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Two members of an HNF1 homeoprotein family are expressed in human liver

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ABSTRACT

HNF1 is a transcriptional activator, required for the liver-specific expression of a variety of genes, that binds to DNA as a dimer via the most diverged homeodomain known so far. We were interested to examine whether HNF1 is a unique homeoprotein example or whether it is the prototype of a new subfamily of homeodomain containing proteins. In this work we describe the isolation of a cDNA clone from a human liver library encoding a protein, highly homologous to HNF1 in three regions, including the homeo- and dimerization domains. We show that this protein can heterodimerize with human HNF1 *in vitro*. Sequence comparison of our clone with a rat variant HNF1 (vHNF1) clone, isolated in parallel in our laboratory from the dedifferentiated H5 hepatoma cell line, identified our cDNA as human vHNF1. vHNF1 is a nuclear protein recognizing the same binding site as HNF1 and previously thought to occur only in dedifferentiated hepatoma cells that fail to express most liver specific genes. Nevertheless, we show by Northern blot analysis that vHNF1 transcripts are present in differentiated human HepG2 hepatoma cells as well as in rat liver and that this transcript level is 10–20 fold lower than that of HNF1. We assigned the *vHNF-1* gene to human chromosome 17 and murine chromosome 11. These chromosomal localizations differ from that of the *HNF-1* gene indicating that both genes are not clustered on the genome.

INTRODUCTION

Tissue-specific gene expression is thought to be conferred by transcription factors which bind to specific promoter and enhancer sites and allow the subsequent transcription of the gene (for review see 1). For the albumin gene it was shown that the Proximal Element (PE) and the TATA-box of its promoter are sufficient to confer hepatocyte specific gene expression in transient transfection assays (2, 3, 4). Hepatic Nuclear Factor I (HNF1, also named HP-1, LF-B1 or APF) binds not only to the PE of the albumin gene but also to the promoters and enhancers of a variety of genes which are expressed in a liver-specific manner (5, 6, 7, and references therein). It has been shown that HNF1 protein can supplement a spleen nuclear extract to drive liver-specific gene transcription (8). Recently, cDNAs encoding rat (7, 9, 10), human (11) and mouse HNF1 (12) have been isolated and it was shown that this transcription factor is highly conserved among these species on amino acid as well as on nucleotide levels (11, 12). HNF1 represents the most diverged homeoprotein so far identified. The homology to homeodomains of other proteins is mainly restricted to the predicted third helix. In addition, a loop of 18–21 amino acids can be found between the putative helices 2 and 3 (13, 14, 10). The protein binds to DNA as a dimer and this dimerization depends on a coiled coil interaction mediated by a short α-helical protein segment located in the N-terminal part of the molecule (14, 10). Surprisingly, HNF1 mRNA and protein can be detected not only in liver, but also in kidney, intestine and stomach (9, 12, 15, 16). In rat hepatoma cell lines isolated by the group of M.Weiss (17), the occurrence of HNF1 transcript seems to be restricted to differentiated cell types like H4II and Fao cells which do still express most hepatocyte-specific marker proteins. HNF1 is not expressed in dedifferentiated hepatoma cell lines like H5 or C2, in which the liver-specific marker proteins are no longer expressed (15). Nevertheless, an activity which binds specifically to the PE of the albumin promoter can be detected by band shift assays in nuclear extracts of dedifferentiated hepatoma cell lines. The protein responsible for this binding, which is distinct from HNF1, has been named variant HNF1 (vHNF1) or variant APF (vAPF) and seemed initially to be restricted to dedifferentiated hepatoma cells (18, 6).

Many homeoproteins are members of gene families, characterized by conservation of certain features within the homeodomain motif. These genes are often organized in clusters on the genome (for review see 19). HNF1 is the only homeoprotein so far known whose homeodomain contains the 18–21 amino acid loop between helices 2 and 3 and in addition

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many key amino acids characteristic for homeodomains are not conserved. Thus, this homeodomain is so divergent with respect to known homeodomains, that it would probably escape detection by 'normal' homeobox containing probes. We wondered whether other members of an 'HNF1 family' would exist besides HNF1.

