

Characterization of a new fatty acid response element that controls the expression of the locust muscle FABP gene

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Abstract

In vertebrate and invertebrate muscles, the expression of fatty acid binding proteins (FABP) is induced by long chain fatty acids. To identify the fatty acid response elements that mediate this up-regulation, the gene of the FABP expressed in locust flight muscle was cloned, and its upstream sequences analyzed for potential regulatory elements. Comparison with other muscle FABP promoters revealed the presence of a 19-bp imperfect inverted repeat sequence that contains two hexanucleotide half sites (AGTGGT and ATGGGA), interspersed by 3 nucleotides. The promoter activity was studied with reporter gene constructs in L6 myoblasts, in which H-FABP expression is stimulated by long-chain fatty acids in a similar manner as in adult cardiomyocytes. The 19 bp element, located 180 bp upstream of the transcription start site, was found to be essential for the fatty acid induction of gene expression, and gel shift analysis confirmed that this fatty acid response element is capable of binding nuclear proteins both from rat myoblasts and locust muscle in the presence of fatty acids. A similar, but reverse sequence that is present upstream of all mammalian H-FABP promoters may modulate the expression of the rat H-FABP gene. (*Mol Cell Biochem* **239**: 173–180, 2002)

Key words: fatty acid binding protein, fatty acid response element, FARE, *Schistocerca gregaria*, inverted repeat, everted repeat, gene regulation, gel shift

Abbreviations: FABP – fatty acid binding protein; L-, A- and H-FABP – liver-, adipocyte- and heart-FABP; FARE – fatty acid response element; PPAR – peroxisome proliferator activated receptor; PPRE – peroxisome proliferator response element; DR-1 – direct repeat separated by 1 nucleotide; IR-3 – inverted repeat separated by 3 nucleotides; ER-3 – everted repeat separated by 3 nucleotides

Introduction

Fatty acid binding proteins are ubiquitous intracellular proteins that participate in fatty acid transport and metabolism. They belong to a conserved multi-gene family that also contains transport proteins for other hydrophobic ligands, such as retinoid acid [1]. In different tissues, distinct FABPs are expressed, reflecting the different metabolic roles of fatty acids. Generally, high levels of FABPs are found in tissues that utilize fatty acids at high rates, and the expression of

FABP appears to increase when fatty acid demand increases [2, 3]. Evidence for such an up-regulation of FABP gene expression upon fatty acid exposure has been observed especially for the adipocyte (A-FABP), liver (L-FABP), and heart isoform of the FABP gene (H-FABP).

The concentration of H-FABP is directly proportional to the fatty acid dependent metabolic rate over the wide range found in various muscles. Fast twitch skeletal muscles that rely mostly on glucose possess relatively small amounts of the protein, while in mammalian heart FABP comprises up

to 6% of all cytosolic proteins [2]. Flight muscles from vertebrates and invertebrates that encounter fatty acid metabolic rates 2 or 3 times higher have been found to contain FABP levels that are elevated by similar factors [4, 5]. Moreover, chronically increased lipid utilization has been found to lead to marked increases in FABP expression in various muscles, both in vertebrates and invertebrates [3–6]. Van Bilsen *et al.* [7, 8] demonstrated *in vitro* that prolonged exposure of cultured neonatal ventricular myocytes to exogenous fatty acids leads to a nearly 4-fold increase in FABP mRNA. All these observations suggest that an increased intracellular fatty acid concentration, maintained over certain time periods, serves as the signal to stimulate FABP expression. Thus, fatty acid itself may induce the expression of its own transport protein in muscle cells.

Fatty acids or their metabolites can modulate the expression of other members of the FABP gene family at the level of transcription initiation [9], by mechanisms similar to lipophilic hormones such as steroids, retinoids, and thyroxins. In these cases, nuclear receptors exist that bind, when complexed with the lipophilic ligand, alone or in concert with other proteins to cis-acting sequences upstream of the genes [10]. It has been demonstrated that both, the liver and adipocyte FABP genes are under the control of peroxisome proliferator activated receptors (PPAR), so called because of their activation by fibrate drugs known to stimulate the formation of peroxisomes [11, 12]. To date, three major forms of PPARs have been characterized; of these, PPAR α is expressed in liver, kidney, and cardiac tissues, while PPAR β is ubiquitously distributed, including in the heart. PPAR γ can be found in adipose tissue, lymphatic tissue, and the intestine [13].

