Organization, chromosomal localization and promoter analysis of the gene encoding human acidic fibroblast growth factor intracellular binding protein

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Acidic fibroblast growth factor (aFGF) intracellular binding protein (FIBP) is a protein found mainly in the nucleus that might be involved in the intracellular function of aFGF. Here we present a comparative analysis of the deduced amino acid sequences of human, murine and *Drosophila* FIBP analogues and demonstrate that FIBP is an evolutionarily conserved protein. The human gene spans more than 5 kb, comprising ten exons and nine introns, and maps to chromosome 11q13.1. Two slightly different splice variants found in different tissues were isolated and characterized. Sequence analysis of the region surrounding the translation start revealed a CpG island, a classical feature of widely expressed genes. Functional studies of the promoter region with a luciferase reporter system suggested a strong transcriptional activity residing within 600 bp of the 5′ flanking region.

Key words: CpG island, gene structure, housekeeping gene, sequence alignment.

INTRODUCTION

It is commonly assumed that the signalling activity of acidic fibroblast growth factor (aFGF) is restricted to binding to cell-surface receptors containing a cytoplasmic tyrosine kinase domain, followed by the activation of a phosphorylation cascade inside the cell. In recent years, evidence has been accumulated that indicates an intracellular function of aFGF as well [1–4]. The growth factor is synthesized as an intracellular protein that is released from the cells by a largely unknown mechanism [1–3]. After binding to its specific FGF receptors, aFGF is partly translocated into the cytosol and nucleus and its nuclear translocation seems to be essential for the mitogenic activity of the growth factor [5]. This mechanism also remains unknown. In an attempt to elucidate the intracellular action of the growth factor, we have identified an intracellular protein interacting with aFGF.

aFGF intracellular binding protein (FIBP) was first cloned as a novel protein binding specifically to the wild-type aFGF, but not to a point mutant, aFGF K132-E [6], which binds to FGF receptors, stimulates tyrosine phosphorylation of the receptors and induces proto-oncogenes and mesoderm formation but is a poor mitogen [7–9]. Since FIBP was first described, no sequence similarity to known human gene products has been found and the biological function of the protein remains to be elucidated. To create a framework for determining the biological role of FIBP we studied the evolutionary sequence conservation and genomic organization of the human *FIBP* gene.

EXPERIMENTAL

Sequence data

The human FIBP cDNA sequence was obtained previously [6] and a sequencing error was corrected (GenBank accession no. NM-004214). Murine FIBP cDNA (GenBank accession no. AF270700), the new splice variant of the human FIBP (GenBank accession no. AF250391) and a human FIBP genomic clone (GenBank accession no. AF250392) were isolated in the present study. The FIBP-like cDNA sequence of *Drosophila melanogaster* was derived from the genomic sequence (GenBank accession no. AE003515) and from an expressed sequence tag (EST) clone (GenBank accession no. AI386501). A genomic *Mse1* fragment corresponding to the 5′ flanking region of the *FIBP* gene was previously cloned as a CpG island (EMBL accession no. Z66504).

Sequence analysis

Database searches were performed by BLAST at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).

Figure 1 was produced with the program CLUSTALW 1.8 run at the Baylor College of Medicine (BCM) (http://dot.imgen.bcm.tmc.edu:9331/multi-align/).

P1-derived artificial chromosome (PAC) subclones were sequenced on an ALF sequencing apparatus (Apbiotech) in accordance with the manufacturer's recommendations.

Isolation of murine FIBP cDNA

The murine FIBP cDNA was generated by PCR amplification from Mouse Brain Marathon Ready cDNA (Clontech) with the primers 5′-ATGACCAGCGAACTAGAC-3′, 5′-ACTTTATTGTCAGCGTGGGG-3′ and Advantage 2 Polymerase Mix (Clontech). The PCR product was purified by agarose-gel electrophoresis and inserted into the pGEM® T-Easy Vector (Promega). After ligation, *Escherichia coli* DH5α were transformed by electroporation. Plasmid DNA was purified from the transformed clones by using a Wizard® Plus SV Minipreps DNA

Abbreviations used: aFGF, acidic fibroblast growth factor; DAPI, 4,6-diamidino-2-phenylindole; EST, expressed sequence tag; FIBP, aFGF intracellular binding protein; FISH, fluorescence *in situ* hybridization; PAC, P1-derived artificial chromosome; RT–PCR; reverse-transcriptase-mediated PCR; SV40, simian virus 40.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers: AF270700 for murine FIBP, AF250391 for human new splice-variant FIBP, and AF250392 for the human FIBP genomic clone.

