Cloning and chromosomal localisation of the murine epidermal-type fatty acid binding protein gene (Fabpe)

Bertram Bleck a, Carsten Hohoff a,b, Bert Binas c, Bernd Rüstow d, Christa Dixkens e, Horst Hameister e, Torsten Börchers b, Friedrich Spener a,*

a Department of Biochemistry, University of Münster, Wilhelm-Klemm-Str. 2, D-48149 Münster, Germany
b Institute of Chemical and Biochemical Sensor Research, Münster, Germany
c Max Delbrück Center for Molecular Medicine, Berlin, Germany
d Department of Neonatology, Charité, Humboldt-University, Berlin, Germany
e Department of Medical Genetics, University of Ulm, Ulm, Germany

Received 3 February 1998; received in revised form 1 May 1998; accepted 1 May 1998; Received by T. Sekiya

Abstract

We succeeded in cloning the gene encoding the murine epidermal-type fatty acid binding protein (E-FABP). To avoid the screening of pseudogenes, the presence of which was shown by PCR, we designed an intron-specific probe and screened a bacterial artificial chromosome library from mouse embryonic stem cells. One of the clones obtained was analysed by restriction with various enzymes and an 11-kb EcoRI fragment with the complete gene was subcloned. The gene revealed the canonical exon/intron FABP structure consisting of four exons (112, 173, 102 and 544 bp, respectively) and three introns (2217, 327 and 546 bp, respectively). The exon sequences were identical with the cDNA encoding mouse E-FABP (Krieg, P., Feil, S., Fürstenberger, G., Bowden, T.G., 1993. Tumor-specific overexpression of a novel keratinocyte lipid-binding protein. Identification and characterisation of a cloned sequence activated during multistage carcinogenesis in mouse skin. J. Biol. Chem. 268, 17362–17369). Of the 5∞ region, 2470 bp were sequenced and searched for transcription factor binding sites. Putative responsive elements within the promoter region were identified that may be responsible for the wide expression observed for E-FABP in mouse tissues. The 11-kb EcoRI fragment was used to localise Fabpe on chromosome 3 in the region 3A1-3 by fluorescence in-situ hybridisation. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Fluorescence in-situ hybridisation; Gene cloning; Gene map; Tissue expression; Transcription factor binding sites

1. Introduction

The epidermal-type fatty acid binding protein (E-FABP) belongs to the multigene family of small intracellular proteins that bind hydrophobic ligands such as fatty acids, eicosanoids and retinoids. E-FABP was first discovered in human psoriatic keratinocytes as one of the up-regulated proteins and was designated as ‘psoriasis-associated fatty acid binding protein’ (Madsen et al., 1992, PA-FABP), whereas Siegenthaler et al. (1993) discovered the same protein in human epidermis and hence named it E-FABP. In human skin xenografts, a ‘melanogenic inhibitor’ (MI) was identified (Nordlund and Farooqui, 1994), whose sequence was found to be identical to that of human E-FABP. Watanabe et al. (1994) purified for the first time a homologous protein from rat skin (‘cutaneous’ FABP) and characterised its cDNA. Krieg et al. (1993) observed up-regulation of the mRNA encoding the mouse homologue of E-FABP (termed ‘mal1’) in squamous cell carcinoma. Since mouse E-FABP bound not only long-chain fatty acids with high affinity, but also eicosanoids (Kane et al., 1996), it was termed keratinocyte lipid binding protein (KLB) by these authors. Sequence alignments of the proteins addressed here reveal these to be orthologues within the family of fatty acid binding proteins.

* Corresponding author. Tel: +49 251 83 33100; Fax: +49 251 83 33206; e-mail: spener@uni-muenster.de

Abbreviations: BAC, bacterial artificial chromosome; bp, base pair(s); cDNA, DNA complementary to RNA; DAPI, 4∞,6∞-diamine-2∞-phenylindole dihydrochloride; ES, embryonic stem; (E-)FABP, (epidermal-type) fatty acid binding protein; Fabpe, murine epidermal-type fatty acid binding protein gene; FISH, fluorescence in-situ hybridisation; kb, kilobase(s) or 1000 bp; PCR, polymerase chain reaction; UTR, untranslated region(s).

