Hepatocyte nuclear factor 6: organization and chromosomal assignment of the rat gene and characterization of its promoter

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Hepatocyte nuclear factor 6 (HNF-6) is the prototype of a family of tissue-specific transcription factors characterized by a bipartite DNA-binding domain consisting of a single cut domain and a novel type of homeodomain. We have previously cloned rat cDNA species coding for two isoforms, HNF-6 α (465 residues) and β (491 residues), which differ only by the length of the spacer between the two DNA-binding domains. We have now localized the rat *Hnf6* gene to chromosome 8q24–q31 by Southern blotting of DNA from somatic cell hybrids and by fluorescence *in situ* hybridization. Cloning and sequencing of the rat gene showed that the two HNF-6 isoforms are generated by alternative splicing

of three exons that are more than 10 kb apart from each other. Exon 1 codes for the N-terminal part and the cut domain, exon 2 codes for the 26 HNF-6 β -specific amino acids, and exon 3 codes for the homeodomain and the C-terminal amino acids. The transcription initiation site was mapped by ribonuclease protection and 5' rapid amplification of cDNA ends. Transfection experiments showed that promoter activity was contained within 0.75 kb upstream of the transcription initiation site. This activity was detected by the transfection of liver-derived HepG2 cells, but not of Rat-1 fibroblasts, suggesting that the promoter is sufficient to confer liver-specific expression.

INTRODUCTION

In multicellular organisms, tissue-specific transcription factors regulate organogenesis and the establishment and maintenance of the differentiated state. A knowledge of the structure of genes coding for such factors is essential to an understanding of their function and the control of their expression. Six families of transcription factors in the liver have been described. These include the CCAAT/enhancer binding proteins, the proline-acid-rich factors, the homeodomain proteins of the hepatocyte nuclear factor (HNF)-1 family, the forkhead proteins HNF-3 α , β and γ , the orphan receptor HNF-4 family (reviewed in [1,2]) and HNF-6, which is the prototype of the sixth family identified recently [3]. HNF-6 (465 residues) contains a bipartite DNA-binding domain consisting of a single cut domain and a homeodomain that differs from the other homeodomains by the nature of its residues 48 and 50. Two isoforms of HNF-6 have been identified. They differ by the presence (HNF-6 β) or absence (HNF-6 α) of a 26 residue-long insert located between the cut domain and the homeodomain [3,4].

HNF-6 was discovered as a transcriptional activator of the gene coding for liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [3,5]. It binds to the promoter of genes coding for enzymes involved in glucose metabolism, for plasma proteins [6,7] and for the liver-enriched transcription factors HNF-4 and HNF-3 β [8]. By controlling the expression of these two factors, HNF-6 might participate in the network of liver-enriched transcription factors that is required for development and differentiation of the liver (reviewed in [2]). Recently it was shown that HNF-6 expression in the liver is induced by growth hormone, and that it is a mediator of sex-dependent effects of growth

hormone [9]. All these observations raise the question of how HNF-6 and its isoforms are controlled at the transcriptional level. To approach this problem we have now characterized the rat *Hnf6* gene and have determined its chromosomal localization.

EXPERIMENTAL

Cloning of rat genomic DNA

A Lambda Dash rat genomic library (Stratagene, Leusden, The Netherlands) was screened in accordance with the supplier's instructions. The probes (see below) were labelled by random priming with [α -³²P]dCTP with the Ready-to-Go labelling kit (Pharmacia Biotech, Roosendaal, The Netherlands). DNA from the genomic clones was purified and subcloned in pSP72 (Promega, Leiden, The Netherlands) or pBluescript (Stratagene), and analysed by restriction digestion. Sequencing of both strands was performed by the dideoxy chain-termination method with the T7 sequencing kit (Pharmacia Biotech).

Chromosomal localization

A panel of rat $\times$ mouse (LB) somatic cell hybrids that segregate rat chromosomes [10] was used and tested in Southern blotting with a 2.5 kb HNF-6 β cDNA probe. Fluorescence *in situ* hybridization (FISH) was done with the same probe, as described [11,12].