To test this possibility we screened a human liver cDNA library with a rat HNF1 homeobox containing probe and detected one clone (HV19), clearly not encoding HNF1 but displaying high sequence homologies to it. To obtain a full length cDNA we used this clone to rescreen the same library and isolated 14 further clones. Analysis of one of these clones (HV17) revealed that it contains a single open reading frame which includes the sequence coded by clone HV19. A homeodomain essentially identical to that of HNF1 was found in both clones. Two more regions of high conservation were identified: the dimerization domain and the B-domain, harboring weak homologies to a POU domain. Outside these regions the homology to HNF1 is much lower.

The high sequence conservations in the dimerization domains led us to investigate whether both proteins might heterodimerize. Thus, by *in vitro* transcription and translation of cDNAs encoding HV17 and human HNF1, we show that both proteins are able to form heterodimers.

Alignment of our sequence with that of a rat vHNF1 clone (20) identifies our clone as the human homologue of vHNF1. Northern blot analysis reveals that HNF1 and vHNF1 transcripts are both present in rat liver as well as in differentiated human HepG2 hepatoma cells.

The *vHNF-1* gene was mapped by *in situ* hybridization to human 17q11.2-q21.1 and murine 11B4-D regions showing that it is not organized as a cluster with the *HNF-1* gene.

MATERIAL AND METHODS

cDNA library screening

1.2×10^6 plaques of a human liver cDNA library constructed in λgt10 (a generous gift of D.Lamy) were screened with the rat HNF1 derived 511 bp NcoI fragment which contains the homeodomain as previously described (11). Rescreening of the same library was carried out with a vHNF1-specific HV19 derived 517 bp PstI/AccI fragment.

Sequencing of positive clones

Phage DNA of positive clones was prepared as described in (21). The phage inserts were subcloned into the EcoRI site of Bluescribe prior to double strand sequencing of both strands with a United States Biochemicals Sequenase Kit and successive primers, synthesized on a Pharmacia gene assembler. Sequence data were analysed on a Data General MV10000 main frame computer.

In vitro transcription and translation

The clones HV17 (vHNF1) and HCL16 (HNF1) subcloned in Bluescribe, both encoding full-length proteins were used directly for *in vitro* transcription. These experiments were carried out with a Stratagene Transcription Kit according to the manufacturer's recommendations.

HVI7 encoding RNA was obtained from the T3 promoter of Bluescribe after linearization of the plasmid with DraI, whereas the HCL16 transcripts were initiated from the T7 promoter of a BamHI linearized plasmid. *In vitro* translations were performed with Promega commercial rabbit reticulocyte lysates and ^{35}S-methionine. The translated proteins were examined on SDS acrylamide (10%) gels prior to the gel retardation assays.

Gel retardation assays

Gel retardation assays were carried out directly with the *in vitro* transcribed proteins. The PE56 double stranded oligonucleotide TGTGGTTAATGATCTACAGTTA, bearing the HNF1 binding site (Proximal Element) of the rat albumin promoter and ^{32}P-labelled by T4 polynucleotide kinase was used as probe. These experiments were performed as described in (6) on 5% polyacrylamide gels.

Northern blot analysis

Total RNA was isolated according to (22). Poly(A)$^+$ RNA was prepared by oligo(dT) cellulose chromatography as described in (21), electrophoresed through a 1.2% agarose−2.2 M formaldehyde gel and blotted onto a Pall Biodyne nylon membrane. The membranes were hybridized with the full-length rat vHNF1-A sequence (20) and a rat HNF1 derived NcoI 626 bp fragment, both ^{32}P-labelled with a multiprime labelling kit (Amersham). These hybridizations were carried out in 50% formamide, 5×SSPE, 5×Denhardt's at 42°C, for 24 h. The membrane was washed in 0.1×SSC, 0.25% SDS at 60°C.

Chromosomal localization of *vHNF-1* by *in situ* hybridization

The *in situ* hybridizations were carried out on metaphase spreads of human or mouse chromosomes as described previously (11). The full-length rat vHNF1-A clone in Bluescript was used as hybridization probe (20).

RESULTS AND DISCUSSION

The existence of homeoprotein families is a common phenomenon in nature. The genes coding for these factors are frequently grouped in clusters on the genome. HNF1 displays unique features not shared by any other known homeoprotein, i.e. the existence of a 18−21 amino acid loop between the putative helices 2 and 3 and the non-conservation of several 'key' amino acids within the homeodomain. For that reason other members of an 'HNF1-family' might have escaped detection in previous screenings which were performed in several laboratories with probes containing canonical homeodomains. We wondered whether HNF1 would be the only member which contains such a highly diverged homeodomain or whether there would be other examples of genes belonging to this family.