PII: 0378-1119(98)00262-5
in accordance with Siegenthaler et al. (1993), we shall refer to these proteins as epidermal-type (E-) FABP.

E-FABP mRNA was found not only in skin but also in brain, skeletal muscle, adipose tissue and mammary gland of mouse (Krieg et al., 1993) and in man on the protein level in kidney, heart and liver (Siegenthaler et al., 1994), thus making E-FABP the FABP-type with the most widespread expression pattern in distinctive mammals.

Moreover the cellular expression of E-FABP appears rather widespread, e.g. human keratinocytes (Siegenthaler et al., 1994) and endothelial cells of the microvasculature (M asouyé et al., 1997), rat alveolar macrophages, type II pneumocytes and lung fibroblasts (Guthmann et al., 1998), rat neurons from different parts of the central nervous system (Liu et al., 1997), sebocytes (Watanabe et al., 1996) and lens epithelial cells (Wen et al., 1993), bovine retinal Müller cells and Sertoli cells of the testis (Kingma et al., 1998) and murine adipocytes (Bernlohr et al., 1997).

The physiological function of E-FABP is not yet clear, but the recent finding that irritation of the human skin by sodium dodecyl sulphate (SDS) slightly increased the expression of E-FABP (Le et al., 1996) indicated a role in the supply of fatty acids for skin repair. Furthermore, an increase of E-FABP expression was observed after hypoglossal nerve crush in rats, suggesting that E-FABP may also be important for fatty acid transport during neuronal regeneration (Owada et al., 1997). To understand the widespread expression profile on the level of gene regulation, we decided to clone the gene encoding murine E-FABP (Fabpe), including its promoter region as a prerequisite for further promoter analysis. The promoter sequence allows identification of possible transcription factor binding sites. Care was taken, not to clone pseudogenes that were reported for other type FABP genes (Treuner et al., 1994; Gerbens et al., 1997) and also found here for Fabpe. Moreover, the chromosomal localization of Fabpe was compared with that of known FABP genes.

2. Materials and methods

2.1. PCR cloning and design of an intron-specific probe

A crude DNA extract was obtained from mouse tail. After shock-freezing in liquid nitrogen and subsequent crushing, the powdered tail was suspended in 10 times the amount (w/v) extraction buffer (0.1 M Tris HCl, pH 8.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.2 M NaCl, 0.2% (w/v) SDS, 100 mg/ml proteinase K) and incubated at 55 °C for 3 h. After inactivation of proteinase K (15 min, 95 °C) and centrifugation (30 min, 4 °C, 13 000 g), the lysate was used as template for PCR (30 cycles). PCR was performed with 5 μM of each primer, 0.2 mM each dNTP, 1.5 mM MgCl2, and 2.5 U of Taq DNA polymerase (GIBCO, Eggenstein, Germany) in 50 μl of reaction volume with the reaction buffer supplied with the enzyme. The primer pairs for amplification of intron 1 (forward: 5′-CATATGGCCACGCCTAAAGGA-3′, reverse: 5′-CCATCTTCTCTAAAGGCGAAGTC-3′), intron 2 (forward: 5′-GACGTGGCTTTAGGAGATG-3′, reverse: 5′-TCTTGGTTATCTGCTTCTCTC-3′) and intron 3 (forward: 5′-GGAAGGAGAGCGCAGATAACAGA-3′, reverse: 5′-CGGATCCTCATGCACTGCTCTCAT-3′) were all designed on the basis of the mal1 cDNA sequence (Krieg et al., 1993) and by alignments of various FABP amino acid sequences using the program MULTalign from the HUSAR package (DKFZ, Heidelberg, Germany). PCR products were separated on a 0.7% (w/v) agarose gel, isolated using the QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany), cloned into pGEM-T easy (Promega, Mannheim, Germany) and sequenced on a single strand using an ABI PRISM sequencer (TopLab, Martinsried, Germany). Using the sequence data obtained for intron 2, a specific primer pair (forward: 5′-ACATCTGTTGAGCAGACGAG-3′, reverse: 5′-CCACACCTCCGCAACCACTG-3′) were designed for preparation of an intron 2 specific probe as well as for subsequent PCR screening.