Figure 1 FIBP amino acid conservation

Alignment of the deduced amino acid sequences of human, mouse and *D. melanogaster* FIBP generated with the CLUSTALW algorithm. Identical residues are boxed in black and similar residues are shaded grey. The similarities between human and mouse FIBP and between human and *D. melanogaster* FIBP are 97% and 46% respectively.

Purification System (Promega) and subjected to automated sequencing.

Human *FIBP* gene isolation and physical mapping

PAC [10] clones were obtained from the RPCI-5 library (http://www.chori.org/bacpac/). High-density colony membranes containing the library clones were screened with an FIBP cDNA probe labelled with [^{32}P]dCTP with the random priming protocol [11]. Hybridizations were performed by the method of Church and Gilbert [12]. PAC DNA was isolated by a modified alkaline extraction protocol [13].

For mapping, DNA from the PACs was digested with *Not*I, separated by pulsed-field gel electrophoresis in a Bio-Rad CHEF Mapper under the following conditions: 7 h 54 min run at 6 V/cm with a linearly increasing pulse time from 0.06 to 17.35 s. For subcloning, 2 μg of RPCI-5-931H5 was digested by *Not*I and *Sac*I, *Bam*HI and *Bgl*II or *Pst*I, inserted into the pNEB193 vector (New England Biolabs) and sequenced automatically.

Isolation of human FIBP splice variants

Splice variants of human FIBP were generated by PCR amplification from a HeLa cDNA library with primers spanning nt 191–210 (5′-ACCATTACCGCACCTTCCAC-3′) and 1111–1130 (5′-ACTTTATTGTCAGCGTGGGC-3′) of the human FIBP cDNA (GenBank accession no. NM-004214). PCR amplification was performed in a DNA thermal cycler Gene Amp® PCR System 970 (PE Applied Biosystems) for 25 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C. PCR products were purified from agarose gels on QIAEX II matrix (Qiagen) and were ligated into the pGEM® T-Easy Vector. *E. coli* DH5α cells were transformed by electroporation with the ligation mixture. Plasmid DNA was purified from the transformed clones with a Wizard® Plus SV Minipreps DNA Purification System (Promega).

Fluorescence *in situ* hybridization (FISH)

PAC DNA (1 μg) was nick-translated and labelled with Cy3-dUTP (Amersham) with the Bio-nick kit from Gibco BRL (Gaithersburg, MD, U.S.A.) in accordance with the manufacturer's recommendations; FISH was performed as described previously [14].

4,6-Diamidino-2-phenylindole (DAPI) fluorescence and probe signals were observed sequentially with a Zeiss Axioplan fluorescence microscope equipped with a 63× (numerical aperture 1.4) objective and a triple-pass emission filter (blue, green and red), a corresponding beam splitter and separate excitation filters (UV for DAPI, 470–490 nm for FITC-labelled centromere probe when employed, 578 nm for Cy3 and Spectrum Orange). All filters ('Pinkel 1' filter set) were obtained from Chroma (Brattleboro, VT, U.S.A.). Images were captured and digitized in a cooled 16-bit CCD camera (Astromed). The localization of the probe signals was determined from the DAPI banding, which gave a resolution of approx. 400 bands.

Transfections and promoter studies

The FIBP promoter sequence (bases 1–1500 in GenBank accession no. AF250392) has been analysed with the CpGplot program [15]. The ratio of observed over expected (obs/exp) CpG was calculated, as described by Gardiner-Garden and Frommer [16], as $N_{CpG}N/N_CN_G$, where N_{CpG} is the number of CpG, N is the total number of nucleotides in the sequence being studied with a 140 bp window ($N = 140$) moving through the sequence at 1 bp intervals, and N_C and N_G are the numbers of C and G respectively. The CpG island definition applied was a region larger than 200 bp, with an average obs/exp CpG higher than 0.6 and an average (G+C) of more than 50% [16].

