Structural and functional characterization of the human and mouse fibulin-1 gene promoters: role of Sp1 and Sp3

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Fibulin-1 is a multifunctional extracellular protein involved in diverse biological processes including cardiovascular development, haemostasis and cancer. To investigate the transcriptional regulation of the gene encoding fibulin-1 we cloned and analysed about 4.0 kb of the 5'-flanking regions of both the human and mouse fibulin-1 genes. The human and mouse fibulin-1 promoters share little sequence similarity except for a short region of approx. 150–170 bp immediately upstream of the translation start site. The conserved region contains a TATA-like sequence (ATAATT) and multiple consensus binding sites for Sp1 and activator protein 2 (AP-2). That the short conserved region in each gene confers basal promoter activity is demonstrated by transient transfections of promoter deletion constructs for

both the human and mouse genes into cells that express fibulin-1 constitutively. Co-transfections of promoter constructs with expression plasmids for Sp1, Sp3 and Sp4 into *Drosophila* SL2 cells indicate that Sp1 and Sp3 are essential for transcriptional activation and that these two factors act synergistically. Electrophoretic mobility-shift assays show that Sp1 and Sp3, but not AP-2, bind to the basal promoter of the human fibulin-1 gene. The results demonstrate the functional importance of Sp1 and Sp3 in regulating the expression of the fibulin-1 gene.

Key words: basement membrane, cardiovascular, DNA binding, extracellular matrix, transcription.

INTRODUCTION

The fibulins comprise a family of extracellular matrix proteins characterized by the presence of repeated calcium-binding epidermal-growth-factor-like modules (domain II) and a homologous C-terminal globular domain (domain III) [1,2]. Five members have been identified so far [3–8]. The first two members, fibulin-1 and fibulin-2, contain an additional protein domain (domain I) at the N-terminus, which consists of three anaphylatoxin-like motifs. Fibulin-1 possesses two unique features not shared by the other family members. First, its domain III is variable because of alternative splicing [1,9]. At least four different forms, A–D, have been reported, of which the C and D forms are the most prevalent. The other four fibulins show no variation in their domain III, which resembles the C form of fibulin-1. Secondly, fibulin-1 is found not only in the extracellular matrix but also in the blood plasma, where it interacts with fibrinogen and might be involved in haemostasis and thrombosis [1,10,11].

As an extracellular matrix protein, fibulin-1 is found in the basement membranes and stroma of most tissues [2,12,13]. It is expressed very early during embryogenesis in the basement membranes and at sites undergoing epithelial–mesenchymal transformation, such as the neural crest, endocardial cushion tissue and the myotome [12,14]. During embryonic development, fibulin-1 is expressed prominently in the outflow tract and endocardial cushion in the heart [15,16] and is also expressed at the region of mesenchymal cell condensation during chondrogenesis [17]. At the adult stage, fibulin-1 remains expressed strongly in the cardiac septa, valves and great vessels and contributes to the structural integrity of these connective tissues. Ultrastructural studies show that fibulin-1 is localized in elastic microfibrils in skin and blood vessel walls [13,18]. Consistent with its tissue localization is the interaction of fibulin-1 with a

variety of extracellular ligands *in vitro*, including elastin, endostatin, fibrinogen, integrins, proteoglycans and various basement-membrane components [11,19–24].

Fibulin-1 has also been shown to have a role in cancer. It is a major protein secreted by the BG-1 ovarian cancer cells after treatment with oestrogen [25]; the protein expression is increased in the stroma of human ovarian epithelial tumours [26]. In contrast, low or no fibulin-1 expression is found in other tumour cell lines, and the overexpression of fibulin-1 suppresses tumour formation and invasion [27]. In addition, fibulin-1 binds NOVH, a member of the connective growth factor family, thus seeming to have a role in growth factor signalling [28]. The involvement of fibulin-1 in cell growth is further supported by a recent study [29] showing that fibulin-1 binds β -amyloid precursor protein and blocks the β -amyloid precursor-protein-mediated proliferation of neural stem cells.

We previously isolated and characterized the entire gene encoding mouse fibulin-1, which spans 90 kb of genomic DNA and comprises 18 exons encoding the two major variants, C and D [30]. We also mapped the gene to human chromosome 22q13 and to mouse chromosome 15, band E–F [31]. In the human fibulin-1 gene, two additional exons encode the A and B forms. To understand the molecular mechanisms responsible for the expression of fibulin-1 gene, we isolated and functionally characterized the promoters of both the human and the mouse fibulin-1 genes.

EXPERIMENTAL

Cell culture

Primary mouse embryonic fibroblasts were established from embryonic day 13 mouse embryos as described [32]. Human

Abbreviations used: AP-2, activator protein 2; EMSA, electrophoretic mobility-shift assay.

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foreskin fibroblasts were established from discarded foreskin tissue of newborn males. Human HeLa cells, mouse NIH 3T3 cells, and *Drosophila* Schneider SL2 cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). Human and mouse cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Gaithersburg, MD, U.S.A.), 50 units/ml penicillin and 50 µg/ml streptomycin sulphate at 37 °C in air/CO₂ (19:1). *Drosophila* SL2 cells were grown at 25 °C without CO₂ in Schneider's medium (Life Technologies) supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin sulphate.

