

A novel fatty acid response element controls the expression of the flight muscle *FABP* gene of the desert locust, *Schistocerca gregaria*

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In many tissues, fatty acid binding protein (FABP) expression is stimulated by exposure to elevated fatty acid levels. In contrast to the *FABP* genes expressed in other tissues, the molecular mechanisms that mediate the upregulation of the muscle *FABP* gene have not been elucidated. We have studied the expression of locust flight muscle FABP, a protein that is highly homologous to the mammalian H-FABPs. A 130-bp promoter fragment of the locust gene, which includes a canonical TATA box and several GC boxes, is sufficient for the transcription of a reporter gene in mammalian L6 myoblasts. Twofold higher expression rates are observed when the promoter contains 280 bp or more of upstream sequence. Treatment of myoblasts with various fatty acids leads to a marked increase of expression in the longer constructs, but not in the minimal promoter. We have identified a 19-bp inverted

repeat (–162/–180) as the element responsible for the fatty acid-mediated induction of gene expression. Deletion of this element eliminates the fatty acid response, and gel shift analysis demonstrates specific binding to nuclear proteins from both L6 myoblasts and locust flight muscle cells. This fatty acid response element bears no similarity to any known transcription factor binding site. A similar palindrome was also found in the promoter of the *Drosophila melanogaster* muscle *FABP* gene, and in reverse orientation upstream of all mammalian heart *FABP* genes. Given the structural and functional conservation of muscle FABPs and their genes, it is possible that this fatty acid response element also modulates the expression of the mammalian *H-FABP* genes.

Keywords: fatty acid binding protein; gene regulation; FARE; myoblast; gel-shift analysis.

Among the most energy-demanding tasks performed by animals is migratory flight of insects that requires many hours of sustained muscle activity [1]. Fatty acids are the preferred substrates to fuel such work, and the extreme metabolic rates encountered during locust migration demand a very large flux of fatty acids through the muscle cell. These muscles appear to be optimally adapted to safely retrieve, transport and metabolize large amounts of fatty acids [2]. One important factor in these processes is the intracellular fatty acid binding protein (FABP), which binds free fatty acids and thus prevents damage due to a fatty acid overload [3,4]. In previous studies we have demonstrated that the expression of FABP in locust flight muscle not only varies with muscle differentiation, but is also upregulated sharply in response to increased lipid flow. Extended flight and increased lipid delivery to the flight muscle lead to a marked increase in FABP expression [5]. This increase parallels similar effects seen for heart *FABP* (*H-FABP*) genes in mammalian species.

In mammalian muscles, H-FABP concentrations generally reflect the rates of fatty acid metabolism, with highest

levels found in cardiomyocytes [6]. *In vivo* and *in vitro* studies have demonstrated that increased fatty acid delivery or utilization lead to elevated FABP levels [7–9]. This increase is due to altered gene expression. In cultured rat cardiomyocytes and L6 myoblasts, fatty acid exposure resulted in the upregulation of *FABP* gene expression, at the level of transcription initiation [6,10].

Fatty acids also induce the expression of other genes involved in lipid transport and metabolism. The promoters for several of these genes contain peroxisome proliferator response elements (PPREs), direct repeats that binds peroxisome proliferator activated receptors (PPARs) [11]. These transcription factors bind, when activated through the binding of long-chain fatty acids, as heterodimers together with the retinoid receptor RXR and stimulate gene expression. Functional PPREs have been demonstrated for various enzymes involved in lipid metabolism (acyl-CoA oxidase [12], acyl-CoA synthetase [13], lipoprotein lipase [14], carnitine palmitoyl transferase [15], mitochondrial uncoupling protein [16]), and also for intracellular fatty acid binding proteins in liver and adipose cells (L-FABP [17], A-FABP [18]).

It has been suggested that similar elements are also functional in the muscle *FABP* gene [9], although this has never been demonstrated. Of all mammalian *H-FABP* genes, only the rodent genes contain direct repeat sequences that have similarity to the consensus PPRE element [19,20]. The cloned upstream region of the locust muscle *FABP* gene also lacks direct repeat elements but contains three copies of a 19-bp imperfect inverted repeat that does not resemble any identified control element [21]. Interestingly, upon re-inspection of the mammalian *H-FABP* genes, a strikingly similar repeat, albeit in reverse orientation, was also found

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Abbreviations: FABP, fatty acid binding protein; PPRE, peroxisome proliferator response element; PPAR, peroxisome proliferator activated receptor; RXR, retinoic acid receptor; ORE, oleate response element; DMEM, Dulbecco's modified Eagle medium.

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in all mammalian *H-FABP* genes that have been cloned to date, as well as in the analogous *Drosophila* gene [21]. We suspected that this element is involved in the control of the muscle FABP expression, and possibly related to its induction by fatty acids.