FIBP 3 (−607 to −203 from ATG) and FIBP 5 (−607 to −4 from ATG) promoter regions were amplified with *Taq* DNA polymerase (Promega) with the primers 5′-GAGCTCGGAGGGGCAGAA-3′ and 5′-AGCACCGCTTCCTCCCGCC-3′, or 5′-GAGCTCGGAGGGGCAGAA-3′ and 5′-GGCGACGCCCGGGGCCGC-3′ respectively. The PCR products were ligated into the pGEM® T-Easy Vector then excised as *Sac*I fragments and cloned into the *Sac*I site of dephosphorylated pGL3® Basic Vector (Promega) containing the Luc$^+$ reporter. U2OS cells maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum were seeded out at 10^5 cells per well (3 cm^2) the day before transfection. On the following day, a mixture containing Fugene® reagent (Boehringer Mann-

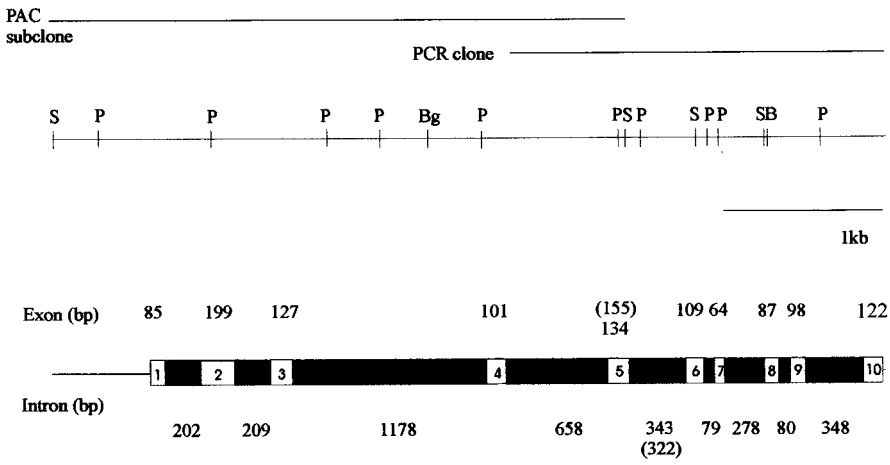

Figure 2 Genomic organization of the human *FIBP* gene

Upper panel: physical map of human *FIBP* gene: B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; S, *Sac*I. Lower panel: exon–intron organization of human *FIBP* gene: black boxes correspond to introns and white boxes represent exons; lengths are indicated in base pairs.

Table 1 Exon–intron junctions of the human *FIBP* gene

Capital and lower-case letters indicate exon and intron sequences respectively. The 3′ end of the alternatively spliced exon 5 is shown in parentheses. Conserved nucleotides to donor acceptor in introns are indicated in bold. Introns interrupt the protein coding sequence between two codons (intron phase 0), after the first nucleotide of a codon (intron phase I) or after the second nucleotide of a codon (intron phase II). The polyadenylation signal is underlined.

No. of exons	3′ intron junction		Size of exon (bp)		5′ intron junction	Size introns (bp)	Intron phase
1		... ATG ACC M T	> 85	GGT TAC TCG G G Y S	**gt**cccgtgag	202	I
2	cgctttcc**ag**	TG ACC GAC V T D	199	CTC ATC GAG AG L I E	**gt**gctcaccc	209	II
3	actccacaa**g**	G TAC TAT R Y Y	127	CGG AGA CAG R R Q	**gt**atgcactg	1178	O
4	tgccctgc**ag**	TTT GAC AAC F D N	101	CGG TTG GCC AG R L A	**gt**gaggagct	658	II
5	gtctgtgc**ag**	G GAC TAT R D Y	134	GGA GCC GTC G G A V	**gt**gaggcccc	343	I
			(155	ACT GAC CCA G T D P	**gt**gcctggac	322	I)
6	ggcctcac**ag**	AC TCA CAG D S Q	109	CTG CAC AAG AG L H K	**tg**gaccttgt	79	II
7	ctctctgc**ag**	C CTG GTG S L V	64	AAC TTC AAG N F K	**gt**ctgtggtc	278	O
8	tgccccac**ag**	AAC CTG TCC N L S	87	GTG GAG AAG V E K	**gt**gagctgtc	80	O
9	gggcccgc**ag**	TTT GTG GAA F V E	98	GAT GGC TTC CG D G F	**gt**gagcagcc	348	II
10	gttcttac**ag**	A CAC CAG R H Q	122	CAT GAC TGA H D *			