2.2. Screening of BAC library

A BAC library from mouse embryonic stem cells (129/Sv) was screened by PCR with the intron 2 specific primers (construction of the library and screening were done by Genome Systems, St. Louis, MO). Two positive clones were obtained, each containing a fragment of about 100 kb.

2.3. Southern blot analysis

Genomic DNA isolated from mouse liver (Sambrook et al., 1989) and DNA isolated from one BAC clone (Birnboim and Doly, 1979) were cut with various restriction enzymes (Boehringer Mannheim, Mannheim, Germany) and transferred on to a positively charged nylon membrane (Hybond N, Amersham, Braunschweig, Germany) by blotting (Birnboim and Doly, 1979) and DNA isolated from one BAC clone (Birnboim and Doly, 1979) were cut with various restriction enzymes (Boehringer Mannheim, Mannheim, Germany) and transferred on to a positively charged nylon membrane (Hybond N, Amersham, Braunschweig, Germany). The Southern blots were hybridised with a 32P-labelled probe, obtained from the intron 2 specific amplicon (see Section 2.1) by random priming using the Prime It II kit (Stratagene, Heidelberg, Germany) as described by Feinberg and Vogelstein (1983) and visualised on a Phosphoimager. For genomic mapping, two further probes were labelled as described above: one corresponding to the 5′ (spanning the whole exon 1, prepared by PCR with primer forward: 5′-CATATGGCCACGCCTTAAGGA-3′, reverse: 5′-GGATACATGAAAGAGCTAG-3′).
sponding to the 3′ (spanning the whole intron 3 with parts of exons 3 and 4 with primers forward: 5′-CGGATCCTCATTGCACCTTCTCAT-3′; reverse: 5′-GCATTCTATTCTGCACTTTCTCAT-3′) end of the coding region, respectively. Fragments obtained were then used in combination with the sequence information (see Section 2.4) to generate a genomic restriction map. Furthermore, from the restriction digest of the BAC DNA with EcoRI, an 11-kb band was identified, containing both ends of the coding region. The band was excised from the gel, purified with the QIAEX G el Extraction Kit and cloned into pBluescript KS (+).

2.4. Sequencing of Fabpe

The subclone containing the 11-kb EcoRI fragment was used for sequencing the gene. A automated sequencing by primer walking was carried out using an ABI PRISM sequencer (TopLab). In addition to the four exons, the three introns and 118 bp of 3′ UTR, 2470 bp of the promoter region were sequenced. The promoter region, the exons and the 3′ region were sequenced on both strands, whereas the introns were sequenced on one strand. The promoter region (bases −2471 to +35) was searched for at least 80% identity to consensus sequences of transcription factor binding sites using the TFSEARCh package v. 1.3 (Yutaka Akiyama, 1995, http://www.tokyo-center.genome.ad.jp/ SIT/TFSEARCH.html).

2.5. Fluorescence in-situ hybridisation (FISH)

New-born WM P/WMP mice, which carry nine pairs of easily identifiable Robertsonian translocation chromosomes (Said et al., 1986), were injected with leukemia virus, and a stable cell line was established, as described in detail by Zornig et al. (1995). From this cell line, metaphases were prepared. After hybridisation with the 11-kb subclone detection via fluoresceinated avidin (FITC-avidin) was performed as reported by Lichter et al. (1988). Chromosomes were counterstained with 4,6-diamine-2-phenylindole dihydrochloride (DAPI). Images of emitted light were captured separately by use of the DAPI and FITC filter set and subsequently merged and aligned.

Other localisation data were retrieved from the Mouse Genome Database (M GD), Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine (http://www.informatics.jax.org/) (December, 1997).

