

# MFH-1, a new member of the fork head domain family, is expressed in developing mesenchyme

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We have isolated a novel mouse gene, MFH-1 (mesenchyme fork head 1) that is related to the *Drosophila* fork head and rat HNF3 genes. MFH-1 encodes a distinct fork head domain that is classified into a distinct subfamily. A recombinant MFH-1 protein could bind to the HNF3 binding site. MFH-1 is expressed temporally in developing embryos, first in the non-notochordal mesoderm and later in areas of mesenchymal condensation in the trunk, head, and limbs. Our results suggest that MFH-1 might be involved in the formation of special mesenchymal tissues.

Brain; Development; Fork head domain; Kidney; Mesoderm; Mesenchyme

## 1. INTRODUCTION

Molecular analysis of mammalian development has progressed rapidly during the last decade. This is mainly the result of the discovery that many of the genes controlling development are members of multigene families. They encode conserved DNA binding domains, such as the homeo domain [1], POU domain [2], and paired domain [3]. Since the development of the polymerase chain reaction (PCR), additional gene families have been isolated from a variety of species, including mouse and man. In the mouse, Hox genes and Pax genes have been well characterized. Results have shown that the former are involved in the formation of the complex body plan and axis [4], while the latter are involved in specification of the developing nervous system [3].

A 110-amino acid sequence of the hepatocyte nuclear factor (HNF) 3 DNA binding domain is conserved in the encoded protein of the region-specific *Drosophila* homeotic gene fork head (fkh) [5]. Mutations of the fkh gene cause homeotic transformation of the ectodermal portion of the gut; that is, the hindgut and foregut are replaced by ectopic head structures in fkh mutant embryos [6]. The conservation of the sequences between the rat and *Drosophila* genes enabled us to search for related genes in mouse brain and embryos. Here we report the isolation and characterization of a novel fork

head domain gene, MFH-1. MFH (mesenchyme fork head)-1 is expressed strongly in developing embryos, first in the non-notochordal mesoderm and later in areas of mesenchymal condensation in the trunk, head and limbs. It is expressed before, but not after, bones are formed. The restricted and temporal expression of MFH-1 in the developing mesenchyme suggests that this gene might be involved in the formation of special mesenchymal tissues.

## 2. EXPERIMENTAL

### 2.1. Cloning of the fork head domain-containing gene

Reverse transcription of mouse brain poly(A)<sup>+</sup> RNA with oligo(dT) was performed using a cDNA Cycle Kit (Invitrogen, San Diego, CA). The PCR [7] with degenerate primers to LITMAIQ and NMFENGCY sequences was performed by 35 cycles consisting of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min per cycle. PCR products were subcloned into the pCR 1000 plasmid using a TA Cloning system (Invitrogen, San Diego, CA). Sequencing revealed that the PCR fragment, named F11A-4, contained the fork head domain-related sequence. We screened lambda gt10 cDNA libraries constructed from mouse brain poly(A)<sup>+</sup> RNA and from 10.5 day post-coitum (dpc) mouse embryo poly(A)<sup>+</sup> RNA with the F11A-4 fragment as a probe and obtained two clones, pBF25 and pEF1. These clones were sequenced in both directions by the chain-termination method using Sequenase (United States Biochemical, Cleveland, OH).

### 2.2. Northern blotting and RNase protection assay

Poly(A)<sup>+</sup> RNAs were electrophoresed in formaldehyde agarose gels and transferred to a Hybond-N nylon membrane (Amersham). The *Pst*I–*Eco*RI fragment (nucleotide 668–1,543) of pBF25 was used as a probe, and the final washing was done in 0.1 × SSPE, 0.1% SDS at 65°C for 10 min.

The *Bgl*II–*Dra*I fragment (nucleotide 1,611–1,880) of pEF1 was subcloned into *Bam*HI, *Sma*I-digested Bluescript SK(+) and named SK-MFH270. A labeled RNA probe was made by *in vitro* transcription in the presence of [ $\alpha$ -<sup>32</sup>P]UTP (800 Ci/mmol) using T7 RNA polymerase (Takara Syuzo, Kyoto) and was purified. Total RNAs (20

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*Abbreviations:* dpc, days post-coitum; HNF, hepatocyte nuclear factor; PAGE, polyacrylamide gel electrophoresis.

