Associations of heart and adipocyte fatty acid-binding protein gene expression with intramuscular fat content in pigs

F. Gerbens*, F. J. Verburg*, H. T. B. Van Moerkerk†, B. Engel‡, W. Buist‡, J. H. Veerkamp†, and M. F. W. te Pas*

*Department of Genetics and Reproduction and ‡Department of Immunology, Pathobiology, and Epidemiology, Institute for Animal Science and Health (ID-Lelystad), Lelystad, The Netherlands and †Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands

ABSTRACT: Intramuscular fat content is a major determinant of meat quality in pigs. Previously, polymorphisms in the adipocyte and heart fatty acid-binding protein genes, A-FABP and H-FABP, have been significantly associated with genetic variation of intramuscular fat content in a Duroc pig population. Further support for the role of H-FABP but not for A-FABP was found in a Meishan crossbred population. However, the effect of closely linked genes could not be excluded in these analyses. To validate the role of A-FABP and H-FABP in intramuscular fat accretion, 153 pigs of a crossbred genotype were evaluated for the A-FABP and H-FABP polymorphisms, mRNA, and protein expression levels of both FABP genes and intramuscular fat content in the longissimus lumborum muscle. For H-FABP, statistical analyses showed significant differences in mRNA but not protein expression levels between H-FABP HaeIII PCR-RFLP genotype classes. Between these genotype classes, significant differences in intramuscular fat content were found within barrows but not in gilts. Moreover, H-FABP mRNA but not protein expression levels were significantly related to intramuscular fat content. For A-FABP genotype classes, no significant differences in mRNA and protein expression levels were found. However, a significant difference in intramuscular fat content was found within barrows but not in gilts. In addition, a significant relationship between A-FABP mRNA but not protein expression levels and intramuscular fat content was found. In conclusion, variation of intramuscular fat content could not be explained by differences in A-FABP and H-FABP mRNA and protein expression levels. However, this may be due to limitations of the assays used and(or) the inappropriateness of the time of sampling. Finally, results suggest that A-FABP and H-FABP expression are translationally rather than transcriptionally regulated.

Key Words: Fat Metabolism, Genetic Markers, Loci, Meat Quality, Pigs

Introduction

Intramuscular fat content is a major determinant of the eating quality of pork and is highly heritable (reviewed by Hovenier et al., 1993). Significant associations between genetic variation at the adipocyte (A-FABP) and heart (H-FABP) fatty acid-binding protein gene loci, FABP4 and FABP3, and intramuscular fat content in purebred Duroc pigs have been identified (Gerbens et al., 1998, 1999). Further analysis of pigs from a crossbred Meishan × Dutch White pig population supports involvement of H-FABP but not A-FABP in intramuscular fat accretion (Gerbens et al., 2000). However, despite these associations, the effect of closely linked genes in both analyses cannot be excluded.

In order to control intramuscular fat, genetic variation in these FABP genes ultimately has to affect the expression level or functionality of the respective mRNA transcripts and(or) proteins. Both A-FABP and H-FABP are members of the fatty acid-binding protein (FABP) family that comprises a group of small cytosolic proteins that specifically bind and intracellularly transport fatty acids and other hydrophobic ligands (Veerkamp and Maatman, 1995). This essential role of FABP in long-chain fatty acid uptake and metabolic homeostasis has been demonstrated for H-FABP by gene disruption experiments (Binas et al., 1999; Schaap et al., 1999). Furthermore, A-FABP is exclusively expressed in adipocytes, whereas H-FABP is expressed in various tissues but predominantly in cardiac and skeletal muscle cells.
The objectives of this research were to 1) validate the involvement of A-FABP and H-FABP in intramuscular fat accretion in pigs through the mRNA and protein expression levels of both FABP genes and 2) clarify the in vivo relationship between mRNA and protein levels for both FABP genes.

Materials and Methods

Animals

The institutional Animal Care and Use Committee approved all procedures involving animal handling. In total, 153 pigs from matings between six Large White boars and 24 Large White × Dutch Landrace crossbred sows were used. These boars and sows were selected based on heterozygosity for the H-FABP genes, sows were used. These boars and sows were selected based on heterozygosity for the H-FABP gene, and the A-FABP microsatellite polymorphism (GenBank: U07786). From each biopsy, at least three randomly selected pigs belonging to different H-FABP HaeIII PCR RFLP genotype classes were included in the analysis. None of the matings or individual pigs were selected based on their H-FABP MspI PCR RFLP genotype. Experimental pigs were housed in groups at a commercial station and were given ad libitum access to feed until a slaughter weight of about 110 kg was reached.