RNA isolation

Total RNA was isolated from cultured cells or from mouse heart tissue with TRIzol reagent in accordance with the protocol suggested by the manufacturer (Life Technologies). Poly(A)⁺ RNA was purified by using the mRNA purification kit from Ambion (Austin, TX, U.S.A.).

Oligonucleotides

Oligonucleotides were synthesized and purified by HPLC at the Nucleic Acid Core Facility of the Kimmel Cancer Center at Thomas Jefferson University (Philadelphia, PA, U.S.A.). Double-stranded oligonucleotides were associated by incubating 10 µg each of the sense and anti-sense strands in a volume of 100 µl in a thermocycler for 5 min at 95 °C, followed by 15 min at each of 75, 55, 45 and 35 °C.

Genomic cloning, Southern blotting and DNA sequencing

To isolate the human fibulin-1 gene, a full-length human fibulin-1 cDNA, HBM8 (T. C. Pan and M.-L. Chu, unpublished work), was used as a probe to screen genomic libraries from Incyte (St Louis, MO, U.S.A.). A positive PAC clone, PACH-32ml, in pADSacBII vector was characterized by Southern hybridization with various subfragments of the cDNA. Purified DNA from PACH-32ml was digested with *Bam*HI and the resulting fragments subcloned into pBluescript II vector (Stratagene, La Jolla, CA, U.S.A.). The subclone library was screened with a 334 bp cDNA probe encoding the most 5' end of the cDNA, yielding a positive subclone with a 13 kb *Bam*HI insert.

A genomic clone, P9, encoding the 5' end of the mouse fibulin-1 gene was previously isolated from a mouse strain 129sv genomic library in λFixII vector (Stratagene) [30]. A 4.9 kb *Pst*I fragment containing exon 1 was subcloned in pBluescript II vector and was completely sequenced.

Southern blotting and subcloning followed standard protocols [33]. PAC DNA was prepared by using KB-100 columns in accordance with the protocol provided by the supplier (Incyte). Plasmid DNA species were purified with Qiagen plasmid kits (Qiagen, Valencia, CA, U.S.A.). DNA probes were randomly prime-labelled with [α -³²P]dCTP (ICN, Costa Mesa, CA, U.S.A.) with the rediPrime II kit (Amersham Pharmacia, Piscataway, NJ, U.S.A.). DNA sequencing was performed with the fluorescent dye-terminator chemistry (Applied Biosystems, Foster City, CA, U.S.A.) and gene-specific primers, and was analysed with an ABI377 automated DNA sequencer at the Nucleic Acid Core Facility of the Kimmel Cancer Center at Thomas Jefferson University.

Potential regulatory elements in the 5' end of the gene were analysed by using the SignalScan computer program [34]. DNA sequences for the human and mouse fibulin-1 promoters were

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-882  tggagcaccat gttgtatgcg ttctgtctga gtgtaaggct gggcggggcg
-832  tcggtggctc aaggggtcca tggtagaact acaggaacac cgctttgagc
-782  tgcccagtgg cgctccatcg cgctccatcc agttccctcg catcttcacc
-732  tgaccatttt tattttcttt ctttggaaaa taaatctacc tgtggttcca
-682  aagcctaact catctcagc aaccccggtg gccatgaatt cctattcaag
-632  aggcctccgc aggcaccagc ccaaaagctg ctcccctgca gccctcggtg
-582  ctcccggtt ctctccctcc ggccctcggtg tcccgggtga gccccagaca
-532  cgttgggatt ttggttggtt gttggcctgg ccgactgtc cctgtctctg
-482  agggcagagt cgggtcccat cccgggacac gccctggggt agccaccgac
-432  ctgcagctc ccggccgagg gtgcgggatg gccggttaag cctgcccag
-382  tgcccccag cagccgggac tcgactgca gccccgggtc ccgctctcc
-332  tcgcgcgga gtcggggg gataaccttt gatcccgagc cctccctcg
-282  agaggggtg gggggagg gggatcggtt ttcacggagt gttggggat
-232  aggactggg gcacccggc cgaaggcgc cctctgtgc cccctccgg
-182  gaagtctggc atccaggccc tcgccccag ccgtgggcgc gcgccctcg
-132  cagccgggt ccggggagg agggctccgc cagggggcgc cggggaggga
-82  ggaccaggag acccgccg ccgcctccgc cgcgcctcc tcccggcg
-32  gataattgaa cggcgcgcc ctggccagc gtTGGCTGCC GAGGCTCGC
+19  CGGAGCGTGG AGCCCGCGCC GCTGCCCGAC GACCGCGCCC GCGCCTTTGT
+69  CCGCCGCGCG CCACCGCCG TCGCCGCGCG CCATGAGC GCGCGCGCC
+119  GTCGCGCGG GTCCCGTTC CGTGTGTGT GTCGCGCGC CTTGCGTGC
+169  TGGCGCGCG AGtaggggc gtcccggtc cgcggccag cttagggtc

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Figure 1 Nucleotide sequence of the human fibulin-1 promoter

An analysis of the sequence of the 5'-flanking region, exon 1 (capital letters) and the 5' end of intron 1 is shown. The transcription start site determined by primer extension (see Figure 2) is denoted as +1 and is shown by an arrow. Filled circles denote the positions of the first nucleotide of the promoter-reporter constructs with the nucleotide positions and restriction enzymes used for generating the constructs indicated. The filled arrowhead denotes the common 3' end of the constructs, which is located immediately upstream of the translation start site, ATG (in bold). Also indicated are potential regulatory elements (underlined) and the TATA-like sequence (boxed).

aligned with the Bestfit program from Genetics Computer Group (Madison, WI, U.S.A.).