3′ UTR GGTGCCTCCCAACGCTCCGCCCACGCTGAC<u>AATAAA</u>GTTGCTCTGAGTTTGG

heim) and plasmid DNA was added. Luciferase (1 μg) and β-galactosidase (1 μg) reporter constructs were used routinely. Transfected cells were harvested and lysed in 250 μl of lysis buffer. The lysates were centrifuged at 18000 ***g*** for 3 min to remove insoluble material. A 50 μl sample was used for determining the protein concentration of the extract with a Protein Assay ESL kit (Boehringer Mannheim), another 50 μl was used to measure luciferase activity with a LucLite Plus kit (Packard) and a further 50 μl was used to estimate the amount of β-galactosidase synthesized from the co-transfected reporter construct, with a chemiluminescent β-Gal Reporter Gene Assay (Roche). Promoter strength was quantified by calculating the ratio of luciferase to β-galactosidase activities.

RESULTS

Sequence conservation of FIBP

To evaluate the amino acid sequence conservation of FIBP, we first amplified murine FIBP cDNA from a mouse brain cDNA library by PCR with FIBP-specific primers. Translated murine FIBP showed 97 % identity with its human counterpart (Figure 1). We extended this observation by an EST database search and found FIBP in many vertebrate species (*Sus scrofa*, *Bos taurus*, *Oryctolagus cuniculus*, *Rattus norvegicus* and *Xenopus laevis*). An additional database search revealed a *Drosophila* FIBP counterpart sharing 46 % amino acid identity with the human protein (Figure 1). However, no obvious FIBP analogues were found in the sequenced genomes of *Caenorhabditis elegans* [17] or *Saccharomyces cerevisiae* [18].

Cloning of the *FIBP* gene from a human PAC library

Screening of the human RPCI-5 PAC library with an FIBP-specific probe resulted in isolation of six positive clones. On the basis of crude restriction mapping and Southern blotting analysis, RPCI-5-931H5, with an insert of approx. 110 kb and at least 8 kb flanking sequence on each side of the *FIBP* gene, was used for further investigations. The insert was digested with either a single restriction enzyme (*Pst*I) or a combination of enzymes (*Not*I and *Sac*I, *Bgl*II and *Bam*HI); the digestion products were subcloned and sequenced. An extended clone encoding the 3′ end of the gene was created by PCR with specific primers from cDNA and PAC DNA as a template and was used for mapping adjacent non-overlapping subclones. On the basis of restriction analysis and genomic sequencing of the subclones, a physical map of the *FIBP* gene was constructed (Figure 2, upper panel). In total, more than 5 kb of the gene was sequenced and submitted to GenBank (accession no. AF250392).

Genomic organization of the *FIBP* gene and its splice variants

A comparison of the genomic sequence of *FIBP* with its cDNA revealed ten exons and nine introns (Figure 2, lower panel). The exact location of the transcription start could not be established by a primer extension technique, probably because of the high GC content of the sequence upstream of the start codon. A canonical polyadenylation signal was located in exon 10, 22 bp upstream of the polyadenylation site. All the exon–intron boundaries followed the GT/AG consensus rule. The sizes determined for exons and introns and their boundary sequences are presented in Table 1.

Although we normally detected what appeared as a single mRNA transcript band on Northern blots [6], an additional band was observed under conditions permitting a better separation of reverse-transcriptase-mediated PCR (RT–PCR) products (results not shown). A double FIBP-specific band was found in a variety of cell lines (HEP2, HeLa, Vero). The sequence analysis of both cDNA forms indicated that the transcript with the higher molecular mass had the fifth exon extended by 21 bp compared with the FIBP cDNA described previously [6], exploiting an alternative 5′ splice junction site further downstream (Table 1). This extension of exon 5 leads to the addition of seven amino acid residues.