Functional analysis of putative fatty acid response elements can be achieved by studying the expression of reporter gene constructs in myocytes in response to fatty acid exposure. Isolated myocytes, however, are difficult to transfect and maintain in culture. As stable cell lines of differentiated muscles are not available from either insects or mammals, rat myoblasts are more suitable for these experiments. These precursor cells are very stable and are easy to transfect. They express H-FABP at lower levels than fully differentiated muscle cells, but respond in a similar manner to fatty acid treatment. H-FABP expression increases more than twofold in both myoblasts and isolated cardiomyocytes, indicating that the factors necessary for the fatty acid response are also present in the precursor cells [10]. Given the conserved nature of the muscle *FABP* promoters, it is not unlikely that the insect promoter is functional in mammalian muscle cells. However, other insect promoters have been shown to function in mammalian cells [22]. This current study was carried out to analyze the activity of the locust muscle *FABP* promoter in L6 myoblasts, and to identify the element that mediates the observed fatty acid response.

EXPERIMENTAL PROCEDURES

Construction of the promoter–luciferase fusion plasmids

With genomic DNA obtained from locust flight muscle as template, a 1.6-kb DNA fragment of the muscle *FABP* gene

and its upstream sequence (−1135/+425) was amplified by PCR (upper primer 5′-GTCATTGCTAATAACTCC-3′ (−1135), lower primer 5′-CGCAATAGCGTAAGCAGGT-3′ (+425)). The PCR product was inserted into the TA 2.1 plasmid (Invitrogen) and served as template for the amplification of the various fragments used in reporter gene construction. Different regions of the muscle *FABP* promoter were amplified 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′-ATGGTACCCATAATCACACTCATGTTA-3′; F−510/−490 5′-ATGGTACCTGACCATTGCAATAAGATTT-3′; F−280/−260 5′-ATGGTACCACTGCGAAACAGATGAATGT-3′; F−130/−100 5′-ATGGTACCTAGCAGTCATGAAACAGAAT-3′; F−23/+5 5′-ATGGTACCGGC CGCCACCGGACGAGC-3′; R+52/+34 5′-AAGCTAGCGCT GCTGTGGTGGCGGT-3′; R+85/+63 5′-ATGCTAGCTACTTGATGCTGCGAAT-3′; R−562/−544 5′-ATGCTAGCCTCAACAAGGAGATTCCG-3′). The PCR products were digested with *Kpn*II and *Nhe*I, gel purified and cloned into the pGL3-basic luciferase vector (Promega). 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*II 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*II 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. 1.

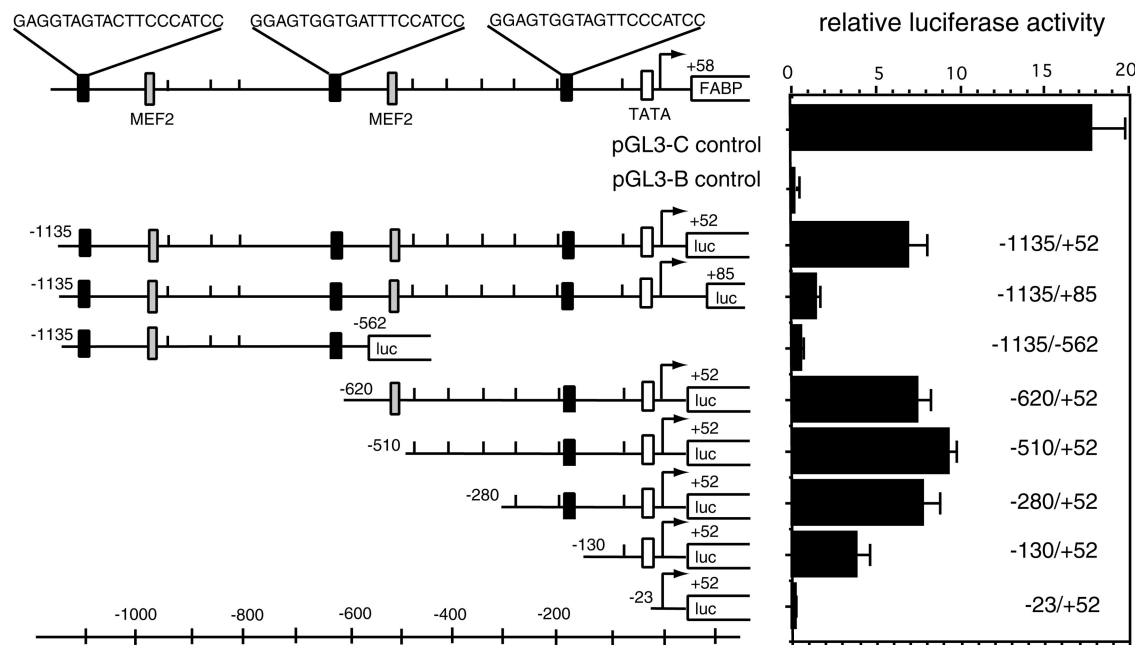


Fig. 1. Reporter gene constructs and their activity in untreated myoblasts. Left panel: reporter gene constructs were prepared as detailed in Experimental procedures. Shown on the schematic drawings of the constructs are the location of the TATA box (open box) and potential regulatory elements (I, E-box; grey boxes, MEF-2 consensus sequence; black boxes, inverted repeat element). The transcription start site is marked with an arrow. Right panel: myoblasts were transfected with reporter constructs, as indicated. Luciferase activity was measured as described in Experimental procedures. Each result represents the average of three to five independent measurements \pm SD.