2.6. Detection of E-FABP in mouse tissues

Tissues were isolated from three 12-week-old female BALB/C mice, suspended in 10 mM TrisHCl, pH 7.4, 30 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride and minced using an Ultra Turrax (Jahnke and Kunkel, Staufen, Germany). This homogenate was spun (5000 × g, 20 min) to remove all debris. Cytoplasm was obtained after centrifugation at 100 000 × g for 90 min. Western blotting was done essentially as described earlier (Guthmann et al., 1998).

3. Results

3.1. Cloning of Fabpe

By alignment of the amino acid sequence of the murine E-FABP and of those derived from the coding regions of closely related genes for adipocyte-, brain- and heart-type FABP, the exon/intron-borders were predicted (Fig. 1). Primer pairs flanking the predicted introns at a distance of about 50 bp were designed to amplify the introns. We confirmed the existence of a pseudogene by PCR with primers flanking the coding region of the cDNA, yielding a fragment with the expected size for an intronless pseudogene (about 400 bp, Fig. 2, lane 9). Sequencing of the amplified pseudogene revealed only seven nucleotide exchanges (data not shown). From the genomic Southern blot, we were able to suggest the existence of four pseudogenes in addition to the Fabpe (data not shown). With primer pairs for the amplification of intron 1, we could only detect a pseudogene-derived amplicon (about 100 bp, Fig. 2, lane 1), whereas amplification of intron 1 (2.2 kb) was not achieved with the PCR conditions applied. With primer pairs for introns 2 and 3, we detected both the pseudogene derived amplicons (about 250 and 110 bp, respectively) and the larger fragments derived from Fabpe amplification (intron 2, about 600 bp; Fig. 2, lane 4–6; intron 3, about 650 bp; Fig. 2, lane 7 and 8). The PCR products containing the introns 2 and 3 were cloned and sequenced, and a primer pair was designed for the amplification of an intron 2 specific probe. This primer pair was then used to screen a BAC library of mouse ES cells for Fabpe in the presence of pseudogenes. Two clones (each about 100 kb) were confirmed by Southern fingerprint analysis using probes located 5′ and 3′ of the coding region (data not shown) to contain at least 20-kb Fabpe sequences flanking each side of the coding region.

The 11-kb fragment, a result of the EcoRI restriction digest, hybridised with the probes located at both ends of the coding region (data not shown) in the same pattern, which suggests that at least the fragment contained the whole coding region. It was excised, cloned into pBluescript and 6.5 kb of the gene, including the promoter region, were sequenced (Fig. 3).
Fig. 1. Alignment of translated murine E-FABP-cDNA sequence to the translated coding region of genomic sequences from closely related murine FABP types. Dots mark identical amino acids. At the border from exon 1/intron 1, the codon for Gly is interrupted after the first nucleotide. Otherwise, the borders are located after the entire codon. Sequences for Fabpb (gene for B-FABP) from Kurtz et al. (1994), Fabph1 (gene for H-FABP) from Treuner et al. (1994) and Ap2 (gene for A-FABP) from Hunt et al. (1986).

3.2. Restriction mapping and structure of Fabpe

Using various restriction endonucleases, we performed restriction mapping of about 28 kb of the gene (Fig. 4), including the 11-kb EcoRI fragment mentioned above.

From this 11-kb fragment, 2470 bp of the 5′ region were sequenced and searched for promoter elements and transcription factor binding sites. The upstream region revealed the typical composition of a core promoter such as a TATA-box at nt −40 (sequence ATATAA), a CCAAT-box at nt −80 (sequence ATTTGG) and some GC-rich regions at nt −110 (sequence GGGGCGG). In addition, we found multiple putative binding sites for MyoD, HNF1, C/EBPs and β, GATA1, MZF1, which may be relevant for the expression pattern of E-FABP (Table 1).

3.3. Chromosomal localisation of Fabpe

For chromosomal localisation of mouse Fabpe, fluorescence in-situ hybridisation using the 11-kb subclone was performed. Double fluorescence signals were detected just distal from the centromere on the long arm of the chromosome Rb (3.12) 4Mpl (Fig. 5). This localises Fabpe to mouse chromosome 3A1-3.