Molecular cloning of cDNAs encoding an HNF1-like protein

To investigate whether other members of a potential HNF1-family are expressed in hepatocytes besides HNF1, we screened a human liver cDNA library with a probe containing the rat HNF1 homeobox. One clone was detected (HV19) which contained sequences, clearly distinct from a human HNF1 cDNA that we have previously identified (11). In an attempt to obtain a full length clone and to confirm our sequence, the same library was rescreened with an HV19 derived 517 bp long PstI/AccI fragment, resulting in 14 positive clones. Clone HV17 with a 2.8 kb insert was chosen for further study. The 2816 bp of this clone contain a single open reading frame of 1671 bp in length, capable of encoding 557 amino acids with a predicted MW of 68 kd. It starts with an ATG start codon at nucleotide 195 and ends at the TGA stop codon at nucleotide 1865. The open reading

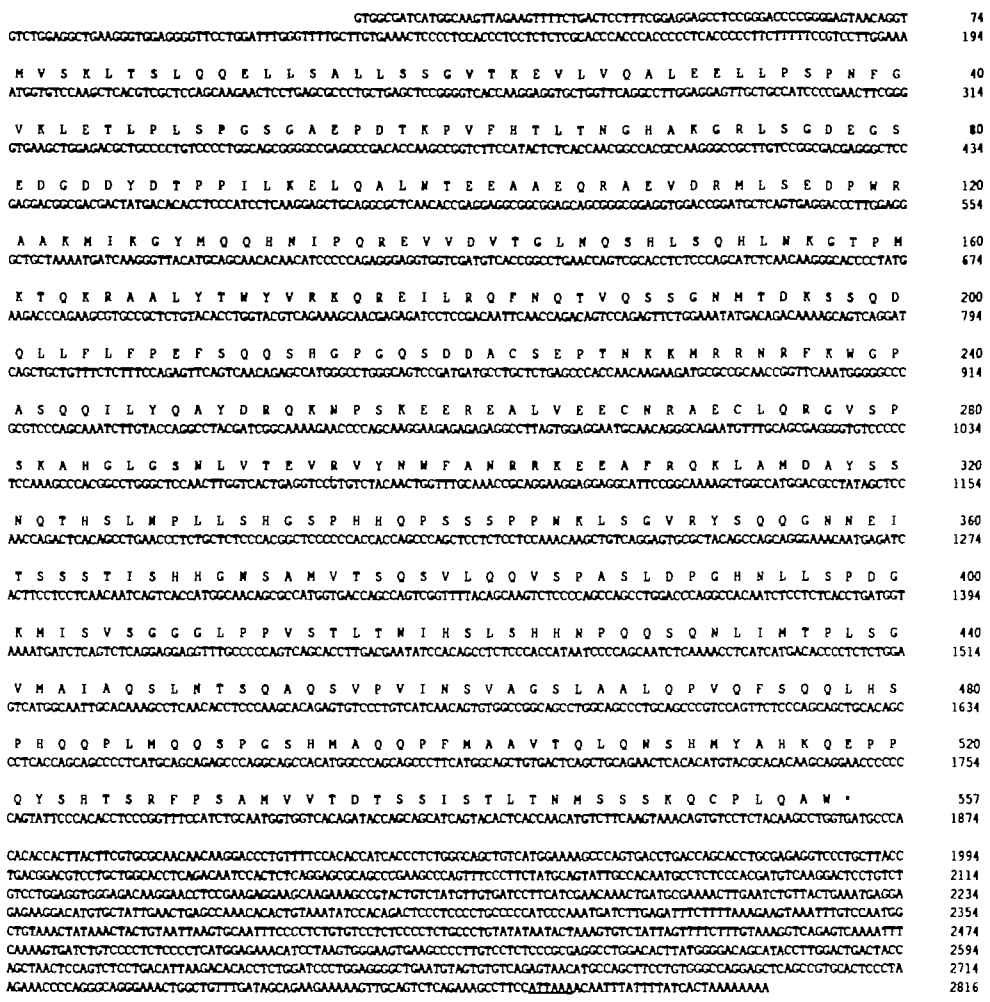

Figure 1. Nucleotide and deduced amino acid sequence of clone HV17. The numbering to the right indicates the last nucleotide or amino acid in the corresponding line. An asterisk marks the first in-frame stop codon. The potential polyadenylation site is underlined.

frame is flanked on the 5′ and 3′ sides by untranslated regions of 194 and 948 nucleotides, respectively. An in frame stop codon is located 72 bp upstream of the putative ATG translational start site. A short half-life of the HV17 transcript is suggested by the presence of an ATTTA motif which is located 15 bp upstream of the poly(A) tail and reported to be responsible for rapid mRNA degradation (23). The 3′ end poly(A) tail is found 24 bp downstream of a potential ATTAAA polyadenylation signal (Figure 1).

