A Concise Promoter Region of the Heart Fatty Acid–Binding Protein Gene Dictates Tissue-Appropriate Expression

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Abstract—The heart fatty acid–binding protein (HFABP) is a member of a family of binding proteins with distinct tissue distributions and diverse roles in fatty acid metabolism, trafficking, and signaling. Other members of this family have been shown to possess concise promoter regions that direct appropriate tissue-specific expression. The basis for the specific expression of the HFABP has not been previously evaluated, and the mechanisms governing expression of metabolic genes in the heart are not completely understood. We used transient and permanent transfections in ventricular myocytes, skeletal myocytes, and nonmyocytic cells to map regulatory elements in the HFABP promoter, and audited results in transgenic mice. Appropriate tissue-specific expression in cell culture and in transgenic mice was dictated by 1.2 kb of the 5′-flanking sequence of FABP3, the HFABP gene. Comparison of orthologous murine and human genomic sequences demonstrated multiple regions of near-identity within this promoter region, including a CArG-like element close to the TATA box. Binding and transactivation studies demonstrated that this element can function as an atypical myocyte enhancer–binding factor 2 site. Interactions with adjacent sites are likely to be necessary for fully appropriate, tissue-specific, developmental and metabolic regulation. (Circ Res. 1999;84:276-289.)

Key Words: fatty acid–binding protein ■ myocyte ■ promoter region ■ transgene

The profound and continuous energy demands of cardiac contraction are associated with a variety of specialized metabolic adaptations, including a preferential use of fatty acids as the energy substrate.1 This requires a high transcytoplasmic flux of these insoluble and relatively toxic molecules. Heart fatty acid–binding protein (HFABP) constitutes 4% to 8% of the soluble cytoplasmic protein in mammalian heart.2 This 14-kDa protein, which binds long-chain fatty acids, and their acyl and carnitine derivatives with 1:1 stoichiometry and high affinity, is thought to play a major role in facilitating the intracellular transport of fatty acids and may be important in protecting the myocyte from their deleterious effects.3,4

HFABP, the product of the FABP3 gene, is a member of a family of intracellular lipid-binding proteins whose gene structures are consistent with an origin by repeated gene duplication.5 These homologous, low molecular weight, soluble cytosolic proteins (identities ranging from 25% to 95%) are commonly named for the tissues from which they were first isolated. Members include the liver, intestinal, ileal, adipocyte, epidermal, retinal, and peripheral myelin lipid-binding proteins, and, more distantly, the cytosolic retinoic and retinol binding proteins.6–12 Germline deletion of the adipocyte fatty acid–binding protein (FABP) by homologous recombination has suggested a major role for this family member in the cellular and systemic regulation of metabolism.13

Differences in the level of the FABP3 gene product in different tissue types and variations in response to environmental stimuli have long been recognized.14–16 In general, mRNA and protein levels change in parallel, and patterns of distribution of mRNA and protein are similar in humans and other species.17,18 The highest relative expression occurs in the heart, with lower levels of expression in “slow” skeletal muscle. Lower but detectable levels of expression occur in “fast” skeletal muscle, the aorta, placenta, adrenal, ovary, and testis. The same gene is expressed in a highly regulated manner in the mammary gland, where it was initially termed mammary-derived growth inhibitor (MDGI).19 MDGI is barely detectable in the quiescent mammary gland but is dramatically upregulated with lactation.

The developmental distribution of HFABP has been evaluated in rats: HFABP was detectable in day 19 rat embryo hearts at 20% of adult levels, increased 3-fold to term, and continued to increase during the postnatal period until weaning.15 These changes parallel developmental increases in mitochondrial number, carnitine level, palmitoyl transferase activity, and β-oxidative capacity. Hormonal milieu and
exercise also modulate HFABP expression, with higher levels in females. Exercise training increases HFABP expression per gram of muscle in heart and skeletal muscle, as does chronic electrostimulation of fast-twitch muscles. Modest changes in HFABP occur with increased dietary-lipid contents. Hearts from diabetic rats show increased HFABP protein and mRNA, and these levels normalize on islet cell transplantation.

The molecular basis for the tissue-specific and regulated expression of HFABP is not known. Although a number of genes expressed at high levels in the heart have been analyzed, the vast majority of these genes encode contractile proteins. The heart has a different substrate preference than most other tissues, abnormalities of metabolism are frequently an early change in many inherited and acquired abnormalities of heart function, and metabolic perturbations can themselves result in cardiomyopathy. The choice of substrate for energy metabolism can also alter the oxygen cost for ATP production, an important consideration in the presence of flow-limiting coronary perfusion. It is important and unknown whether similar mechanisms govern the expression of metabolic and contractile genes in the heart. The high levels of expression of HFABP in heart and the compact and highly informative promoter regions present in other FABPs prompted us to investigate transcriptional regulation of cardiac FABP.

We report that a 1.2 kb region of the 5'-flanking sequence of the HFABP gene is sufficient to direct cell-type specific expression in tissue culture and tissue-appropriate patterns of expression in transgenic mice. Comparison of orthogonal human and mouse sequences demonstrates multiple regions of striking conservation within this short region. A completely conserved CArG-like element close to the TATA box constitutes a myocyte enhancer–binding factor 2 (MEF2) site on the basis of binding and transactivation. Although this site does not correspond to an established MEF2 consensus sequence, it can be recognized by a threshold relaxation of the probability weight matrix method. Other sites are likely to be important in factor-factor interactions that enhance specific expression and couple tissue-specific and metabolic regulation.