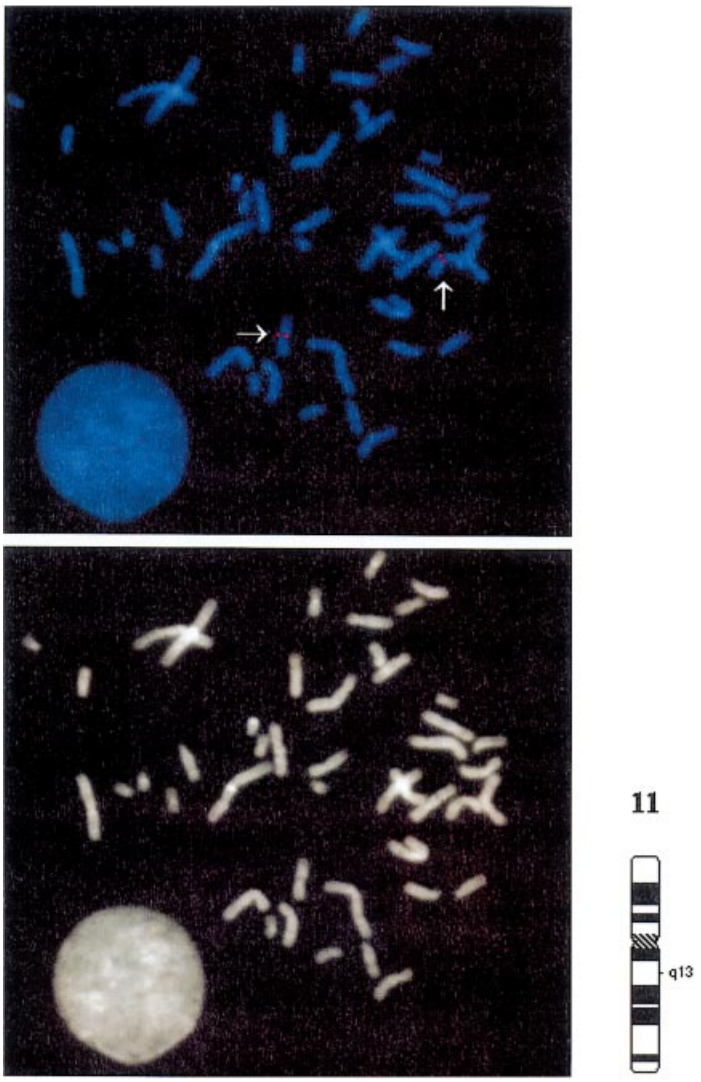

Figure 3 Chromosomal localization of the human *FIBP* gene

Upper panel: FISH staining of *FIBP* loci in human metaphase lymphocytes; chromosomes 11 are indicated by arrows. Lower panel: the same mitotic image stained with DAPI. A diagram of chromosome 11 with band q13 indicated is shown at the right.

Chromosomal localization of the *FIBP* gene

Chromosomal assignment of the gene was performed on normal human lymphocytes by using FISH, with the RPCI-5-931H5 PAC clone of *FIBP* as a probe (Figure 3, upper panel). Hybridization of RPCI-5-931H5 DNA to human metaphase chromosomes revealed labelling in the q13 region of chromosome 11. The identity of the chromosomes was determined from the DAPI banding (Figure 3, lower panel), which gave a resolution