Cell culture and treatment

Rat L6 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum, 150 $\mu\text{g}\cdot\text{mL}^{-1}$ penicillin and 150 $\mu\text{g}\cdot\text{mL}^{-1}$ streptomycin, as previously described [10]. Prior to transfection, cells were grown for 3 days to reach 80–90% confluency. After disposal of the medium, the cells were rinsed twice with NaCl/P_i (0.01 M phosphate, 0.9% NaCl, pH 7.4). After addition of 2 mL of serum-free medium (Opti-MEM, Life Technologies, Burlington, ON, USA), transient transfections were performed using LipofectAMINE 2000 reagent (Life Technologies) according to the manufacturer's instructions. For each dish, 5 μg luciferase reporter gene construct, 500 ng internal control vector pRL-TK (Promega) and 12 μL Lipofect reagent were gently mixed in 400 μL Opti-MEM and left at room temperature for 15 min. Subsequently, the transfection mixture was added to the cells. After 6 h incubation at 37 °C, 5% CO₂, the medium was changed back to DMEM.

Fatty acid treatment of transfected cells was carried out as described previously [10]. Briefly, a complex of 600 μM of fatty acid/200 μM BSA was prepared by dissolving essentially fatty-acid-free BSA and palmitic acid (16:0), oleic acid (18:1) or linoleic acid (18:2) (Sigma) in 0.9% NaCl. The fatty acid–BSA complex solution was sterile filtered and added to cell culture media following the 6 h transfection period, at a final concentration of 60 μM fatty acid. As a control, the same volume of a 200- μM BSA solution was used. Incubations were carried out for 18 h.

Luciferase assay

Cells were harvested and lysed with 200 μL passive lysis buffer (Promega), and cell debris was removed by centrifugation. A dual-luciferase reporter assay system (Promega) was employed to evaluate the relative luciferase activity, using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). The assay distinguishes between firefly (*Photinus pyralis*) luciferase activity (derived from pGL3-based constructs) as an indicator for the promoter activity, and sea pansy (*Renilla reniformis*) luciferase activity (derived from cotransfected plasmid pRL-TK, under control of the herpes simplex virus thymidine kinase promoter). The latter enzyme was used as internal control to adjust for variations in transfection efficiency and lumino-metric detection.

Preparation of nuclear extracts

All steps were carried out at 4 °C or on ice. Flight muscle was homogenized under liquid nitrogen, and the frozen muscle powder suspended in 4 vol. 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 phenylmethanesulfonyl fluoride, 0.15 mM spermine, 0.5 mM spermidine, 2 $\mu\text{g}\cdot\text{mL}^{-1}$ leupeptin and 2 $\mu\text{g}\cdot\text{mL}^{-1}$ aprotinin; all from Sigma). The tissue was homogenized by 15–20 strokes in a Potter–Elvehjem glass homogenizer with a Teflon pestle. After addition of 0.1% Nonident P-40 the homogenate was filtered through two layers of cheesecloth and centrifuged for

10 min at 1300 g. The pelleted nuclei were washed three times with homogenization buffer.

Nuclear extracts were prepared using the Nu-CLEAR extraction kit (Sigma). The nuclei were suspended in 3 vol. 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 phenylmethanesulfonyl fluoride, 2 $\mu\text{g}\cdot\text{mL}^{-1}$ leupeptin and 2 $\mu\text{g}\cdot\text{mL}^{-1}$ aprotinin). After the addition of 400 mM NaCl, the suspension was stirred for 30 min and centrifuged for 15 min at 13000 g. The supernatant was dialyzed for 3 h against 100 vol. 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 phenylmethanesulfonyl fluoride). The dialysate was then centrifuged at 13 000 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 NaCl/P_i, and cells were harvested. The pooled cells were centrifuged at 800 g for 10 min, suspended in 3 vol. of homogenization buffer, and processed further as described above.

Electrophoretic mobility shift assay

DNA fragments for electrophoretic mobility-shift assays were amplified by PCR from the cloned reporter gene constructs –280/+53 (wild-type) and –280/+53Δ–180/–162 (deletion mutant), with primers annealing at –280/–260 bp (5'-ACTGCGAAA CAGATGAATGT-3') and at –135/–154 bp (5'-GTCCAAACATCGAGTGTGA-3'). The PCR products (145 bp for wild-type, 126 bp for mutant) were end-labeled with [γ -³²P]ATP (3000 Ci·mmol^{–1}, NEN) and T4 polynucleotide kinase. Nuclear extracts (\approx 2 μg of protein) were preincubated 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 $\mu\text{g}\cdot\text{mL}^{-1}$ poly(dI-dC)/poly(dI-dC) and 5% glycerol) for 10 min at 24 °C. The labelled probe (\approx 15 ng) was added and incubated at 24 °C for 30 min. Subsequently, the reaction mixture was loaded onto nondenaturing polyacrylamide gels (5% T, 3.3% C) and electrophoresed at 20 V·cm^{–1} for 30 min in 0.5 × NaCl/Tris (45 mM Tris, 45 mM boric acid, 1 mM EDTA). The gels were dried and analyzed by autoradiography. For the competition experiments, the preincubation was performed in the presence of unlabelled competitor DNA at the molar excess indicated in the figure legends.