Materials and Methods

Genomic Cloning

A randomly primed [32P]-dCTP labeled Kpn1-EcoR1 fragment of the rat HFABP cDNA (Dr Jeff Gordon, Washington University School of Medicine), encompassing nucleotides 69 to 447 of the coding region, was used to screen 1×10^6 plaques of a λ-EMBL3 human placental genomic library (Stratagene) by hybridization. Clones H1 and H5 were obtained, restriction maps generated, and inserts subcloned in plasmid vectors for further analysis. Dideoxy chain termination sequencing was performed on both strands with universal and specific sequencing primers (DNA International); when necessary, nested deletions were created according to the EcoIII/mung bean nuclease method (Erase-a-base, Promega). Recombinations of this same library together with an additional 6×10^6 plaques of a λ-FIX II human lymphocyte genomic library (Stratagene) with probes prepared from (1) the 266 bp intrinsic sequence at the 5'-end of clone H1, (2) the 331 bp from the 5'-end (Kpn1-NcoI) fragment of the rat HFABP cDNA, and (3) a full-length human HFABP cDNA obtained from a λ-ZAP II human heart library (Stratagene) completed the genomic cloning. Six additional overlapping clones (H8, H10, H11, H13, H14, and H15) were obtained. A similar approach was used to clone the 5'-flanking sequence of the mouse HFABP gene from a FIX II mouse genomic library (129SV, Stratagene).

HFABP Promoter Constructs

Constructs were derived from the human HFABP promoter. An Xho1 site was introduced in the wild-type HFABP promoter at +29 relative to the primary transcription start site by converting CCTAGA to TCTAGA. Cassettes of 0.4 kb (Spel site), 0.6 kb (Hind3), 1.2 kb (Spel), and 4.6 kb (Hind3) were cloned between the Hind3 and Xho1 sites in the polylinker region of the promoterless expression vectors pCAT-basic (Promega) and p0GH (Nichols Institute), which created the chimeric expression plasmids pCAT-HFABP0.4–pCAT-HFABP4.6 and p0GH-HFABP0.4–p0GH-HFABP4.6, respectively.

For mutagenesis, the 0.4 kb HFABP promoter was cloned into pALTER-1 (Promega), and the mutation was introduced with the use of the Altered Sites II mutagenesis system (Promega) with the complementary oligonucleotide (change underlined): (−68) 5'-GCTCCGAAATGAGGACCCCGA3'. Dideoxy chain termination sequencing confirmed the presence of only the desired mutation.

Tissue Culture and Transfections

C2C12 and Sol8 cells, permanent murine cell lines of fast and slow skeletal myocyte derivation, respectively, were maintained in 14% FCS in DMEM in the presence of penicillin/streptomycin (growth media) and passed at 80% confluence. Differentiation was induced with the change to 4% heat-inactivated horse serum in DMEM (differentiation media) and was visually monitored by the appearance of multinucleated myotubes. Only cells with a low passage number were used. CaCo-2 cells were maintained in 20% FCS in DMEM.

Primary ventricular myocytes were prepared from 1-day-old rat pups with the use of a procedure that has been previously shown to produce a >95% separation of myocytic and nonmyocytic cells. Spontaneous rhythmic contraction occurred within 12 hours in the myocytes.

Calcium phosphate coprecipitates or lipofectin were used for the transient and permanent transfections of Sol8, C2C12, and CaCo-2 cells. Primary cardiomyocytes were transfected with 30 μL of DOTAP (Boehringer-Mannheim) per 35-mm plate at 12 hours after initial plating. When chloramphenicol acetyl transferase (CAT) was used as a reporter, a constitutive expression plasmid for growth hormone (pXGH5) was cotransfected to control for variations in transfection efficiency. Levels of expression among different cell types were compared by normalizing the expression of test constructs relative to the expression of CAT in the same cell type driven by the SV40 promoter/enhancer (pCAT-Control, Promega).

Transgenic Mice

Transgenic mice were generated by pronuclear injection of the linearized pCAT-1,2 HFABP construct exclusive of vector sequence (U.A.B. N.I.C.H.D. center). Positive animals were identified by the polymerase chain reaction (PCR) screening with the use of CAT-specific primers and confirmed through Southern blotting. All animal studies were conducted according to protocols approved by institutional animal studies committees.

Reporter Assays

CAT activity in cell lysates and tissue homogenates was analyzed with the use of a diffusion assay with [3H]-labeled chloramphenicol, with multiple aqueous back extractions to minimize background. hGH (human growth hormone) level in tissue culture media was analyzed with the use of the hGH-TGES 100T sandwich RIA kit (Nichols Institute). A regression with the use of known hGH standards was computed, and counts were converted into ng hGH/mL media equivalents. For tissue samples, activity was normalized to total protein content (Biorad).
Nuclear Extracts and Electrophoretic Mobility

Nuclear extracts were prepared from ~100 mg of tissue snap-frozen in liquid nitrogen. Double-stranded DNA fragments for electrophoretic mobility shift assay (EMSA) were synthesized as oligonucleotides and annealed. The sequence of the 42-bp fragment encompassing the CArG-like element (see infra) from HFABP was (−115) 5′-CCCCAGGCTGGGGCTTATCCCTGAGGGGCGGCGGCTGTG GCCCAAGCTCT-3′; the shorter 20-bp element that encompassed the same core sequence was 5′-CTTCCTATTTT CCGGGAGCCGGG-3′. The 44-bp fragment that encompassed the adjacent E boxes and TATA boxes had sequence 5′-CCGGGCTGGG CTGCCGCTTT AATAAGGCTCC GATCCACAT GAGG-3′. The ME2F site from the muscle creatine kinase (MCK) promoter contained the core sequence 5′-CCGTCTAAAA ATAACCCCT-3′. Adjacent GATC linkers were present, which permitted labeling with the Klenow fragment of DNA polymerase.