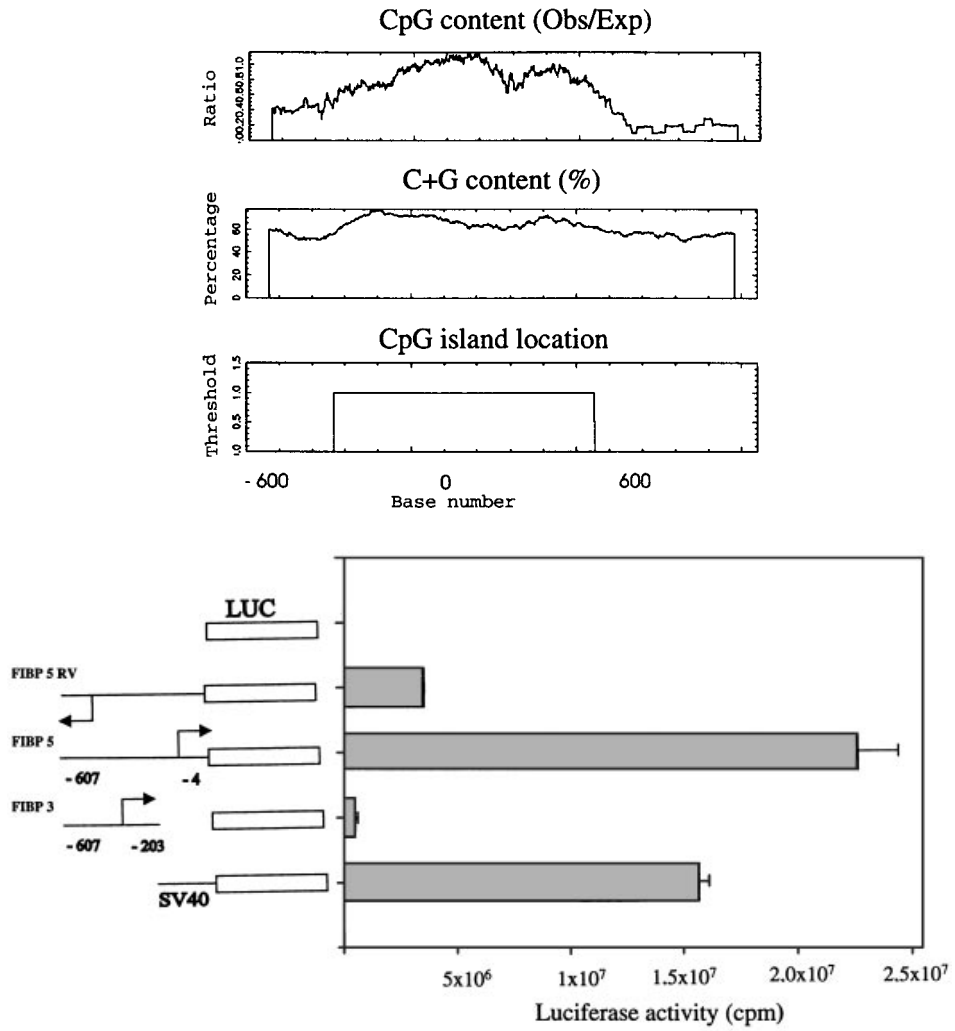

Figure 4 Structural and functional analysis of the human FIBP promoter

Upper panel: position and characteristics of the CpG island associated with the FIBP promoter region, showing obs/exp CpG, (G + C) content and the location of the CpG island. Lower panel: promoter activity of the luciferase reporter gene fused to FIBP 5′ flanking regions of different sizes. A promoterless luciferase construct and a construct encoding the luciferase gene under the SV40 promoter were used as negative and positive controls respectively.

of approx. 400 bands. No other chromosome region showed specific labelling.

Sequence analysis and functional characterization of the FIBP promoter

Because FIBP is expressed in all tissues that we investigated, it is possible that *FIBP* is a 'housekeeping' gene. To investigate this, we sequenced the region surrounding the start codon, analysed the CpG content and expressed it in terms of obs/exp CpG and percentage (G + C) content by using the CpGplot program [15]. The estimated values for obs/exp CpG and percentage (G + C) were plotted against the position in the analysed sequence. On the basis of this analysis, a region of approx. 800 bp fulfilled the criteria for a CpG island with an average obs/exp CpG of higher than 0.6 and an average (G + C) of more than 50 % (Figure 4, upper panel).

Because we did not manage to identify the exact transcription initiation site of the *FIBP* gene by the primer extension method, probably because of its GC-rich character, we decided to study the promoter activity of the region within the CpG island. To determine whether the 5′ flanking region of the *FIBP* gene was sufficient for the initiation of basal transcription, a fragment of 603 bp upstream of the translation start was inserted in both orientations into a luciferase reporter vector and its promoter activity was measured in transiently transfected U2OS cells. We performed a comparative analysis of the luciferase activity in cells transfected with constructs in which the FIBP promoter was inserted in the correct or the reverse orientation. A construct in which the luciferase gene transcription was driven by the simian virus 40 (SV40) promoter and enhancer was used as a control.

Fusion of the 603 bp 5′ flanking region of the *FIBP* gene resulted in a 314-fold increase in luciferase activity compared with the promoterless luciferase construct (Figure 4, lower panel). When the promoter had been cloned in the reverse orientation, its promoter activity was strongly decreased, whereas a construct with a deletion of 199 bp from the 3′ end of the promoter showed an almost complete loss of promoter function. A plasmid containing the SV40 promoter/enhancer used as a positive

control exhibited a 217-fold increase in transcription activity in comparison with the promoterless construct in the cell line used. Clearly, the 5′ flanking region of the *FIBP* gene has a very potent promoter activity.