Comparing the deduced amino acid sequence encoded by clone HV17 with that of human HNF1 using the amino acid equivalence matrix of Dayhoff reveals striking homologies (Figure 2). Three regions in the N-terminal part of the proteins show a very high degree of conservation: the dimerization domain (aa 1−32), the B-region which displays weak homologies to a POU sequence (aa 83−172) and the homeodomain (aa 213−287). All three regions were shown to be essential for DNA binding in the case of HNF1 (14, 10). The high amino acid conservation in the homeodomains of HV17 and HNF1 (88% aa identity) and secondary structure predictions suggests that HV17 protein also contains the 18−21 amino acids loop between helices 2 and 3 (13, 14). Interestingly, a segment of 23 amino acids can be found in the HV17 protein (aa 202−225) located between the B-region and the homeodomain which does not exist in HNF1 (see Figure 2). The physiological role of this region is not clear. The glycine and proline rich region which was found in HNF1 (aa 288−310) does not exist in the HV17 protein. A lower degree of conservation is seen in the C-terminal part of the proteins, although this region is rich in serine and threonine residues in both factors. Taken together these results show that the protein encoded by clone HV17 is clearly a new representative of an HNF1 homeodomain family.

HNF1 can form heterodimers with other family members

It has been shown recently, that the N-terminal segment of HNF1 possesses an α-helical conformation that promotes dimerization by formation of a coiled coil structure (14). Since this domain of HNF1 displays extensive sequence homology to the N-terminus of the HV17 protein, we investigated whether these two proteins are able to heterodimerize. We approached this question by *in vitro* transcription and translation of both factors and subsequent gel retardation assays with the resulting products (24). The oligonucleotide PE56 which contains the Proximal Element sequence of the rat albumin promoter was used as binding probe. After *in vitro* transcription of HV17 and human HNF1 the resulting RNAs were either translated separately or together.