3.4. Occurrence of E-FABP in mouse tissues

The widespread expression of E-FABP can be deduced from the Western blot shown in Fig. 6. All tissues investigated contain E-FABP, though in variable amounts. It was barely detected in ileum and heart, whereas small amounts were found in tongue, intestine, liver, kidney, brain and bone marrow. The highest E-FABP levels were observed in lens, stomach and lung.

4. Discussion

The genomic structure of Fabpe shows the canonical order of four exons with three introns known for the members of the family of intracellular lipid binding proteins. The sizes of the introns are comparable to...
Fig. 3. Sequence of Fabpe. The putative TATA-box (nt -40), CCAAT-box (nt -80, sequence ATTGG), GC rich region (nt -110), putative transcription start (nt +1) of the promoter region and polyadenylation signals (nt +3727 and nt +4001) are shown in italics. Intron 1 interrupts the codon for Gly, indicated by G- - -G. In accordance with the enumeration by Krieg et al. (1993), we set the first base of the mRNA as the putative transcription start (nt +1) and capitalized the transcribed sequence. The Fabpe DNA sequence has been deposited in the EMBL database under Accession No. AJ223066.
Fig. 4. Restriction map of Fabpe: (A) region from nt −9500 to nt +18000; (B) sequenced region (see Fig. 3), shown schematically. This region spans from nt −2471 to nt +4124, exons are symbolised by black boxes, exon 1 starts at nt +1 (putative transcription start) and exon 4 ends after the second polyadenylating signal at nt +4022.

Table 1

<table>
<thead>
<tr>
<th>Factor</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyoD</td>
<td>−2137, −2062, −1559, −1154, −1076, −1062, −1050, −1042, −1030, −976, −888, −870, −849, −799, −697, −598, −462, −435, −405, −394, −351, −372, −304, −271, −238, −206, −181, −158, −137, −118, −100, −83, −69, −57, −47, −38, −30, −23, −18, −14, −9, −5, −2, −1, −1</td>
</tr>
<tr>
<td>E47</td>
<td>−2136, −1557, −1520, −1285, −1079, −1061, −1050, −1042, −1030, −976, −888, −870, −849, −799, −697, −598, −462, −435, −405, −394, −351, −304, −271, −238, −206, −181, −158, −137, −118, −100, −83, −69, −57, −47, −38, −30, −23, −18, −14, −9, −5, −2, −1, −1</td>
</tr>
<tr>
<td>AP1</td>
<td>−1778, −1489, −1286, −1136, −950, −783, −755</td>
</tr>
<tr>
<td>C/EBP</td>
<td>−2441, −2354, −2122, −1807, −1836, −1662, −1571, −1551, −1520, −1285, −1079, −1061, −1050, −1042, −1030, −976, −888, −870, −849, −799, −697, −598, −462, −435, −405, −394, −351, −304, −271, −238, −206, −181, −158, −137, −118, −100, −83, −69, −57, −47, −38, −30, −23, −18, −14, −9, −5, −2, −1, −1</td>
</tr>
<tr>
<td>HNF1</td>
<td>−453</td>
</tr>
<tr>
<td>MZF1</td>
<td>−2285, −1803, −1124, −896, −693, −517, −308, −62, −8</td>
</tr>
<tr>
<td>GATA1</td>
<td>−2495, −2417, −2271, −2216, −2155, −2106, −1906, −1840, −1769, −1660, −1521, −1371, −1198, −1159, −1042, −921, −887, −870, −849, −799, −697, −598, −462, −435, −405, −394, −351, −372, −304, −271, −238, −206, −181, −158, −137, −118, −100, −83, −69, −57, −47, −38, −30, −23, −18, −14, −9, −5, −2, −1, −1</td>
</tr>
</tbody>
</table>

Pseudogenes were reported for the closely related H-FABP genes from mouse and pig, respectively (Treuner et al., 1994; Gerbens et al., 1997). In our study, we proved the existence of at least one pseudogene by PCR, and a total of four pseudogenes were deduced from Southern blot analysis.