1	T	GGG	AGT	GGT	ACC	CTA	TTT	GAG	TGA	GCA	AAA	CTA	CTA	CCG	GGC	GGC	CGC	AGC	TAC	GGC	58	
59	GGC	ATG	GCC	AGC	CCC	ATG	GGC	GTC	TAC	TCC	GGC	CAC	CCG	GAG	CAG	TAC	GGC	GCC	GCC	ATG	118	
119	GGC	CGC	TCC	TAC	GCG	CCC	TAC	CAC	CAC	CAG	CCC	GCG	GCG	CCC	AAG	GAC	CTG	GTG	AAG	CCG	178	
20	G	R	S	Y	A	P	Y	H	Q	P	A	A	P	K	D	L	V	K	P		39	
179	CCC	TAC	AGC	TAT	ATA	GCG	CTC	ATC	ACC	ATG	GGC	ATC	CAG	AAC	GCG	CCA	GAG	AAG	AAG	ATC	238	
40	P	Y	S	Y	I	A	L	I	T	M	A	I	Q	N	A	P	E	K	K	I	59	
239	ACT	CTG	AAC	GGC	ATC	TAC	CAG	TTC	ATG	GAC	CGT	TTC	CCC	TTC	TAC	CGC	GAG	AAC	AAG		298	
60	T	L	N	G	I	Y	Q	F	I	M	D	R	F	P	F	Y	R	E	N	K	79	
299	CAG	GGC	TGG	CAG	AAC	AGC	ATC	CGC	CAC	AAC	CTG	TCA	CTC	AAT	GAG	TGC	TTC	GTG	AAA	GTG	358	
80	Q	G	W	Q	N	S	I	R	H	N	L	S	L	N	E	C	F	V	K	V	89	
359	CCG	CGC	GAC	GAC	AAG	AAG	CCG	GGC	AAG	GGC	AGC	TAC	TGG	ACG	CTC	GAC	CCG	GAC	TCC	TAC	418	
100	P	R	D	D	K	K	P	G	K	G	S	Y	W	T	L	D	P	D	S	Y	119	
419	AAC	ATG	TTC	GAG	AAT	GGC	AGC	TTC	CTG	CGG	CGG	CGG	CGG	CGC	TTC	AAG	AAG	AAG	GAT	GTG	478	
120	N	M	F	E	N	G	S	F	L	R	R	R	R	R	F	K	K	K	D	V	139	
479	CCC	AAG	GAC	AAG	GAG	GAG	CGG	GCC	CAC	CTC	AAG	GAG	CCG	CCC	TCG	ACC	ACG	GCC	AAG	GGC	538	
140	P	K	D	K	E	E	R	A	H	L	K	E	P	P	S	T	T	A	K	G	159	
539	GCT	CCG	ACA	GGG	ACC	CCG	GTA	GCT	GAC	GGG	CCC	AAG	GAG	GCC	GAG	AAG	AAA	GTC	GTG	GTT	598	
160	A	P	T	G	T	P	V	A	D	G	P	K	E	A	E	K	K	V	V	V	179	
599	AAG	AGC	GAG	GCG	GCG	TCC	CCC	GCG	CTG	CCG	GTC	ATC	ACC	AAG	GTG	GAG	ACG	CTG	AGC	CCC	658	
180	K	S	E	A	A	S	P	A	I	P	V	I	T	K	V	E	T	L	S	P	199	
659	GAG	GGA	GCG	CTG	CAG	GCC	AGT	CCG	GCG	AGC	GCA	TCC	TCC	ACG	CCC	GCA	GGT	TCC	CCA	GAC	718	
200	E	G	A	I	Q	A	S	R	S	A	T	S	T	S	P	A	D	P	D		219	
719	GGC	TCG	CTG	CCG	GAG	CAC	CAC	GCC	GCG	GCG	CCT	AAC	GGG	CTG	CCC	GGC	TTC	AGC	GTG	GAG	778	
220	G	S	I	P	E	H	H	A	A	P	N	G	I	P	G	F	S	V	E		239	
779	ACC	ATC	ATG	ACG	CTG	CGG	ACG	TCC	CCT	CCG	GGC	GGC	GAT	CTG	AGC	CCA	GCG	GCC	CCG	CGC	838	
240	T	I	M	T	L	R	T	S	P	P	G	G	D	I	S	P	A	A	A	R	259	
839	GCC	GGC	CTG	GTG	GTG	CCA	CCG	CTG	GCA	CTG	CCA	TAC	GCC	GCA	GCG	CCA	CCC	GCC	GCT	TAC	898	
260	A	G	L	V	V	P	P	I	A	I	P	Y	A	A	A	P	P	A	A	Y	279	
899	ACG	CAG	CCG	TGC	GCG	CAG	GGC	CTG	GAG	GCT	GCG	GGC	TCC	SAC	GGC	TAC	CAG	TGC	AGT	ATG	958	
280	T	Q	P	C	A	Q	G	T	E	A	A	G	S	A	G	Y	Q	C	S	M	299	
959	CGG	GCT	ATG	AGT	CTG	TAC	TAC	ACC	GGG	GCC	GAG	CGG	CCC	GCG	CAC	GTG	TGC	GTT	CCG	CCC	CGG	1018
300	R	A	M	S	L	Y	A	S	E	R	P	A	H	V	C	P	P	P	A		319	
1019	CTG	GAC	GAG	GCT	CTG	TCG	GAC	CAC	CCG	AGC	GGC	CCC	GGC	TCC	CCG	CTC	GGC	GCC	CTC	AAC	1078	
320	L	D	E	A	L	S	D	H	P	S	G	P	G	S	P	L	G	A	L	N	339	
1079	CTC	GCA	GCG	GGT	CAG	GAG	GGC	GCG	TTG	GGG	GCC	TCG	GGT	CAC	CAC	CAC	CAG	CAT	CAC	GGC	1138	
340	L	A	A	G	Q	E	G	A	L	G	A	S	G	H	H	H	Q	H	H	G	359	
1139	CAC	CTC	CAC	CCG	CAG	GCG	CCA	CCG	CCC	GCC	CCG	CAG	CCC	GCT	CCC	GGC	CCG	CAG	CCC	GCC	1198	
360	H	L	H	P	Q	A	P	P	P	A	P	Q	P	P	P	A	P	Q	P	A	379	
1199	ACC	CAG	GCC	ACC	TCC	TGG	TAT	CTG	AAC	CAC	GGC	GGG	GAC	CTG	AGC	SAC	CTC	CCC	GGC	CAC	1258	
380	T	Q	A	T	S	W	Y	L	N	H	G	G	D	L	S	H	L	P	G	H	399	
1259	ACG	TTT	GCA	ACC	CAA	CAG	CAA	ACT	TTC	CCC	AAC	GTC	CGG	GAG	ATG	TTC	AAC	TCG	CAC	CGG	1318	
400	T	F	A	T	Q	Q	Q	T	F	P	N	V	R	E	M	F	N	S	H	R	419	
1319	CTA	GGA	CTG	GAC	AAC	TCG	TCC	CTC	GGG	GAG	TCC	CAG	GTG	AGC	AAT	GCG	AGC	TGT	CAG	CTG	1378	
420	L	G	L	D	N	S	S	I	G	E	S	Q	V	S	N	A	S	C	Q	L	439	
1379	CCC	TAT	YGA	GCT	ACG	CCG	TCC	CTC	TAC	CGC	CAC	GCA	GCC	CCC	TAC	TCT	TAC	GAC	TGC	ACC	1438	
440	P	Y	R	A	T	P	S	L	Y	R	H	A	A	P	Y	S	Y	D	C	T	459	
1439	AAA	TAC	TGA	GGC	TGT	CCA	GTC	CGC	TCC	AGC	CCC	AGG	ACC	GCA	CCG	GCT	TCG	CCT	CCT	CCA	1498	
460	K	Y	*																	462		
1499	TGG	GAA	CCT	TCT	TCG	ACG	GAG	CCG	CAG	AAA	GCG	ACG	GAA	AGC	GCC	CCT	CTC	TCA	GAA	CCA	1558	
1559	GGA	GCA	GAG	AGC	TCC	GTG	CAA	CTC	GCA	GGT	AAC	TTA	TCC	GCA	GCT	CAG	TTT	GAG	ATC	TCA	1618	
1619	GCG	AGT	CCC	TCT	AAG	GGG	GAT	GCA	GCC	CAG	CAA	AAC	GAA	ATA	CAG	ATT	TTT	TTT	TTA	ATT	1678	
1679	CCT	TCC	CCT	ACC	CAG	ATG	CTG	CGC	CTG	CTC	CCT	TGG	GGC	TTC	ATA	GAT	TAG	CTT	ATG	GAC	1738	
1739	CAA	ACC	CAT	AGG	GAC	CCC	TAA	TGA	CTT	CTG	TGG	AGA	TTC	TCC	ACG	GGC	GCA	AGA	GGT	CTC	1798	
1799	TCC	GGA	TAA	GGT	GCC	TTC	TGT	AAA	CGA	GTG	CGG	ATT	TGT	AAC	CAG	GCT	ATT	TTG	TTC	TTG	1858	
1859	CCC	AGA	GCC	TTT	AAT	ATA	ATA	TTT	AAA	GTT	GTG	TCC	ACT	GGA	TAA	GGT	TTC	GTC	TTG	CCC	1918	
1919	AAC	TGT	TAC	TGC	CAA	ATT	GAA	TTC												1942		

