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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 form head (fkh) [5]. Mutations of the fkh gene cause homeotic transformation of the ectodermal portion of the gut; that is, the hindgut and forefut 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.

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

2. EXPERIMENTAL

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

Reverse transcription of mouse brain poly(A)$^+$ 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)$^+$ RNA and from 10.5 day post-coitum (dpc) mouse embryo poly(A)$^+$ 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)$^+$ 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 [α-^{32}P]UTP (800 Ci/mmol) using T7 RNA polymerase (Takara Syuzo, Kyoto) and was purified. Total RNAs (20

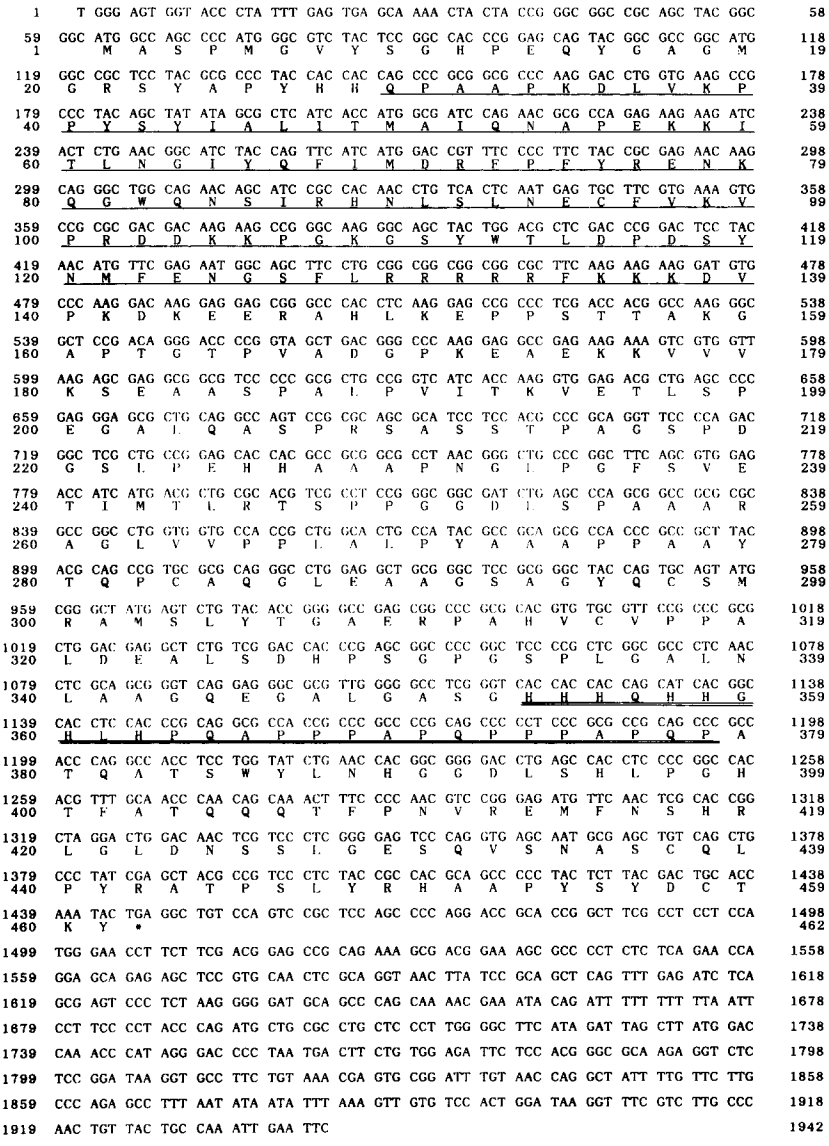

Fig. 1. Nucleotide and predicted amino acid sequence of th MFH-1 cDNA. Amino acids are shown in the single letter code. The fork head domain is underlined and the histidine- and proline-rich region is double underlined.

μg) 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 promotor. 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}S]methionine. The following oligonucleotides were synthesized in a 391 PCR-MATE DNA synthesizer (Applied Biosystems, CA): site 3, tcgacTTTGTTGACTAAGTCAATAATCAGAATCAGgatc; site 1, agcttCAAACTGTCAAATATTAACTAAAGgatc.