RESULTS

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 < 12-fold higher than for a promoterless 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 three times within the first thousand basepairs upstream of the TATA box (Fig. 1). Similar levels of

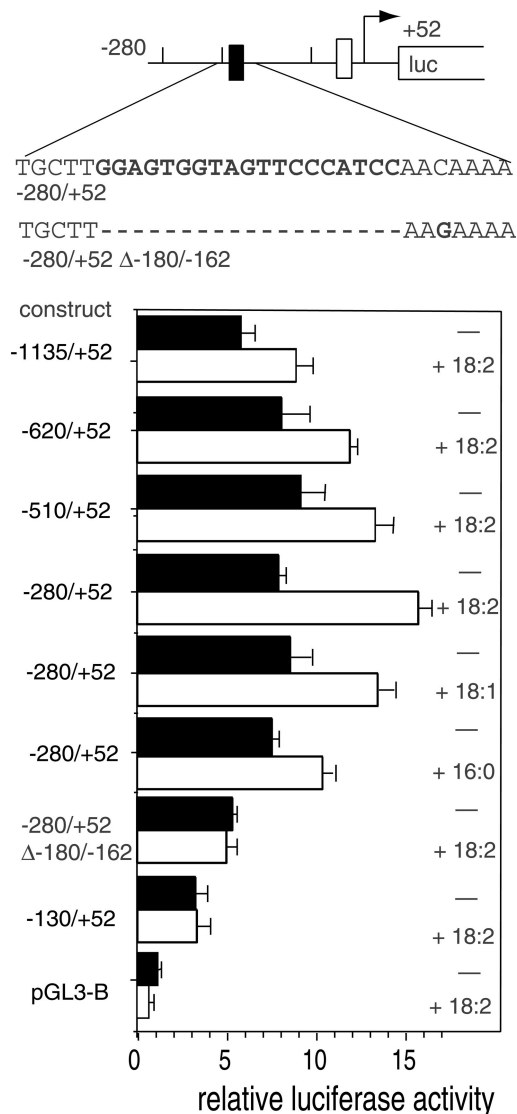


Fig. 2. Fatty acid induction of reporter gene expression. Myoblasts were transfected with reporter gene constructs, as indicated. The transfected cells were incubated for 18 h with BSA alone (black bars), or with 60 μ M fatty acid/BSA (white bars) as described in Experimental procedures. 16:0, palmitic acid; 18:1, oleic acid; 18 : 2, linoleic acid. Each result represents the average of three to six independent measurements \pm SD. The differences between the native construct $-280/+52$ and the deletion mutant $-280/+52 \Delta-180/-162$ are depicted above the chart. The other reporter gene constructs used are illustrated in Fig. 1.

luciferase activity were measured for all constructs that contained at least 280 bp of upstream sequence. A shorter construct that started just downstream of the inverted repeat sequence also expressed luciferase, albeit at much weaker levels ($< 50\%$) 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 of the inverted repeat element, a deletion mutant was constructed that contained

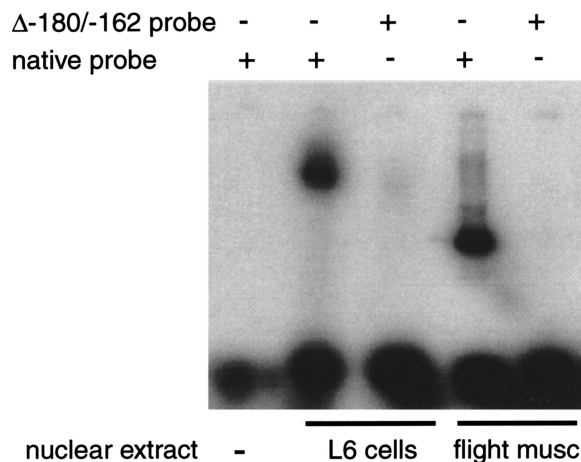


Fig. 3. Binding of nuclear proteins to the inverted repeat element. Electrophoretic mobility shift assays were carried out with nuclear extracts from L6 myoblasts and locust flight muscle. Each extract was treated with a radiolabeled 145-bp probe (representing the sequence $-280/-135$) that included 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.

the entire promoter ($-280/+52$), but omitted the 19-bp repeat ($\Delta-180/-162$). One nucleotide substitution in the promoter sequence was necessary ($-159 C \rightarrow G$) in order to introduce a restriction site needed to construct this mutant. The deletion-mutant promoter continued to strongly stimulate reporter gene expression; luciferase activity was approximately one-third lower than for the wild-type promoter, but still remained higher than that of the minimal active promoter construct ($-130/+52$) (Fig. 2).

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 twofold) 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 monounsaturated fatty acids also led to a clear increase in luciferase expression (≈ 1.5 -fold). 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 (Fig. 2).

