binding affinity of both HNF1 and vHNF1 and/or their localization to the nucleus appear not to be affected. Moreover, transient transfection studies with either HNF1, vHNF1-A or vHNF1-B expression vectors do not indicate that they are unstable proteins. Thus, the incongruity between transcript accumulation and binding activity in mouse embryo extracts suggests a posttranscriptional level of control of vHNF1 and HNF1.

One attractive hypothesis is either translational activation or repression of vHNF1 and HNF1 transcripts during development. In certain situations, i.e. the yolk sac, the result is a reciprocal expression of each activity, in others only one species is modulated (i.e. HNF1 in developing kidney). Indeed, there are some mammalian regulatory proteins whose expression appears to be controlled posttranscriptionally during development. The appearance of the pituitary-specific transcription GHF-1/pit-1 factor (Dollié et al., 1990; Simmons et al., 1990) and the myogenic factor myogenin (Cusella-De Angelis et al., 1992) is delayed by 1 or 2 days relative to the respective transcripts. The actual
mechanisms are still unknown. The expression of different members of the C/EBP family of transcription factors is also controlled posttranscriptionally (Mueller et al., 1990). In particular, the gene encoding the liver enriched transcriptional activator protein LAP is expressed in several tissues, but the protein is considerably more abundant in terminally differentiated parenchymal hepatocytes (Descombes et al., 1990). Additional complexity is achieved by the generation of a repressor form, LIP, by the use of an alternative translational start site (Descombes and Schibler, 1991).

Clearly, further studies, in addition to the subcellular localization of HNF1 and vHNF1 in the developing embryo, are required to elucidate the various mechanisms that mediate the observed discrepancies between transcript accumulation and nuclear binding proteins. Moreover, an accessory factor (DCoH: Dimerization Cofactor of HNF1) that does not bind DNA, but acts to stabilize dimerization of both HNF1 and vHNF1, has been recently characterized (Mendel et al., 1991b). We have observed that in all the cells where vHNF1 is induced or present (i.e. visceral and parietal F9 cells, 3TDM, PYS-2) co-expression of DCoH mRNA occurs (S. Cereghini, unpublished results). Yet, the developmental regulatory activity of either HNF1 or vHNF1 can be modulated by variations in the levels of the DCoH protein. Furthermore, it has been recently reported that nascent in vitro elongated HNF1 transcripts are present in certain adult tissues, such as lung and spleen that contain neither HNF1 mRNA nor protein (Xanthopoulos et al., 1991). Thus, the mechanisms that control the expression of vHNF1, and particularly HNF1, seem unexpectedly complex.

Finally, it is tempting to speculate that the distinct temporal and spatial expression patterns of vHNF1 and HNF1 during development are consistent with the suggestion that they may participate in the regulation of different and sequential steps of the visceral endodermal, hepatic and kidney differentiation.

Separate regulatory mechanisms for the establishment and subsequent maintenance of tissue-specific gene transcription have been suggested in several mammalian differentiation systems (Vogt et al., 1988; Mueller et al., 1990; Cascio and Zaret, 1991). It is possible that vHNF1 and HNF1 proteins are both required to ensure the progression through the terminally differentiated phenotype. It remains to be established, however whether HNF1 can functionally replace vHNF1 during development. Gene disruption may answer this question.

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References


Expression patterns of HNF1 homeoproteins in mouse embryo