Primer extension and RNase protection analysis

Primer extension analysis was performed with the Primer Extension System from Promega (Madison, WI, U.S.A.). In brief, 20 ng of a primer (5'-TCCATGGGCGGCGGGCGACG-3', nt +106 to +86; see Figure 1) labelled with [γ -³²P]ATP (ICN) was hybridized with either 1 µg of human foreskin fibroblast poly(A)⁺ RNA or 10 µg of yeast total RNA in 15 µl of 150 mM KCl/10 mM Tris/HCl (pH 8.3) for 3 h at 50 °C. Primer extension reactions were performed in a final volume of 50 µl with 2.5 units of avian-myeloblastosis-virus reverse transcriptase and 20 units of RNase inhibitor at 42 °C for 1 h. The reaction products were analysed on a 5% (w/v) polyacrylamide/8 M urea gel.

For the RNase protection assay, a 580 bp *Sma*I-*Sac*I fragment flanking exon 1 of the mouse fibulin-1 gene was isolated from the 4.9 kb *Pst*I subclone and then cloned into the *Sac*I and *Sma*I sites of pBluescript II KS (Stratagene). The clone was cut with *Pvu*II and used as template in an *in vitro* transcription system with T3 polymerase (Ambion) to prepare a 344 nt [γ -³²P]UTP-labelled RNA probe. The RNA probe was loaded on a 5% (w/v) polyacrylamide/8 M urea gel and subsequently eluted into a

buffer containing 0.5 M ammonium acetate, 1 mM EDTA and 0.2% SDS. Approximately $(1-2) \times 10^5$ c.p.m. of the probe was annealed to 1 μ g of poly(A)⁺ RNA from mouse embryonic fibroblasts or from mouse heart tissue, or to 10 μ g of yeast tRNA species in 20 μ l of 80% (v/v) deionized formamide/100 mM sodium citrate (pH 6.4)/300 mM sodium acetate/1 mM EDTA for 16–18 h at 44 °C. After hybridization, the annealed probe–RNA complexes were treated with RNase A/T1 (Ambion) and analysed on a 5% (w/v) polyacrylamide/8 M urea gel.

Promoter–reporter constructs

All fibulin-1 promoter–reporter constructs were prepared by subcloning various genomic fragments prepared by restriction-enzyme digestion into the polylinker of the pGL2-basic vector (Promega), which contains a firefly luciferase gene without a promoter and enhancer. The cloning sites of all reporter plasmids were verified by DNA sequencing to ensure nucleotide fidelity and directionality.

Mouse fibulin-1 promoter fragments were generated by restriction-enzyme digestion of the 4.9 kb *PstI* subclone, using *NcoI* or *XhoI* at the 3' end and *XhoI*, *SacII*, *SacI* or *PstI* at the 5' end. The 3' ends of the constructs corresponded to nt +76 or +19; their 5' ends corresponded to +19, –69, –418, –3622 and –3972.

Human fibulin-1 deletion constructs were generated from the 13 kb *BamHI* subclone by digestion at the 3' end with *NcoI*, and at the 5' end with *SmaI*, *SacII*, *XhoI* or *NcoI*. The common 3' end of the constructs corresponded to +101; their 5' ends corresponded to –38, –68, –285 and –814.

Transient transfections and reporter assays

Approximately 10^7 HeLa cells and 2×10^6 mouse embryonic fibroblasts or NIH 3T3 cells were plated in six-well plates 24 h before transfection. Cells were co-transfected with 2 μ g of each firefly luciferase reporter plasmid and with either 200 ng of the pRL-SV40, in which SV40 stands for simian virus 40, or pRL-null vector (Promega), which served as an internal control for normalizing the expression of the firefly luciferase reporter [35]. The pRL-SV40 plasmid contains early SV40 enhancer/promoter-driven *Renilla* luciferase; the pRL-null plasmid contains *Renilla* luciferase without a promoter and enhancer. Transfections were performed with either Eugene 6 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) or Superfect (Qiagen) in accordance with the manufacturers' protocols. Cells were harvested 48 h after transfection and cell extracts were prepared. The luciferase activity was assayed with a dual luciferase reporter assay system (Promega) and was measured in a luminometer (model FB12; Zylux, Maryville, TN, U.S.A.). The firefly luciferase reading was normalized against that of the co-transfected *Renilla* luciferase. All transfections were performed in duplicate or triplicate and repeated at least three times.

Co-transfections of Sp expression plasmids

Approximately 3×10^6 *Drosophila* SL2 cells were plated in 60 mm dishes 24 h before transfection. Cells were transfected by the calcium phosphate precipitation method [36]. Each plate was transfected with 1 μ g of a specific luciferase reporter plasmid, 0.1–1.0 μ g of pPacSp1 [37], pPacSp3 or pPacSp4 expression plasmids [38–40] and 0.2 μ g of pRL-null vector. The total amount of expression plasmid in each plate was adjusted to 1 μ g with the pPac vector, which contains only the *Drosophila* actin promoter. The pPacSp1 plasmid, which expresses Sp1 from the *Drosophila* actin promoter, was generously provided by Dr R. Tjian (De-

Table 1 Oligonucleotides used in PCR and EMSA

For double-stranded oligonucleotides used in EMSA, only the sense strand is shown. Binding sites in the Sp1 and AP-2 oligonucleotides are underlined with mutations in bold.