To find out whether the inverted repeat element binds 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. 3). 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 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 (Fig. 4).

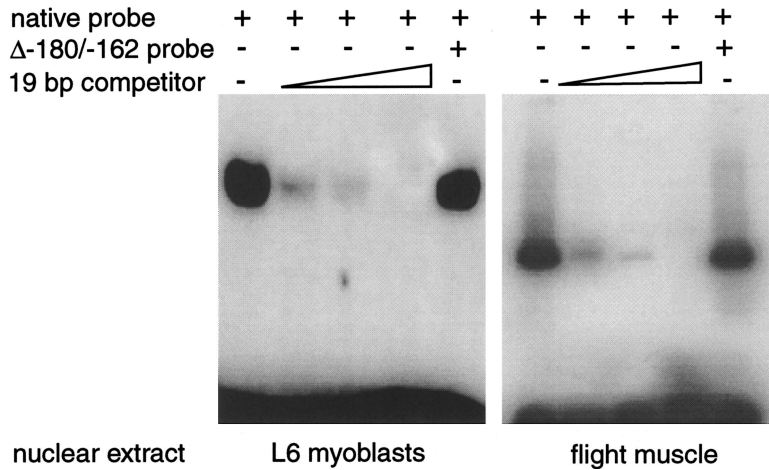


Fig. 4. Influence of unlabelled competitors on the binding of nuclear proteins. Electrophoretic mobility shift assays were carried out with nuclear extracts from L6 myoblasts (left panel) and locust flight muscle (right panel) in absence and presence of competitor probe. Each extract was treated with the radiolabeled 145-bp native probe, as specified in Fig. 3. Increasing amounts (5 ×, 10 × and 25 × molar excess) of the double stranded 19-bp element (−180/−162) were added to the extract. The right lane of each panel shows the pattern after adding 10 × molar excess of unlabelled probe that lacks the 19-bp element.

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. However, the band appeared at a different location (Fig. 3). 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 (Fig. 4).

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 μ M linoleic acid complexed to BSA, the labeled band moved to the same location as observed with rat extract, possibly indicating a change in protein binding induced by fatty acids (Fig. 5, right panel). BSA alone, without fatty acids, did not alter the gel-shift pattern. Furthermore, fatty acids did not induce any binding to the deletion mutant nucleotide. No changes were visible with rat myoblast proteins, other than a smear

between the loading well and the gel-shift band, probably due to aggregation or denaturation of binding proteins (Fig. 5, left panel). Aggregation was much more pronounced with larger fatty acid concentrations (3 μ M, data not shown).

DISCUSSION

In this study we have demonstrated that the promoter of the locust muscle *FABP* gene is sufficient for its expression in a vertebrate myoblast cell line. The basic promoter sequence contains a canonical TATA box and adjacent GC-rich regions that are common in strong promoters. Longer promoter constructs yielded twofold higher expression rates, indicating the presence of additional regulatory elements shared by insect and mammals. The overall expression rate of the full-length promoter is in the same order of magnitude as seen with the universal SV40 enhancer/promoter. Most muscle-specific regulatory factors are expressed only in differentiated muscle cells [23], and therefore the promoter should be much stronger in muscle tissue. Previous studies have shown that the native *H-FABP* genes are expressed at low rates in rat L6 or mouse C2C12 myoblasts, but much more strongly in skeletal or cardiac muscle [10,24]. Reporter genes under the control of the human *H-FABP* promoter are also expressed at much lower levels in mouse C2C12 myoblasts than in cardiac myocytes [25]. The relatively low expression rate caused by the absence of muscle-specific transcription factors in myoblasts allowed us to focus on the elements responsible for fatty acid-mediated induction of gene expression.

All promoter constructs containing at least 280 bp of upstream sequence responded to fatty acid treatment. Using the small fatty acid concentrations that have previously been shown to induce the expression of the native *FABP* gene in L6 myoblasts twofold to threefold [10], a similar increase was also observed for the locust *FABP* promoter with 280 bp or more of upstream sequence, but not for the minimal promoter. Treatment of L6 myoblasts with 60 μ M linoleic acid, well within the physiological range, resulted in a twofold increase in expression. Palmitate and oleate also stimulated reporter gene expression, a finding that suggests that the element is activated by fatty acids independent of their degree of desaturation. Through deletion mutant