Plasmid constructions

pSPCla-Xho/0.4 contains a 358 bp *Hnf6* genomic fragment

Abbreviations used: FISH, fluorescence *in situ* hybridization; HNF-6, hepatocyte nuclear factor 6; RACE, rapid amplification of cDNA ends.

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extending from the *Cla*I site (+161) to the *Xho*I site (−197) cloned in the *Cla*I and *Xho*I sites of pSP72 (Promega). *Hnf6*-firefly luciferase constructs were made by subcloning a 2.3 kb *Eco*RI–*Cla*I fragment (−2150 to +161; pNF/2.1 luc), a 0.9 kb *Stu*I–*Cla*I fragment (−752 to +161; pNF/0.75 luc), or a 0.5 kb *Sac*I–*Cla*I fragment (−328 to +161; pNF/0.32 luc) of the rat *Hnf6* gene subcloned in pGL3 basic (Promega). pNF/2.1 invluc contains the 2.3 kb *Eco*RI–*Cla*I (−2150 to +161) fragment in the reverse orientation. pRL138 contains 138 bp of the liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene promoter cloned upstream of the *Renilla* luciferase coding sequences in pRL null (Promega).

RNase protection and rapid amplification of 5' cDNA ends–PCR (5' RACE–PCR)

Extraction of total RNA from rat liver was performed by the guanidium isothiocyanate method [13]. RNase protection was performed with a probe 206 bases long synthesized *in vitro* with T7 RNA polymerase and [α - 32 P]CTP. The probe was derived from the *Bam*HI-linearized pSPC1a-Xho/0.4 plasmid. The probe was incubated overnight at 45 °C with 20 μ g of total liver RNA or yeast tRNA, as negative control, and digested with RNase A and RNase T1 as described [13]. 5' RACE–PCR was performed with the RACE kit (Gibco BRL, Merelbeke, Belgium) in accordance with the supplier's instructions with the following primers: LPGATG, 5'-CGCCTCCATGGTCAGCTGTG-3' (reverse transcription); HNFMR1, 5'-CGTGATGCGGGTGAGCGGGCTG-3' (first PCR); HNFMR2, 5'-GAGTCCAGTCTTCACATCGGCTGC-3' (second PCR). The specificity of the 5' RACE–PCR products was verified by Southern blotting with the LP5 SOU 5'-CAGAGGGGAAGGTAGGAGCAAGAGA-3' oligonucleotide. The position of the oligonucleotides used is indicated in Figure 4.

Cell culture and transient transfection

HepG2 and Rat-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. For transfection, 8×10^5 HepG2 cells or 3×10^5 Rat-1 cells were plated on 60 mm dishes and transfected overnight with *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-triethylammonium methyl sulphate ('DOTAP'; Boehringer Mannheim) with 8 μ g of an *Hnf6* gene promoter-driven firefly luciferase expression vector and 2 μ g of pRL138 as internal control (*Renilla* luciferase). At 24 h after transfection, luciferase activities were measured with the dual-luciferase kit (Promega) in accordance with the supplier's instructions. Results are expressed as the ratios of firefly and *Renilla* luciferase activities. The activity of the firefly luciferase reporter constructs was compared with that of the promoterless vector pGL3 basic (Promega).

RESULTS

Chromosomal localization of the rat *Hnf6* gene

The gene was first assigned to a rat chromosome by using a well-characterized panel of rat $\times$ mouse somatic cell hybrids segregating rat chromosomes. The rat probe detected two *Bam*HI-generated fragments (12.0 and 2.2 kb), which were easily distinguishable from the mouse fragments (19.0 and 4.5 kb) (results not shown). The rat fragments co-segregated with chromosome 8. The regional localization of the rat gene was then determined by FISH. The results are illustrated in Figure 1. Double spots

Figure 1 Regional localization of the rat *Hnf6* gene by FISH

A region of a metaphase spread is shown with one copy of rat chromosome 8 labelled by two fluorescent signals at 8q24–q31 (arrow). A diagram of rat chromosome 8 is also shown, where the average position of the *Hnf6* signals is indicated (arrowhead).

(two labelled sister chromatids) due to the HNF-6 probe were found only on chromosome 8, at 58–69% of the chromosome length, the average position being 62%. These values correspond to bands 8q24–q31, the average position being in the lower part of 8q24. The *Hnf6* gene was thus assigned to 8q24–q31.