The double-stranded oligonucleotides were labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (6,000 Ci/mmol). Then 0.5 ng of the labeled probe was mixed with 5 μl of the in vitro reticulocyte translate in 20 μl 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 μg/μl) 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 × 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}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

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                    10        20        30        40        50        60        70        80        90       100       110
group1 [ MFH-1      QPAAPKDLVKPPYSYIALITMAIQNAPEKKITLNGIYQFIMDRFPFYRENKQGWQNSIRHNLSLNECFVKVPRDDKKPGKGSYWTLDPDSYNMFENGSFLRRRRRFKKKDV
       [ fkh        TYRRSYTHA-------S--------N-TRML--SE-------L-----Q-Q-R-------S--F-D----I--TPD------F---H---G------CY---QK---CEKK
       [ HNF3α      TFKRSYPHA-------S--------Q--S-ML--SE---W---L--Y--Q-Q-R-------S--F-A-----A-SPD----------H---G------CY---QK---CEKQ
group2 [ HNF3ß      TYRRSYTHA-------S--------QS-N-ML--SE---W---L-----Q-Q-R-------S--F-D--L----APD------F---H---G------CY---QK---CEKQ
       [ HNF3γ      GYRRPLAHA-------S--------Q--G-ML--SE---W---L--Y----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
       [ BF-1       GDKKNGKYE---F--N---M---RQS---RL------E---KN--Y--------------------K-------HYDD----N--M---S-DDV-IG-TTGKL---STTSRA
group3 [ slp1       KMT-GS-TK------N---M----DS--QRL-------YLIN---YFKA--R-------------K--I-I--SYDD----N--I---SAEEV-IGETTGKL--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
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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]; HNF3α, rat hepatocyte nuclear factor 3α [11]; HNF3β and HNF3γ, 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)$^+$ RNA and from 10.5 dpc mouse embryo poly(A)$^+$ 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

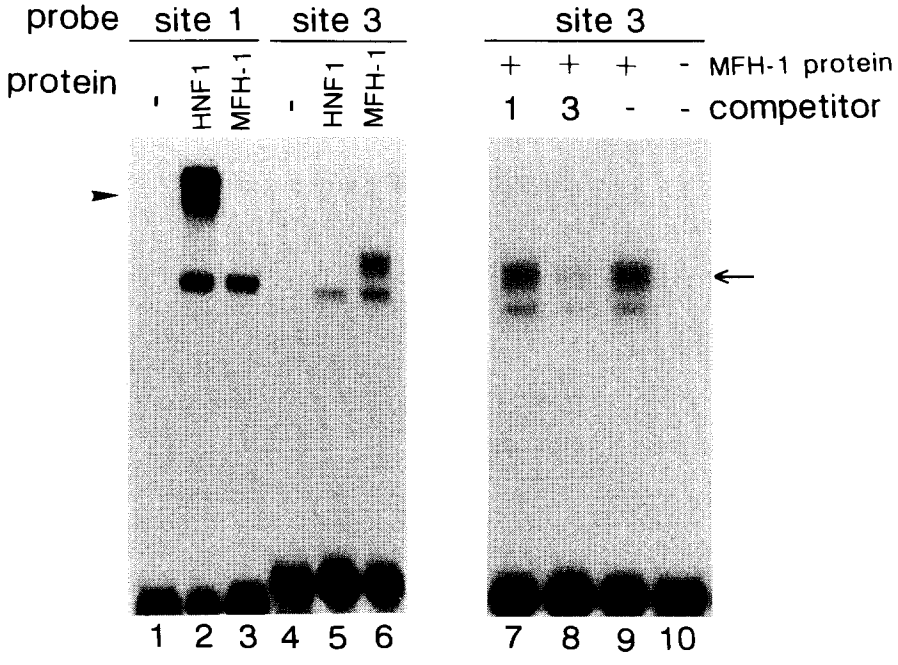

Fig. 3. Electrophoretic mobility shift assay of a recombinant MFH-1 protein. Recombinant MFH-1 and HNF1 proteins were obtained by in vitro translation of RNA transcribed in vitro from the corresponding cDNAs as described in section 2. Labeled site 3 (lanes 4–10) and site 1 (lanes 1–3) oligonucleotides were incubated without (lanes 1, 4 and 10) and with 5 μl 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 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.