DISCUSSION

In the present study the structural organization of the human *FIBP* gene has been determined by genomic sequence analysis, revealing a gene spanning 5 kb and consisting of ten exons and nine introns. The exons vary in size from 64 to 199 bp and all exon–intron boundaries follow the GT/AG consensus-sequence rule. A classical polyadenylation signal is located 22 bp upstream of the polyadenylation site.

Our observation of a double FIBP-positive band after RT–PCR amplification with two gene-specific primers and mRNA from several human and simian cell lines prompted us to study the possibility of alternative splicing. One isolated cDNA form was identical with that previously reported [6], whereas exon 5 of the other form turned out to be extended by 21 bp (GenBank accession no. AF250391). These findings could explain the double band of endogenous FIBP recognized by FIBP-specific antibodies on Western blots [6]. The two splice forms were found in the human EST database in ESTs isolated from a variety of tissues. Therefore a strict tissue specificity of each form is unlikely; the biological significance of this diversity remains to be determined. Nevertheless, the new splice variant was clearly under-represented in the EST databases. A similar distribution of the two splice variants was found in mouse EST databases.

During the study we were able to map the *FIBP* gene to the long arm of chromosome 11 by exploiting the FISH technique. This localization is in agreement with the findings that the reported genomic sequence of cathepsin W, previously localized to chromosome 11q13.1 by FISH [19], contains the last exon of the *FIBP* gene. Moreover, several FIBP ESTs were mapped to 11q13 by radiation hybrid mapping [20]. This location seems to be a promiscuous site involved in reciprocal translocations with different chromosomes in myeloid and lymphoid malignancies [21]. Moreover, the genes localized to the 11q13 region are amplified in 30–50% of patients with human head and neck squamous cell carcinomas and in a large proportion of breast and bladder carcinomas [22–24]. Although there are several potential proto-oncogenes in this chromosome region, such as those for cyclin D, FGF3 and FGF4 and the multiple endocrine neoplasia type 1 gene (*MEN1*) [25–27], the possibility remains that FIBP is involved in neoplasia.

As we have reported previously [6], mRNA for *FIBP* is present in all tissues that we have investigated, suggesting that it is a 'housekeeping' gene. The analysis of the 5′ flanking area of the *FIBP* gene revealed that the region satisfies the criteria for a CpG island [16], a classical feature of widely expressed genes.

It is believed that most CpG islands are normally unmethylated, that they have a decondensed chromatin structure and that they contain binding sites for a variety of transcription factors [28]. Although we have not performed any methylation studies on the CpG island covering the putative FIBP transcription start, there is an indication that it is unmethylated. Thus, employing a BLAST homology search [29], we found part of it to be identical with a sequence in a human CpG island library that was based on CpG-enriched DNA inserts in the unmethylated state [30].

To study the promoter properties of the 600 bp sequence upstream of the functional translation start of FIBP we constructed fusion genes of different-sized 5′ flanking fragments with the luciferase gene. The results indicate that the full-length construct (-607 to -4) possesses strong promoter activity in the U2OS cell line, whereas such activity was almost completely abolished by the deletion of -203 to -4, a region in which we assume the transcription start to be located. The FIBP promoter proved to be more powerful than the SV40 promoter, yielding 144% of the activity measured for the latter in U2OS cells.

The cDNA and amino acid sequence comparison of FIBP analogues derived from different species ranging from *D. melanogaster* to *Homo sapiens* presented in this study strongly suggests a strong evolutionary conservation of the protein, which in turn implies an important biological function. However, a further protein structure analysis of the most conserved regions of FIBP will be needed for a full understanding of its intracellular role.

aFGF introduced into cells with diphtheria toxin as a vector was able to stimulate DNA synthesis in cells lacking FGF receptors [2], suggesting a mitogenic function in addition to the activation of phosphorylation cascades through the cell surface receptors. It is possible that FIBP is involved in this action, which is consistent with its being a primarily nuclear protein coded for by a 'housekeeping' gene.

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