Fig. 1. Nucleotide and predicted amino acid sequence of the MFH-I cDNA. Amino acids are shown in the single letter code. The fork head domain is underlined and the histidine- and proline-rich region is double underlined.

$\mu\text{g}$ ) from embryos at various stages of gestation were used for RNase protection assays according to the recommendations in the RPA II kit (#1410; Ambion, Austin, TX), and the undigested products were analyzed in denaturing gels.

### 2.3. Electrophoretic mobility shift assay

The *Eco*RI insert of pEF1 was subcloned under the direction of the T7 promoter. pHF22.1 contained an open reading frame of HNF1 under the SP6 promoter [6]. In vitro transcripts were obtained as described previously [9] and translated in vitro with a rabbit reticulocyte lysate (Amersham N90) in the presence of [ $^{35}\text{S}$ ]methionine. The following oligonucleotides were synthesized in a 391 PCR-MATE DNA synthesizer (Applied Biosystems, CA): site 3, tcgacTTTGTT-GACTAAGTCAATAATCAGAATCAGgac; site 1, agcttCAAAC-TGTCAAATATTAATAAGgac.

The double-stranded oligonucleotides were labeled using T4 polynucleotide kinase and [ $\gamma$ - $^{32}\text{P}$ ]ATP (6,000 Ci/mmol). Then 0.5 ng of the labeled probe was mixed with 5  $\mu\text{l}$  of the in vitro reticulocyte translate in 20  $\mu\text{l}$  of 10 mM HEPES-KOH (pH 7.9), 50 mM KCl, 2 mM EDTA,

1 mM DTT, 10% glycerol and poly(dI-dC)/(dI-dC) (0.25  $\mu\text{g}/\mu\text{l}$ ) and incubated at 30°C for 30 min. The reaction mixtures were applied to 5% native polyacrylamide gels (80:1 acrylamide/bisacrylamide) in 0.25  $\times$  Tris-borate-EDTA, and after electrophoresis the gels were dried and autoradiographed.