Each EMSA binding reaction contained 4% Ficoll, 5 mmol/L MgCl₂, 50 mmol/L KCl, 5 mmol/L DTT, 25 mmol/L HEPES, pH 7.4, 1 μg sonicated Herring sperm DNA, 4 μg nuclear extract, and 25 000 counts of labeled probe. Reactions that contained reticulocyte lysate also contained 0.1 μg of single-stranded nonspecific oligonucleotide to reduce lane background. A 40-fold molar excess of cold competitor was added unless otherwise indicated. After a 20-minute incubation at room temperature, complexes were resolved with 4% polyacrylamide gel in a buffer containing 45 mmol/L Tris, 45 mmol/L boric acid, and 1 mmol/L EDTA, pH 8.0. Phosphorimaging and quantification were performed on the dried gel.

For supershifts, 0.5 μL of rabbit polyclonal antibody to nonspecific rabbit serum was preincubated for 10 minutes with the nuclear extract.

DNAase I Footprint Analysis

Each reaction mixture that contained 25 000 cpm of end-labeled DNA (−351 to +29, SphI to XbaI), 10 μg of poly-dIdC, 30 μg of nuclear extract, 10 mmol/L Tris, pH 8, 5 mmol/L MgCl₂, 1 mmol/L CaCl₂, 2 mmol/L DTT, 50 μg/mL BSA, 2 μg/mL salmon sperm DNA, and 100 mmol/L KCl in a final volume of 200 μL was equilibrated at room temperature for 10 minutes before the addition of 0.001 to 0.03 U of DNAase I. After digestion at 37°C for 2 minutes, DNAase I activity was inhibited by the addition of stop solution (645 μL 100% ethanol, 5 μL 1 mg/mL tRNA, and 50 μL saturated ammonium acetate), and the DNA fragments precipitated. Samples were resuspended in 95% formamide, 20 mmol/L EDTA with dye markers, heated to 80°C, resolved on a 6% denaturing gel, and autoradiographed.

Computation of the Probability Weight Matrix

A computer program was written (J.N.R.) to apply arbitrary position weight matrices (PWM) to DNA sequences, with numerical PWM values from Fickett. Predicted probability values were normalized to a linear 0% to 100% scale as described. Because this PWM is not symmetric with respect to reverse complementation, 2 values were computed at each residue that consisted of the application of PWM to the sense strand starting at that residue and the application of the PWM to the complementary strand terminating at that residue.

Results

Organization and Genomic Sequence of Human Cardiac Fatty Acid–Binding Protein Gene

Genomic clones that encompass the entire human HFABP coding region and the 5′-flanking and 3′-flanking regions were isolated. A schematic of the gene structure and cloning strategy is shown in Figure 1: 4 exons and 3 introns span ~10 kb. Exon 1 contains the complete 5′-untranslated region and codes for the first 24 amino acids. Exons 2 and 3 code for 59 and 34 amino acids, respectively; exon 4 codes for the final 16 amino acids and the 3′-untranslated region. The first consensus polyadenylation signal AATAAA falls 228 nucleotides after the termination codon. All intron-exon boundaries correspond to the usual GT-AG rules for eukaryotic splice donors and acceptors, and the intron-exon structure is conserved among FABP3, the FABP gene, and the other members of the FABP family whose genomic structures have been determined. A highly informative dinucleotide (GT) repeat polymorphism was identified in the third intron.

During the cloning of the FABP3 gene, a highly similar (80% identity) intronless genomic sequence, suggestive of a processed pseudogene, was identified. These 2 genomic sequences, FABP3 and the putative pseudogene, were the only genomic sequences detected on high-stringency Southern blotting (data not shown) consistent with the identity of the human HFABP and the highly related (>95% identical) MDGI.

Although several transcription start sites from 45 to 63 nucleotides 5′ to the ATG encoding the initiator methionine were observed on primer extension studies (data not shown), transcription initiated predominantly at the start site closest to the translation initiation ATG. This site (designated as +1 subsequently) closely corresponded to the transcription start site previously identified for HFABP in the rat.

Human and Mouse HFABP 5′-Flanking Sequences Are Highly Conserved

The expression patterns of HFABP protein and mRNA are very similar between human and mouse. Thus, although the order, context, and even sequences of important regulatory elements may differ between these 2 orthologs, highly conserved genomic sequence might signal important transcriptional regulatory motifs. A mouse genomic library was screened with the murine HFABP coding sequence. Clones corresponding to the gene 5′- and 3′-ends were identified by PCR, restriction mapped, and sequenced. The human and mouse 5′-flanking sequence were compared with the program MACAW to identify regions of statistically significant evolutionary conservation, which are identified as the shaded sequences in Figure 1b. Within the proximal 1200 bp of the 5′-flanking sequence, the order of the conserved sequences was maintained although the spacing differed. Some of these regions were similar to known motifs such as an 11/14 match for the chicken troponin T CArG box occurring 40 bp upstream of the TATA box, although others did not correspond to established skeletal muscle or cardiac transcriptional motifs. Canonical GATA or peroxisome proliferator–activated receptors (PPARs) direct repeat (DR) sites were not present.