Structure of the rat *Hnf6* gene

A rat genomic phage library was screened with a 410 bp cDNA probe coding for residues 162–297. This yielded a 16.8 kb clone (λ 1) that contained the liver promoter (see below), the first exon and a portion of the first intron. This showed that the first exon codes for the first 368 residues of HNF-6, including the cut domain. A second genomic clone (λ 6) was obtained by screening the library with a 180 bp cDNA probe encompassing the homeodomain (residues 385–444). The 3' end of this clone contained in a single exon the sequence coding for residues 369–465, encompassing the entire homeodomain and the C-terminus of HNF-6. An overlapping clone was obtained by screening the library with a 880 bp probe derived from the 5' end of clone λ 6. This clone (λ 8) contained the exon coding for the 26 residues specific for HNF-6 β . Figure 2 summarizes the structure of the rat *Hnf6* gene and shows its restriction map. Table 1 shows the nucleotide sequences of the splice donor and acceptor sites. These junctions match the consensus for splice junctions [14].

Identification of the liver promoter

To identify the transcription initiation site we resorted to a combination of RNase mapping and 5' RACE–PCR. RNase mapping (Figure 3) was performed by incubating total RNA from rat liver and the 206-base probe indicated in Figure 4. After digestion with RNase, protected fragments of 161, 158 and 152 bases were obtained. To confirm that the RNase-protected fragments indicated the location of a transcription initiation site, we performed 5' RACE–PCR experiments with the primers described in the Experimental section and indicated in Figure 4. Subcloning and sequencing of the 5' RACE–PCR product revealed that a 129 bp HNF-6 cDNA fragment had been amplified with primer HNFMR2. The sequence was in agreement with the 161-base band detected in the RNase protection experiment. This transcription initiation site is indicated as +1 in Figure 4. The 158-base and 152-base bands detected by RNase

Figure 2 Organization and restriction endonuclease map of the rat *Hnf6* gene

Black boxes represent exons; cut and hd refer to the cut domain and the homeodomain respectively. Labels: A, *Asp7181*; B, *Bam*HI; E, *Eco*RI; H, *Hind*III.

Table 1 DNA sequences of the exon/intron boundaries of the rat *Hnf6* gene

Exon	Exon size (bp)	5' Splice site	Intron size (kb)	3' Splice site
1	1296	CAG/GTGAAG	> 15	CTTACCCAG/A
2	78	CTG/GTGAGT	10	TCITTCAG/C
3	> 1018			
Rodent consensus		^C AG/GT ^A _G AGT		TTTTT _{CCCC} N ^T _C AG/G

protection correspond to the initiation sites indicated by smaller solid arrows in Figure 4. These initiation sites are located 26 bp downstream of a sequence compatible with a TATA box.

We then functionally delineated the promoter by subcloning genomic fragments upstream of the luciferase-coding sequences and transfecting the constructs in cells that express HNF-6 (HepG2 hepatoma) or not (Rat-1 fibroblasts). Figure 5 shows that pNF/2.1 luc, which contains nt -2150 to +161 relative to the transcription initiation site, induced in HepG2 cells a 25-fold stimulation of luciferase activity compared with the promoterless vector pGL3 basic. When the same genomic fragment was cloned in the reverse orientation upstream of the luciferase coding sequences (pNF/2.1 invluc), no induction of luciferase activity was seen. This demonstrated that a promoter was located within this 2.3 kb region. To delineate the promoter further, 5' deletion mutants of the 2.3 kb *Hnf6* gene fragment were made and tested for promoter activity by transient transfection of HepG2 cells. Figure 5 shows that maximal promoter activity was located within a 913 bp region (-752 to +161; pNF/0.75 luc). Furthermore transfection of the latter construct in Rat-1 fibroblasts produced no increase in relative luciferase activity compared with that of the promoterless vector (0.5 ± 0.05 -fold induction, $n = 3$). Internal control values (*Renilla* luciferase) were 1.2-fold higher in Rat-1 cells than in HepG2 cells when the same amount of pRL138 control plasmid was used. These results demonstrate that transfection efficiencies were satisfactory in Rat-1 cells and

Figure 3 Mapping of the transcription start site of the rat *Hnf6* gene

A ³²P-labelled 206-base RNA probe was used in the RNase protection assay with 20 µg of total RNA from rat liver. Three protected bands were seen. The 161-base band corresponds to nt +1 in Figure 4.

that the promoter located between +1 and -752 functions in a liver-specific way. The nucleotide sequence of the liver-specific promoter is shown in Figure 4.