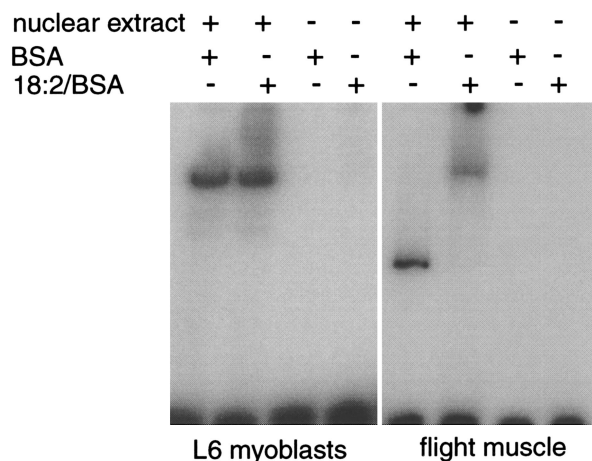


Fig. 5. Influence of fatty acids on the binding of nuclear proteins. Electrophoretic mobility shift assays were carried out with nuclear extracts from L6 myoblasts (left panel) and locust flight muscle (right panel) in absence and presence of BSA and fatty acids. Each extract was treated with the radiolabeled 145-bp native probe, as specified in Fig. 3. BSA (1 μ M) or 3 μ M linoleic acid (18:2)/1 μ M BSA solution was added to the nuclear extracts (final concentration 0.3 μ M fatty acid, 0.1 μ M BSA), as indicated above the lanes.

analysis we tracked down the fatty acid response element to a 19-bp inverted repeat, a potential candidate because of its symmetry and conservation among other *H-FABP* genes [19]. Transcription factor database searches failed to identify any known control element within this sequence. The 19-bp palindromic sequence is unlike other fatty acid receptor binding sites that are generally direct repeats of a consensus element [26]. The fatty acid response element found here is obviously not related to PPREs, direct repeat elements with a consensus sequence 5'-AGGNCAAAGGTCA-3' [11]. In mammalian tissues, these elements are activated by PPAR, a transcription factor that binds long-chain fatty acids and other hydrophobic substrates. Functional PPREs have not been described in insect genes and no close homologue to the PPAR transcription factor has been found in the *Drosophila* genome. Another direct repeat element (5'-TGGCTGCTGGCTG-3') was recently identified in the promoter of the human plasminogen activator inhibitor-1 gene [27]. Other known mammalian transcription factors may be targets for fatty acid control (e.g. HNF4, thyroid receptors) [11], but their binding sites are also unrelated to the identified fatty acid response element. Some structural similarity with an oleate response element (ORE) identified in yeast [28] is noteworthy. The ORE element is a 23-bp imperfect inverted repeat that binds two Zn-cluster transcription factors, Pip2p and Oaf1p [29]. Activation of gene expression occurs in the presence of oleic acid, which binds exclusively to Oaf1p. No homologues to these transcription factors are present in multicellular eukaryotes. It is conceivable, however, that the 19-bp fatty acid response element also interacts with two proteins, as do all nuclear binding sites activated by the steroid hormone receptor superfamily [26]. If activation of the locust fatty acid response element is mediated in a similar manner, binding would require two hexanucleotide half-sites interspersed by several nucleotides. By comparing the sequences of the three copies of the element upstream of the locust *FABP* gene, as well as the putative element in the *D. melanogaster FABP* promoter, two potential half-sites become clear: 5'-GGAGTGGT NNN TCCCATCC-3'. Thus, the 19-bp fatty acid response element may contain an inverted repeat element separated by three nucleotides (IR-3) with the half-site sequences 5'-AGTGGT-3' and 5'-ATGGGA-3'. While neither of these sequences have been shown to bind to steroid hormone receptors, their sequences have some similarity to such elements (consensus sequences 5'-AGAACA-3' or 5'-AGGTCA-3'). At this time, it is impossible to predict the nature of the transcription factors responsible for the fatty acid-induction of the insect *FABP* gene expression.

The existence of such factors, however, has been clearly demonstrated in gel-shift assays. Proteins that specifically interact with the fatty acid response element are present in nuclear extracts of both rat myoblasts and insect flight muscles, indicating that the fatty acid response element is also active within insect muscles. Interestingly, the oligonucleotide is retarded less by insect muscle extract, suggesting that a smaller protein, or fewer protein molecules, bind to this element. Symmetrical elements often bind two separate molecules of transcription factors, either as homodimers or heterodimers, and it is therefore possible that both factors are bound in nuclear extract of rats, but only one in the locust extract. Binding to the fatty

acid response element requires that at least one of these factors is activated by free fatty acid. Because of the low water solubility, however, the fatty acids present in the cell are mostly bound by FABP, and hence their availability depends on the FABP concentration. Using immuno-gold labeling techniques, we have previously demonstrated that the FABP concentrations in the cytosol and the nuclear lumen increase similarly during locust muscle development, as expected for a small protein that can diffuse freely through nuclear pores [30]. Thus, high cytosolic levels of FABP lead to similar nuclear levels of the protein, thereby providing large numbers of binding sites for fatty acids. While locust flight muscle is the tissue with the greatest known FABP concentration, myoblasts contain only minimal amounts of FABP [24]. A nuclear fatty acid receptor would therefore compete with FABP in locust muscle extracts, but not in the myoblasts. A similar competition between L-FABP and PPAR has been previously suggested [31]. In our experiments, nuclear protein concentrations were adjusted to $0.1 \text{ mg}\cdot\text{mL}^{-1}$, and assuming that the relative FABP content in the nucleus is similar as in the cytosol, the concentration of this protein may be less than 1 nM in the nuclear extract from myoblasts [24], but approach 10 μM for the extract from locust muscle [30]. Therefore, larger amounts of fatty acids should be required to activate a fatty acid receptor in the insect cells. Indeed, when the fatty acid concentration in the locust extract was raised, a gel-shift pattern similar to that of the rat extract was observed (Fig. 5). It appears that FABP is indeed an integral player of the nuclear events that control its expression. It will be of great interest to study the effect of fatty acids and FABP in electrophoretic mobility shift assays in more detail.

