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Structure and chromosomal location of the rat gene encoding the heart fatty acid-binding protein

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The gene coding for rat heart fatty acid-binding protein (FABP), along with 1.2 kb of its 5′-untranscribed region, was amplified by PCR, cloned and sequenced. As in other FABP genes, the coding sequence is interrupted by three introns of 3.4, 1.4 and 1.1 kb, respectively. Fluorescence *in situ* hybridization mapping revealed that the gene is located on chromosome 5q36. Using intron-specific primers flanking exon 2, unspliced primary transcript RNA of the FABP gene was detected in a preparation of total RNA isolated from rat heart, proving that the cloned gene is expressed in adult cardiac tissue.

Keywords: FABP; FISH mapping; heart; MDGI; RT-PCR.

Cardiac fatty acid-binding protein (H-FABP) belongs to a multigene family of intracellular proteins involved in fatty acid transport and utilization [1,2]. The protein has been shown to be expressed in cardiac and various skeletal muscles of vertebrates and invertebrates. Its concentration in various muscles seems to reflect their metabolic capacity for fatty acids [3,4]. In mammals, FABP is most abundant in cardiac muscle, followed by other red muscles that depend largely on fatty acid as fuel. Even higher concentrations have been reported in specialized muscles that sustain high metabolic rates, such as the flight muscles of migratory birds [5] and locusts [6]. Hence, it is generally assumed that FABP plays a role in fatty acid transport and utilization in these muscles. It has also been proposed that FABP keeps the cytosolic concentration of unbound, free fatty acids low, thus preventing potential damage of cellular structures by the amphiphilic fatty acid molecules. Cardiac FABP, in addition to its functions in lipid metabolism, may also suppress the proliferation of certain tumor cells. The protein is identical to the mammary-derived growth inhibitor (MDGI) originally identified in mouse mammary epithelium [7], which has been shown to inhibit the growth of mammary tumor cell cultures [8].

It has been demonstrated that the expression of FABP can be induced by an elevated fatty acid-dependent metabolism, for example during chronic electrical stimulation of rat muscle [9] or migratory flight in locusts [10] and birds [11]. In both invertebrates and vertebrates FABP expression is low or absent in immature muscles, but rises rapidly as cells begin to utilize fatty acids to a larger extent [6,12]. The concentration of FABP increases several-fold during rat heart development, from 1.2 $mg \cdot g^{-1}$ protein in neonatal heart to 5.7 $mg \cdot g^{-1}$ protein in adults, also reflecting increased fatty acid utilization [12]. Recently, van Bilsen *et al.* [13] demonstrated that prolonged exposure of cultured neonatal ventricular myocytes to exogenous fatty acids led to an almost fourfold increase in FABP mRNA. However, because FABP and its messenger RNA are present in relatively large amounts in adult cardiac tissue, it is difficult to detect transient changes in expression rates in response to physiological stimuli.

While the genes for cardiac FABP or MDGI from mouse [14], human [15] and pig [16] have been cloned, little is known about the molecular mechanisms that control the expression of this gene, possibly because these organisms are not optimal for linking physiological experiments with molecular analysis. For metabolic studies, the rat is the preferred model system, where well-defined *in vitro* and *in vivo* protocols have been developed. Detailed molecular analysis of such experiments, however, requires the availability of the cardiac FABP gene. Here we report the cloning and sequencing of the cardiac FABP gene from *Rattus norwegicus*, and its localization in the rat genome.

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Abbreviations: DAPI, 4′,6-diamino-2-phenylindole; ERRα, estrogen related receptor α; FABP, fatty acid-binding protein; FISH, fluorescence *in situ* hybridization; FITC, fluorescein isothiocyanate; H-FABP, cardiac fatty acid-binding protein; MDGI, mammary derived growth inhibitor; NRRE 1, nuclear receptor response element 1; PPAR, peroxisome proliferator-activated receptors.

Note: the novel nucleotide sequence data published here have been submitted to the EMBL sequence data bank and are available under accession number AF144090.

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MATERIALS AND METHODS

DNA and RNA isolation

Male Wistar rats were housed in the animal care facility of SFU. Prior to heart dissection, rats were anesthetized with sodium pentabarbital. For the isolation of genomic DNA, freshly excised or frozen tissue was minced and powdered under liquid nitrogen. Genomic DNA was isolated by an alkaline lysis procedure [17]. Plasmid DNA was isolated using mini prep columns (Qiagen).