Although PPARs are involved in the regulation of several muscle specific genes, they may not play a role in the fatty acid mediated up-regulation of the H-FABP gene. Several mammalian H-FABP genes lack peroxisome proliferator response elements (PPREs), and the PPRE-like sequence found upstream of the rodent genes [14] appears to be non-functional (Spener, personal communication). Thus, the involvement of a different, hitherto unknown fatty acid response element was suspected. The conserved gene structure of FABP genes, coupled with similar physiological responses to elevated fatty acid levels, suggests that analogous mechanisms control the expression of the mammalian and insect muscle FABP. Therefore, we reasoned that the suspected fatty acid response elements should be conserved as well, and that it may be possible to identify candidate sequences by comparing sequences upstream of the invertebrate and vertebrate muscle FABP promoters. If such sequences were found, they could be linked to reporter genes, and their expression studied in a suitable cell line. This paper summarizes our studies that led to the discovery of a novel fatty acid response elements in the insect muscle FABP gene, and shows additional experiments

that point to a similar function of a related element found upstream of the mammalian FABP genes.

Materials and methods

Cloning and sequencing

The cloning of the locust muscle FABP gene is described in [15]. Sequencing was carried out on carried an ABI sequencer or with ABI's AmpliTaq DyeDeoxy terminator Cycle sequencing chemistry (PE Biosystems, Foster City, CA, USA) on the automatic ABI Model 373 Stretch DNA sequencer at Biotechnology Laboratory/Nucleic Acid Service Unit of the University of British Columbia.

Cell culturing and isolation

Male Sprague–Dawley rats were housed in the Animal Care facility of Simon Fraser University. Prior to heart dissection, rats were intra-peritoneally anesthetized with sodium pentobarbital. The heart was rapidly excised, placed into ice cold dissecting solution, and via the aorta attached to a water-jacketed Langendorff perfusion apparatus. Following perfusion first with aerated dissecting solution and then with collagenase solution, the digested tissue was gently disintegrated, and cells were transferred to culture dishes [16]. The fresh cardiomyocytes were cultured at approximately 60–80% confluency in Delbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 150 μ g/ml penicillin and 150 μ g/ml streptomycin. L6 myoblasts were grown in the same medium for 3 days to approximately 80% confluency.

Fatty acid treatment

Fatty acid (600 μ M)/BSA (200 μ M) solutions were prepared by dissolving fatty acid free BSA (Sigma, Oakville, ON, Canada) and sodium salts of fatty acids (Sigma, Oakville, ON, Canada) in 0.9% NaCl. The filter-sterilized fatty acids–BSA complex (0.5 ml) was added to 4.5 ml fresh DMEM medium supplemented with 1% fetal bovine serum (final concentration of fatty acids 60 μ M). Cells incubated with DMEM supplemented with 1% fetal bovine serum but without fatty acids were used as control samples.

Quantification of rat H-FABP expression

Prior to RNA extraction, cells were detached by 0.25% trypsin in phosphate buffered saline (0.01 M phosphate, 0.9% NaCl, pH 7.4) and collected by centrifugation. Total RNA was iso-

lated by Totally-RNA isolation kit from Ambion (Austin, TX, USA). RNA was stored in ethanol at -80°C until used for RT-PCR. Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were brought to final volume of $50\ \mu\text{l}$ and $500\text{--}800\ \text{ng}$ of total RNA were added. The reverse transcription reaction was carried out for 10 min at 25°C , 10 min at 60°C , and 15 min at 42°C , with random hexanucleotides as primers. The cDNA was denatured at 95°C for 1 min and amplified for 31 cycles of 30 sec at 95°C , 30 sec at 58°C , 1 min at 72°C . PCR-primers specific for exon 1 (upper primer R1, 5'-TAGCATGACCAAGCCGACCACAATC-3') and exon 3 (lower primer R4, 5'-GTTCCCGTGTAAGCTTAGTCTCTCTG-3') of the rat H-FABP gene were used to amplify a 224 bp fragment of H-FABP mRNA. For the simultaneous amplification of 18 S internal standard RNA, a mix of natural and inactivated 18 S primers was added, yielding a 324 bp PCR product (18 S:competimer ratio 2:8 for myoblasts, 6:4 for cardiomyocytes; QuantumRNA 18s Internal Standard kit, Ambion, Austin, TX, USA).