When L6 myoblasts were treated with fatty acids, the expression of the locust reporter gene increased in a very similar manner as the *FABP* gene that is native to these cells [10]. Rat H-FABP is expressed only at small levels in myoblasts, but its mRNA increases \approx twofold after fatty acid treatment. The increase of the mRNA follows a similar rise of its primary transcript that can be seen within minutes after fatty acid exposure. This effect is also elicited by fatty acids of varying chain-length and degree of desaturation.

Given the absence of a PPRE in at least some of the mammalian promoters, and the presence of nuclear proteins that bind to the fatty acid response element characterized in the locust promoter, it may well be possible that the mammalian genes are regulated by similar elements. The high degree of conservation found for the *H-FABP* genes may also include regulatory elements in their promoters. It has been proposed that two major subfamilies of the *FABP* gene branched off a common ancestor more than 900 million years ago, well before the vertebrate/invertebrate divergence [32]. Related fatty acid response elements may have been conserved in all muscle *FABP* genes. In this respect, it should be noted that the mammalian *FABP* genes also possess a strikingly similar element, but in reverse orientation [21]. Rat and mouse *H-FABP* have a perfect inverted repeat 510 and 524 bp upstream, respectively, and closely related repeats are also present upstream of the human [33] and porcine *H-FABP* genes [34]. It remains to be seen whether the sequences also bind to nuclear proteins, and whether these or other elements are involved in the

fatty-acid-mediated control of *H-FABP* expression in vertebrates.

