Genetic control of intramuscular fat accretion in pigs.

The role of heart and adipocyte fatty acid-binding proteins.

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Oan Heit en Mem,
Foar Petra

Abbreviations

aa	amino acids
A-FABP	adipocyte fatty acid-binding protein
BFT	backfat thickness
bp	base pairs
BW	body weight
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DRIP	water-holding capacity of meat by drip method
ELISA	enzyme-linked immunosorbent assay
FAAR	fatty acid-activated receptor
FABP	fatty acid-binding protein
FABP3	heart/muscle fatty acid-binding protein encoding gene
FABP4	adipocyte fatty acid-binding protein encoding gene
FAT	fatty acid translocase
FATP	fatty acid-transport protein
FISH	fluorescence *in situ* hybridisation
H-FABP	heart/muscle fatty acid-binding protein
HSC1	human (*Homo sapiens*) chromosome 1
HSC8	human (*Homo sapiens*) chromosome 8
I-FABP	intestinal fatty acid-binding protein
IMF	intramuscular fat
kb	kilobase pairs or 1000 base pairs
L-FABP	liver fatty acid-binding protein
M-FABP	myelin fatty acid-binding protein
MAS	marker-assisted selection
MDGI	mammary-derived growth inhibitor
MMC3	murine (*Mus musculus*) chromosome 3
MMC4	murine (*Mus musculus*) chromosome 4
mRNA	messenger ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PPAR	peroxisome proliferator-activated receptor
QTL	quantitative trait locus/loci
RNA	ribonucleic acid
RFLP	restriction fragment length polymorphism
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
SINE	short interspersed repeat unit
SNP	single nucleotide polymorphism
SSC4	porcine (*Sus scrofa*) chromosome 4
SSC6	porcine (*Sus scrofa*) chromosome 6
TG	triacylglycerol
VNTR	variable number of tandem repeats

Contents

Chapter 1

General introduction

Introduction

Pigs are mainly raised for the human consumption of fresh meat and processed meat products. For decades strategies applied in pig breeding have successfully concentrated on quantitative aspects of pig production like performance and carcass traits. Annual improvement rates have been 1 to 2% (De Vries and Kanis, 1994). Besides the ongoing improvement in quantitative traits, qualitative aspects, in particular meat quality are attracting increasingly more attention. This is mainly due to the increased consumer awareness with respect to eating quality and nutritional aspects of meat. Several studies involving consumer and trained panels revealed that intramuscular fat (IMF) content is one of the important traits that influences eating quality traits like meat tenderness, juiciness and taste (reviewed by Hovenier et al., 1993). Although some studies were unable to detect this relationship it is now generally agreed that variation in IMF content explains an important part of the genetic variation in eating quality of porcine meat (Wood and Cameron, 1994). The IMF content of porcine meat is an optimum trait with respect to the influence of meat tenderness (reviewed by Hovenier et al., 1993). An IMF content below the recommended optimum range of 2.5 to 3% diminishes eating quality whereas a higher IMF content will not further improve this parameter and have adverse effects on consumer acceptability due to increased visibility of fat in the meat i.e. marbling.

In the early nineties, IMF content was already well below the desired range of 2.5 to 3% in European pigs (Casteels et al., 1995, De Vries et al., 1994) but not in American pigs (NPPC, 1995). In addition, IMF content in pigs is decreasing ever since as a result of unfavourable genetic correlations with the selection criteria higher lean meat content and reduced backfat thickness. For example, each percentage improvement in carcass lean percentage would be accompanied by a reduction in IMF content of 0.07% (De Vries et al., 1994). Therefore, strategies to improve IMF content especially in European pigs need to be investigated.

Strategies to improve intramuscular fat content

In order to improve IMF content of pigs, different strategies can be exploited. These strategies are based on the observation that IMF content can be improved by selection because heritability estimates indicate substantial genetic variation in this trait. Heritability estimates (h^2) of IMF content of pigs differ among studies, and range from 0.26 to as high as 0.86 with an average of 0.5 (reviewed by Hovenier et al., 1993, De Vries et al., 1994).