Construction of the promoter-luciferase fusion plasmids

Different regions of the locust muscle FABP promoter were amplified from a genomic clone with forward primers (F), modified to contain a *Kpn*II restriction site, and reverse primers (R) containing a *Nhe*I restriction site (F-1135/-1115 5'-ATGGTACCTGCTAATAACTCCAATTGTC-3'; F-620/-600 5'-ATGGTACCCATAATCACACTCATGTTA3'; F-510/-490 5'-ATGGTACCTGACCATTGCAATAAGATTT-3'; F-280/-260 5'-ATGGTACCACTGCGAAACAGATGAATGT-3';

F-130/-100 5'-ATGGTACCTAGCAGTCATGAAACAGAAT-3'; F-23/+5 5'-ATGGTACCGGCCG CCACCGGACGAGC-3'; R+52/+34 5'-AAGCTAGCGCTGCTGTGGTGGCCGGT-3'; R+85/+63 5'-ATGCTAGCTACTTGATGCCTGCGAAT-3'; R-562/-544 5'-ATGCTAGCCTCAACAAGGAGATTCCG-3'). The PCR products were double digested with *Kpn*II and *Nhe*I, gel purified and cloned into the pGL3-basic luciferase vector (Promega, Madison, WI, USA). A 19-bp deletion mutant (Δ -180/-162) was constructed by amplifying the 5' and 3' flanking DNA, using PCR primers slightly modified to contain an *Afl*III restriction site (F-1135/-1115, R-280/-260 5'-ATGGTACCACTGCGAAACACAGATGAATGT-3'; F-161/-143 5'-ATGGTACCACTGCGAAACAGATGAATGT-3', R+52/+34). Following digestion of the two PCR products with *Afl*III and ligation, the product was used as template for the PCR-based construction of deletion mutant vectors, as described above. All reporter constructs were sequenced to confirm the correct sequence and orientation. The various reporter gene constructs are depicted in Fig. 3.

Luciferase reporter gene assay

L6 were transiently transfected with the various reporter gene constructs, using LipofectAMINE 2000 reagent (Life Technologies, Burlington, ON, Canada) according to the manufacturer's instructions. For each dish, $5\ \mu\text{g}$ luciferase reporter gene construct, 500 ng internal control vector pRL-TK (Promega, Madison, WI, USA) and $12\ \mu\text{l}$ Lipofect reagent were gently mixed in $400\ \mu\text{l}$ Opti-MEM and left at room temperature for 15 min. Subsequently, the transfection mixture was

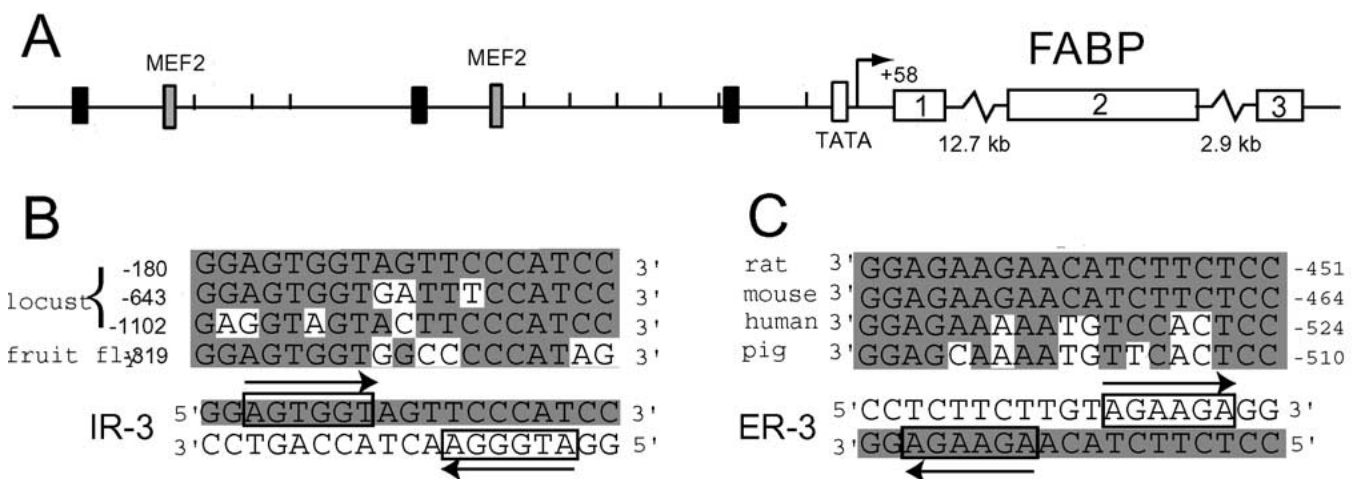


Fig. 1. The locust FABP gene and the conserved elements. (A) A schematic drawing of the muscle FABP gene from the desert locust, *Schistocerca gregaria*. The numbered open boxes depict the three introns of the gene. The arrow indicates the transcription start site. Shown is also the TATA box ($-23\ \text{bp}$), 2 sequences with similarity to binding sites for MEF2, and the inverted repeat sequences (black boxes at -180 , -643 , -1102). The individual inverted repeat sequences, their analogue in the *Drosophila* FABP gene, and the consensus sequence with the two inward facing hexanucleotide half sites (IR-3) are shown in (B). (C) shows the elements found upstream of the cloned mammalian H-FABP genes, as well as the consensus sequence with two outward facing hexanucleotide half sites (ER-3).