Transient Transfections Suggest a Compact Promoter Region of HFABP Confers Specific Expression

Previous data that concerned tissue distribution of HFABP expression was based on antibodies with cross-reactivity to other members of the FABP family or mRNA hybridization with cross-species probes under differing degrees of stringency. High-stringency Northern blots with species-specific probes in rat and mouse confirmed ~8-fold higher expression in heart, both atrium and ventricle, than in slow skeletal...
Figure 1. a. FABP3 structure and cloning strategy. Positions of exons are displayed below the genomic restriction map; B: BamH1, S: SacI, X: XbaI, E: EcoRI. The outlined B indicates the site of a single-base BamH1 polymorphism, and the circled GT indicates the region of a dinucleotide repeat polymorphism. The extents of the clones are displayed below the map.

b, Sequence of the 5'-flanking region of FABP3 (HFABP). Possible target sites for transcription factors are labeled; Ebox denotes the sequence CAnnTG that is associated with binding to specific myogenic factors. The SRF/CArG-like element is discussed in the text. Transcription initiation sites are indicated by "*". The bold ATG marks the site at which translation commences. Sequences from the mouse 5'-flanking region identified as significantly conserved by the alignment program MACAW are placed below the corresponding human sequences. The order of conserved elements is maintained between human and mouse 5'-flanking sequences, although the spacing differs.
muscle, with far lower expression in fast skeletal muscle. Low levels of expression were detected in ovary, testis, adrenal, and kidney; expression was not detected in adipose tissue, brain, liver, or intestine. Endogenous HFABP mRNA was readily detected in primary neonatal ventricular myocytes.

Primary ventricular myocytes were therefore used as a permissive cell type in transient transfection assays to suggest essential promoter sequences. Reporter constructs with regions of the FABP3 5’-flanking sequence coupled to the bacterial CAT reporter were constructed and transiently transfected. Expression of a 0.4 kb HFABP 5’-sequence/CAT reporter construct was at 120-fold over basal CAT expression, which corresponded to 74% of the level of a CAT-SV40 enhancer-promoter construct (Figure 2a). A decrease in CAT activity was observed with an addition of 200 bp more of promoter context, which suggested the presence of a negative regulatory element in this region (Figure 2a, P<0.05 versus 0.4 construct). The addition of another 600 bp of promoter context resulted in a significant increase in CAT activity to twice that of the 0.4 kb construct or 125% of CAT-SV40 promoter-enhancer (Figure 2a, P<0.05 versus other constructs), which suggested the presence of a positive element in the cardiac ventricular myocytes within this 600 bp region.

In contrast, no significant expression was observed in CaCo-2 cells, which support the expression of analogous constructs derived similarly from the intestinal FABP (Figure 2b), or in passaged fibroblasts co-isolated with the primary ventricular myocytes (data not shown).

Primary ventricular myocytes permitted the comparison of transgene and endogenous HFABP expression. However, their unsuitability for permanent transfections would hinder the mapping of elements underlying metabolic regulation. To determine whether other cell lines might permit the comparison of endogenous HFABP and transgene expression, Northern blots containing mRNA from a variety of permanent cell lines were hybridized with a specific HFABP probe. No signal was observed in any permanent cell line tested, including the myoblast/myotube lines C2C12, Sol8, and LE9, the atrial tumor-derived line AT1 in either proliferative or contracting conditions, and a variety of noncardiac/non-muscle cell lines (CaCo-2, HepG2, and fibroblast; data not shown) under conditions in which expression in primary ventricular myocytes were readily detected. Although the expression of HFABP in C2C12 cells has recently been reported, protein expression was observed at <1/40th the level observed in primary skeletal myocytes, and this level of expression required especially long (>6 days) periods of differentiation with insulin supplementation.

During an analogous situation, no established enterocytic cell line expressed significant levels of intestinal FABP, but it was still possible to use CaCo-2 cells as a transcriptionally

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Figure 2. Transient transfections assays. Indicated regions of the HFABP 5’-flanking sequence linked to a CAT reporter were transiently transfected into the indicated cell types. Expression was normalized for variations in transfection efficiency with a cotransfected constitutively expressed hGH plasmid. Data for each cell type are expressed relative to that expression of CAT driven by the SV40 promoter and enhancer in that same cell type. Data represent the mean±SD of 3 or more independent transfections. In (c) and (d) tube is differentiated myotube and blast is myoblast. Note the 3-fold higher scale in panel a, primary ventricular myocytes.
permissive environment that correlated closely with in vivo transgenic expression. With this in mind, expression of HFABP 5'-flanking region/CAT reporter chimeric reporter constructs was evaluated in transient transfection studies in Sol8 and C2C12 skeletal myocyte-derived cells. Four hundred bases of the HFABP 5'-flanking region demonstrated promoter activity in both Sol8 and C2C12 myoblasts, with CAT activity more than 40-fold greater than basal (promoterless) activity (Figure 2c and 2d) at 16% (C2D12) to 36% (Sol8) of the levels of CAT activity observed under the transcriptional control of the SV40 promoter and enhancer. Constructs based on 600 bp of the HFABP 5'-flanking sequence showed reduced but comparable levels of CAT activity relative to the 400 bp construct (Figure 2c and 2d, P<0.05 for comparison between 400 bp and 600 bp) in both Sol8 and C2C12 cells, which suggests the presence of a negative cis regulatory element for these cell types between −400 and −600 in the HFABP promoter. In C2C12 and Sol8 cells, the addition of another 600 bp of promoter context resulted in a significant decrease in CAT activity to less than half that observed with the 0.4 kb construct (Figure 2c and 2d; P<0.05 versus 0.4 HFABP construct). The contrast between these skeletal muscle-derived cell lines and primary ventricular myocytes suggests that the region between −600 and −1200 contains cis sequences that differentially activate or inhibit in heart muscle compared with skeletal muscle.