### 2.4. In situ hybridization in developing mice

ICR mice were mated and timed pregnant mice (e9.5, e13.5, e17.5) and 3-day-old mice were used. Embryos from the uterus and neonatal mice were rapidly frozen with powdered dry ice. Frozen sections were cut on a cryostat, thaw-mounted on 3-aminopropyltriethoxysilane-treated slides and subjected to in situ hybridization.

$^{35}\text{S}$ -Labeled antisense and sense (control) riboprobes were transcribed from the SK-MFH270 mentioned above, which contained 270 bp of the 3' untranslated region, and used to minimize cross-hybridization to other members of the fork head domain family. In situ hybridization was performed as described previously [10]. The specificity of the hybridization signal was checked by comparing sections treated with the antisense probe with those treated with the sense

		10	20	30	40	50	60	70	80	90	100	110
group1	MFH-1	QPAAPKDLVKPPYSYIALITMAIQNAPEKIKITLNGIYQFIMDRFFFYRENKQGWQNSIRHNLSINECFVKVPRDDKKPKGKGSYWTLDPSYNYMFENGSLRRRRRFFKKKDV										
	fkh	TYRRSYTHA-----S-----N-TTML--SE-----L-----Q-Q-R-----S-F-D-----I---TPD-----F---H---G-----CY--QK--CEKK										
group2	HNF3 $\alpha$	TFKRSYPHA-----S-----Q-S-ML--SE--W--L-Y--Q-Q-R-----S-F-A-----A-SPD-----H---G-----CY--QK--CEKQ										
	HNF3 $\beta$	TYRRSYTHA-----S-----QS-N-ML--SE--W--L-Y--Q-Q-R-----S-F-D-L---APD-----F---H---G-----CY--QK--CEKQ										
	HNF3 $\gamma$	GYRRPLAHA-----S-----Q-G-ML--SE--W--L-Y--Q-Q-R-----S-F-D-----A-SPD-----A-H-S-G-----CY--QK--LEEK										
	XFKH1	TYRRN-SHA-----S-----Q-N-MM--E--W-V-L-Y--Q-Q-R-----S-F-D-I---SPE-----H-E-G-----CY--QK--CERS										
	pinta	TYRRNYSHA-----S-----Q-N-MM--E--W-I-L-Y--Q-Q-R-----S-F-D-----SPE-----H---G-----CY--QK--CERS										
group3	BF-1	GDKKNGKYE--F--N--M--RQS--RL-----E--KN--Y-----K-----HYDD--N--M--S-DDV-IG--TTGKL--STTSRA										
	slp1	KMT-GS-TK-----N--M--DS--QRL-----YLIN--YFKA--R-----K--I-I--SYDD--N--I--SAEEV-IGETTGL--KNPGASR										
	slp2	PVKDK-GNE-----N--M--RQSS--RL-----EY--TNH-Y--D-----K-----HYDD--N--M--SAEDV-IG--TGKL--TTAASR										
group4	ILF	GDSPK--DS-----AQ--VQ--TM--D-QL-----TH-TKNY-Y--TADK-----RY-I--SQEE--F--RI--A-ESKLIEQA-RK--P-GVPCFR										

Fig. 2. Comparison of the amino acid sequences of the fork head domain proteins. Dashes indicate identity with residues in MFH-1. fkh, *Drosophila* fork head [6]; HNF3 $\alpha$ , rat hepatocyte nuclear factor 3 $\alpha$  [11]; HNF3 $\beta$  and HNF3 $\gamma$ , rat [12]; XFKH1, *Xenopus* [15]; pinta, *Xenopus Pintallavis* [16]; BF-1, rat brain factor 1 [17]; slp1 and slp2, *Drosophila* sloppy paired 1 and 2 [18]; ILF, human interleukin enhancer binding factor [20].

probe. In the present study, we did not detect any significant labeling of sections treated with the sense probe (Fig. 5D).

### 3. RESULTS AND DISCUSSION

*Drosophila* homeotic gene fork head (fkh) is expressed in the central nervous system and cells destined to form gut structures [6]. Interestingly, the liver transcription factor, HNF3, has a high degree of amino acid sequence similarity over 110 amino acids with the fkh protein [5] and is not expressed in the brain [11,12]. We expected that there is a large gene family encoding the fork head domain (110 amino acids) and tried to isolate novel new genes encoding the fork head domain in the

brain by RT-PCR. One PCR fragment of 260 bp, named F11A-4, had a new fork head domain-related sequence. Northern blotting using this fragment as a probe revealed that this gene is expressed in mouse brain and strongly in mouse embryos (data not shown), so we screened lambda gt10 libraries constructed from mouse brain poly(A)<sup>+</sup> RNA and from 10.5 dpc mouse embryo poly(A)<sup>+</sup> RNA with the F11A-4 fragment as a probe and obtained two clones, pBF25 (1.6 kbp; nucleotide 24–1,543) and pEF1 (2.0 kbp; nucleotide 1–1,942). Sequencing of these two overlapping clones revealed an open reading frame encoding a protein of 461 amino acids that is related to the HNF3 and fkh proteins (Figs. 1 and 2). The sequence surrounding the first methionine