added to the cells. After 6 h incubation at 37°C, 5% CO₂, the medium was changed back to DMEM. Following the 6 h transfection period, the fatty acid/BSA complex solution was added to cell culture media at final concentrations of 60 µM fatty acid. As control, the same volume of a 200 µM BSA solution was used. Incubations were carried out for 18 h. Cells were harvested and lysed with 200 µl passive lysis buffer (Promega, Madison, WI, USA), and cell debris was removed by centrifugation. The dual-luciferase reporter assay system (Promega, Madison, WI, USA) was employed to evaluate the relative luciferase activity, using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA).

Preparation of nuclear extracts

Flight muscle was homogenized under liquid nitrogen, and the frozen muscle powder suspended in 4 volumes of homogenization buffer (10 mM Hepes-NaOH, pH 7.9, 0.35 M sucrose, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.15 mM spermine, 0.5 mM spermidine, 2 µg/ml leupeptin and 2 µg/ml aprotinin; all from Sigma, Oakville, ON, Canada). The tissue was homogenized by 15–20 strokes in a Potter–Elvehjem glass homogenizer with a Teflon pestle. After addition of 0.1% Nonidet P-40 the homogenate was filtered through two layers of cheesecloth and centrifuged for 10 min at 1300 × g. The pelleted nuclei were washed 3 times with homogenization buffer.

Nuclear extracts were prepared using the Nu-CLEAR extraction kit (Sigma, Oakville, ON, Canada). The nuclei were suspended in 3 volumes of 10 mM Hepes buffer (10 mM Hepes-NaOH, pH 7.9, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin and 2 µg/ml aprotinin). After addition of 400 mM NaCl, the suspension was stirred 30 min and centrifuged for 15 min at 13000 × g. The supernatant was dialyzed for 3 h against 100 volumes of 20 mM Hepes buffer (20 mM Hepes-NaOH, pH 7.9, 75 mM KCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride). The dialysate was then centrifuged at 13000 × g for 15 min, divided into aliquots and stored at –80°C.

Nuclei from cultured L6 cells were prepared in a similar manner. Culture dishes were rinsed with phosphate buffered saline, and cells were harvested. The pooled cells were centrifuged at 3000 rpm for 10 min, suspended in 3 volumes of homogenization buffer, and processed further as described above.

Electrophoretic mobility shift assay

DNA fragments of the locust FABP promoter for electrophoretic mobility-shift assays were amplified by PCR from the cloned reporter gene constructs –280/+53 (wild-type) and

–280/+53 Δ–180/–162 (deletion mutant), with primers annealing at –280/–260 (5'-ACTGCGAAACAGATGAATGT-3') and at –135/–154 (5'-GTCCAAACATCGAG TGTGA-3'). The PCR products (145 bp for wild-type, 126 bp for mutant) were end-labeled with (γ-³²P)-ATP (3000 Ci/mmol, NEN, Markham, ON, Canada) and T4 polynucleotide kinase. Nuclear extracts (~ 2 µg of protein) were pre-incubated in 20 µl of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 50 µg/ml poly(dI-dC)/poly (dI-dC) and 5% glycerol) for 10 min at 24°C. The labelled probe (~ 15 ng) was added and incubated at 24°C for 30 min. Subsequently, the reaction mixture was loaded onto non-denaturing polyacrylamide gels (5% T, 3.3% C) and electrophoresed at 20 V/cm for 30 min in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA). The gels were dried and analyzed by autoradiography. For the competition experiments, the pre-incubation was performed in the presence of unlabelled competitor DNA at the molar excess indicated in the figure legends. Rat H-FABP (gift from Dr. F. Spener, University of Münster) was added in one experiment, as indicated. Gelshifts with the rat H-FABP element were carried out in the same manner, except that the double stranded synthetic oligonucleotide (5' CCTCTTCTGTGTCAGAAGAG-G 3', –510 → –529) was used as probe.