**Expression of HFABP Promoter Constructs Is Not Significantly Increased With Myotube Formation**

Sol8 and C2C12 cells undergo a myoblast to myotube differentiation process when removed from FBS. The expression of constructs based on the promoters of a number of sarcomeric proteins, such as the β-myosin heavy chain, significantly, reproducibly, and rapidly increase during this in vitro model of myocyte terminal differentiation. During conditions in which these changes occurred, no significant change was observed with these 3 HFABP promoter constructs between myoblasts and myotubes (Figure 2c and 2d, light versus dark cross-hatching).

To investigate further this apparent lack of differentiation dependence, pooled permanent C2C12 and Sol8 transfectants harboring HFABP 5'-flanking sequences coupled to a hGH reporter were constructed. This secreted reporter allowed the continuous readout of activity in the same population of cells during differentiation. Measurable hGH activity could not be detected in media in the absence of transfected hGH reporter constructs, and typical activity in the media from the pooled permanent transfectants was >100-fold above levels required for accurate detection by radioimmunoassay. Activity in the media was normalized to that of parallel platings of the same number and type of cells permanently transfected with a constitutive construct pXGH5 under the same differentiating or nondifferentiating conditions. Expression is shown relative to time 0. The average of 3 experiments is shown; error bars represent the standard deviation.

**Figure 3. Permanent transfections assays.** Pooled Sol8 permanent transfectants harboring 400 bp of HFABP 5’-flanking sequences coupled to a hGH reporter were selected by cotransfections with a neomycin resistance plasmid and selection in G418 (400 μg/mL active). At time 0, cells were either switched to differentiation media (tube) or maintained in growth media (blast) although additional growth was prevented by contact inhibition. hGH, which is efficiently secreted and represents a continuous readout of transcriptional activity, was assayed in the media, expressed in ng/mL. hGH was normalized to relative to parallel platings of the same number and type of cells permanently transfected with a constitutive construct pXGH5 under the same differentiating or nondifferentiating conditions. Expression is shown relative to time 0. The average of 3 experiments is shown; error bars represent the standard deviation.

relative levels of expression of 0.4 HFABP-hGH between these cell populations. Similar results were observed with constructs based on 0.6 kb and 1.2 kb of the HFABP promoter in Sol8 and with these 3 constructs in C2C12 cells (data not shown). These data contrast with the strong induction observed with differentiation in these same cell types with some well-characterized, sarcomeric tissue-specific promoters such as the β-myosin heavy chain (data not shown).

**Expression of the 1.2-kb HFABP Promoter Is Tissue-Specific In Vivo**

The expression in tissue culture may or may not accurately reflect true in vivo expression for FABPs. Audits in transgenic mice were used to confirm the apparent tissue-specificity of the 1.2 kb HFABP promoter region. Three independent lines with germline transmission were generated in C57BL/6 mice. Quantitative Southern blotting revealed 5 to 10 copies of the reporter per germline (data not shown). Relative levels of expression in different tissues in these lines were similar, and levels of expression among lines were roughly proportional to copy number (data not shown).

The pattern of transgene expression observed was similar to the tissue distribution of endogenous HFABP. Representative data from one line are shown in Figure 4. Expression in the heart was 20-fold higher than in any other tissue and >3 orders of magnitude above background levels of the assay. Separate dissections of the septum, right and left ventricular free walls, and atria showed minimal differences in expression of both the transgene and the endogenous protein (data not shown). The next highest levels of expression were observed in the lung. Lower levels of expression were observed in brown fat and skeletal muscle. Expression in
most other tissues samples was at levels that approached the limits of detection by the assay.

Point Mutation in a Conserved CArG-Like Element Reduces Expression in Permissive Cells

The transgenic data provided compelling confirmation that 1.2 kb of the HFABP promoter contained most or all of the elements required for tissue-specific expression. The large number of highly conserved motifs within this relatively short region suggested that expression might depend on synergistic interactions. We therefore initiated a sequential analysis of the conserved elements. The TATA-box sequence itself is identical between mouse and human and is immediately adjacent to an E box (CATGTG, consensus CANNTG) that also resembles a high-affinity Nkx-2.5 site (TCATGTG, consensus TNNATGT). The next identical region between human and mouse (Figure 1b, nt −68 to −107) contained a GC-rich palindromic CACG sequence and a 17-base sequence that is also found in the porcine HFABP-flanking sequence (5'-CTT CCT ATT TCG GGA GC-3'). This latter CArG-like element, an 11/14 match for the chicken troponin T CArG box, matches the yeast MADS-box transcription factor MCM1 consensus. SRF and MEF2, other members of the MADS-box family that also bind to specific A/T-rich sites, have been extensively implicated in muscle-specific and heart-specific gene expression. Because the transient transfection experiments suggested that the proximal promoter region might direct specific expression in muscle, with more upstream regions responsible for preferential expression in the heart, we studied this element.