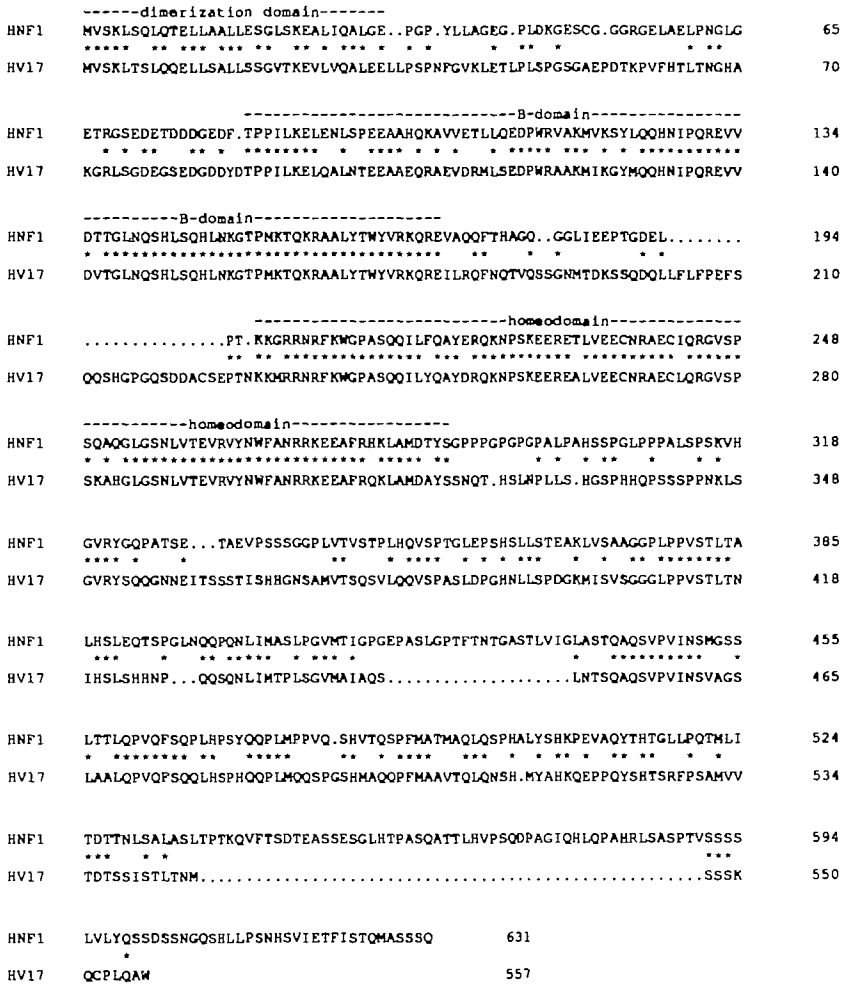

Figure 2. Alignment of predicted human HNF1 and HV17 amino acid sequences. Asterisks indicate amino acid identities. The numbers indicate the last amino acid of each line. This alignment was done by using the amino acid equivalence matrix of Dayhoff.

Translation products were examined on 10% SDS gels to monitor the correct molecular weight sizes (not shown) and subsequently used for the gel shift binding assays. While HV17 and HNF1 separately translated resulted in a single band each, a new intermediate band appeared when both RNAs were cotranslated or when the translation products were mixed before the binding assay (Figure 3). These results demonstrate that both proteins can form heterodimers and that HV17 protein, similarly to HNF1, binds to DNA as dimer. The fact that both proteins can heterodimerize *in vitro* and that both mRNAs can be found in the same tissue suggests that heterodimers may also exist *in vivo*.

Heterodimerization between two different helix-loop-helix proteins has been shown to be an important cellular tool to alter the activity and binding specificity of a given transcription factor (for review see 25). An example of heterodimerization between two homeoproteins, postulated previously and experimentally confirmed only recently, concerns the yeast mating type factors **a**1 and α2 (26, 27). In this case, the heterodimerization of Mat **a**1 to Mat α2 changes its binding specificity. Thus, heterodimerization might be a common phenomenon in regulating activity and binding specificity of homeoproteins. It will be interesting to decipher the functional role of the HV17/HNF1 heterodimerization and to elucidate whether there is any change in the recognition specificity of the heterodimer.

HV17 protein is the human homologue of rat vHNF1

In an attempt to clone the rat gene coding for the variant binding activity to the albumin proximal element (vHNF1), present in dedifferentiated rat hepatoma cells, Rey-Campos *et al.* (20) screened a cDNA library derived from the H5 dedifferentiated hepatoma cell line. A clone encoding rat vHNF1 was isolated which is homologous to HNF1 in its N-terminal part. Sequence alignment of HV17 and this clone reveals a very high degree of conservation (92% on nucleotide level) in the coding region. This homology is almost as high as the one between human, rat and mouse HNF1 (11, 12). The 3′ noncoding regions of both clones display 73% of homology, with several gaps (data not shown). Comparison of the deduced amino acid sequences shows a remarkable 97% overall identity. The regions in human and rat vHNF1 corresponding to the HNF1 dimerization domains, the B-regions and the homeodomains are identical underlining their importance for protein function (not shown). These results argue strongly that the protein product of our HV17 clone is the human homologue of rat vHNF1.

As has already been observed for other protein families, i.e. steroid/thyroid hormone nuclear receptor superfamily (for review see 28, 29), the HNF1/vHNF1 homeoprotein family also has the property that a particular member protein is more highly