Results and discussion

The locust muscle FABP gene

The gene coding for locust muscle FABP was amplified by PCR and cloned, together with 1.2 kb of upstream sequence [15]. The sequence coding for the 607 bp cDNA is interrupted by two introns of 12.7 and 2.9 kb, inserted in analogous positions as the first and third intron of the mammalian homologues: one intron is found following the sequence coding for glycine 26, which is located in the turn of the helix-turn helix motif that shields the binding cavity, the other intron before threonine 117. An additional intron found in all mammalian FABP genes (following lysine 84), is absent in the locust FABP gene (Fig. 1). Both introns contain repetitive sequences also found in other locust genes, and the second intron contains a GT-microsatellite. The promoter sequence includes a canonical TATA box 24 bp upstream of the transcription start site. The upstream sequence contains various potential myocyte enhancer sequences and a 160 bp segment that is repeated 3 times. This finding raised the possibility that the repeats contain regulatory elements which could act more efficiently in multiple copies. A comprehensive sequence analysis did not reveal any known transcription factor binding sites within these repeats. Noteworthy, however, is the presence of a 19 bp inverted repeat sequence, 5'-GGAGTGGTA N TTCCATCC-3'. A similar, partially pal-

indromic sequence is also found upstream of the putative *Drosophila* FABP promoter (Fig. 1b). While this sequence is not recognized as a transcription factor binding site, it contains inverted repeats of two hexanucleotide half-sites: 5' gg AGTGGT nnn TCCCAT cc 3'. This sequence is similar to the response elements for steroid hormone receptors, which generally bind to inverted repeat element separated by 3 nucleotides (IR-3) with the half-site sequences 5' AGAACA 3' or 5' AGGTCA 3'.

Interestingly, a strikingly similar, but reversed palindromic sequence is found within 600 bp upstream of the promoter of all mammalian heart FABP genes (Fig. 1c), however with consensus half-sites facing outward (everted repeat, ER-3).

The fact that this conserved 19 bp sequence is found within the promoter region of all heart and muscle FABP genes cloned so far, which otherwise does not show obvious areas of sequence conservation, suggested its involvement in the regulation of the FABP gene. Given its similarity to previously characterized response elements, it appeared possible that this sequence is involved in the induction of gene expression by fatty acids. In order to investigate this, a cell system was needed that is suitable to measure changes in the expression of reporter gene constructs of the FABP promoter. Suitable cells should be easily transfected with reporter gene constructs, and respond to fatty acid treatment in a similar manner as differentiated muscle cells. Since muscle cells are terminally differentiated and hence not suitable for protocols involving transfection with reporter gene constructs and lengthy fatty acid incubation periods, we decided to investigate whether undifferentiated myoblasts can be used for these studies.

Rat myoblasts are suitable for expression studies

In order to compare the expression of the H-FABP gene in undifferentiated and differentiated myocytes, H-FABP mRNA was measured by reverse-transcription PCR, yielding PCR products of 224 bp. To account for variations in template amount, 18 S RNA sequence was used as internal standard. A mixture of regular and blocked primers (competimers) was necessary to achieve similar intensities between the gene specific PCR products and the 324 bp 18S PCR product. H-FABP mRNA can be found in myoblasts and adult cardiomyocytes, albeit at different levels (Fig. 2). Quantitative comparison with the PCR products from myoblasts indicate far higher mRNA levels in cardiomyocytes. Because of the relative abundance of H-FABP mRNA in the heart, 18S RNA could only be seen when the concentration of the unblocked ribosomal RNA primers was increased. Thus, we established that the H-FABP is expressed in myoblasts, but at a lower level than in cardiomyocytes. To study the induction of H-FABP expression by fatty acids, cultured myoblasts and isolated cardiomyocytes were treated with linoleic acid. H-FABP

Table 1. Induction of H-FABP expression by long chain fatty acids

| Fatty acid | mRNA levels (\times control) |
|-----------------------|---------------------------------|
| <i>Myoblasts</i> | |
| 16:0 | 3.0 \pm 0.7 |
| 18:1 | 1.9 \pm 0.2 |
| 18:2 | 2.0 \pm 0.5 |
| 18:3 | 2.7 \pm 0.5 |
| 20:4 | 2.2 \pm 0.8 |
| <i>Cardiomyocytes</i> | |
| 18:2 | 2.4 \pm 0.3 |

Values are the mean of 3–6 independent determinations \pm S.D.

mRNA increased in myoblasts and cardiomyocytes following 10 h of incubation. Quantitative analysis revealed a similar increase in myoblasts and cardiomyocytes. Saturated and unsaturated long chain-fatty acids all stimulated H-FABP gene expression to similar degrees (Table 1). Induction of gene expression was visible already after 30 min incubation in both cell types, when the primary transcript was quantified in an analogous manner [17]. These findings show that fatty acid treatment affects gene expression similarly in L6 cells and fully differentiated myocytes; thus, myoblasts can be useful to study the activity of the FABP promoter *in vitro*.