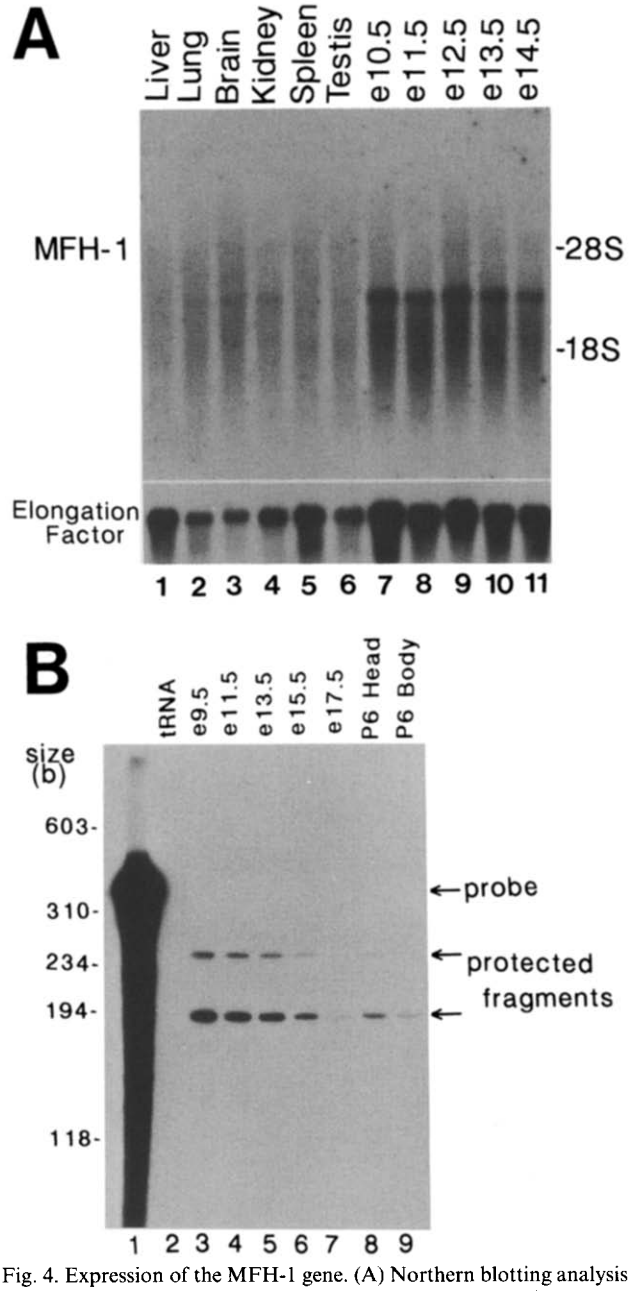

Fig. 4. Expression of the MFH-1 gene. (A) Northern blotting analysis of poly(A)$^+$ RNA from adult tissues and embryos. Poly(A)$^+$ RNAs (5 μg) from adult liver (lane 1), lung (lane 2), brain (lane 3), kidney (lane 4), spleen (lane 5), and testis (lane 6) and from 10.5 dpc (lane 7), 11.5 dpc (lane 8), 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 μg 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.

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 were 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.