Animal Performance and Meat Quality Data

Age and body weight were recorded for each pig at slaughter. Furthermore, backfat thickness was measured by ultrasound with the LEAN-MEATER (Renco Corporation, Minneapolis, MN) at four defined points on each side of the back, 5 cm lateral from the dorsal line. Twenty-four hours after slaughter, a slice of the longissimus lumborum muscle was isolated from the right carcass half at the third lumbar vertebra to assess intramuscular fat content. A muscle sample was taken from this slice, carefully avoiding intermuscular fat deposits surrounding the muscle. The intramuscular fat content was determined using Soxhlet petroleum-ether extraction and expressed as the weight percentage of wet muscle tissue.

Genotype Data

All experimental pigs were genotyped for the H-FABP MspI and HaeIII PCR-RFLP (Gerbens et al., 1997) and the A-FABP microsatellite polymorphism (Gerbens et al., 1998) as described.

Muscle Biopsies

A day before slaughter at 1600, a muscle biopsy sample was taken from the longissimus lumborum muscle using a shot biopsy method as described by Geverink et al. (1999). Biopsies were performed at a standardized site (i.e., at the third to fourth lumbar vertebra, 6 cm lateral from the dorsal line at the right-hand side of the body). The entire biopsy sample was immediately frozen in liquid nitrogen and stored at −80°C for further analysis.

RNA Extraction

From each biopsy sample, about 100 mg of muscle tissue was isolated that was free from contaminating adipose tissue originating from the backfat layer. Total RNA was extracted from the muscle samples essentially as described by Chomczynski and Sacchi (1987). All 153 RNA extractions were performed in 2 d in batches of 18 samples each. The RNA concentration was quantified spectrophotometrically at 260 nm. All RNA samples had A260/A280 ratios between 1.7 and 1.9, indicating pure and clean RNA isolates. The RNA integrity was checked by agarose gel electrophoresis to ensure that RNA was intact. Genomic DNA contamination of the RNA extractions was negligible as determined by PCR on identical equivalents of cDNA and RNA lacking reverse transcription. Amplification products with similar intensity were observed on agarose gel only after ten additional PCR amplification cycles on RNA lacking reverse transcription as compared with its respective equivalent of cDNA.

PCR Amplification and mRNA Level Quantification

Messenger RNA expression levels were quantified using RT-PCR-ELISA. An equivalent of 1 μg of total RNA was reverse-transcribed using the GeneAmp RNA PCR Core kit (PE Biosystems, Foster City, CA), and the resulting cDNA solution was diluted 10-fold before further use.

The RT-PCR-ELISA technique (Roche Diagnostics, Mannheim, Germany) was performed essentially as described before (Van den Hemel-Grooten et al., 1997; Te Pas et al., 1999, 2000). For the A-FABP, H-FABP, and β-actin genes, specific PCR amplifications were developed that discriminate between amplification from cDNA or genomic DNA template. For A-FABP, forward (5′CTG AGATTGCCTTCAAAATTG) and reverse (5′CCCTGGTCTCTCCTCATA) primers were designed to amplify a 225-bp cDNA fragment that hybridizes to a biotin-labeled capture probe (5′CTGGTACAGGTCGAGAAGTGG) and reverse (5′CTTGGCTCATGTGCTCTCCTATA) primers were designed to amplify a 188-bp fragment that hybridizes to the capture probe (5′CTTGTCACACCTGCGAGAAGTGG). For β-actin, forward (5′GGACTTCCACCTGCGAGAAGTGG) and reverse (5′GCACCCTGTTGGCCTGAGG) primers were described by Baarsch (1994) and the respective capture probe (5′GAGTCCTGCGCATCCACAGCAG) was designed according to the porcine β-actin mRNA sequence (GenBank: U07786).