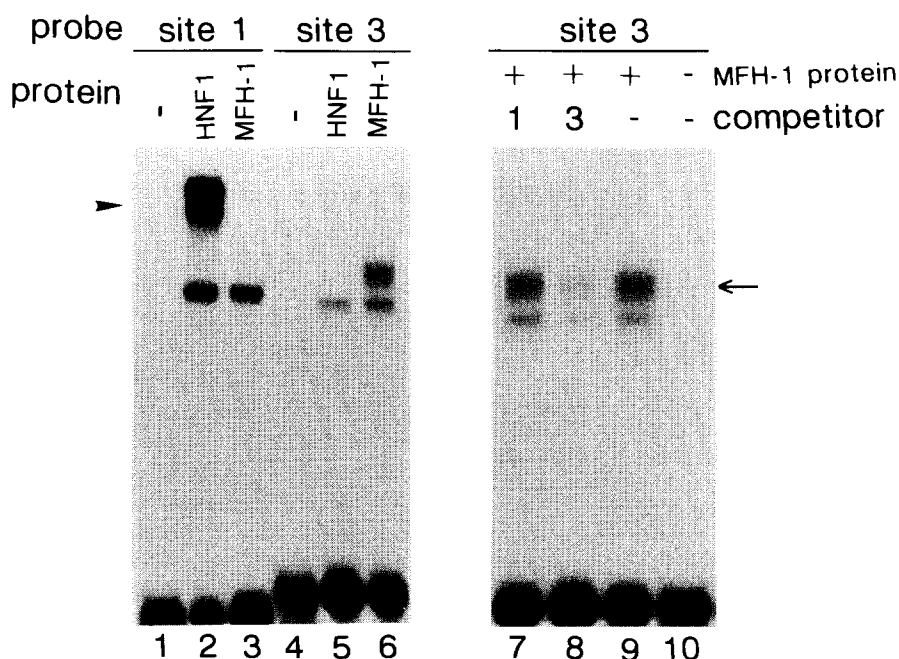


Fig. 3. Electrophoretic mobility shift assay of a recombinant MFH-1 protein. Recombinant MFH-1 and HNF1 proteins were obtained by in vitro translation of RNA transcribed in vitro from the corresponding cDNAs as described in section 2. Labeled site 3 (lanes 4–10) and site 1 (lanes 1–3) oligonucleotides were incubated without (lanes 1, 4 and 10) and with 5  $\mu$ l of the in vitro translation products of MFH-1 (lanes 3 and 6–9) and HNF1 (lanes 2 and 5). The mixtures were analyzed in 5% native polyacrylamide gels. In lanes 7 and 8, the reaction was performed in the presence of a 100-fold excess of the site 1 and site 3 oligonucleotides, respectively. The specific complexes retarded by the recombinant MFH-1 and HNF1 proteins are indicated by arrows and an arrowhead, respectively.

at nucleotide 62–64 agrees well with the consensus sequence derived from eukaryotic translational initiation