Analysis of the locust FABP promoter

We used a dual luciferase reporter gene assay for the sensitive detection of the expression under the control of the insect promoter [18]. Transfection of rat myoblasts with a reporter gene construct containing the full locust FABP promoter resulted in strong expression of luciferase. The enzyme activity detected 18 h post transfection was more than 12-fold higher than found for a promoter-less luciferase vector, and nearly half as strong as for a control vector containing the strong, universal SV 40 promoter, demonstrating that the locust muscle FABP promoter is effective in a heterologous system. Reporter gene expression was measured with various constructs of decreasing length, gradually eliminating potential regulatory elements such as MEF2 and an inverted repeat that is present 3 times within the first thousand basepairs upstream of the TATA box. Similar levels of luciferase activity were measured for all constructs that contained at least 280 bp of upstream sequence (Fig. 3). A shorter construct that started just downstream of the inverted repeat sequence also expressed luciferase, however much weaker (< 50%) than the longer constructs. Constructs that did not contain the TATA box (–1153/–562, –23/+52), or expressed luciferase out of frame (–1135/+85, luciferase cDNA inserted 29 bp downstream of the FABP start codon) led to drastically reduced levels of luciferase activity. To further determine the impact

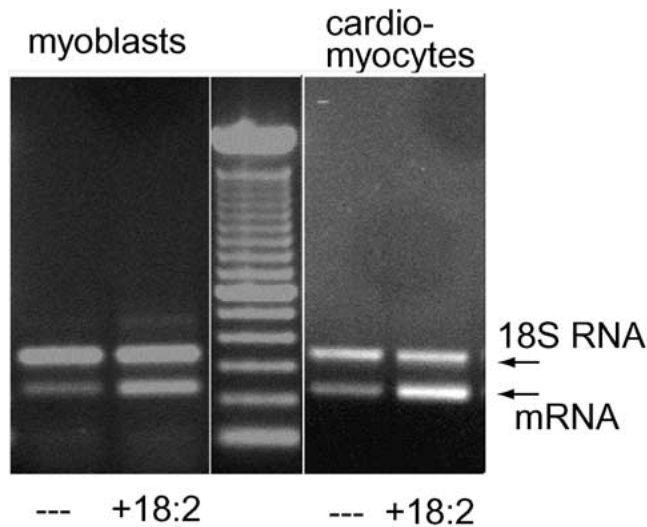


Fig. 2. RT-PCR of rat H-FABP mRNA. Cells were incubated for 10 h with culture media containing 20 μ M BSA or 60 μ M linoleic acid complexed to 20 μ M BSA, as described in Materials and methods. Total RNA (500 ng) was used as template for multiplex PCR, with primers specific for 18 S RNA and H-FABP mRNA. The specific primer: competitor ratio was 2:8 for myotubes, and 4:6 for cardiomyocytes.

of the inverted repeat element, a deletion mutant was constructed that contained the entire promoter ($-280/+52$), but omitted the 19-bp repeat ($\Delta-180/-162$). The deletion mutant promoter continued to strongly stimulate reporter gene ex-

pression; luciferase activity was approximately one third lower than for the wild-type promoter, but remained still higher than for the minimal active promoter construct ($-130/+52$). When transformed cells were treated with fatty acids, expression rates were unaltered for control constructs, but markedly increased for constructs containing the FABP promoter with the inverted repeat elements. The increase was highest (more than 2-fold) for the $-280/+52$ construct that contained one copy of the inverted repeat element, and somewhat lower (~ 1.5 -fold) for longer constructs. Treatment with saturated and mono-unsaturated fatty acids also led to a clear increase in luciferase expression (~ 1.5 -fold, [18]). However, neither the minimal promoter ($-130/+52$) nor the construct in which the inverted repeat had been deleted ($\Delta-180/-162$) responded to fatty acid treatment, indicating that the inverted repeat is indeed involved in the fatty acid response.