The PCR amplifications were performed on 10 μL of cDNA template (i.e., equivalent to 0.05 μg of total RNA)
in a total volume of 50 μL containing 0.2 unit of AmpliTaq DNA polymerase (PE Biosystems, Foster City, CA) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.5 μM of each primer, 0.2 mM of dATP, dCTP and dGTP, 0.19 mM dTTP, and 0.01 mM of digoxigenin (DIG)-dUTP. For the A-FABP and H-FABP genes, after 3 min of denaturation at 94°C, amplification cycling was carried out at 92°C for 45 s, 57°C for 45 s, and 72°C for 45 s with a final extension step at 72°C for 10 min. For the β-actin gene, the annealing temperature was 65°C and AmpliTaq Gold (PE Biosystems, Foster City, CA) was used for amplification. The number of cycles was optimized empirically to allow quantitative amplification by PCR and evaluation by ELISA (i.e., 23 and 25 cycles for both the A-FABP and H-FABP genes and 24 and 26 cycles for the β-actin gene).

The A-FABP, H-FABP, and β-actin gene RT-PCR amplifications were each very specific because PCR reactions allowed amplification of a single fragment of the expected size (data not shown).

After PCR amplification, PCR products were hybridized to the respective biotin-labeled capture probes and immobilized to streptavidin-coated microtiterplate wells. Subsequent incubation with anti-DIG-peroxidase (0.01 unit/mL) and its substrate ABTS allowed detection and colorimetical quantification of the bound hybrid at 405 nm. The entire procedure included several controls: two separate PCR cycles to evaluate proper PCR amplification and several spectrophotometrical measurements in time to evaluate linearity of ELISA. Furthermore, several controls were evaluated within each microtiterplate, such as PCR reactions without sample cDNA as well as H₂O and ABTS to account for background and standard controls to correct data from different microtiterplates. Results from different microtiterplates were assembled after correction for respective background and differences in the standard controls. Linearity and quantitativeness of the RT-PCR ELISA procedure have been described by Te Pas et al. (2000). The entire PCR-ELISA procedure was performed at a single day for all cDNA samples. To determine the repeatability of these PCR-ELISA assays, a number of cDNA samples were tested in duplicate either in the same assay, as for the H-FABP gene (n = 22), or in a separate assay 3 d later, as for the A-FABP gene (n = 46). The repeatability estimate (see the statistical analysis subsection) of the entire PCR-ELISA procedure for H-FABP was 0.97, which addresses the intraassay variability, whereas, for A-FABP, the repeatability estimate of 0.87 addresses the interassay variability. These repeatability estimates agree well with the estimates from literature that range from 0.80 to 0.98 (Te Pas et al., 1999, 2000).

Antisera Preparation

Recombinant rat A-FABP (Prinsen and Veerkamp, 1998) and porcine H-FABP (Paulussen et al., 1989) were isolated and purified as described. Rabbit-anti-rat-A-FABP and rabbit-anti-porcine-H-FABP antisera were obtained after injection of multiple doses of 100 μg recombinant rat A-FABP or porcine H-FABP (Paulussen et al., 1989). Prior to usage, both rabbit-anti-rat-A-FABP and rabbit-anti-porcine-H-FABP antisera were affinity-purified. These antisera specifically bind either porcine A-FABP or H-FABP, respectively, and showed no cross-hybridization as determined by Western blot analysis (Figure 1).

Protein Quantification

The FABP content was quantified as described by Paulussen et al. (1989). Briefly, about 100 mg of muscle tissue was isolated from each muscle biopsy sample and homogenized in phosphate-buffered saline (5%, wt/vol) using a Potter-Elvehjem tissue homogenizer. Homogenates were centrifuged for 1 h at 105,000 × g at 4°C and the supernatant was stored at −80°C. Protein content was determined according to Lowry et al. (1951) with bovine serum albumin as standard and standardized at 0.5 mg/mL. The A-FABP and H-FABP content were quantified in six samples of 25 ng of each cytosolic protein preparation by ELISA. Calibration and cytosolic protein samples were adsorbed to microtiter plates by incubation for 16 h at 4°C. Porcine H-FABP or A-FABP was probed with affinity-purified rabbit-anti-porcine-H-FABP or rabbit-anti-rat-A-FABP antibodies, respectively, and horseradish peroxidase-conjugated mouse-anti-rabbit IgG monoclonal (Sigma, St. Louis, MO) was used as a second antibody. The bound peroxidase was assessed with o-phenylenediamine dichloride as a substrate and this reaction was terminated by addition of H₂SO₄ (12.5% vol/vol). The product of the reaction was determined at 492 nm. The amount of immune-reactive protein in each sample was calculated by linear regression using a standard curve (0 to 1.0 ng of rat A-FABP or porcine H-FABP) and expressed in nanograms of FABP per microgram of cytosolic protein. To determine repeatability (see next subsection) of these protein ELISA assays, a number of cytosolic protein preparations were tested in two independent experiments. The repeatability estimates of the H-FABP ELISA assay (n = 46) was 0.95, and this addresses both intra- and interassay variability, whereas the repeatability estimate of the A-FABP ELISA assay (n = 159) was 0.82, which addresses interassay variability.