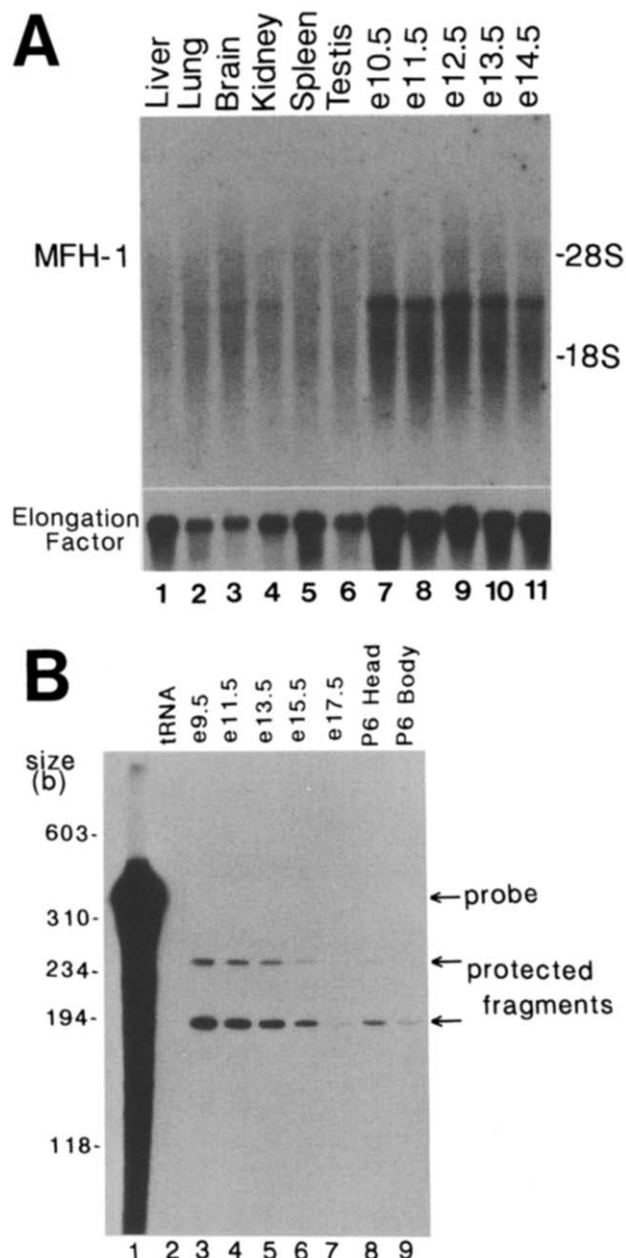


Fig. 4. Expression of the MFH-1 gene. (A) Northern blotting analysis of poly(A)<sup>+</sup> RNA from adult tissues and embryos. Poly(A)<sup>+</sup> RNAs (5  $\mu$ g) from adult liver (lane 1), lung (lane 2), brain (lane 3), kidney (lane 4), spleen (lane 5), and testis (lane 6) and from 10.5 dpc (lane 7), 11.5 dpc (lane 8), 12.5 dpc (lane 9), 13.5 dpc (lane 10) and 14.5 dpc (lane 11) whole embryos were size-separated in formaldehyde agarose gels and transferred to nylon membranes. The fragment not containing the fork head domain was used as a probe. One major mRNA species is detected at 3.0 kb. The same blot was re-probed with elongation factor-1 cDNA [21] to assess the quantity of RNA. (B) Ribonuclease protection assays of total RNAs from embryos at various stages of gestation. Probe only (lane 1). The probes were hybridized with 20  $\mu$ g of yeast tRNA (lane 2) and total RNAs from 9.5 dpc (lane 3), 11.5 dpc (lane 4), 13.5 dpc (lane 5), 15.5 dpc (lane 6), 17.5 dpc (lane 7) embryos and from the head (lane 8) and the body (lane 9) of a neonatal mouse (6 days after birth). The undigested products were separated in denaturing gels.

sites [13]. SDS-PAGE analysis showed that the in vitro translated protein had a molecular mass corresponding to 53 kDa (data not shown), indicating that this open reading frame was translated. The amino acid sequence (Fig. 1) showed that the amino-terminal part contained the fork head domain (amino acid 29–139), the putative DNA binding domain [11], while the carboxyterminal part encoded a histidine- and proline-rich region (amino acid 353–378), the putative transactivating domain [14]. The functional significances of these domains must be tested by detailed mutational analyses.

The amino acid sequence of the fork head domain of the MFH-1 protein is compared with those of other fork head domain proteins in Fig. 2. Although there are several variations, these proteins can be classified into 4 groups. HNF3 proteins are considered to be rat homologues at *Drosophila* fork head protein. The recently isolated XFKH1 [15] and *Pintallavis* [16] proteins are very similar to HNF3 and fkh, and form group 2. The sequence of telencephalon-restricted BF-1 protein [17] is more similar to those of the *Drosophila* slp1 and slp2 proteins [18] (group 3). Our clone, MFH-1, which was isolated from mouse brain and mouse embryo libraries, differs at several sites within the fork head domain and constitutes a distinct subfamily (group 1). As shown later, differences in the expression patterns of MFH-1 and the group 2 and group 3 proteins are correlated with differences in the amino acid sequences in these groups.

As HNF3 protein binds to the HNF3 sequence (–111 to –85) from the transthyretin promoter [19], we tested whether MFH-1 protein could also bind to this site. As the recombinant MFH-1 protein expressed in *E. coli* was easily degraded for some unknown reason, we used proteins translated in vitro in reticulocyte lysates from RNAs transcribed in vitro as recombinant proteins. When a recombinant HNF1 protein (control protein) was incubated with oligonucleotides corresponding to the HNF3 site (site 3), only a non-specific complex was formed (Fig. 3, lane 5). When the recombinant MFH-1 protein was incubated with the site 3 oligonucleotides, however, a specific complex was formed (lane 6, arrow). Furthermore, incubation of the recombinant MFH-1 protein with the oligonucleotides corresponding to the HNF1 site (site 1) formed only a non-specific complex, and no specific complexes (lane 3). When the recombinant MFH-1 protein was incubated with labeled site 3 oligonucleotides in the presence of an excess amount of unlabeled site 3 oligonucleotides, the amount of radioactivity in the band of the specific complex was greatly diminished (lane 8 vs. lane 9). When incubation was carried out in the presence of an excess amount of the site 1 oligonucleotides, however, the formation of the specific complex was not affected (lane 7). This finding, that a recombinant MFH-1 protein can bind to the HNF-3 site, indicates that MFH-1 is a sequence-specific DNA binding protein.