CAGGGTATACTGCTCCCAAGTCCCTTAGGACAATCATTTAAGTACAGG -703
NFI
AACGGACTGGCTCCAGAGACTCTCCAGGAATAAAGCCCAATGGCGAA -653
HNF-4
CGGGCAAGGCCATGGCATAGTCTCCAGCTCCAGCCGATTAACCTGCACC -603
GGGAACAGCACTCCGACCCAGGGCTCTAGTTCTCTAACCTTGCTGGC -553
GAGA box
CCACGGTGCAGAAAGCAGGCAAGAGAGTCCGGCTGGGAAGAGAGAGGGA -503
GGAAGTAAGGAAGACAGGAGGTAGCAAAATCTCCAGGACTGTGGCGCT -453
USF
CCAGTGTGGCTGTGGCGCTCCGCTGCACTGCTCACCGATCCCGGGC -403
CATTCGGGCGCGCTGAGAAAGCTCTACTCGGCCCCCACCCTTCTAG -353
HNF-6 CREB **Sp1**
TTTCATTTGTGCTGAGCTCAGGAGCTCTGGGACTGAGGGGCGGGGC -303
TGGCAGCGCATGCGCTGCGCGCGCGGGCGGTGAGGGTGGCAGCGG -253
CAGCGCAGCGGCTACCGAATCTCAGCCACAGTCTGCAACAGTAACGAG -203
CCATGGCTGAGCTGCGGGCGGCACAGGACAGCAGCGCGCGCAGGC -153
STAT
ACAGGGCGCGCTCAAGGAGCGCGGGCGCAGGATTTCTAAGAAAGAGA -103
Sp1
GGGGCGAGGGGGCGCGCTGGGGGGCTAGGGGACCCCGCTCTCCCA -53
BamHI **STAT**
ACAGCAGCGATCTTTTGGAAATTAATTTATTAAGAAAAAAGAAA -3
LP5 SOU
AGAAAAAGGCAGAGGGAGGTAGAGCAGAGAGGAGGACAGACACAC -48
AGAGAGAGCGAAAGACAGATCCCACTGAGAGGAGAAAGGCCACA -98
GTCCGAGCGCATGATGTGAAGACTTGAGACTCTGTGGCCCTCGCCGCT -148
HNFMR2
CTGCCCGGCACATCGATGTGACGCCCGCTCACCGCATCAGTAAC -198
Clal **HNFMR1**
GCACAGCTGACCATGAGCGCATTCGGCGAGCTGCACGGGTGAGCCATGA -248
LP5ATG
GCCGGTGCCTCGCCCTGTGACTGTCTGGCGGGAGCCCTCACGCGCGCA -298
GCTCCGT

Figure 4 Sequence of the rat *Hnf6* gene promoter

Transcription initiation sites mapped by RNase protection and 5' RACE-PCR are shown by broken arrows. The sequence between the *Bam*HI and *Clal* sites shown by open triangles corresponds to the probe used in RNase protection. The sequences of the oligonucleotides used in 5' RACE-PCR are underlined; their names are indicated in italics. The TATA box is indicated and putative binding sites for transcription factors are underlined and named in bold.

Figure 5 Functional delineation of the rat *Hnf6* gene promoter

HepG2 hepatoma cells were transiently transfected with the *Hnf6* gene promoter-luciferase reporter constructs indicated. Results are means $\pm$ S.E.M. for three independent experiments.