For the isolation of total RNA, tissue was homogenized in extraction buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), followed by a one-step extraction [18]. Subsequently, the RNA sample was treated for 15 min at 37 °C with 1 U of DNAse I (Ambion) to remove contaminating DNA and kept at 95 °C for 15 min to inactivate the enzyme.

Amplification and cloning

Initial analysis of intron localization and size was carried out with PCR primer combinations specific for the conserved exons in all FABP genes. The following primers were used: upper primers M272 (CAGGAGACAGAGGCAGGAGAATCT, 1248 bp upstream of transcription start site in the mouse FABP gene), M727 (AGGCGCAGGCGGGAGACATTC, 793 bp upstream of transcription start site in the mouse FABP gene), R3 (CTGGAAGCTTGTGGACAGCAAGAAT, exon 1), R1 (TAGCATGACCAAGCCGACCACAATC, exon 2), R4C (CAGGAGACTACGCTTACACGGGAAC, exon 3); lower primers R305 (CCACGTATCAAACTGGGGTCGTCAG, 305 bp upstream of transcription start site in the rat FABP gene, H2 (TCATCAAAATTCTTGCTGTCCAC, exon 1), R6 (GATTGTGGTCGGCTTGGTCATGCTA, exon 2), R4 (GTTCCCGTGTAAGCTTAGTCTCCTG, exon 3) and R2 (CAGGAAAAGCTTAACCAAAGAGTAT, exon 4). Primers R3, R4 and R2 have a slightly altered sequence to incorporate a *Hin*dIII restriction site.

Fragments up to 3.5 kb were amplified by PCR for 35 cycles (30 s denaturation at 94 °C, 3 min annealing and 5 min extension), using Advantage Tth polymerase (Clontech). Smaller upstream fragments were amplified using the Ready-To-Go PCR Beads (Amersham Pharmacia Biotech) and slightly different cycling conditions (30 s denaturation, 30 s annealing at 65 °C followed by 1 min extension). The longer fragments resulting from primer combinations R3 and R2 or R3 and R6 were obtained by long-range PCR amplification of genomic DNA using the Expand system (Boehringer Mannheim). The following conditions were used in long-range PCR: denaturation for 2 min at 94 °C, 10 cycles of 10 s at 94 °C, 30 s at 59.5 °C and 8 min at 68 °C. For each of the following 20 cycles, the extension time was increased by increments of 20 s over each previous cycle. After a total of 30 cycles had been completed, the sample was kept for 7 min at 68 °C. The 3.5 kb R3–R6 PCR product was cloned directly into the pCR 2.1 vector (Invitrogen), following the manufacturer's instructions. The PCR product M727-H2 which contains exon 1 and 0.7 kb of upstream sequence was also cloned into pCR 2.1. The 7-kb amplification product R3–R2 was double digested with *Hin*dIII (restriction site in the lower primer) and *Eco*RI (restriction site 500 bp downstream of exon 1) and directionally cloned into Bluescript KS (Stratagene).

Sequencing

Sequencing was carried out by primer walking with ABI's AmpliTaq DyeDeoxy terminator Cycle sequencing chemistry (Perkin-Elmer Biosystems) on the automatic ABI Model 373 Stretch DNA sequencer at Biotechnology Laboratory/Nucleic Acid Service Unit of the University of British Columbia. The entire gene was sequenced in both directions and ambiguous areas were re-sequenced with alternative primers. Sequences were compared against Genbank and the upstream sequence as well as individual introns were aligned with the corresponding sequences from the cardiac FABP genes from mouse (Genbank accession number U02884), man (Genbank accession numbers U57623, U17081) and pig (Genbank accession numbers X98558, Y16180, Y16181).