Statistical Analysis

Repeatability of A-FABP and H-FABP RT-PCR-ELISA and protein ELISA analysis is defined as the correlation between duplicate analyses of the same cDNA or cytosolic protein samples, respectively. Repeatability was estimated as the ratio of the estimated sample variance component and the total variance in a linear mixed model. In all mixed models, components of variance were estimated by restricted maximum like-
Figure 1. Specificity of affinity-purified anti-porcine H-FABP (p-HFABP) and anti-rat A-FABP (r-AFABP) antisera by Western blot analysis. Lanes 1 and 2: 50 (20 in right panel) ng of rat A-FABP and porcine H-FABP, respectively; Lanes 3 and 4: 50 μg of porcine muscle cytosolic proteins, Lane 5 and 6: 20 (50 in right panel) ng of rat A-FABP and porcine H-FABP, respectively.

likelihood (Patterson and Thompson, 1971; Searle et al., 1992) with the statistical programming language Genstat 5 (Numerical Algorithms Group Inc., Downers Grove, IL).

The A-FABP and H-FABP mRNA and protein expression levels were analyzed with a mixed analysis of variance model (Searle et al., 1992). Random effects in the model accounted for possible correlation between observations from the same slaughter day and litter. Although the experiment was designed to perform a within-litter analysis, the effect of litter did not contribute significantly in any of the analyses and was discarded from the final model. Fixed effects in the model accounted for differences between sex and genotype classes (both as factors with main effects and interaction terms) and slaughter weight (with linear and quadratic terms as covariables). The sex × genotype class interaction and the quadratic term of slaughter weight did not contribute significantly in any of the analyses and were discarded from the model in the final analysis. Furthermore, analyses were performed on log-transformed data because diagnostic plots suggested that the variance increased with the mean. In the first analysis of mRNA expression level, the logarithm of β-actin mRNA expression level was an additional covariable in the model to evaluate differences in mRNA isolation procedures. However, β-actin mRNA expression level showed no significant effect and was, therefore, discarded from the model in the final analyses.

Intramuscular fat content was also analyzed with a mixed analysis of variance model, with random effects for slaughter days and fixed effects for sex, genotype (both main effects and interaction terms), age, and backfat thickness. Backfat thickness was included to account for its considerable genetic correlation with intramuscular fat content in pigs.

The relationship between intramuscular fat content and H-FABP or A-FABP mRNA and protein expression levels was studied with a mixed linear model, with intramuscular fat as the dependent variable and mRNA or protein expression level as explanatory variables. Random effects for slaughter days and fixed effects for sex, age, and weight were included in the model as well. Correlations between H-FABP or A-FABP mRNA and protein expression levels were calculated, accounting for differences between slaughter days and sex of the animals, that is, a pooled within-sex and slaughter-day correlation.

Results

H-FABP Analyses

Three H-FABP PCR-RFLP (HaeIII, HinfI, and MspI) have been described for pigs (Gerbens et al., 1997) that are all located within 10 kb of genomic DNA. These polymorphisms are present in almost all common pig breeds, but in the current experiment only the HaeIII and MspI PCR-RFLP alleles were segregating. Designed matings were used based on the HaeIII PCR-RFLP to obtain expected balanced sample sizes and to estimate genotype effects within litters. The MspI PCR-RFLP yielded unbalanced sample sizes because the linkage phase between the alleles of the HaeIII and MspI PCR-RFLP differs between experimental pigs.