Oligonucleotide	Nucleotide position	Sequence of the sense strand (5' → 3')
HEX1	+106 to +87	TCC ATG GGC GGC GGG CGA C
HEX2	–4 to +16	GCG TTG GCT GCC GAG GCT C
HEX3	+17 to –3	CCG AGC CTC GGC AGC CAA CG
HEX4	–92 to –71	CGG GGA GGG AGG ACC AGG AG
O2A	–21 to +9	GGC GCG GCC CTG GCC CAG CGT TGG CTG CCG
O2B	–46 to –18	CTC CTC CCG GGC GGG ATA ATT GAA CGG CGC
O2C	–81 to –43	ACC CGC GGC CCC GCC TCC GCC GCG CCC TCC
AP2W	Wild-type AP-2	GAT CGA ACT GAC CGC CCG CGG CCC GT
AP2M	Mutated AP-2	GAT CGA ACT GAC CGC TTG CGG CCC GT
SP1W	Wild-type Sp1	ATT CGA TCG GGG CGG GGC GAG C
SP1M	Mutated Sp1	ATT CGA TCG GTT CGG GGC GAG C

partment of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California, Berkeley, U.S.A.). The pPacSp3 and pPacSp4 plasmids, which express Sp3 and Sp4 respectively from the *Drosophila* actin promoter, were generously provided by Dr G. Suske (Institut für Molekularbiologie und Tumorforschung, Klinikum der Phillips-Universität Marburg, Marburg, Germany). Cells were harvested 48 h after transfection and the luciferase activity was determined as described above.

Electrophoretic mobility-shift assay (EMSA)

Nuclear proteins were prepared from HeLa cells as described [41]. Two double-stranded probes covering the basal promoter of the human fibulin-1 gene were prepared by PCR amplification of the 13 kb *BamHI* genomic subclone. Specifically, PCR1 (from –4 to +106) was amplified with Hex1 and Hex2 primers, and PCR2 (from –92 to +17) was amplified with Hex3 and Hex4 primers (Table 1). Three double-stranded oligonucleotides, O2A, O2B and O2C, within PCR2 were prepared by annealing the sense and anti-sense synthetic oligonucleotides (Table 1). DNA probes were 5'-end-labelled with T4 polynucleotide kinase and [γ -³²P]ATP (ICN). A typical EMSA reaction contained 15–30 μ g of nuclear proteins, 1 ng $[(2-5) \times 10^4$ c.p.m.] of labelled probe, 25 mM Hepes, pH 7.9, 5 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol and 1 μ g of poly(dI-dC) in a final volume of 25 μ l. The reactions were preincubated at 25 °C for 15 min before the addition of the labelled probe, followed by a 30 min incubation at 25 °C. Competitor oligonucleotides (Table 1) and antibodies were added at the same time as nuclear extracts. For supershift experiments, 1 μ l of polyclonal antibodies specific for Sp1, Sp3 or Sp4 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) were used. The bound and free probes were resolved on a 4–20% (w/v) polyacrylamide gel (BioWhittaker, Walkersville, MD, U.S.A.) in 1 × Tris/borate/EDTA buffer at 15 mA for 90 min at 4 °C. Gels were dried and bands were revealed with a Phosphor-Imager (Amersham Pharmacia).

RESULTS

Cloning of the 5'-flanking region of the human fibulin-1 gene and determination of the transcription start site

A PAC clone, PACH-32ml, containing the human fibulin-1 gene was isolated by screening a genomic library with a full-length cDNA. Southern blot analysis with the 5' end of cDNA as a probe revealed that both the PAC clone and the human genomic DNA hybridized with a 13 kb *BamHI* fragment (results not

Figure 2 Analysis of the transcription start site of the human fibulin-1 gene

Primer extension was performed with 1 μ g of poly(A)⁺ RNA (lane 7) from human foreskin fibroblasts or with 10 μ g of yeast tRNA (lane 6) as a negative control, by using a ³²P-labelled oligonucleotide within the first exon as described in the Experimental section. The transcription start site (marked with an asterisk) was identified by running sequencing reactions of a genomic clone containing exon 1 with the same primer on the same gel (lanes 2–5). The sequence surrounding the transcription start site is shown on the right. A DNA size marker end-labelled with ³²P indicates the length of the nucleotide sequence (lane 1).

shown), suggesting that there was no DNA rearrangement within this region of the PAC clone. The 13 kb *Bam*HI fragment was subcloned and a 5 kb region surrounding exon 1 was sequenced (Figure 1).

The transcription start site of the human fibulin-1 gene was determined by primer extension with an anti-sense oligonucleotide located in exon 1. A single band of 106 nt was obtained in mRNA from human foreskin fibroblasts but not in control tRNA from yeast (Figure 2). A comparison with the DNA sequencing ladder suggested that transcription started from a T base 101 nt upstream of the translation start site.