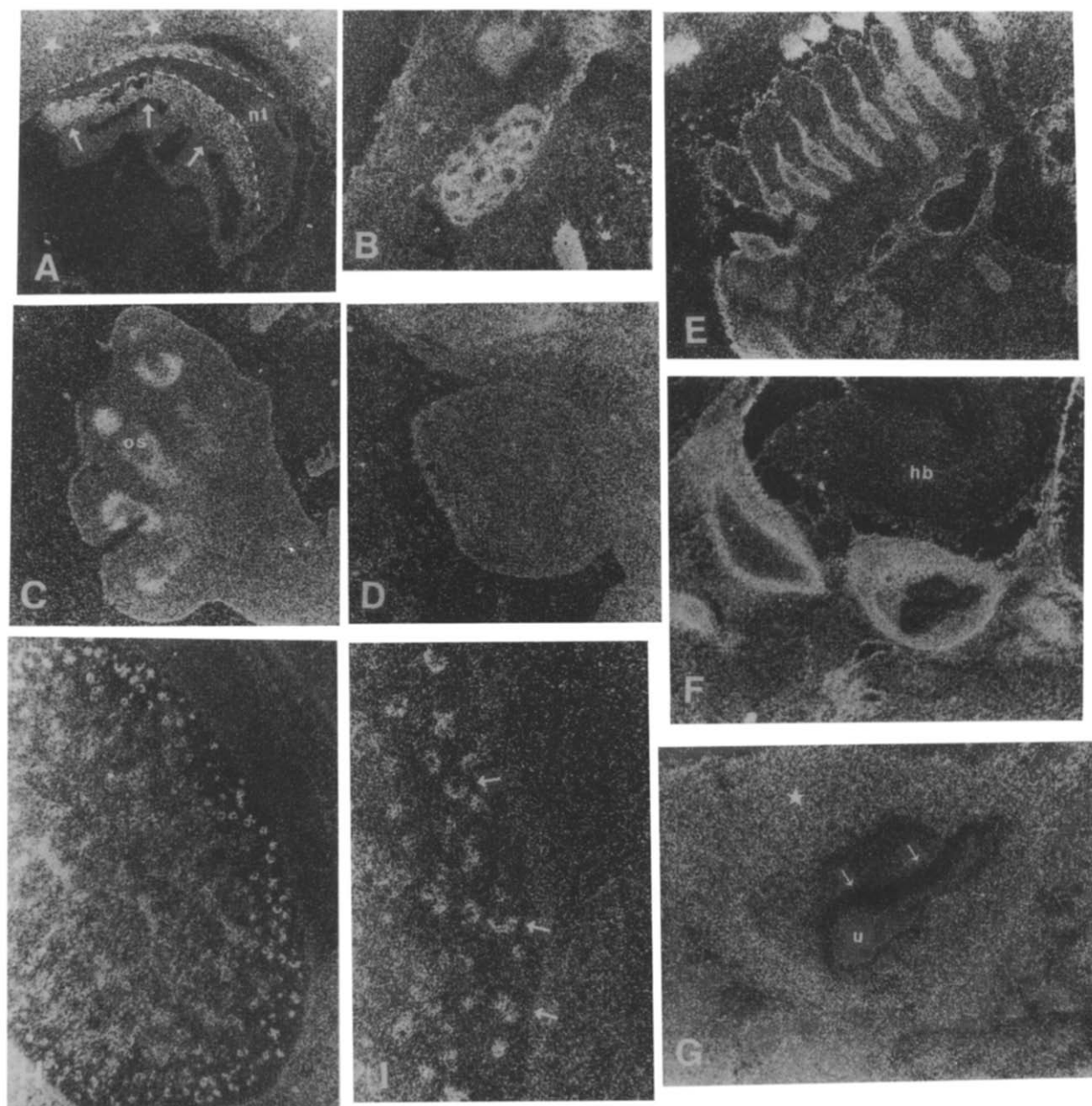


Fig. 5. Dark-field photomicrographs showing the locations of MFH-1 mRNA expression in embryonic and postnatal mouse tissues. (A) e9.5 embryo. MFH-1 messages are observed in the somites (arrows), while the neural tube (nt) is not labeled. Asterisks indicate the uterus. (B) Metanephros of an e13.5 embryo. Note intense labeling over the compact mesenchyme surrounding the collecting ducts. (C) Limb bud of an e13.5 embryo. The limb primordium is labeled. Note that the ossification center (os) is devoid of signals. (D) Section of an e13.5 limb bud treated with the sense probe. No significant labeling is observed. (E) Cervical vertebrae of an e13.5 embryo. The head is at the bottom. (F,G) Neck portion of an e13.5 embryo. The cartilaginous tissue surrounding the inner ear is strongly labeled, while the hindbrain (hb) shows little labeling. At higher magnification of the inner ear (G), MFH-1 mRNA is detectable exclusively in the cartilaginous tissue (asterisk). Note the absence of MFH-1 mRNA expression in the sensory epithelium (arrows) of the utricle (u). (H,I) Kidney of a 3-day-old mouse. MFH-1 mRNA is mainly expressed in the renal cortex. At higher magnification (I), the message is seen in the compact mesenchyme surrounding the glomerulus.

As the MFH-1 protein has a fork head domain and might play a role in development, we next examined the expression pattern of MFH-1 mRNA. First, we performed Northern blotting of poly(A)<sup>+</sup> RNA from various adult tissues and tissues at several stages of gestation (Fig. 4A). In adult tissues, MFH-1 mRNA was

detected in the brain and kidney as weak bands of 3.0 kb (lanes 3 and 4). No message was detected in the liver, spleen and testis (lanes 1, 5 and 6) and only a trace amount was detected in the lung (lane 2). In contrast, MFH-1 was expressed at high levels in mouse embryos (lanes 7–11). To determine the exact expression profile

during development, we performed RNase protection of total RNA from embryos at various gestational stages (Fig. 4B). The RNA probe (340 b, lane 1) was chosen from the 3'-untranslated region and was expected to be protected to produce 270 b. When the probe mixed with tRNA was digested, no bands were protected (lane 2). When total RNA from embryos was used, two bands (270 b and 190 b) were unexpectedly protected, however, as the amounts of these two bands were proportional, we concluded that expression of MFH-1 was highest on 9.5 days of gestation and then gradually decreased with progress of gestation. Its expression was detectable in 17.5 dpc embryos and neonates at only low levels (lanes 7-9).