Chromosomal mapping

Fluorescence *in situ* hybridization (FISH) analysis, carried out in the laboratory of G. Levan at Göteborg University, was essentially as published previously [19]. Two probes (plasmids containing R3–R2 and R3–R6, 1 μg each) were labeled with digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation to obtain probe fragments with a final size of 200–400 bp. Labeled probes were mixed with hybridization buffer (50% formamide, 2 × NaCl/Cit and 10% dextran sulfate). After denaturation the mixture was placed on metaphase chromosome slides of normal rat skin fibroblasts which had been denatured at 72 °C for 2 min in 70% formamide, 2 × NaCl/Cit. After hybridization for 48 h at 37 °C, preparations were washed for 15 min in 50% foramide, 2 × NaCl/Cit. The digoxigenin-labeled probe molecules were detected with fluorescein isothiocyanate (FITC)-anti-digoxigenin (Oncor, Gaithersburg, MD, USA). The slides were washed for 15 min each in 1× PBD (Oncor). Finally, chromosome spreads were counterstained with 0.1 $\mu g \cdot mL^{-1}$ 4′,6-diamino-2-phenylindole (DAPI) in an antifade solution (Vectashield, Vector Laboratories Burlingame, CA, USA).

Microscopy was performed using a Leica DM RXA microscope. Metaphase chromosomes stained with DAPI were screened through a UV-A filter (Exciter, 340–380 nm) for visualization of the banding pattern. FITC signals visualized through a band pass G filter (exciter, 490/20 nm). The images were captured using the Leica DM RXA microscope in combination with the QW-FISH software for microphotography.

RT-PCR of primary transcript

A pair of primers that flanks exon 2 was designed to amplify part of the primary transcript of the FABP gene by RT-PCR (GeneAmp PCR kit, Perkin-Elmer) in a GeneAmp PCR System 2400. Reverse transcription was carried out from primer R13 (TCCCAGCACTGAGCAGGCTTTATGA) which anneals to a sequence 78 bp downstream of exon 2. The 10 μL reaction mixture contained PCR buffer II, 5 mM $MgCl_2$, 1 mM each of dNTP, 1 U of RNase inhibitor, 2.5 U of MuLV reverse transcriptase, 2.5 μM lower primer and 200 ng total RNA isolated from adult rat heart tissue. Following incubation for 10 min at 25 °C, 10 min at 60 °C and 15 min at 42 °C, the reaction was terminated by heating to 95 °C for 5 min and cooled to 4 °C. The reverse transcription product (5 μL) was added immediately to 20 μL PCR reaction mixture containing 2 mM $MgCl_2$, PCR buffer II, 2.5 U AmpliTaq DNA polymerase, 0.5 μM upper primer R12 (GTTGCCAACCTTCCCAGACATCCAC) which anneals 194 bp upstream of exon 2. The mixture was denatured at 95 °C for 2 min and amplified for 35 cycles of 30 s at 95 °C, 30 s at 59 °C and 1 min at 72 °C. Following 10 min at 72 °C, the reaction mixture was cooled to 4 °C.

RESULTS AND DISCUSSION

In this study, we took advantage of the conserved gene structure of all FABP genes. Using exon-specific primer combinations that flank the individual introns, we were able to confirm the gene structure and determine the intron size of each of the three introns (3.5, 1.5 and 1.2 kb; Fig. 1). In each case, only one amplification product was obtained, indicating that H-FABP is encoded by a single copy gene. To generate the entire sequence of the gene, we cloned fragments produced by long-range PCR. An ≈ 7 kb amplification product was cloned into Bluescript after digestion with *Eco*RI, which cleaves in the lower PCR primer and at a unique site 0.5 kb downstream of the 5′-end. A second, overlapping clone was produced by TA cloning from

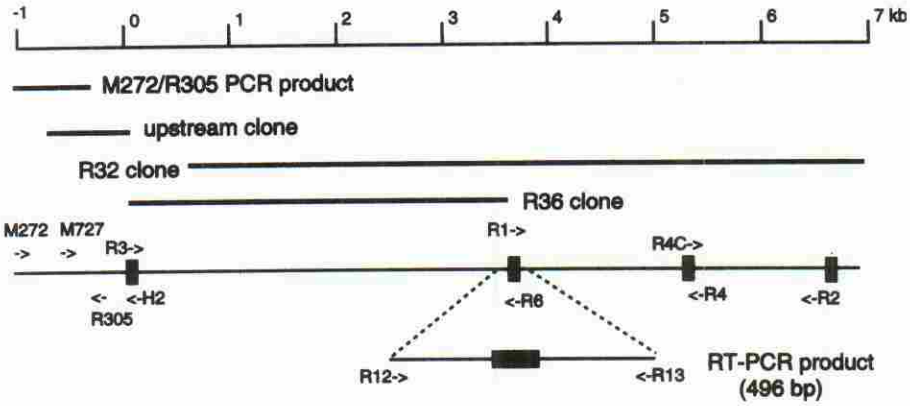

Fig. 1. Schematic representation of the rat H-FABP gene. The exons are shown as filled boxes. The PCR product and the three clones that served as templates for sequencing are depicted above the gene. Also shown are the upper (→) and lower (←) PCR primers mentioned in the text. The primer combination used for RT-PCR of the primary transcript of the FABP gene and the PCR product obtained (Fig. 4) are shown below.