DNAase protection with the use of the 0.4 kb fragment of the HFABP 5'-flanking sequence revealed a difference in the protection conferred by heart nuclear extracts in the region around the CArG-like element compared with that observed in the absence of nuclear protein (Figure 5a). DNAase hypersensitive bases flanked the region of protection: a pattern that is often associated with transcription factor binding. Differences between heart and liver nuclear extracts were less clear (Figure 5a), but this was not surprising because the tissue distribution of MADS-box factors is broader than would be suggested by their role in tissue-specific expression.

We constructed a one-base mutation in this element, CCTATTTCGG→CCGATTTCGG, in which the intrinsic spacing and context was maintained. Transfections of the single-base change CArG-box mutant into ventricular myocytes resulted in a consistent and statistically significant (Figure 5b, P<0.01) reduction in CAT activity compared with the unmutated construct. Similar decreases in activity were observed with the use of this altered promoter with an hGH rather than a CAT reporter (data not shown) and with transient transfection of these promoter constructs into C2C12 cells (Figure 5b). Thus, the CArG-like element is necessary for full promoter activity in both ventricular myocytes and skeletal muscle cells. In contrast, no change in the levels of expression between the native and mutant constructs was observed in the far lower relative levels of expression observed with transfections into nonmyogenic 3T3 cells (data not shown).

MEF2 Overexpression Transactivates the HFABP Minigene Expression

Serum response factor (SRF), a MADS-family transcription factor, transactivates a variety of promoters that contain a serum response element (SRE). Because of the similarity of the CArG-like element to an SRE, on the basis of its similarity to the troponin element, and the length of its A/T rich core, we determined whether exogenous SRF could transactivate the 0.4 HFABP promoter. Expression of exogenous SRF by cotransfections of 1 μg of an expression plasmid: 6 μg reporter resulted in a 50% to 60% decrease in
the CAT activity from pCAT-0.4HFABP in both C2C12 and 3T3 cells used as models of permissive and nonpermissive cell types (Figure 6b, CAT activity expressed relative to basal expression for each cell type). Lesser degrees of downregulation were observed with transfections of smaller quantities of SRF expression plasmid (data not shown). Although squelching has often been observed with overexpression of SRF, its exogenous overexpression is capable of transactivation of other promoters and elements.43–45 Neither cotransfections of the null expression plasmid with pCAT-0.4 HFABP nor cotransfections of SRF with an indifferent positive CAT control resulted in significant downregulation of corrected activity (data not shown). Cotransfection of SRF with a weak transactivable SRE (SRE.LP)46,47 resulted in upregulation in both 3T3 and C2C12 cells (data not shown). Surprisingly, cotransfections of the MADS-box factor MEF2A, which has a canonical consensus target (C/T)TA(A/T)(A/T)(G/A),41 resulted in a strong and dose-related increase in CAT activity in 3T3 cells, with up to a 14-fold increase (Figure 6a, solid line, left vertical axis). These experiments used an isolate of 3T3 that has little endogenous MEF2 binding activity and normally expresses pCAT-0.4 HFABP at relatively low levels. A smaller relative increase compared with basal levels of expression of the pCAT-0.4 HFABP construct was observed in C2C12 cells (Figure 6a, dashed line, right vertical axis), which express significant endogenous levels of MEF2 isoforms. Higher ratios of MEF2A plasmid: reporter plasmid were also required for increased activity in C2C12 cells, which possibly reflect higher endogenous levels, although possible differences in the efficiency of pMT2-MEF2A expression preclude comparison of true transcription factor dose-response. MEF2A overexpression increased activity of constructs based on longer HFABP 5'-flanking sequences (Figure 6d), which demonstrates that this transactivation was not an artifact of a specific element removed from its endogenous context. The single-base mutation in the CArG-like element, which decreased promoter expression in muscle/myogenic cells, abrogated MEF2A-dependent transactivation in 3T3 cells (Figure 6c) and C2C12 cells (data not shown). Thus, the CArG-like element was necessary and sufficient for MEF2A transactivation.

MEF2A Specifically Binds to the HFABP CArG-Like Element

The MEF2A transactivation could be indirect or direct. We determined whether MEF2A could bind directly and specifically to the HFABP CArG-like element. MEF2 binds avidly and specifically to an A/T rich element from the MCK upstream promoter. Both 40 and 20 bp double-stranded oligonucleotides that contained the core HFABP CArG-like element competed binding of the ventricular nuclear extracts to the canonical MCK-MEF2 sequence (Figure 7a, lanes 4 and 5). Competition was less effective than with an equimolar cold MCK-MEF2 sequence (Figure 7a, lane 3), which suggested a lower binding affinity for MEF2 to the HFABP site. Similar binding and competition were observed with nuclear extracts derived from differentiated C2C12 cells, which possess high MEF2A binding activity.29 This difference in affinity was explored further in a titration-competition experiment (Figure 7b). Least-squares modeling of retarded counts fit to a logistic equation was
consistent with a 14-fold difference in dissociation constants between the MCK and HFABP MEF2 sites.