Significant differences in intramuscular fat content were observed between genotype classes of both H-FABP PCR-RFLP in barrows but not in gilts (Table 1). However, the high intramuscular fat content for the MspI PCR-RFLP aa genotype class was due to data from only two barrows, of which one contained the highest intramuscular fat content of all experimental pigs.

Significantly lower H-FABP mRNA expression levels were found for the HaeIII PCR-RFLP dd genotype class compared with the other genotype classes. This effect was irrespective of the gender of the animals. For comparison with the intramuscular fat data, in Table 1 the H-FABP mRNA expression levels for each genotype class are, however, presented for each sex separately and significant differences within sexes are indicated as well. No significant differences in H-FABP mRNA expression level were found between genotype classes
Table 1. Means and standard errors of H-FABP mRNA and protein expression levels and intramuscular fat content in longissimus muscle of pigs from different H-FABP RFLP genotype classes

<table>
<thead>
<tr>
<th>RFLP and Class</th>
<th>n</th>
<th>Barrows</th>
<th>n</th>
<th>Gilts</th>
<th>n</th>
<th>Barrows</th>
<th>n</th>
<th>Gilts</th>
<th>n</th>
<th>Barrows</th>
<th>n</th>
<th>Gilts</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaeIII</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td>12</td>
<td>0.25 (0.02)</td>
<td>25</td>
<td>0.26 (0.02)</td>
<td>13</td>
<td>4.28 (0.38)</td>
<td>25</td>
<td>4.08 (0.36)</td>
<td>13</td>
<td>1.07 (0.44)</td>
<td>24</td>
<td>0.76 (0.39)</td>
</tr>
<tr>
<td>Dd</td>
<td>27</td>
<td>0.24 (0.01)</td>
<td>27</td>
<td>0.25 (0.01)</td>
<td>27</td>
<td>4.75 (0.38)</td>
<td>30</td>
<td>4.53 (0.37)</td>
<td>27</td>
<td>0.90 (0.33)</td>
<td>27</td>
<td>0.80 (0.28)</td>
</tr>
<tr>
<td>dd</td>
<td>9</td>
<td>0.21 (0.02)</td>
<td>18</td>
<td>0.22 (0.02)</td>
<td>10</td>
<td>4.38 (0.42)</td>
<td>18</td>
<td>4.18 (0.39)</td>
<td>9</td>
<td>1.31 (0.88)</td>
<td>16</td>
<td>0.78 (0.19)</td>
</tr>
<tr>
<td>MspI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>27</td>
<td>0.24 (0.01)</td>
<td>40</td>
<td>0.25 (0.01)</td>
<td>28</td>
<td>4.54 (0.37)</td>
<td>42</td>
<td>4.30 (0.35)</td>
<td>28</td>
<td>0.87 (0.07)</td>
<td>40</td>
<td>0.85 (0.06)</td>
</tr>
<tr>
<td>Aa</td>
<td>18</td>
<td>0.23 (0.01)</td>
<td>25</td>
<td>0.24 (0.01)</td>
<td>19</td>
<td>4.54 (0.40)</td>
<td>26</td>
<td>4.30 (0.38)</td>
<td>19</td>
<td>0.92 (0.08)</td>
<td>23</td>
<td>0.74 (0.07)</td>
</tr>
<tr>
<td>aa</td>
<td>3</td>
<td>0.24 (0.03)</td>
<td>5</td>
<td>0.24 (0.03)</td>
<td>3</td>
<td>4.77 (0.69)</td>
<td>5</td>
<td>4.52 (0.65)</td>
<td>2</td>
<td>2.60 (0.24)</td>
<td>4</td>
<td>0.94 (0.17)</td>
</tr>
</tbody>
</table>

mRNA expression levels are expressed in relative ELISA values per 0.05 µg of total RNA and protein expression levels are expressed in nanograms of H-FABP per microgram of cytosolic protein.

Within a column, for each H-FABP RFLP, means without a common superscript letter differ (P < 0.05).

of the MspI PCR-RFLP (Table 1). In addition, for H-FABP protein expression levels, no significant differences were observed between genotype classes of both H-FABP PCR-RFLP (Table 1).

In an overall analysis, a significant relationship between H-FABP mRNA expression levels and intramuscular fat content was found, whereas no relationship was detectable between H-FABP protein expression levels and intramuscular fat content. The correlation between H-FABP mRNA and protein expression levels within the muscle sample was extremely low (0.05).