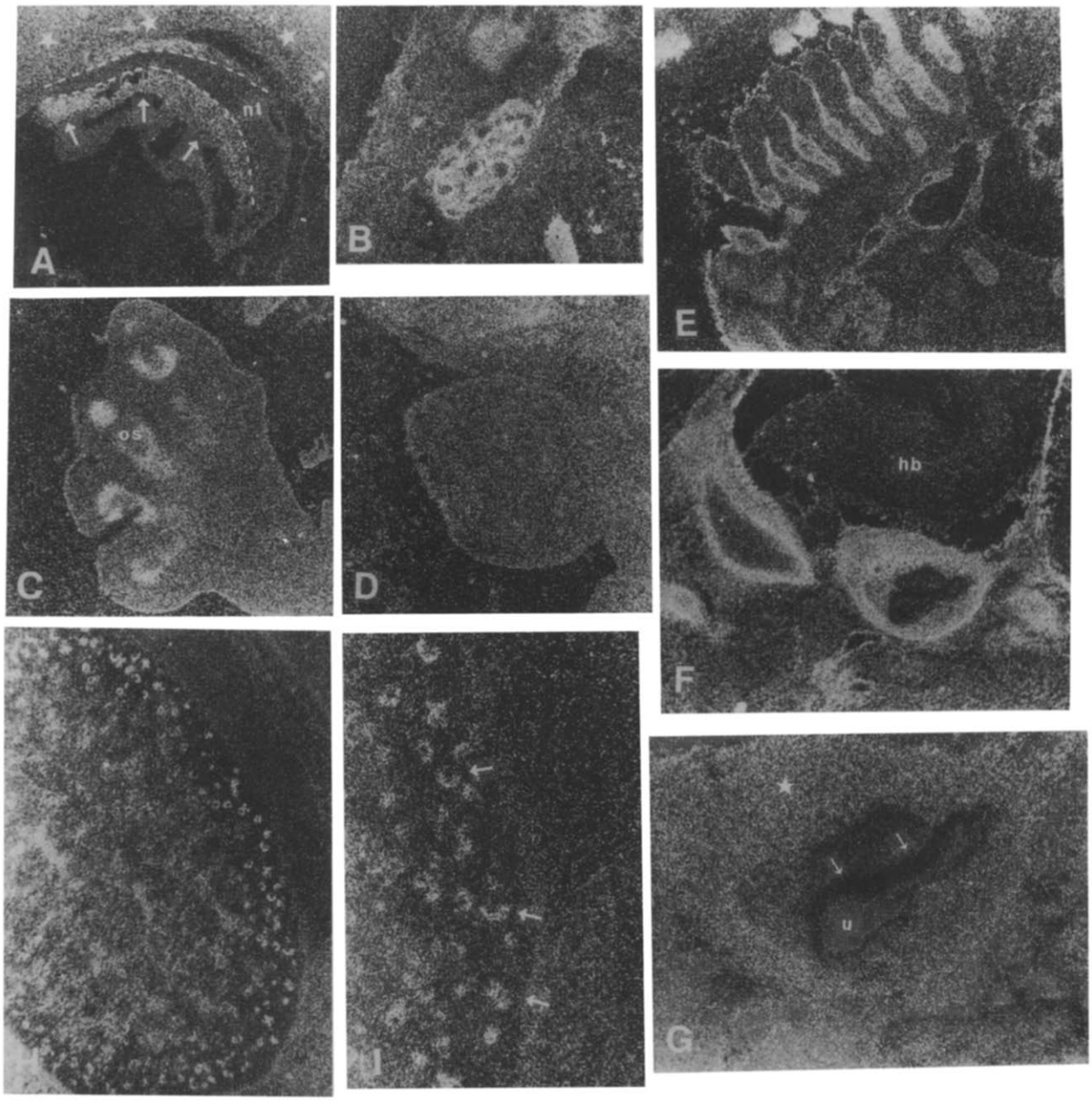

Fig. 5. Dark-field photomicrographs showing the locations of MFH-1 mRNA expression in embryonic and postnatal mouse tissues. (A) e9.5 embryo. MFH-1 messages are observed in the somites (arrows), while the neural tube (nt) is not labeled. Asterisks indicate the uterus. (B) Metanephros of an e13.5 embryo. Note intense labeling over the compact mesenchyme surrounding the collecting ducts. (C) Limb bud of an e13.5 embryo. The bond 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 MHF-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)$^+$ 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]. HFN3α, -β, and -γ seem to be restricted to endotherm-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 identication of the target genes and inactivation of the MFH-1 gene in mice by homologous recombination.

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