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REFERENCES

- Crabtree, B. & Newsholme, E.A. (1975) Comparative aspects of fuel utilization and metabolism by muscle. In *Insect Muscle* (Usherwood, P.N.R., ed.), pp. 405–491. Academic Press, London.
- Haunerland, N.H. (1997) Transport and utilization of lipids in insect flight muscles. *Comp. Biochem. Physiol.* **117B**, 475–482.
- Vogel Hertzler, A. & Bernlohr, D.A. (2000) The mammalian fatty acid-binding protein multigene family: molecular and genetic insights into function. *Trends Endocrinol. Metab.* **11**, 175–180.
- Glatz, J.F., Van Breda, E. & Van der Vusse, G.J. (1998) Intracellular transport of fatty acids in muscle. Role of cytoplasmic fatty acid-binding protein. *Adv. Exp. Med. Biol.* **441**, 207–218.
- Chen, X. & Haunerland, N.H. (1994) Fatty acid binding protein expression in locust flight muscle. Induction by flight, adipokinetic hormone and low density lipophorin. *Insect Biochem.* **24**, 573–579.
- Veerkamp, J.H. & van Moerkerk, H.T.B. (1993) Fatty acid binding protein and its relation to fatty acid oxidation. *Mol. Cell. Biochem.* **123**, 101–106.
- van Breda, E., Keizer, H.A., Vork, M.M., Surtel, D.A.M., de Jong, Y.F., van der Vusse, G.J.K. & Glatz, J.F.C. (1992) Modulation of fatty-acid-binding protein content of rat heart and skeletal muscle by endurance training and testosterone treatment. *Pflügers Arch.* **421**, 274–279.
- van der Lee, K.A., Vork, M.M., De Vries, J.E., Willemsen, P.H., Glatz, J.F., Reneman, R.S., Van der Vusse, G.J. & Van Bilsen, M. (2000) Long-chain fatty acid-induced changes in gene expression in neonatal cardiac myocytes. *J. Lipid Res.* **41**, 41–47.
- Kempen, K.P., Saris, W.H., Kuipers, H., Glatz, J.F. & Van Der Vusse, G.J. (1998) Skeletal muscle metabolic characteristics before and after energy restriction in human obesity: fibre type, enzymatic beta-oxidative capacity and fatty acid-binding protein content. *Eur. J. Clin. Invest.* **28**, 1030–1037.
- Chang, W., Rickers-Haunerland, J. & Haunerland, N.H. (2001) Induction of cardiac *FABP* gene expression by long chain fatty acids in cultured rat muscle cells. *Mol. Cell. Biochem.* **221**, 127–132.
- Jump, D.B. & Clarke, S.D. (1999) Regulation of gene expression by dietary fat. *Annu. Rev. Nutr.* **19**, 63–90.
- Osumi, T., Wen, J.K. & Hashimoto, T. (1991) Two cis-acting regulatory sequences in the peroxisome proliferator-responsive enhancer region of rat acyl-CoA oxidase gene. *Biochem. Biophys. Res. Commun.* **175**, 866–871.
- Tugwood, J.D., Isseemann, I., Anderson, R.G., Bundell, K.R., McPheat, W.L. & Green, S. (1992) The mouse peroxisome proliferator activated receptor recognizes a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. *EMBO J.* **11**, 433–439.
- Amri, E.Z., Teboul, L., Vannier, C., Grimaldi, P.A. & Ailhaud, G. (1996) Fatty acids regulate the expression of lipoprotein lipase gene and activity in preadipose and adipose cells. *Biochem. J.* **314**, 541–546.
- Brandt, J.M., Djouadi, F. & Kelly, D.P. (1998) Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha. *J. Biol. Chem.* **273**, 23786–23792.
- Van der Lee, K.A.J.M., Willemsen, P.H.M., van der Vusse, G.J. & van Bilsen, M. (2000) Effects of fatty acids on uncoupling protein-2 expression in the rat heart. *FASEB J.* **14**, 495–502.
- Wolfrum, C., Ellinghaus, P., Fobker, M., Seedorf, U., Assmann, G., Börchers, T. & Spener, F. (1999) Phytanic acid is ligand and transcriptional activator of murine liver fatty acid binding protein. *J. Lipid Res.* **40**, 708–714.
- Frohnert, B.I., Hui, T.Y. & Bernlohr, D.A. (1999) Identification of a functional peroxisome proliferator-responsive element in the murine fatty acid transport protein gene. *J. Biol. Chem.* **274**, 3970–3977.
- Zhang, J., Rickers-Haunerland, J., Dawe, I. & Haunerland, N.H. (1999) Structure and chromosomal location of the rat gene encoding the heart fatty acid-binding protein. *Eur. J. Biochem.* **266**, 347–351.
- Treuner, M., Kozak, C.A., Gallahan, D., Grosse, R. & Müller, T. (1994) Cloning and characterization of the mouse gene encoding mammary-derived growth inhibitor/heart-fatty acid-binding protein. *Gene* **147**, 237–242.
- Wu, Q., Andolfatto, P. & Haunerland, N.H. (2001) Cloning and sequence of the gene encoding the muscle fatty acid binding protein from the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol.* **31**, 553–562.
- Fatyol, K., Illes, K. & Szalay, A.A. (1999) An alternative intronic promoter of the *Bombyx mori* A3 cytoplasmic actin gene exhibits high level of transcriptional activity in mammalian cells. *Mol. Gen. Genet.* **261**, 337–345.
- Black, B.L. & Olson, E.N. (1998) Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu. Rev. Cell Dev. Biol.* **14**, 167–196.
- Rump, R., Buhlmann, C., Börchers, T. & Spener, F. (1996) Differentiation-dependent expression of heart type fatty acid-binding protein in C2C12 muscle cells. *Eur. J. Cell Biol.* **69**, 135–142.
- Qian, Q., Kuo, L., Yu, Y.T. & Rottman, J.N. (1999) A concise promoter region of the heart fatty acid-binding protein gene dictates tissue-appropriate expression. *Circ. Res.* **84**, 276–289.
- Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. et al. (1995) The nuclear receptor superfamily: the second decade. *Cell* **83**, 835–839.
- Chen, Y., Billadello, J.J. & Schneider, D.J. (2000) Identification and localization of a fatty acid response region in the human plasminogen activator inhibitor-1 gene. *Arterioscler. Thromb. Vasc. Biol.* **20**, 2696–2701.
- Einerhand, A.W., Kos, W.T., Distel, B. & Tabak, H.F. (1993) Characterization of a transcriptional control element involved in proliferation of peroxisomes in yeast in response to oleate. *Eur. J. Biochem.* **214**, 323–331.
- Baumgartner, U., Hamilton, B., Piskacek, M., Ruis, H. & Rottensteiner, H. (1999) Functional analysis of the Zn(2) Cys(6) transcription factors Oaf1p and Pip2p. Different roles in fatty acid induction of beta-oxidation in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **274**, 22208–22216.
- Haunerland, N.H., Andolfatto, P., Chisholm, J.M., Wang, Z. & Chen, X. (1992) Fatty-acid-binding protein in locust flight muscle: Developmental changes of expression, concentration and intracellular distribution. *Eur. J. Biochem.* **210**, 1045–1051.
- Desvergne, B. & Wahli, W. (1995) PPAR: a key nuclear factor in nutrient/gene interaction? In *Inducible Gene Expression* (Baeuerle, P.A., ed.), Vol. 1, pp. 142–176. Birkhäuser, Boston.
- Matarese, V., Stone, R.L., Waggoner, D.W. & Bernlohr, D.A. (1989) Intracellular fatty acid trafficking and the role of cytosolic lipid binding proteins. *Prog. Lipid Res.* **28**, 245–272.
- Phelan, C., Morgan, K., Baird, S., Korneluk, K., Narod, S. & Pollak, M. (1996) The human mammary-derived growth inhibitor (MDGI) gene: genomic structure and mutation analysis in human breast tumors. *Genomics* **34**, 63–68.
- Gerbens, F., Rettenberger, G., Lenstra, J.A., Veerkamp, J.H. & te Pas, M.F. (1997) Characterization, chromosomal localization, and genetic variation of the porcine heart fatty acid-binding protein gene. *Mamm. Genome* **8**, 328–332.