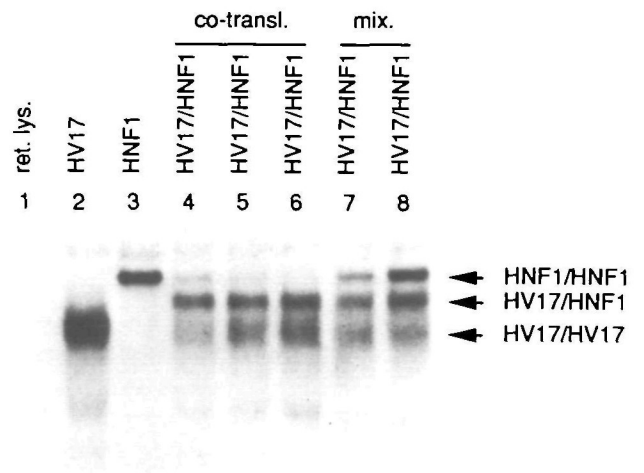

Figure 3. Full length HV17 and human HNF1 were transcribed *in vitro*. The resulting RNAs were either translated separately or together. Translations were checked on SDS polyacrylamide gels (not shown). The gel retardation assays were carried out with ^{32}P-labelled PE56 as probe directly on the translation products. Lane 1 corresponds to a translation without adding RNA. Equal amounts of the HV17 and human HNF1 translations were loaded in lanes 2 and 3, respectively. Lanes 4–6 correspond to the cotranslations done with constant amounts of HNF1 RNA and increasing amounts of HV17 RNA. For lanes 7 and 8 products from separate HV17 and HNF1 translations were mixed and incubated at room temperature for 20 min before doing the gel shift. Constant amounts of the HV17 translation were mixed with increasing amounts of the HNF1 translation. The bands migrating faster or slower than the HNF1 or vHNF1 complexes are due to non-specific binding since they also appear in the control sample where no RNA was added to the reticulocyte lysate (lane 1).

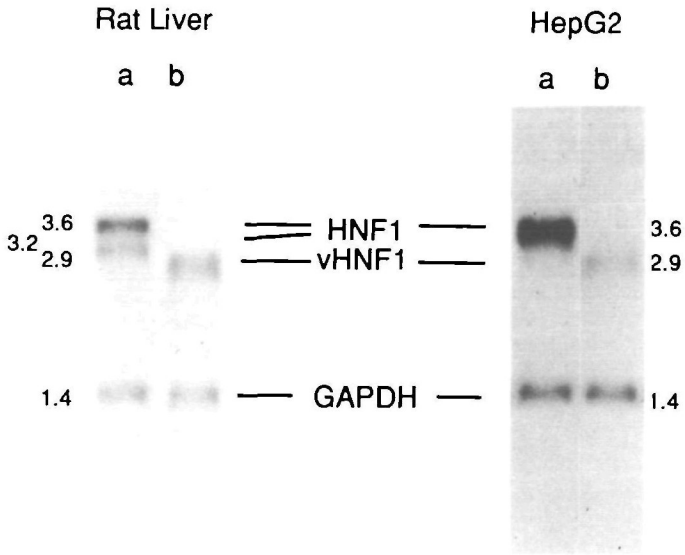

Figure 4. Northern blot analysis with mRNAs of rat liver and HepG2 cells using HNF1 (a) or vHNF1 (b) probes. Numbers indicate the lengths of the visible bands in kb.

conserved between species than it is to a second member protein in the same organism.

HNF1 and vHNF1 mRNAs are expressed in liver and human HepG2 hepatoma cells

A first indication that vHNF1 and HNF1 mRNAs are both expressed in human liver was the fact that cDNAs of both proteins could be isolated from the same human liver cDNA library. However, liver contains more than one cell-type and cDNA clones from a total liver library might originate from mRNA expressed in endothelial cells, Kupfer cells etc. To verify if vHNF1 is expressed also in differentiated human hepatoma cells as well as in rat liver and to evaluate the relative abundance of vHNF1 and HNF1 we performed Northern blot analysis on mRNA prepared from both cell types. Figure 4 demonstrates that transcripts coding for both proteins are present in these cells although vHNF1 mRNA is 10 to 20 fold less abundant compared to the HNF1 mRNA. The two bands obtained upon hybridization with the HNF1 probe in rat liver mRNA is consistent with the finding that different polyadenylation sites are used by HNF1 transcripts in this tissue (10).

These results suggest that both genes are transcribed in hepatocytes as well. This argument is strengthened by two observations: 1) vHNF1 encoding mRNA can also be detected in differentiated rat (H4II, C2Rev7, and Fao) and murine (BW1G) hepatoma cell lines (20, S.C., A.Rollier, unpublished results) and 2) *in situ* hybridizations on mouse sections show that HNF1 mRNA as well as the vHNF1 transcript is homogeneously distributed in the liver (16; M.-O.Ott *et al.*, manuscript in preparation). If vHNF1 was not expressed in hepatocytes but in other cell types occurring in the liver one would expect a more punctate distribution of the vHNF1 transcript. However, further studies need to be carried out to clarify if vHNF1 is really co-expressed together with HNF1 in the same hepatocyte.