Cloning of the 5'-flanking region of the mouse fibulin-1 gene and determination of the transcription start site

We previously isolated a genomic phage clone, P9, encoding the 5' end of the mouse fibulin-1 gene [30]. Southern blot analysis revealed that a 4.9 kb *Pst*I fragment in P9 contained exon 1. DNA sequencing indicated that the fragment contained 4.0 kb of the 5'-flanking region, exon 1 of 155 bp and 0.8 kb of intron 1 (Figure 3). Several attempts with primer extension to map the 5' end of the gene were unsuccessful, probably because the DNA

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-422  tttgagctca ggtcctcttg tttccacagc aagtgcttta tccactgagc
      SacI
-372  catcgagcga gccattttcc cagcttttaa aaaaaaatt tttattgtct
-322  tttttttttt ttttaataaa tcagagagtc ctctaaactg tctgcgggct
-272  gccaggaggt ctacacaagc aggtctctgc tggcttagcc tacggctgcc
-222  cgctatcctg acaattttag ttagacactt ttcacgtgac tttctgcctg
      E-Box
-172  taggtgggtg gcacgtctct gagcttttgc ctctgcctgc atccccgcag
-122  gagagtcctg gtggcggcgt gctccagctg cgcagtgcgt aaggaggagg
      AP-2 -69 AP-2/Sp1
-72  ccccgccggc cccgccccc cgcgcctcc tccggcggg ataattgaac
      SacII AP-2/Sp1
-22  gggcgccgca ggaagctcgc gtTGGCTGCC CAGGCTTGGC TCGAGCGTGT
      Sp1 +2 AP-2 +19 XhoI
+29  AGCCGCTGCC CGCGTGCCT TTGTCCGCC CCGAGCCCG CAGGCCCAT
      Sp1 NcoI
+79  GAGAGCGCCC GTGCCGTCG GCCTCTGCC GCTGCCGCT CTGCTGCTCA
      AP-2 AP-2
+129 GCAGCCTCTC GCTGCTGGCA GCCGAGGta ggggagcccc gggccacagg
+179 gccgcactag gtcccaccac aggccccaag ctccctggaa gtttgcaagg

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Figure 3 Nucleotide sequence of the mouse fibulin-1 gene promoter

An analysis of the sequence of the 5'-flanking region, exon 1 (capital letters) and the 5' end of intron 1 is shown. Solid and broken arrows indicate the major and minor transcription start sites respectively, as determined by the RNase protection assay (see Figure 4). The T corresponding to the transcription start site of the human fibulin-1 gene is assigned as +1 (see the text). Filled circles denote the positions of the first nucleotide of the promoter-reporter constructs with the nucleotide positions and restriction enzymes used for generating the constructs indicated. The filled arrowhead denotes the 3' end of all constructs, except for two constructs that terminated at +19. Also indicated are the positions of potential regulatory elements (underlined) and the TATA-like sequence (boxed).

sequence was extremely GC-rich. We therefore performed an RNase protection assay with an anti-sense RNA probe. As shown in Figure 4, one major and three minor protected fragments between 167–147 bp were obtained with poly(A)⁺ RNA from either mouse embryonic fibroblasts or heart tissue. The major protected band of about 164–167 bp mapped to a region approx. 70 bp upstream of the translation start codon and corresponded well to the transcription start site of the human fibulin-1 gene determined by primer extension described above. We therefore arbitrarily assigned a T, which is homologous to the transcription start site in the human fibulin-1 gene, as +1.

Sequence analysis of the human and mouse fibulin-1 promoters

A comparison of the human and mouse fibulin-1 promoters revealed little sequence similarity in the 4.0 kb 5'-flanking region, except for a short stretch of approx. 150–170 bp in the proximal promoter region (Figure 5). The conserved stretch started approx. 70 bp upstream of the transcription start site to the translation start site. High sequence similarity was also seen in the translated region of exon 1 and in the first 100 bp of intron 1. The proximal promoters of both species were extremely GC-rich. Specifically, the 170 bp region upstream of the translation start site in the human gene was 84% GC and the homologous region of 150 bp in the mouse gene was 79% GC (Figure 5). The coding regions in exon 1 as well as the 5' ends of intron 1 in both human and mouse genes were also GC-rich (Figure 5). As a consequence of this high content of GC, the regions upstream and downstream of the transcription start sites in both human and mouse genes contained numerous CpG dinucleotides. It is known that the C residues in the CpG dinucleotides are the major sites at which DNA methylation occurs and that the state of methylation of the 5' end of the gene is linked to the control of gene transcription. There were 58 CpG dinucleotides in the region from –72 to +349 in the human gene and 38 CpG dinucleotides in the homologous region of the mouse gene (–71 to +328; Figure 5).

Figure 4 Analysis of the transcription start site of the mouse fibulin-1 gene

An RNase protection assay was performed with 1 μ g of poly(A)⁺ RNA from mouse heart (lane 7) and mouse embryonic fibroblasts (lane 6), or with 10 μ g of yeast tRNA (lane 5) by using a 344 bp [α -³²P]UTP-labelled anti-sense probe (lane 2) as described in the Experimental section. The protected fragments were run on an 8% (w/v) sequencing gel together with a DNA size marker (lane 1), a 172 nt RNA size marker (lane 3) and a 123 nt RNA size marker (lane 4). RNA size markers were generated by the transcription *in vitro*, with T7 polymerase, of a control plasmid linearized with two different restriction enzymes. Arrowheads on the right indicate major and minor transcription start sites.

Figure 5 Alignment of the nucleotide sequences of the human (H) and mouse (M) fibulin-1 gene promoters

The alignment was performed with the Bestfit program of the GCG DNA analysis software; the region with high similarity is shown. The sequence of exon 1 is shown in bold. The translation start sites and the ATAATT sequences are circled. Potential binding sites for Sp transcription factors are boxed. Arrows indicate the transcription start sites.