To determine the precise location of MFH-1 expression in the developing embryos, we examined sections of mouse embryos and neonates by in situ hybridization (Fig. 5). In general, MFH-1 mRNA was expressed strongly in mesoderm-derived tissues in the embryonic period. In e9.5 (9.5 dpc) embryos, a diffuse hybridization signal of moderate intensity was detected in the somites (Fig. 5A, arrows). During further development, signals of MFH-1 mRNA became stronger and were restricted to the cartilaginous tissues and metanephros. Representative results are shown in Fig. 5B-G. In e13.5 embryos, the compact mesenchyme of the metanephros was intensely labeled (Fig. 5B). The bone primordium of the limb buds where chondrocytes are actively dividing and synthesizing hyaline matrix also showed an intense hybridization signal (Fig. 5C). Characteristically MFH-1 mRNA was mainly observed in the perichondrium and was not detected in the ossification center (Fig. 5C, os). This pattern persisted throughout the embryonic period (data not shown). The vertebrae (Fig. 5E) and the cartilaginous bones of the inner ear (Figs. 5F and G) were other regions showing MFH-1 mRNA expression. In contrast, the central nervous system (Fig. 5F), heart, lung, liver, and gut (data not shown) were devoid of hybridization signals. In neonatal mice (3 days after birth), MFH-1 mRNA expression was mainly detected in the kidney (Fig. 5H and I) and the skin. In the kidney, the hybridization signal was observed in the compact mesenchyme surrounding the glomeruli (Fig. 5I, arrows). Incidentally the meningeal cells and the dura mater in the head were labeled by the hybridization signal, whereas the brain was not (data not shown). Thus, in general, MFH-1 mRNA was mainly expressed in actively dividing cells of mesoderm-derived tissues.

In the present study, we isolated a novel member of the fork head domain family which is temporally expressed in non-notochordal mesenchyme in developing embryos. The expression pattern of MFH-1 is different from that of other members of the fork head domain

family. XFKH1 and *Pintallavis* are expressed in the dorsal blastopore lip (organizer region) and later in the notochord and neural floor plate [15,16]. HFN3 $\alpha$ , - $\beta$ , and - $\gamma$  seem to be restricted to endoderm-derived tissues [11,12]. In contrast, BF-1 is expressed in the rostral neuroepithelium and later in the telencephalon [17]. Each member could be involved in the genesis of distinct tissues. In our simplified model, group 2 members might be involved in the formation of endoderm-derived tissues and notochord-related tissues. The group 3 member (BF-1) might be involved in the formation of rostral ectoderm. MFH-1 (group 1 member) might be involved in the formation of special mesenchymal tissues. Unidentified members of the fork head domain family could contribute to developmental decisions in other compartments. For determination of the developmental roles of the MFH-1 gene, further studies are necessary, including identification of the target genes and inactivation of the MFH-1 gene in mice by homologous recombination.

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## REFERENCES

- [1] Scott, M.P., Tamkun, J.W. and Hartzell III, G.W. (1989) *Biochim. Biophys. Acta* 989, 25-48.
- [2] Rosenfeld, M.G. (1991) *Genes Dev.* 5, 897-907.
- [3] Gruss, P. and Walther, C. (1992) *Cell* 69, 719-722.
- [4] Gaunt, S.J. (1991) *BioEssays* 13, 505-513.
- [5] Weigel, D. and Jäckle, H. (1990) *Cell* 63, 455-456.
- [6] Weigel, D., Jurgens, G., Küttner, F., Seifert, E. and Jäckle, H. (1989) *Cell* 57, 645-658.
- [7] Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Ehrlich, H.A. (1988) *Science* 239, 487-491.
- [8] Miura, N., Iwai, K. and Miyamoto, I. (1993) *Eur. J. Cell Biol.* (in press).
- [9] Struhl, K. (1987) *Current Protocols in Molecular Biology*, pp. 10.17-17.5, Wiley, New York.
- [10] Wanaka, A., Johnson Jr., E.M. and Milbrant, J. (1990) *Neuron* 5, 267-281.
- [11] Lai, E., Prezioso, V.R., Smith, E., Litvin, O., Costa, R.H. and Darnell Jr., J.E. (1990) *Genes Dev.* 4, 1427-1436.
- [12] Lai, E., Prezioso, V.R., Tao, W., Chen, W.S. and Darnell Jr., J.E. (1991) *Genes Dev.* 5, 416-427.
- [13] Kozak, M. (1987) *Nucleic Acids Res.* 15, 8125-8146.
- [14] Mitchell, P.J. and Tjian, R. (1989) *Science* 245, 371-378.
- [15] Dirksen, M.L. and Jamrich, M. (1992) *Genes Dev.* 6, 599-608.
- [16] Ruiz i Altaba, A. and Jessel, T.M. (1992) *Development* 116, 81-93.
- [17] Tao, W. and Lai, E. (1992) *Neuron* 8, 957-966.
- [18] Grossniklaus, U., Pearson, R.K. and Gehring, W.J. (1992) *Genes Dev.* 6, 1030-1051.
- [19] Costa, R.H., Grayson, D.R. and Darnall Jr., E. (1989) *Mol. Cell Biol.* 9, 1415-1425.
- [20] Li, C., Lai, C., Sigman, D.S. and Gaynor, R.B. (1991) *Proc. Natl. Acad. Sci. USA* 88, 7739-7743.
- [21] Uetsuki, T., Naito, A., Nagata, S. and Kaziro, Y. (1989) *J. Biol. Chem.* 264, 5791-5798.