the 5 kb PCR product of exon 1 to exon 3. Finally, a clone containing 750 bp of upstream sequence was obtained from the PCR product between primers H2 and M727, which had been designed to match a string of conserved sequence upstream of the human and mouse MDGI genes. A primer (M272) specific for another conserved sequence string ≈ 1 kb upstream of the transcription start site was used in combination with primer R305 (which anneals in the upstream region) to amplify a 0.8 kb PCR product of upstream sequence. The sequence of the clones and PCR products (Fig. 2) confirms that the 7 kb gene contains three introns, located at exactly the same positions as those in other FABP genes [2]. The protein sequence encoded by the exons is 100% identical to the published cDNA sequence of rat heart FABP (GenBank accession number AAA41137).

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ctacggtcaa gcccggctca cattgagatg ccgacttggt gataattaaa cagaaactgc tgaactaagg aacgggctcc    -1120

actgagcttc ctttactcac ctatgaaaag gagaggatac cacctaccaa tggaaaagtt cagtgggtcc atgtcctgaa    -1040

agccggctac aaccatccat gaagtgggtc ttatttaata aagtgcttta aggtgtcaaa cagtctagtg gcagaagtca    -960

gagataaaac tgactccaga aaagggtcgc ggctttccgg cagttaaggt ggccgaggcc agaagaacta tctgaataga    -880

caaactttct acgcggagtg aagaacgacc ctggcgctag ctcagaggtc agaaaagcct ggagcgcttt caggcagcgg    -800
                                                DR-1
tggggagtgg gactgcggag agaggcgcag gcaggagaca ttccgcaggg aggggcaagc acgtgtgggg ctagcatgag    -720

ggaagcaagg tcatgttctc ccagagcagg tgaggcgctg ggcagctcag cctcggcggt gtccaaggca actcttctcc    -640
        ERRα
acttgtctgg atgaagcaag aaggctcaag ggccactaga ccacgttctc tgtccgggct ccaaatcttt tctacttatg    -560

gtgaccgcgt cattcccatt cgagcctccg cgcctcttct acaagaagag gacatagggc cgttgaaatg ggtcattagg    -480

taaaggcatt tgctgccaaa gctgacgacc ccagtttgat acgtgggacc cacacggtgg aaaggagaga aatgactccc    -400
  E-box
gcgagttgca acgccccagt ttccccctcc ccaataagcg cgcctacacg tccacgcaca ctgtaataaa accacagtga    -320

aacaaggcgc cagaaggatc aggcggcgtt tccccagcgt gacgaaagct caagagcgag tttcctttca gtatggcggt    -240

gggatggatg ctctatttgg gttgcgggga gcgccgggca atcgggcagg gatgggttag agggcaccgg cgggaccgcg    -160

ggcgccgctg acgtaggcgc ccggagggct gtgggggatg ggcgctagcc ccttgaggga gtgcaagccc gggcttccta    -80
            E-box                                                                  CArG-
tttcgggagc aaggggtgtg ggccactttc atcatgtgat gcgagggcta ttaaagaga ctctccaccc gggagccgcg    0
element                                              tata box
ATTCTCACTG CCTGCACGCT CCTTTCTCAT TGCACCATGG CGGACGCCTT TGTCGGTACC TGGAAGCTAG TGGACAGCAA    80
                                        M  A  D  A  F  V  G  T  W  K  L  V  D  S  K
GAATTTTGAT GACTACATGAA GTCACTCGGT ggtgagcgaa cgactggcgc aggatccaa gggtcaggag ggcccgcgag    160
  N  F  D  D  Y  M  K  S  L
gaaatc-------------------3390 bp of intron I-----------------gcta atggagtctc aaccttctgc    3580