A-FABP Analyses

For A-FABP, six microsatellite alleles were detected in the experimental population resulting in 10 different genotype classes. Because mating of parental animals was based on H-FABP HaeIII PCR-RFLP genotype, the frequency of each A-FABP genotype varies considerably. In Table 2, only A-FABP genotype classes represented by more than four pigs are shown.

The A-FABP A6A9 genotype class significantly differed in intramuscular fat content from the other genotype classes in barrows but not in gilts. Coincidentally, this genotype class is represented by the same two barrows as for the H-FABP MspI PCR-RFLP aa genotype class, one of which has the highest intramuscular fat content. No significant differences in A-FABP mRNA and protein levels were found between A-FABP genotype classes (Table 2). Similar as with H-FABP, a significant overall relationship between intramuscular fat content and A-FABP mRNA but not protein expression levels was found. In addition, the correlation between A-FABP mRNA and protein expression levels was very low (0.09).

Discussion

Previous studies have demonstrated associations between polymorphisms in the A-FABP and H-FABP genes and intramuscular fat content in pigs (Gerbens et al, 1998, 1999). However, these analyses do not exclude other closely linked genes from being responsible for these effects on intramuscular fat content. Obviously, to exert an effect, a particular mutation needs to alter the function (quality) or the abundance (quantity) of the respective gene products (i.e., mRNA or protein molecules). This paper explicitly investigates A-FABP and H-FABP mRNA and protein quantity and not the quality of the respective mRNA or protein molecules. To our knowledge, this paper reports for the first time results from a study as to the validation of previous associations of a genetic polymorphism with a quantitative trait. To validate the role of the FABP genes, rela-

Table 2. Means and standard errors of A-FABP mRNA and protein expression levels and intramuscular fat content in longissimus muscle of pigs from different A-FABP microsatellite genotype classes

<table>
<thead>
<tr>
<th>Class</th>
<th>n</th>
<th>Barrows</th>
<th>n</th>
<th>Gilts</th>
<th>n</th>
<th>Barrows</th>
<th>n</th>
<th>Gilts</th>
<th>n</th>
<th>Barrows</th>
<th>n</th>
<th>Gilts</th>
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<tbody>
<tr>
<td>A1A4</td>
<td>2</td>
<td>0.26 (0.04)</td>
<td>4</td>
<td>0.25 (0.04)</td>
<td>2</td>
<td>2.34 (0.28)</td>
<td>4</td>
<td>2.18 (0.26)</td>
<td>2</td>
<td>1.32 (0.24)</td>
<td>5</td>
<td>0.74 (0.15)</td>
</tr>
<tr>
<td>A1A9</td>
<td>9</td>
<td>0.21 (0.02)</td>
<td>5</td>
<td>0.20 (0.03)</td>
<td>9</td>
<td>2.14 (0.19)</td>
<td>5</td>
<td>1.99 (0.19)</td>
<td>10</td>
<td>1.08 (0.10)</td>
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<td>0.70 (0.15)</td>
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<tr>
<td>A4A9</td>
<td>20</td>
<td>0.23 (0.02)</td>
<td>25</td>
<td>0.23 (0.02)</td>
<td>20</td>
<td>2.29 (0.18)</td>
<td>26</td>
<td>2.13 (0.17)</td>
<td>23</td>
<td>0.77 (0.07)</td>
<td>25</td>
<td>0.86 (0.07)</td>
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<tr>
<td>A6A9</td>
<td>3</td>
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<td>8</td>
<td>0.24 (0.03)</td>
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<td>2.56 (0.27)</td>
<td>8</td>
<td>2.39 (0.24)</td>
<td>2</td>
<td>2.60 (0.24)</td>
<td>8</td>
<td>0.95 (0.12)</td>
</tr>
<tr>
<td>A9A9</td>
<td>9</td>
<td>0.20 (0.02)</td>
<td>22</td>
<td>0.22 (0.02)</td>
<td>9</td>
<td>2.38 (0.21)</td>
<td>22</td>
<td>2.21 (0.18)</td>
<td>7</td>
<td>0.91 (0.12)</td>
<td>18</td>
<td>0.81 (0.08)</td>
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</table>

mRNA expression levels are expressed in relative ELISA values per 0.05 µg of total RNA and protein expression levels are expressed in nanograms of A-FABP per microgram of cytosolic protein.