MEF2A produced by in vitro translation with reticulocyte lysate produced a retarded complex with the HFABP CArG-like site, although no such complex was observed with unprogrammed reticulocyte lysate (Figure 7c, lanes 1 and 2, respectively). With the same HFABP CArG-like element probe, a complex of similar mobility was observed with nuclear extracts prepared from ventricular muscle (Figure 7d, lane 2) or C2C12 myotubes (Figure 7e, lane 2). In both cases the complex was specifically competed by either excess cold HFABP CArG site or the MCK-MEF2 site (Figure 7d and 7e, lanes 3 to 5). Finally, the HFABP CArG-element complex formed with nuclear extracts from ventricular muscle, C2C12 myotubes, or programmed reticulocyte lysate was supershifted by preincubation with a highly specific MEF2 antibody (Figure 7f, lane 2, 5, and 15). No supershift was observed with nonspecific rabbit serum in parallel reactions (Figure 7f, lanes 3, 6, and 17) or with unprogrammed reticulocyte lysate (Figure 7f, lane 16). Increasing antibody resulted in near total shift of the complex (data not shown), and antibody to SRF (SC194x, Santa Cruz) did not shift or alter the complex (data not shown). The supershift mobility corresponded to that observed with the same antibody and nuclear extracts bound to the MCK-MEF2 site (Figure 7f, compare lanes 7 to 12). Total counts present in the unshifted and supershifted bands typically exceeded those seen in control and preimmune reactions, which suggested that the antibody stabilized the DNA/protein complex (Figure 7f).29

Quantitative Modeling of the CArG-Like Element as an MEF2 Site

These data suggested that the CArG-like element was a bona fide MEF2 response element, capable of both specific protein-DNA binding and transactivation. This element did not correspond to any of the suggested MEF2 consensus sequences: YTAAAAATAACYY, TWWWAATAR, CTAWWWWTAG, YTWWAAATAR, or YTAAAWA-
TARCY. The PWM has been suggested as a more precise and quantitative approach to define transcription factor target sites, and a matrix has been defined for MEF2. In this approach, a numerical weight is assigned to each base in each position (first, second, third base, etc) of a putative element in proportion to the frequency of that base in that position in a population of documented response elements, generalizing the use of degenerate codes in consensus elements and allowing quantitative assessment. The CArG-like element scored 68%, similar to the myoglobin gene MEF2/ATF35 site, but well below the 80% cutoff suggested for identification of typical MEF2 sites. We applied this same weight matrix computation to the 1400 bps that surround the HFABP promoter (a portion is illustrated in Figure 8). Of the 16 sites that scored at the same or higher level, only 2 were conserved between human and mouse: the CArG-like element and the HFABP TATA box. The highest score of 88% was for the HFABP TATA box. MEF2 is known to bind to a number of TATA box sites, and we have confirmed that the HFABP TATA box effectively competes for MEF2 binding on EMSA with both rat ventricular myocyte nuclear extract and C2C12 nuclear extract (data not shown). However, the mutagenesis studies described are inconsistent with the HFABP TATA box mediating MEF2 transactivation.

Discussion

This study demonstrates that the heart FABP3, like other members of the FABP family, possesses a concise promoter region that is sufficient to direct cell-type specific expression in cell culture and in vivo. Thus, the HFABP 1.2 kb promoter joins a modest list of promoters, most from contractile genes, known to be capable of directing cardiac-specific or preferential expression of a heterologous protein product. These promoters differ in terms of level and homogeneity of
expression and regional and developmental specificity. Different promoters, with potentially independent regulatory mechanisms, represent useful reagents as these sequences are used as vehicles for directing the expression of exogenous proteins with cell-biological effects. Additional characterization of the exact developmental and cell-specific pattern of HFABP transgene expression is currently underway. These experiments will define whether the expression observed in other tissues is low level but uniform or restricted to limited cell populations such as the myocardial-like cells found in the pulmonary vasculature of the mouse.

The in vitro pattern described here differs in one important aspect from what has been commonly observed with contractile genes such as the myosin heavy chains: expression appears to be cell-type specific but is not substantially upregulated with differentiation of myoblasts to myotubes. A recent report describes the induction of endogenous HFABP mRNA in C2C12 cells with differentiation. However, the extremely low level of expression, at 1/40th of the level of primary skeletal myocytes, and the modest level of induction, 3-fold, complicate the interpretation of this finding. The time course of this induction, 6 to 8 days after the change to differentiation medium, is substantially longer than is typically required (eg, myosin). Finally, the addition of insulin to the differentiation medium was required, which suggested that differentiation may have been permissive for transduction of a metabolic signal rather than directly causal. The high density of conserved elements in the HFABP promoter suggests that it will be a useful system in which to study specific transcription factor interactions, particularly the interaction of tissue-specific and metabolic signals. The in vivo and in vitro data, taken together, suggest that the 0.4 kb promoter suffices to specify muscle expression, although elements farther upstream in the 1.2 kb promoter are required for the highly enriched expression in the heart. Auditing of additional constructs in vivo will be required to confirm this hypothesis.