Gel retardation assays with HepG2 nuclear extracts and the radiolabeled PE oligonucleotide suggest that the vHNF1 protein exists in these cells at low levels compared to HNF1. All vHNF1 is found in the form of HNF1/vHNF1 heterodimers (not shown).

Chromosomal localization of the *vHNF-1* gene by *in situ* hybridization

Genetic studies in *Drosophila* suggested that homeotic genes are arranged in complexes on the genome (30). Identification of the homeodomain at the DNA level followed by cloning of many of these genes confirmed this early conclusion (for review see 31). Later, it was discovered that vertebrate homeobox containing genes are also clustered and their expression along the antero-posterior developing body axis follows a positional hierarchy, which reflects their respective physical position within the gene cluster (19, 32). To investigate whether the *HNF-1* and *vHNF-1* genes would be arranged similarily, we localized the *vHNF-1* gene on chromosomal spreads of human and mouse metaphase chromosomes. The full length rat vHNF1-A clone (92% homology to the human clone) in Bluescript plasmid (20) was used as probe for these *in situ* hybridizations. In the 100 human metaphase cells examined, 243 silver grains were associated with the chromosomes and 58 of those (= 23.8%) were located on chromosome 17. 75.8% of the silver grains associated to chromosome 17 mapped to the q11.2-q21.1 region with a maximum in the q12 band. These results allowed us to map the *vHNF-1* gene to the q11.2-q21.1 region in the long arm of chromosome 17 (Figure 5) in the human genome. In 100 mouse metaphase spreads 311 grains were associated with the chromosomes and 75 of them (= 24%) were located on chromosome 11. Of those, 85.3% mapped to the B4-D region with a maximum in the C band (Figure 6). Thus, the most probable localization of mouse *vHnf-1* is the 11B4-D region of murine chromosome 11. These two loci are included in a previously established large segmental homology between human and mouse genomes (33).

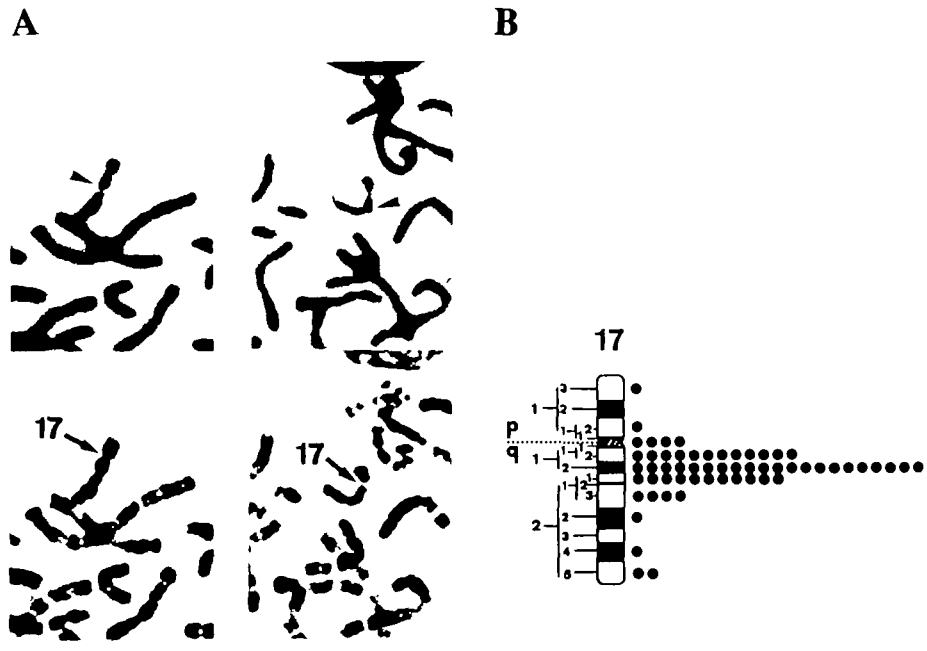

Figure 5. Localization of the *vHNF-1* gene to human chromosome 17 by *in situ* hybridization. A) Specific site of hybridization of the vHNF1 probe on two partial metaphases Upper: Silver grains on Giemsa-stained chromosomes. Lower: Identification of the chromosomes with silver grains by R-banding. B) Distribution of the labelled sites on an idiogram of human chromosome 17.