Within a column, means without a common superscript letter differ (P < 0.05).
tionships were investigated between A-FABP and H-FABP polymorphisms, mRNA and protein expression levels and intramuscular fat content in pigs.

**H-FABP and A-FABP Analyses**

With respect to the H-FABP analyses, results show that both H-FABP polymorphisms have significant associations with intramuscular fat content in barrows but not in gilts. These results are consistent with other association studies with the H-FABP polymorphisms in pigs (Gerbens et al., 1999; Grindflek et al., 2000) apart from the significant sex effect. Interestingly, the dd and aa genotype classes of the H-FABP HaeIII and MspI PCR-RFLP, respectively, also had the highest intramuscular fat content in these previous studies (Gerbens et al., 1999; Grindflek et al., 2000). With respect to the H-FABP HaeIII PCR-RFLP, the dd genotype class has a significantly lower H-FABP mRNA expression level and a significantly higher intramuscular fat content in the longissimus lumborum muscle of barrows as compared with the DD genotype class. Although these findings may suggest a causal relationship between H-FABP mRNA expression level and intramuscular fat content, it is generally protein molecules, and not mRNA molecules, that determine phenotype. However, at the level of H-FABP protein expression, no corresponding significant differences were detected between genotype classes and no relationship with intramuscular fat content was found. Therefore, these results do not explain the association between H-FABP and intramuscular fat content in pigs. However, several reasons may be responsible for these inconclusive results, which will be described below.

With respect to the A-FABP analyses, the A6A9 genotype class has a significantly higher intramuscular fat content than the other genotype classes in barrows but not in gilts. However, due to the large number of alleles segregating in the population and the mating strategy based on another unlinked polymorphism, the sample sizes of the genotype classes were small and unbalanced. Therefore, these genotype classes may not represent the population as a whole. Previously, a considerable and significant contrast in intramuscular fat content between A-FABP genotype classes was detected in Duroc pigs (Gerbens et al., 1998). Although, the A1, A2, and A3 alleles that were segregating in the Duroc population are, except for A1, completely different from alleles in the current experimental population (Table 2), the A-FABP gene may still be involved in genetic variation of intramuscular fat content in this latter population.

No significant differences in A-FABP mRNA and protein expression levels were detected between A-FABP genotype classes. This may be due to the absence of an association between A-FABP and intramuscular fat content in these pigs or the small sample sizes of the genotype classes. Furthermore, because no direct relationship between A-FABP protein expression levels and intramuscular fat content was detectable, these results suggest no role for A-FABP in intramuscular fat content in this pig population and do not explain previous results in Duroc pigs (Gerbens et al., 1998).

Surprisingly for both A-FABP and H-FABP genes, a significant relationship between FABP mRNA expression levels and intramuscular fat content was demonstrated. Because no further evidence was found for the involvement of these FABP genes in intramuscular fat content, this relationship is most likely a consequence of higher fatty acid metabolism in the longissimus lumborum muscle. Probably, expression of genes involved in fatty acid metabolism like FABP is up-regulated in cells or tissue containing a higher concentration of fatty acids (i.e., intramuscular fat content). This up-regulation of the mRNA expression level of the FABP genes does not result in up-regulation of the protein expression level of the FABP genes due to the suggested translational control of H-FABP and A-FABP gene expression.

Results from the current study do not support the involvement of either A-FABP or H-FABP mRNA or protein expression level in intramuscular fat content as determined in previous studies (Gerbens et al., 1998, 1999). This lack of support could be due to the pig population under investigation, which may not be segregating for the previously identified effects of the FABP genes. Obviously, the ideal population for this experiment would have been the Duroc pig population used in previous studies (Gerbens et al., 1998, 1999). However, this population was eradicated due to a classical swine fever outbreak at this herd in 1997.

Another reason may be that the effect of FABP genes on intramuscular fat content is caused by differences in protein functionality (i.e., fatty acid-binding capacity, ligand specificity, etc.) rather than protein expression levels. In general, proteins affect phenotype and not mRNA molecules. Because no significant differences in protein expression levels were observed, a role for mRNA transcript quality as a possible explanation for genetic variation in intramuscular fat content can be excluded.