DISCUSSION

The present study provides the first characterization of an *Hnf6* gene. It demonstrates that the 465-residue HNF-6 α protein is coded by only two exons separated by at least 25 kb. Thus the two DNA-binding domains of HNF-6, namely the cut domain and the homeodomain, are coded by different exons. The results also show how alternative splicing of a third, 78 bp, exon located between the two giant introns gives rise to the β isoform of HNF-6. Our sequencing of the gene showed that the poly(A) stretch found at the 3' end of the rat liver cDNA described previously [3] belongs to exon 3 and is therefore not a poly(A) tail. The sequence of the HNF-6 protein is highly conserved in rats, humans and mice [3,15], suggesting that the organizations of the corresponding genes are identical in the three species. Sequence

comparisons revealed that HNF-6 is the founding member of a new class of homeoproteins characterized by a single cut domain and by a homeodomain containing phenylalanine and methionine residues at positions 48 and 50 respectively [4]. Open reading frames and cDNA species from *Caenorhabditis elegans* code for proteins of the same class [4]. The analysis of these *C. elegans* genes indicates that the cut domain and the homeodomain are each encoded by a single exon, as with HNF-6. Because the location of introns in homeobox genes is often conserved between members of the same family, the similarity in structure of the genes of this new class reinforces their evolutionary relationship.

Our transfection experiments indicated that the *Hnf6* gene promoter is contained within 0.75 kb upstream of the transcription initiation site. Consistent with the liver-enriched expression pattern of HNF-6, the promoter displayed hepatoma-specific activity. Earlier work showed that HNF-6 expression in mouse embryonic liver is initiated at the onset of liver differentiation [8,15]. In addition, Landry et al. [8] showed that HNF-6 activates the transcription of the *Hnf4* and *Hnf3 β* genes. This suggests that HNF-6 participates in the regulatory network of liver-enriched transcription factors. The analysis of the nucleotide sequence of the *Hnf6* gene promoter (Figure 4) revealed, in addition to sites for the ubiquitous factors nuclear factor-1, upstream stimulatory factor, cAMP-response-element-binding protein/activating transcription factor and Sp1 [16], the presence at -645 of an HNF-4-binding site that matches the consensus proposed by Sladek et al. [17]. In addition, an HNF-6-binding site consensus [4] is found at -340 on the *Hnf6* gene itself (Figure 4). These observations suggest that HNF-6 might regulate its own expression directly via the binding of HNF-6 to its promoter and indirectly via a stimulation of *Hnf4* gene expression.

Growth hormone stimulates the expression of the *Hnf6* gene in liver [9]. This hormone can control gene transcription via the STAT family of transcription factors [18-22]. Also, Legraverend et al. [23] identified in the promoter of the serine protease inhibitor genes a GAGA box that mediates the stimulation of this promoter by growth hormone. It is noteworthy that the *Hnf6* gene promoter contains at -115 and at -35 a STAT binding consensus and at -510 a GAGA-rich nucleotide sequence similar to that of the serine protease inhibitor genes (Figure 4). These sequences might therefore be targeted by factors that mediate the effect of growth hormone on the *Hnf6* gene.

The assignment of the HNF6/*Hnf6* gene to human chromosome 15q21.1-q21.2 [24], rat chromosome 8q24-q31 (this work) and mouse chromosome 9 [15] [MGD, Mouse Genome Database, Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine; WWW URL <http://www.informatics.jax.org/> (April 1997); and RATMAP, Rat Genome Database, Department of Genetics, University of Göteborg, Göteborg, Sweden; WWW URL <http://ratmap.gen.gu.se/> (April 1997)] adds this gene to the list of genes known to be syntenic on these three chromosomes (reviewed in [25]). All human genes of this conserved synteny group are located between 15q21 and 15qter [GDB, Genome Database (database online), Johns Hopkins University, Baltimore, MD, U.S.A.; WWW URL <http://gdbwww.gdb.org/> (January 1998)], whereas the homologous mouse genes are clustered in the middle of chromosome 9. Taking into account the morphological similarity of mouse chromosome 9 and rat chromosome 8 [26], it is likely that the rat homologues will also be clustered and will map at 8q24-q31, like *Hnf6*. The available genetic linkage data are compatible with this prediction. Indeed, the rat *Cyp1a1*, *Cyp1a2*, *Cyp19* and *Tpm1* genes, which are members of the conserved synteny group, can be placed between *Sm22* (8q24) and *Rbp2* (8q31) [27-29].

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