ccctgccctt agGTGTGGGC TTTGCCACCA GACAGGTGGC TAGCATGACC AAGCCGACCA CAATCATTGA GAAGAATGGG    3660
             G  V  G  F  A  T  R  Q  V  A  S  M  T  K  P  T  T  I  I  E  K  N  G
GATACCATCA CCATAAAGAC ACACAGTACC TTCAAGAACA CAGAGATCAG CTTTCAGCTG GGAGTAGAGT TTGACGAGGT    3720
  D  T  I  T  I  K  T  H  S  T  F  K  N  T  E  I  S  F  Q  L  G  V  E  F  D  E
CACAGCAGAT GACAGGAAGG TCAAGgtgag tcagagaaag gggtttggat agtaagaagc tggttc--------1396 bp
V  T  A  D  D  R  K  V  K
of intron II----------gctctggtca caacttaggc tctcccttct ttctacagTC GGTCGTGACA CTGGACGGAG    5262
                                                              S  V  V  T  L  D  G
GCAAACTGGT CCATGTGCAG AAGTGGGACG GGCAGGAGAC TACGCTTACA CGGGAACTAA GTGATGGGAA ACTCATCctg    5342
G  K  L  V  H  V  Q  K  W  D  G  Q  E  T  T  L  T  R  E  L  S  D  G  K  L  I
gtaagatggg caacgacagg aaccg------1060 bp of intron III------tcctg ctctgcccca tctgaccctg    6462

tctttccctt gCTGACTCTC ACCCATGGCA ATGTGGTGAG CACTCGGACT TACGAGAAGG AGGCGTGACC TGGCTGCCCC    6542
             L  T  L  T  H  G  N  V  V  S  T  R  T  Y  E  K  E  A Stop
GTCACTGACT GCTCCTCTGC CAATGGCTAC CCCTAACTCA GCACCACGTT GCCTCATGTT TCTCCCCTCT GACGTTTTAT    6622

ATAAATACTC TTTGGTTGGG CTTTTCCTG                                                        6652
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Fig. 2. Sequence of the *H-fabp* gene. Exons are shown as capital letters, while the introns and the 5′ and 3′-untranscribed regions are shown as lower case letters. The putative transcription start site, TATA box and potential regulatory elements are marked.

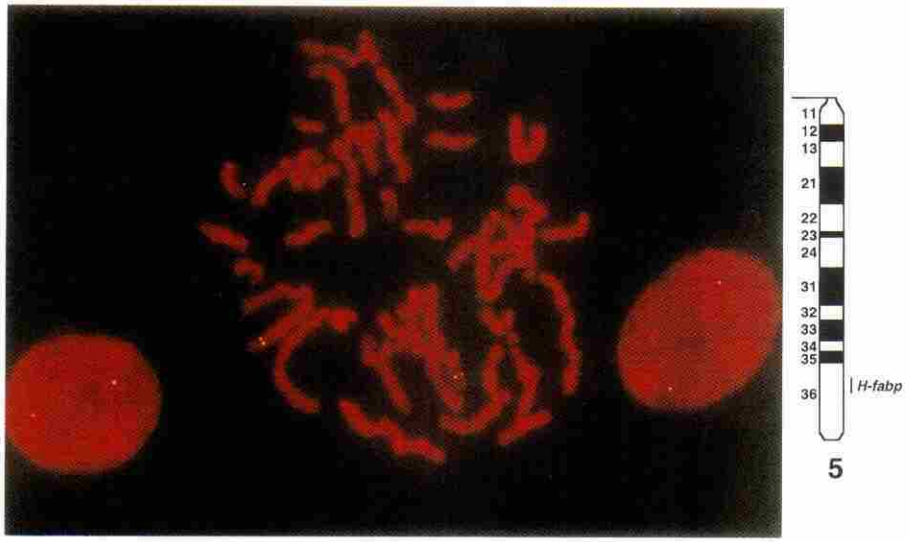

Fig. 3. Localization of the H-FABP gene to rat chromosome 5q36. Fluorescence *in situ* hybridization was carried out as described in the text.

Extensive sequence homology with the other sequenced mammalian heart FABP genes was detected in the promoter region. The 1.2 kb of upstream sequence has > 80% homology to the mouse gene, and also shows extended regions of sequence similarity with the human analog. The TATA signal, located 80 bp upstream of the translation start site (1238) is TTTAAA, a common muscle promoter also found in the mouse, human and pig genes. Naturally, the overall gene organization and sequence is very similar to that of the mouse, another rodent species. However, little sequence similarity with the other mammalian H-FABP/MDGI genes can be detected in the noncoding regions of the gene, suggesting that no control regions are contained in the introns.