Characterization of the locust fatty acid response element

To see whether the inverted repeat element does bind a nuclear transcription factor, electrophoretic mobility shift assays were carried out. A 145-bp fragment of the upstream region containing the repeat element ($-280/-135$) was shifted to lower mobility in the presence of nuclear proteins isolated from rat myoblasts (Fig. 4). The shift, however, did not occur with an analogous fragment without the 19-bp sequence ($-280/-135 \Delta-180/-162$). Increasing amounts of the double-stranded

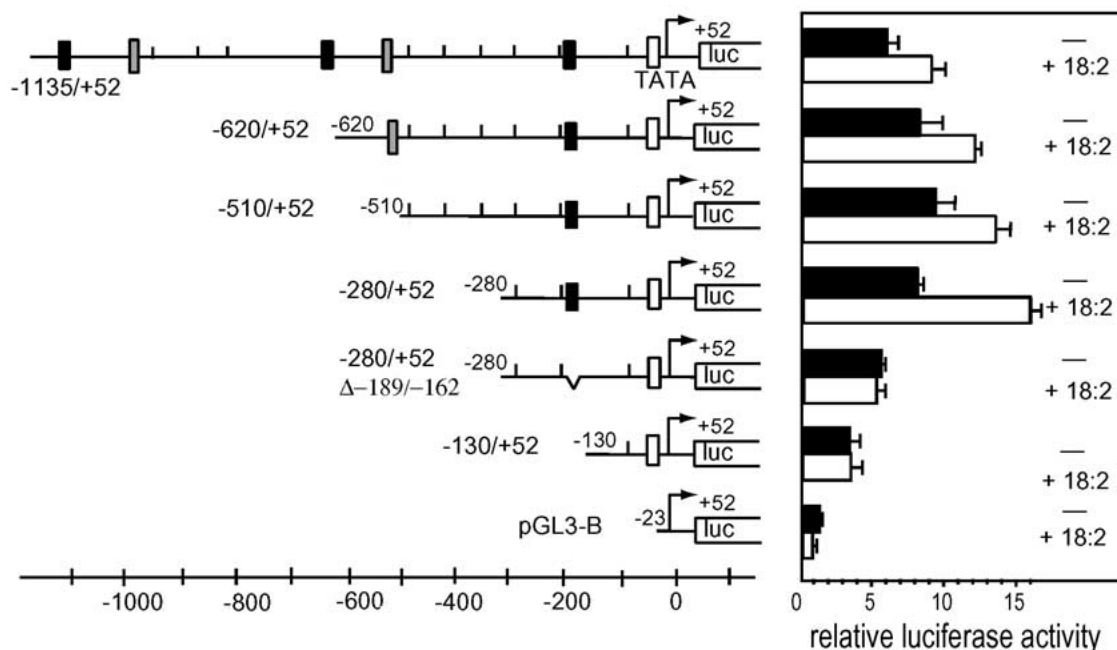


Fig. 3. Luciferase expression from the locust muscle FABP promoter. Myoblasts were transfected with reporter gene constructs, as indicated. The transfected cells were incubated for 18 h with BSA alone (black bars), or with 60 μ M linoleic acid/BSA (white bars) as described in Experimental procedures. Each result represents the average of 3–6 independent measurements \pm S.D.

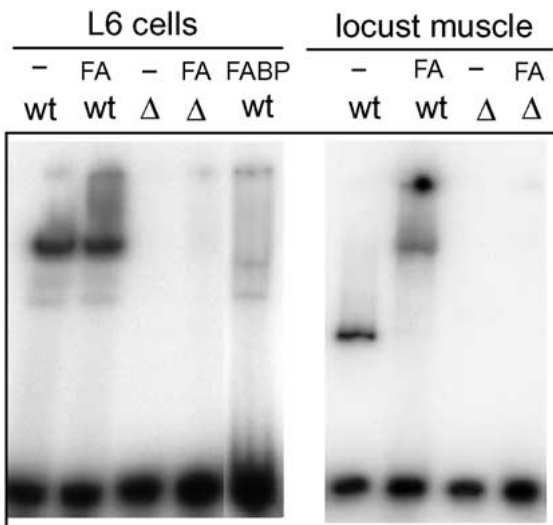


Fig. 4. Binding of nuclear proteins to the locust inverted repeat element. Electrophoretic mobility shift assays were carried out with nuclear extracts from L6 myoblasts and locust flight muscle. Each extract was treated with a radiolabeled 145-bp probe (representing the sequence $-280/-135$) that included the inverted repeat element (native probe) or an identical 126-bp probe where the element had been deleted ($\Delta-180/-162$ probe), as shown above the lanes. BSA (-, final concentration $0.1 \mu\text{M}$), fatty acid (FA, final concentration $0.3 \mu\text{M}$ linoleic acid/ $0.1 \mu\text{M}$ BSA solution (FA), or FABP (FABP, $\sim 1 \mu\text{g}$) was added to the nuclear extracts, as indicated above the lanes.