It is conceivable that methylation of some CpG dinucleotides might regulate fibulin-1 gene expression. Both promoters contained a TATA-like sequence, ATAATT, 30 bp upstream of the transcription start site, but lacked a canonical CAAT box and initiator element.

Figure 6 Functional analysis of the mouse fibulin-1 gene promoter

Upper panel: a schematic representation of deletion constructs, identified by nucleotide positions at the 5' and 3' ends of the constructs. Thin lines indicate promoter sequences and boxes indicate the firefly luciferase reporter. The putative *cis*-acting elements and restriction enzymes used for preparing the deletion constructs are indicated at the top. Lower panel: luciferase activities of the deletion constructs in mouse embryonic fibroblasts (open bars) and in NIH 3T3 cells (filled bars). Each construct was co-transfected with the pRL-TK vector, which harbours the *Renilla* luciferase reporter driven by the thymidine kinase promoter. The activities were measured with the dual luciferase reporter system and the values for the firefly luciferase were normalized to those of *Renilla* luciferase. The results are means $\pm$ S.D. for at least three separate transfections performed in duplicate or triplicate. Values are expressed as percentages of that of the pGL2-Control vector, which contains the SV40 promoter and enhancer. The promoterless pGL2-Basic vector was used as a negative control.

The 5 kb regions encompassing the human and mouse promoters contained numerous potential binding sites for Sp and activator protein 2 (AP-2) transcription factors (partial sequences are shown in Figures 1 and 3) [42]. There were also several E-boxes (CANNTG), which bind basic helix-loop-helix transcription factors. An AP-1 consensus sequence was located at -2270 of the mouse promoter (results not shown). In both promoters the Sp and AP-2 sites were concentrated in the region approx. 500 bp upstream of exon 1 (Figures 1 and 3). Several Sp sites were also clustered within exon 1 immediately upstream of the translation start site and at the 5' end of intron 1. There were four Alu repetitive sequences of approx. 300 bp each in the region from -1130 to -3430 of the human promoter and three repetitive sequences of 50-100 bp each in the region from -550 to -3400 of the mouse promoter. A 53 bp homopyrimidine stretch was located from -2747 to -2695 of the mouse promoter. Previous studies suggest that oestrogen might up-regulate expression of the fibulin-1 gene [25]. However, there was no consensus oestrogen response element in the region of the human or mouse fibulin-1 promoters analysed, except for three or four half sites (AGGTCA) in the distal promoter and the 5' end of intron 1 in both genes. The AGGTCA sequences were located at -3382, -2549, -1454 and -1192 in the human fibulin-1 gene and at -3622, -1097 and +543 in the mouse gene. These half sites were not conserved between the human and mouse genes. Because the half sites were not clustered within each gene, they are unlikely to serve as oestrogen response elements.

Functional analysis of the mouse fibulin-1 promoter

To assess the promoter activity of the mouse fibulin-1 gene, we prepared several 5' deletion fragments with their 3' ends at either

We prepared four human fibulin-1 promoter deletion constructs. They ranged between 0.14 and 0.9 kb in size and their common 3' end was located immediately upstream of the translation start site at +101 (Figure 7). Transient transfection of these constructs into HeLa cells, which express fibulin-1 mRNA, showed that a short construct, pHF1(−68/+101), of 0.17 kb, conferred significant promoter activity. Two longer constructs, pHF1(−285/

To investigate the role of Sp factors on fibulin-1 transcription, we determined the activity of the human fibulin-1 promoter in *Drosophila* Schneider (SL2) cells, which lack endogenous Sp factors. In SL2 cells, the pHF1(-814/+101) construct did not exhibit significant promoter activity, even though it showed the highest activity in HeLa cells (Figure 8A). Co-transfection with the expression vectors pPacSp1 or pPacSp3 led to marked increases in promoter activities, whereas the pPacSp4 expression vector had little effect. The increases in promoter activities by Sp1 and Sp3 were dose-dependent. For pPacSp1, maximal induction was observed with 0.1 μ g of the expression plasmid; higher concentrations resulted in less stimulation. In contrast, the increases were proportional to the amount of pPacSp3 expression vector, with maximal stimulation at 1 μ g. The results suggested that Sp1 was more effective than Sp3 in activating

Figure 9 EMSAs with the -92 to $+106$ region of the human fibulin-1 promoter

(A) DNA sequence of the -92 to $+106$ region of the human fibulin-1 promoter. Two overlapping DNA fragments, PCR2 (-92 to $+17$) and PCR1 ($+4$ to $+106$), were used in EMSA. Potential binding sites for Sp1 and AP-2 are indicated. The translation start codon (ATG) and the TATA-like sequence are shown in bold. (B) EMSA with the PCR2 ($-92/+17$) probe. 32 P-labelled probe was incubated without (lane 1) or with $15 \mu\text{g}$ (lane 2) or $30 \mu\text{g}$ (lanes 3–9) of nuclear extracts (NE). The competition experiment was performed with 10-fold, 50-fold and 100-fold molar excesses of mutated Sp1 oligonucleotide (M) (lanes 4–6), and with 10-fold, 50-fold and 100-fold molar excesses of wild-type Sp1 oligonucleotide (W) (lanes 7–9). (C) EMSA with the $-4/+106$ probe. 32 P-labelled probe was incubated without (lane 1) or with (lanes 2–5) $30 \mu\text{g}$ of nuclear extracts. The competition experiment was performed with 10-fold, 50-fold and 100-fold molar excesses of wild-type Sp1 oligonucleotide (W) (lanes 4–6) and with mutated Sp1 oligonucleotide (M) (lane 6).

fibulin-1 promoter. The mouse construct pMF1($-418/+76$) showed similar stimulation by the Sp1 and Sp3 expression vectors (results not shown). Co-transfection of pPacSp1 and pPacSp3 expression vectors into SL2 cells showed that they activated fibulin-1 promoter synergistically (Figure 8B).