The 5′ region contains a core promoter region typical for eukaryotic genes (Maniatis et al., 1987) with a putative TATA-box at nt −40, a CCAAT-box at nt −80 and a GC-rich region at nt −110. Various cis-acting elements were identified by comparison with consensus sequences of transcription factor binding sites. At the level of 80% identity, 560 binding sites for 40 transcription factors were found; some of them are well characterised and might be related to the expression pattern of E-FABP (Fig. 6). As shown in Table 1, among them were multiple E-boxes for binding of MyoD, one of the transcription factors responsible for activation of muscle-specific genes and other regulatory elements (e.g. E47). This is interesting with respect to our recent detection of E-FABP mRNA and corre-
Fig. 6. Occurrence of E-FABP in mouse tissues. Cytosolic proteins were separated by SDS–PAGE (15% T, 2.7% C), transferred on to nitrocellulose membranes and decorated with affinity-purified anti-rat E-FABP antibodies and a secondary antibody-alkaline phosphatase conjugate for colorimetric detection. Lane 1, tongue (25 mg); lane 2, 5 ng of recombinant rat E-FABP; lane 3, lens (10 mg); lane 4, stomach (25 mg); lane 5, intestine (25 mg); lane 6, ileum (25 mg); lane 7, liver (20 mg); lane 8, lung (20 mg); lane 9, kidney (20 mg); lane 10, heart (25 mg); lane 11, brain (20 mg); lane 12, bone marrow (15 mg).

A corresponding protein in the C2C12 murine muscle cell line (C. Buhlmann, F. Spener, manuscript in preparation). Several a- and b-type C/EBP binding sites were identified. C/EBPα was shown to induce adipocyte-specific genes and is essential for the differentiation of preadipocytes to adipocytes (Samuelsson et al., 1991). Binding of C/EBPα to the Fabpe promoter would explain the expression of E-FABP in adipocytes, which is increased in mice lacking the gene encoding adipocyte FABP (Ap2) as found by Bernlohr et al. (1997). C/EBPβ, however, is found also in hepatocytes. Another transcription factor inducing liver-specific genes for which binding sites are found on the Fabpe promoter is HNF-1 (Courtois et al., 1988). Indeed, we could demonstrate the expression of E-FABP in hepatic tissue by Western blotting (Fig. 6). The influence of the transcription factors MZF1 and GATA1, both playing a role in the regulation of erythropoietic genes (Hromas et al., 1991; Peyv et al., 1991) on the Fabpe expression remains to be investigated, but could be related to E-FABP expression in bone marrow (Fig. 6) and in alveolar macrophages (Guthmann et al., 1998). Several sites for the ubiquitous transcription factor AP1 were found that are reported to be important in epithelial promoters (Eckert et al., 1997). Although E-FABP is found in a variety of tissues, in some cases possibly originating from microvascular endothelial cells (M assouyé et al., 1997), Fabpe is no ‘classical’ housekeeping gene, as the promoter contains a highly conserved TATA box, which is not present in housekeeping genes, and only a minor GC-rich region.

Using FISH, Fabpe was localised on mouse chromosome 3A1-3 (Fig 5). This chromosome also harbours other genes of this protein family, i.e. on distal mouse chromosome 3 at 72 cm Fabpi, Ap2 and Pmp2 in the region 3A1-3. Whereas the human homologue of Fabpi, namely FABP2, resides on human chromosome 4q28–q31, the human homologues of Ap2 and Pmp2 have both been localised to human chromosome 8q21–q22. It is interesting to note the close proximity of Fabpe to Ap2 and Pmp2 on proximal mouse chromosome 3. A genetic analysis will clarify the relationship between these three members of the same protein family.

A genetic linkage group common to mouse chromosome 3 and human chromosome 8q21–q22 has been described, it is suggested that the human homologue of Fabpe might reside also on human chromosome 8q21–q22.

Acknowledgement

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Sp 135/10-1, Ru 517/2-1) and is part of the Ph.D. thesis of C. Hohoff.

References