19-bp oligonucleotide competed with the labeled nucleotide, and gradually eliminated the gel-shift band. An excess of the unlabeled full fragment used in the gel-shift experiments also eliminated the band, but an otherwise identical fragment that lacked the element did not reduce binding. Nuclear proteins from locust flight muscle also interacted with the nucleotide fragments. A strong gel-shift band was seen when the labeled 145-bp probe was mixed with locust nuclear proteins; the band, however, appeared at a different location. Similar as in the rat extract, the binding was eliminated by an excess of the unlabeled 19-bp element, but not affected by the fragment devoid of the element, clearly indicating specific interactions. The addition of fatty acids to the nuclear extracts prior to gel-shift analysis led to a shift of the band seen for locust proteins. After treatment with $0.3 \mu\text{M}$ linoleic acid complexed to BSA, the labeled band moved to the same location as seen with rat extract, possibly indicating a change in protein binding induced by fatty acids. BSA alone, without fatty acids, did not alter the gel-shift pattern. Fatty acids also did not induce any binding to the deletion mutant nucleotide. The addition of FABP to the gel shift mixture, however, eliminated either band, indicating that fatty acid is essential for the binding of nuclear proteins to the fatty acid response element (Fig. 4).

Model for the regulation of the locust muscle FABP gene

From the results presented above, it is likely that two different factors bind to the fatty acid response element. At least one of these, but possibly both are activated by the binding of long chain fatty acids. This behavior is similar to the other known fatty acid response elements, which bind heterodimers of the transcription factors PPAR and RXR. However, in contrast to PPREs which are direct repeat elements (DR-1), the fatty acid response element in locust flight muscle is an inverted repeat (IR-3), similar to steroid hormone response elements. Fatty acids are required for full activity, and their availability to nuclear proteins depends on the intracellular concentration of FABP. FABP appears to have free access to the nuclear lumen [19], and thus this protein's contribution must be considered. An analogous participation of L-FABP in the PPAR mediated control of expression of the L-FABP gene has recently been shown by Wolfrum *et al.* [20].

Evidence for a fatty acid response element in the rat H-FABP promoter

The presence of a related sequence within 600 bp of upstream sequence of all mammalian heart FABP genes suggests an analogous function. Indeed, preliminary data confirm that fatty acids stimulate the expression of a reporter gene under the control of the rat promoter that contains the elements,

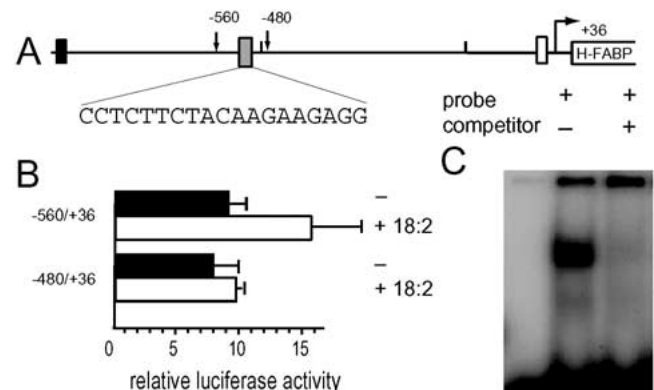


Fig. 5. Characterization of the rat H-FABP element. The promoter region of the rat H-FABP gene is shown in (A). White box: TATA box; black box: PPRE-like element; grey box: 19 bp inverted repeat element. The 5' end of the two reporter gene constructs are marked with an arrow. (B) Myoblasts were transfected with the luciferase reporter gene constructs, as described in Materials and methods. Luciferase activity was measured after incubation for 18 h with BSA (black bars) or the BSA/fatty acid complex (open bars). Each value is the mean of 3 determinations \pm S.D. (C) The double-stranded 19 bp inverted repeat element was radiolabeled and incubated with nuclear extracts from L6 cells, in the absence and presence of a 10-fold molar excess of the unlabelled 19-bp element, as indicated above the lanes.

while the response is lost in shorter constructs (Fig. 5b). Moreover, in gel shift assays the mammalian 19 bp element binds strongly to proteins from an L6 nuclear extract (Fig. 5c). However, it remains to be seen whether this element serves any regulatory function *in vivo*.

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