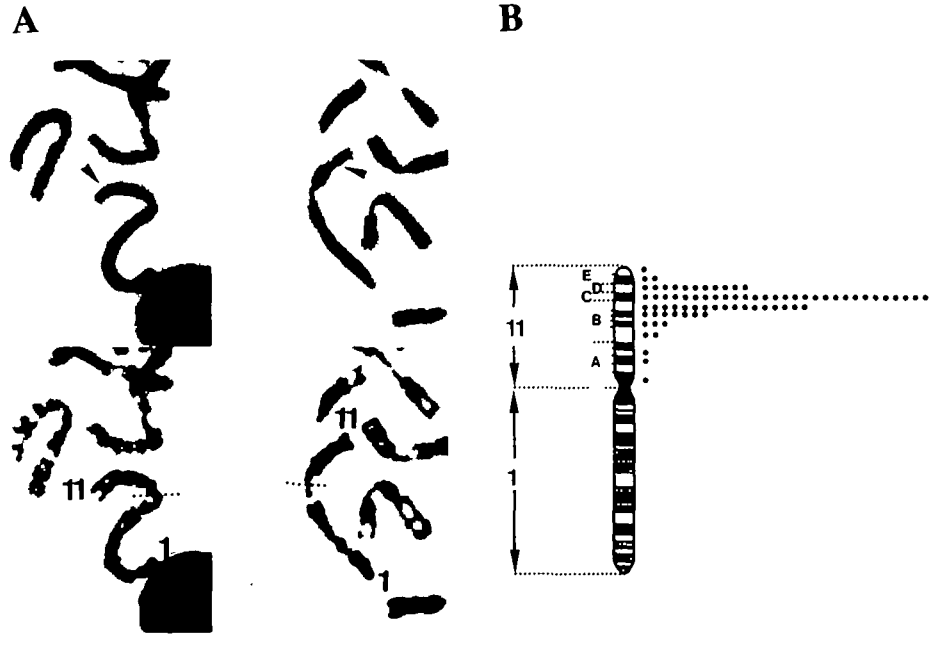

Figure 6. Localization of the *vHnf-1* gene to mouse chromosome 11. A) Two partial metaphases showing the specific site of hybridization. Upper: Silver grains on Giemsa-stained chromosomes. Lower: R-banding of those chromosomes. B) Diagram of WMP mouse Rb (1;11) chromosome illustrating the distribution of the labelled sites.

Since the gene coding for HNF1 is located on chromosomes 12q24.3 in human and 5F in mouse, respectively (11), the *HNF-1* and *vHNF-1* genes are located on different chromosomes. This demonstrates that two homeoproteins that probably evolved from a common ancestor gene by gene duplication, are not grouped at the same chromosomal locus.

Whilst our studies were in progress another group isolated a genomic clone containing a short segment homologous to HNF1 (34). This DNA fragment, called TCF2 was mapped by a PCR based approach to human chromosome 17 as well. Even though the amino acid sequence of the 66 residues that they published differs in two positions from our sequence (an aspartic acid in position 140 and a leucine in position 151 whereas we find a valine and a histidine residue at these positions, respectively) it is probable that both represent the same gene. Both amino acid mismatches can be traced back to a single bp change. Thus, these

errors might have occurred by unfaithful trancription by the Taq-polymerase during the PCR or are simply sequencing errors.

Finally, the observation that human liver harbours two homeoproteins with an identical or very similar DNA binding specificity is reminiscent of the situation observed in B-lymphocytes. These cells contain two closely related homeoproteins Oct1 and Oct2 with identical DNA binding specificities. Oct1 is a ubiquitously expressed transcription factor (35) whereas Oct2 gene expression is restricted to B-lymphocytes, brain, kidney and sperm cells (36). Even though both proteins can bind to the octamer sites in the immunoglobulin promoter and enhancer, the expression of these genes is dependent on the presence of Oct2 (37). Similarly, differentiated hepatoma cell lines that do express liver-specific genes like the albumin gene are dependent on the presence of HNF1 whereas dedifferentated hepatoma cells or extinguished somatic cell hybrids that fail to express the liver specific genes continue to express vHNF1. The observation that only vHNF1 and not HNF1 mRNA is induced upon in vitro differentiation of F9 cells into primitive endoderm cells (S.C., in preparation) argues that vHNF1 might be involved in the early differentiation of endodermal cells. The appearance of HNF1 expression would then occur during the final differentiation of hepatocytes.

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