There are various potential control regions in the upstream sequence, as indicated in Fig. 2. This includes muscle E-boxes, and a CArG-like element that was recently demonstrated to function as an atypical myocyte enhancer-binding factor 2 site [20]. Another intriguing element, present at position −713, resembles the binding site for the orphan receptor estrogen-related receptor α (ERRα), named nuclear receptor response element 1 (NRRE-1; 21). This element has been described in several genes involved in fatty acid metabolism, most notably medium-chain acyl CoA dehydrogenase, and because the receptor's ligand has yet to be found, it may turn out to play a role in fatty acid-mediated transcription.

Also noteworthy is a direct repeat element (AGCTCA-GAGGTCA) with strong homology to a peroxisome proliferator response element or a retinoic acid receptor binding site at position −842. This element is conserved in the mouse sequence, but not in the human, and is interesting because the peroxisome proliferator-activated receptors (PPAR) are known to mediate the effects of fatty acids on gene transcription in other contexts [22]. Indeed, a member of the PPAR family has been described as a fatty acid activated nuclear receptor in adipocytes [23]. This element therefore presents a candidate worthy of further study. Finally, a 35-bp element located at position −770 is absolutely conserved in mouse, human and rat, and has no homology to any known transcription factor response element. Further investigation will elucidate the possible role of all these elements in fatty acid-mediated transcription.

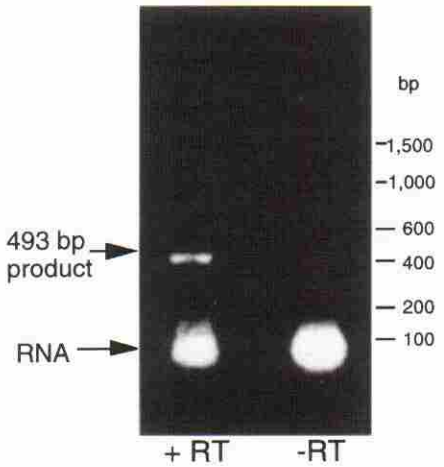

Fig. 4. RT-PCR of the primary transcript. Total RNA from adult rat heart (200 ng) was used as template for RT-PCR amplification of the 493 segment between R12 and R13 (Fig. 1). Left lane, RT was carried out prior to PCR, as described in Materials and methods. Right lane, PCR was carried out without a preceding RT, to prove that no contaminating genomic DNA is present in the sample.

Chromosomal location

The chromosomal localization of the gene was determined by FISH. Both plasmids (R3–R2 and R3–R6) were used by themselves and were also mapped together. Identical results were obtained with each probe. A total of ≈ 100 cells were studied and all exhibited labeling of both probes of rat chromosome (RNO) 5 (Fig. 3). The probes were assigned unequivocally to RNO5q36. The heart FABP gene has been found in mouse on chromosome MMU4-61.0 [14], and in the human genome on chromosome 1p32-p35 [24]. The rat locus 5q36 has been found to contain various other homologous genes that can also be found on mouse chromosome 4 and human chromosome 1.

Expression

To prove that the cloned gene is indeed expressed in cardiac muscle, the presence of the primary transcript of heart FABP in cardiac tissue was analyzed. Intron-specific PCR primers were designed that flank exon 2 and used in RT-PCR amplification of

total RNA preparations that were previously treated with DNAse I to remove genomic DNA contamination. Using these primers, the expected 493-bp product was obtained from total RNA following reverse transcription, but not when the reverse transcription reaction was omitted (Fig. 4). This proves that no contamination of genomic DNA, which yields the same PCR fragment without the reverse transcription reaction, was present, and that the gene is actively expressed in adult cardiac muscle.

We have demonstrated earlier in locust muscle [25] that the RT-PCR detection of FABP-primary transcript can be used as a sensitive and quantitative measure for the expression of the FABP gene. A similar approach should allow detection of transient changes in gene expression of the rat FABP gene, for example in response to physiological stimuli. Thus, the characterization of the FABP gene reported here will allow study of how FABP expression is controlled, and may lead to experimental approaches that result in increased intracellular concentrations of this protein. As FABP has been suspected to act as a cytoprotectant agent against fatty acid overload, this may have therapeutic applications in the treatment of heart disease.

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