Sp1 and Sp3 bind to the basal promoter of the human fibulin-1 gene

Transient transfections indicated that the basal promoters of both human and mouse fibulin-1 genes lay between a conserved *Sac*II site and the translation start codon (see Figures 1, 3 and 5). As described above, the DNA sequences of the basal promoters were highly conserved between the two species, except that the mouse minimal promoter was 25 bp shorter. To examine the protein-binding activity of the basal promoter, we performed EMSA experiments with two overlapping DNA fragments covering the 170 bp human fibulin-1 basal promoter (Figure 9A). The addition of nuclear proteins to 32 P-labelled PCR2 (from -92 to $+17$) or PCR1 (from -4 to $+106$) resulted in the formation of

two DNA–protein complexes, C1 and C2 (Figures 9B and 9C). We consistently noted that the complexes formed with the PCR1 fragment were significantly weaker than those formed with the PCR2 fragment. Both C1 and C2 complexes competed with an unlabelled Sp1 consensus oligonucleotide (SP1W), whereas a mutated Sp1 oligonucleotide (SP1M), consensus and mutated AP-2 oligonucleotides were not able to affect protein binding even at 100-fold molar excess. The results suggested that the Sp family of proteins participated in binding to the basal promoter.

We next analysed proteins binding to three overlapping double-stranded oligonucleotides (O2A, O2B and O2C) within PCR2 (Figure 10A). As shown in Figure 10(B), only O2C was able to form the C1 and C2 protein complexes. Both complexes could be abrogated by unlabelled O2C itself or by the consensus Sp1 oligonucleotide, but could not be abrogated by the mutated Sp1 oligonucleotide or the consensus or mutated AP-2 oligonucleotides (Figure 10C). To determine the identity of the proteins binding to O2C, supershift assays were performed with antibodies specific for different Sp proteins (Figure 10D). DNA–protein complex C1 could be supershifted by an antibody against Sp1; the formation of complex C2 was inhibited by an antibody

Figure 10 EMSAs with the -81 to +9 region of the human fibulin-1 promoter

(A) DNA sequence of the -82 to +9 region with three synthetic oligonucleotide probes, O2A, O2B and O2C, indicated. (B) EMSA with O2A, O2B and O2C probes. 32 P-labelled O2C (lanes 1 and 2), O2B (lanes 3 and 4) and O2A (lanes 5 and 6) probes were incubated without (lanes 1, 3 and 5) or with (lanes 2, 4 and 6) 30 μ g of nuclear extracts (NE). (C) EMSA with O2C probe and oligonucleotide competitors. 32 P-labelled O2C probe was incubated without (lane 1) or with 30 μ g (lanes 2–10) of nuclear extracts. Reactions were competed with 50-fold and 100-fold molar excesses of unlabelled wild-type Sp1 oligonucleotide (lanes 3 and 4), a 50-fold molar excess of mutated Sp1 oligonucleotide (lane 5), 50-fold and 100-fold molar excesses of AP-2 oligonucleotide (lanes 6 and 7), a 50-fold molar excess of mutated AP-2 oligonucleotide (lane 8) and 50-fold and 100-fold molar excesses of unlabelled O2C oligonucleotide. (D) EMSA with the O2C probe and Sp antibodies. 32 P-labelled O2C probe was incubated without (lane 1) or with (lanes 2–5) 30 μ g of nuclear extracts. Reactions included 1 μ l of antibodies specific for Sp1 (lane 3), Sp3 (lane 4) and Sp4 (lane 5).

against Sp3. The formation of either C1 or C2 was not significantly affected by an antibody against Sp4, although a weak supershifted band was observed.

DISCUSSION

The fibulin-1 gene has previously been shown to be involved in cardiovascular development as well as in tumour formation and invasion. However, the transcriptional mechanisms regulating its expression have yet to be investigated. Here we report the cloning and initial characterization of both human and mouse fibulin-1 gene promoters.

We previously showed that human and mouse fibulin-1 genes share many conserved structural features, having a translation start site in exon 1 and a very large first intron more than 15 kb long [30]. However, a sequence comparison of the approx. 4.0 kb 5'-flanking regions of the human and mouse fibulin-1 genes has shown only a very small region with sequence conservation. The conserved region is located immediately upstream of the translation start site. The DNA sequence is extraordinarily GC-rich, with a TATA-like sequence, ATAATT, and multiple potential binding sites for the Sp family of transcription factors, several of which overlap with AP-2 sites. Primer extension shows that the human fibulin-1 gene initiates transcription predominantly at a single site 30 bp downstream of the ATAATT sequence. This suggests that the sequence is probably involved in positioning the transcription initiation complex, similarly to a canonical TATA box [43]. In the mouse fibulin-1 gene, although the predominant transcription start site determined by the RNase protection assay is approx. 30 bp downstream of the ATAATT sequence, transcription is also initiated at additional minor sites (Figure 4). Both human and mouse fibulin-1 promoters

also share structural features characteristic of the TATA-less promoters, e.g. high GC content and multiple Sp1-binding sites. It is known that GC-rich TATA-less promoters initiate transcription from multiple sites and Sp1 has a role in their transcription initiation [44]. Thus both the atypical TATA box and the multiple Sp1-binding sites seem to be involved in transcription initiation in the mouse fibulin-1 gene.