The sensitivity of the RT-PCR-ELISA and protein ELISA assays may also affect the outcome of the study. Functional differences in A-FABP and H-FABP mRNA and protein expression levels may still be present but too small to be detected with these assays. Furthermore, the moment of sampling of the pigs may also have been inappropriate. Namely, intramuscular fat content is a trait that results from fat accretion over the lifetime, whereas the FABP mRNA and protein expression levels are measured at a single moment during the life of the pig. The different fat depots in pig develop in a specific gradient from subcutaneous to inter- to intramuscular fat (Lee and Kauffman, 1974) and this latter fat depot increases with age surely beyond the common slaughter age of 180 d (Catchpole and Lawrie, 1972). Unfortunately, it is not known which period during the lifetime of a growing pig is responsible for ge-
netic variation in intramuscular fat content and hence the most appropriate moment to assess FABP mRNA and protein expression levels. Thus, FABP mRNA or protein expression level may still be responsible for genetic variation in intramuscular fat content but either or both may have been measured at an inappropriate time.

Finally, H-FABP protein expression levels differ between muscles with different fiber type composition for rats and pigs (Veer Kamp and Moerkerk, 1993; our unpublished observations). Although between-animal variation in porcine longissimus lumborum muscle fiber type composition is relatively small (Brocks et al., 1998), differences in fiber type composition between our experimental pigs may have influenced results for H-FABP. Interestingly, the intramuscular fat content of muscles is only to a minor extent related to differences in myofiber type composition in rabbits (Gondret et al., 1998).

Gene Expression Analysis

To our best knowledge, this is the first report of A-FABP and H-FABP gene expression in pigs. The extremely low correlation between mRNA and protein expression levels suggests that A-FABP and H-FABP expression are translationally rather than transcriptionally regulated. In this respect, the fact that FABP inhibit cell-free protein synthesis (Zimmerman and Veerkamp, 1998) and thus regulate their own expression may also be of importance. On the other hand, gene disruption experiments in mice suggest transcriptional regulation. For example, mice hemizygous for the A-FABP gene express A-FABP mRNA and protein in adipocytes at a significantly lower level than wild-type mice (Hotamisligil et al., 1996); however, some compensatory up-regulation of the keratinocyte FABP (E-FABP) was observed (Ribarik Coe et al., 1999). Moreover, in human bladder carcinomas, A-FABP is transcriptionally regulated (Gromova et al., 1998). In addition, mice hemizygous for the H-FABP gene express H-FABP mRNA transcripts at about 50% of the wild-type level in the heart, but, unfortunately, no data on H-FABP protein expression were presented (Binas et al., 1999). The H-FABP-deficient mice show no compensatory expression of other members (A-FABP, brain FABP, E-FABP, or liver FABP) of the FABP family (Binas et al., 1999). However, results from knockout experiments or from cancer cells may be difficult to extrapolate to normal physiological circumstances. In addition, differences in fat metabolism between species may also explain the differences between studies.

In conclusion, the H-FABP polymorphisms were associated with intramuscular fat content in barrows of this crossbred pig population. However, these effects could not be explained by differences in H-FABP mRNA and protein expression levels. This lack of support for the role of the H-FABP gene in genetic variation of intramuscular fat accretion may be due to limitations of the assays used and/or the inappropriateness of the time of sampling. For A-FABP, no clear relationship with intramuscular fat content, either by genetic association or directly through protein expression was found. Results suggest translational control of both porcine A-FABP and H-FABP expression, and therefore evaluation of FABP protein expression levels may suffice in future association studies.

Implications

Findings showed no clear explanations for the involvement of adipocyte and heart fatty acid-binding protein genes with genetic variation in intramuscular fat content in pigs by means of gene transcript or protein expression level analysis. Despite these results, adipocyte and heart fatty acid-binding protein may still be involved in genetic variation of intramuscular fat content in this and other pig populations by other genetic mechanisms. Providing the association of adipocyte and/or heart fatty acid-binding protein gene polymorphisms with intramuscular fat content exists in the respective population, these polymorphisms can at least be used for marker-assisted selection for improved intramuscular fat content and hence meat quality in pigs.

Literature Cited