Some of the motifs and factors responsible for directing the expression of HFABP are reminiscent of those known from contractile genes. MEF2 has been identified in the promoter regions of a number of genes expressed at high level in the heart, including muscle creatine kinase, α-myosin heavy chain, myosin light chains, desmin, aldolase A, cytochrome C oxidase Vla, and the glucose facilitative transporter GLUT4. Interestingly, although only a few metabolic genes have been studied, MEF2 sites seem to be disproportionately represented in this population. The MEF2 site in the HFABP promoter does not correspond to any of the established MEF2 consensus sequences. However, several lines of evidence point to a direct role of MEF2 or a closely related molecule: the binding of in vitro translated MEF2 to the site, the cross-competition between the HFABP CArG-like site and the MCK-MEF2 site, and the supershifts with a highly specific antibody. Although the atypical MEF2 (or MEF2-related) binding site is necessary for full expression of the HFABP construct in the myocytic cell lines tested, it cannot by itself be sufficient. First, the various isoforms of MEF2 are widely expressed in a variety of permissive and nonpermissive cell types by the time of definitive organogenesis, and available functional assays suggest considerable overlap and redundancy in isofrom-specific pathways. Second, point mutations of the MEF2 site that eliminate MEF2 transactivation do not reduce promoter activity to background levels of expression. Third, the 0.4 kb region, which encompasses the MEF2 site, appears not to provide the appropriate gradient of cardiac and skeletal muscle expression. The lower affinity for direct MEF2 binding and the conserved presence of an E-box site at 2.5 helical turns suggest that combinatorial factor

Figure 8. MEF2 PWM applied to the FABP3 5′-flanking sequence. Because the matrix is not symmetric, results of application in both the forward (darker line) and reverse (lighter line) directions are shown. For the forward direction the computation starts at the indicated base; for the reverse direction, the computation on the reverse complementary strand ends at the indicated base. Values are normalized to linear 0% to 100% scale as in Fickett.
interactions involving MEF2 or an MEF2-like protein with shared epitopes may be necessary for full in vivo effect. The minimal changes in expression observed with the differentiation of C2C12 myoblasts to myotubes, despite the substantial upregulation of MEF2 during this process, favors a balanced interaction requiring multiple factors. Liu et al. defined a typical MEF2 site in the promoter of the GLUT4 gene that was necessary for full-level expression in C2C12 myotubes. Full expression of constructs that contain this element was also dependent on flanking sequence; the differences in differentiation-dependent alterations in level of expression between the GLUT4 and HFABP constructs may reflect either differences between their MEF2 sites per se or the flanking elements.

These other factors may include Sp family members. Increasing evidence implicates Sp family transcription factors in the regulation of metabolically important genes in the heart. The GC-rich element immediately adjacent to the CARG-like element satisfies the Sp1-related motif C(C/G)(A/T)(C/G)(C/G)(C/G). Both the human and mouse HFABP promoters contain a potential response element for ERα, an orphan receptor implicated in the transcriptional regulation of the important cardiac metabolic gene medium-chain acyl coenzyme A dehydrogenase, in a highly conserved region at \( \sim 700 \). No canonical GATA or PPAR response elements are present in the conserved regions in the proximal HFABP promoter. A consistent relationship between MEF2 sites and myogenic E-box sites at a spacing of \( n+\frac{1}{2} \) helical turns has been noted. A conserved E box, immediately adjacent to the CARG-like element, with exactly this spacing resembles the high-affinity Nkx-2.5 site 5'TNNAGTG-3'. Although MEF2 and Nkx-2.5 interactions have not yet been described, the related MADS-box factor SRF interacts with the homeobox factor Nkx-2.5, and this interaction did not require DNA binding of both partners. Detailed quantitative studies with combinatorial collections of site mutations and in vivo footprinting will help elucidate important factor-factor interactions. PWM have been suggested as a more quantitative and generally applicable method of predicting factor-binding sites. PWM, which yield a continuous measure of the similarity of a site to those previously established, are more informative for those sites that seem similar to, but fail to meet, traditional consensus measures. Although a modest relaxation of the suggested threshold for MEF2 sites by PWM would include the HFABP site established here, on a genome-wide basis, even a small change would result in an exponential decrease in specificity. Thus, coupling a lowered PWM threshold with other criteria, such as evolutionary conservation or appropriate spacing to correlated sites, may be necessary for realistic use in exploratory genomics.

Effective cardiac contraction is dependent on the coordinate expression of specific contractile, channel, and metabolic genes. The factors and motifs that direct expression of contractile genes have been intensively studied. The metabolic demands of cardiac contraction are extraordinary, and substrate preference and characteristics of energy flux differ substantially between heart and skeletal muscle. This is associated with the expression of certain metabolic genes like the HFABP at substantially higher levels in heart muscle than in other striated muscle. The choice of metabolic substrate by the heart has physiological and clinical significance and is an important determinant of oxygen requirements for constant workload. The HFABP promoter may be a useful tool to direct the expression of exogenous gene products in the heart. In turn, we expect that additional studies can use the compact HFABP promoter to explore in greater detail the interactions of the transcriptional signals that specify tissue identity, metabolic substrate availability, and energy demand: the essential governing elements of cardiac metabolism.

Acknowledgments

This study was supported by a Grant-in-Aid from the American Heart Association (to J.N.R.). We thank Drs Dan Kelly, David Bader, Joey Burnett, and Thomas Quertermous and members of their laboratories for their thoughtful comments and suggestions.

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Qinghua Qian, Lyn Kuo, Yie-Teh Yu and Jeffrey N. Rottman

Circ Res. 1999;84:276-289
doi: 10.1161/01.RES.84.3.276

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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