Functional analyses of the human and mouse fibulin-1 promoters indicate that the -68/+101 region of the human gene and the -69/+76 region of the mouse gene confer basal promoter activity (Figures 6 and 7). The regions that confer basal promoter activity correspond precisely to the regions highly conserved between the two species. DNA elements within the basal promoters potentially contributing to transcriptional activation are the ATAATT sequence and multiple Sp and AP-2 sites. Of note is that binding sites for transcription factors are also present in the 5'-untranslated regions. Deletion of the DNA sequence in the 5'-untranslated region of the mouse gene abrogates transcription in mouse embryonic fibroblasts (Figure 6), suggesting that the 5'-untranslated region of the fibulin-1 gene is essential in transcriptional activation. However, the 5'-untranslated region has little effect on transcription in NIH 3T3 cells (Figure 6). The cell-type difference might be related to the high transcriptional activities of all fibulin-1 promoter constructs in NIH 3T3 cells in comparison with those in primary fibroblasts.

EMSA and competition experiments with human fibulin-1 promoter demonstrate that both the 5'-flanking region (-92/+17) and the 5'-untranslated region (-4/+106) bind to the Sp family of proteins but not to AP-2 (Figure 9). It is unclear why the 5'-flanking region shows stronger protein binding than the 5'-untranslated region, because both regions have comparable

numbers of potential Sp-binding sites. Subsequent EMSA analyses performed with three overlapping oligonucleotides covering the 5'-flanking region show that only the most 5' oligonucleotide, O2C, which contains several possible Sp sites, is capable of interacting with nuclear proteins. Unexpectedly, O2B, which contains a typical Sp1 site and the adjacent TATA-like sequence, does not form any DNA-protein complex under the same binding conditions. The results suggest distinct binding affinities for the potential Sp1-binding sites.

Sp1 is a ubiquitous DNA binding protein with three zinc fingers at its C-terminus that activates the transcription of many cellular and viral genes [45]. Sp1 binds not only GC-box motifs (GGGCGG) but also GT (GGGTGTGC) and CTC (CTCCTC-CTC) motifs [46,47]. There are three additional nuclear proteins, Sp2, Sp3 and Sp4, that share a similar modular structure and conserved DNA-binding domain to that of Sp1. Sp3 and Sp4 are closely related to Sp1 and recognize GC/GT boxes with similar affinity. However, Sp2 has a low affinity for a GC box and binds preferentially to GT sequences. Supershift experiments with antibodies against individual Sp proteins demonstrate that Sp1 and Sp3 are able to bind sites in O2C (Figure 10). The involvement of Sp1 and Sp3, but not Sp4, in the transcription of the fibulin-1 gene is also shown by the co-transfection of Sp expression vectors in *Drosophila* SL2 cells, which lack endogenous Sp proteins (Figure 8). Sp1 and Sp3 are ubiquitously distributed, whereas Sp4 has a very restricted expression pattern, being present predominantly in neuronal cells and certain epithelia [48,49]. Fibulin-1 is expressed by several different cell types, including epithelial cells and mesenchymal cells. The findings that fibulin-1 gene transcription is activated by the ubiquitously expressed Sp1 and Sp3, but not by the highly tissue-specific Sp4 are consistent with the expression pattern of fibulin-1. Co-transfections with increasing amounts of the expression plasmids indicate that Sp1 is a more potent transcriptional activator of the fibulin-1 gene than Sp3. It has been shown that Sp3 can either activate or repress Sp1-mediated transcriptional activation [38, 50]. Simultaneous transfection with Sp1 and Sp3 demonstrates that they act synergistically (Figure 8). In this regard, Sp1 and Sp3 regulate fibulin-1 gene expression in a manner similar to their regulation of matrix metalloproteinase 2, a matrix-degrading enzyme implicated in angiogenesis and tumour invasion [51].

The distal regions of both genes also contain binding sites for transcription factors Sp1 and AP-2. Besides participating in basic transcription activation, Sp1 might also have a role in tissue-specific expression by interacting with other transcription factors. In addition, the level of gene expression might vary depending on the ratio of Sp1 to Sp3 in different tissues. AP-2 is a developmentally regulated transcription factor expressed primarily in the neural crest lineage [52]. It is of interest that fibulin-2, which shares overlapping expression patterns with fibulin-1 in the cardiovascular system, also possesses multiple Sp1 sites and numerous potential AP-2-binding sites [53].

In summary, we have characterized the human and mouse fibulin-1 promoters and have identified conserved features that are likely to be important for transcriptional activation. We have delineated the minimal regions in the human and mouse fibulin-1 genes that are necessary for basal transcription and demonstrated essential roles for Sp1 and Sp3. Our studies provide a step towards the future understanding of the *cis*- and *trans*-acting elements involved in the developmental and tissue-specific regulation of the fibulin-1 gene.

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