Besides unfavourable genetic correlations with other production traits, conventional selection for IMF content is not very effective for two additional reasons. First, IMF content is determined in slaughtered animals excluding these animals for further selection. Therefore, selection is based on data from slaughtered siblings and hence genetic improvement is less optimal and accurate. Furthermore, the currently applied fat extraction methods to determine IMF content are laborious and expensive. These limitations can be circumvented by developing and introducing accurate, noninvasive, cost effective techniques to assess IMF content in pigs.

One such technique is the direct exploitation of the genetic control of this trait. The size of the genetic correlations between IMF content and production traits as mentioned before, indicate that part of the genetic variation is independently inherited. In other words, these traits are partially controlled by different genes. Hence, traits may be treated independently when the respective genes that contribute to the genetic variation of IMF content but not of production traits are identified. Evidence for the existence of genes with a substantial effect on IMF content has been provided by segregation analysis in pigs (Janss et al., 1997). Upon identification, these polymorphic genes or genomic regions may be applied in breeding by eliminating detrimental allele(s) from the population, by marker-assisted selection (MAS; see Meuwissen and Goddard, 1996; Spelman and Bovenhuis, 1998) or by introgression of beneficial allele(s) in populations or breeds lacking these alleles (Visscher et al., 1996). MAS can considerably increase the selection response in particular for traits with a low heritability and/or for carcass and meat quality traits, like IMF content (Meuwissen and Goddard, 1996).

Molecular-genetic methods to improve intramuscular fat content

Two approaches can be employed to identify genomic regions influencing quantitative traits. The genome scan approach uses large numbers of available genetic markers and linkage maps to identify loci affecting quantitative traits (QTL). This approach relies on the premise that trait-affecting alleles will be in linkage disequilibrium with flanking genetic markers over a sufficiently large distance that association can be detected. However, the relatively large marker intervals (typically 10-20 cM) used allow only linkage disequilibrium mapping within families, as opposed to across families in a population, with inherently low accuracy to locate genes.

Several genome scans are underway in livestock animals and QTLs for several traits have already been reported for pigs (Andersson et al., 1994), and cattle (Georges et al., 1995). The advantage of the genome scan approach is that all genomic regions that have a substantial contribution to the genetic variation of the trait of interest will be identified.

However, the reliability of identifying real QTL is of main concern due to the large number of tests performed in the analysis which are also not independent because markers or marker brackets are situated on the same chromosome. Moreover, the size of QTL regions is of major concern, because smaller QTL regions increase the genetic response from MAS (Spelman and Bovenhuis, 1998), and reduce the number of positional candidate genes substantially.

The alternative approach is the candidate gene approach, that uses existing knowledge of physiological and biochemical processes from the species under investigation or other species, to identify genes that may be expected to influence the trait of interest. From a statistical genetic point of view, the candidate gene approach is a linkage disequilibrium mapping approach using linkage disequilibrium across families in a population. As an example, the estrogen receptor (ESR) was presumed to be involved in the genetic variation of reproductive traits in pigs. A polymorphism was identified in the ESR encoding gene and associated with litter size in pigs (Rothschild et al., 1996; Short et al., 1997). Benefits of the candidate gene approach are the relative straightforwardness and low costs. However, success largely depends on the amount and quality of prior information to identify candidate genes and the pig population under investigation.

Aim and outline of thesis

The aim of this thesis is to investigate the genes encoding for heart and adipocyte fatty acid-binding proteins, H-FABP and A-FABP, as candidate genes for genetic variation in IMF content in pigs. In other words, polymorphisms in these genes may affect the expression of these genes or the function of the proteins and hence influence intracellular fatty acid transport or trafficking, ultimately resulting in differences in IMF accretion between pigs. Therefore, this thesis starts with an literature overview regarding fatty acid-binding proteins (FABPs) and their role in fat deposition, in particular IMF deposition (Chapter 2). Chapter 2 also includes more recent literature and data that have been gathered during investigations that are described in subsequent chapters of this thesis.

In Chapters 3 and 6 the isolation and characterization of the genes encoding porcine H-FABP and A-FABP, *FABP3* and *FABP4*, are described. In addition, DNA polymorphisms were identified in these genes (Chapters 3, 4, 6 and 7).

To investigate the role of the H-FABP and A-FABP genes in genetic variation of IMF content in pigs, the identified polymorphisms were tested for association with IMF content in Duroc pigs as decribed in Chapters 5 and 6. In addition, in Chapter 8 a similar investigation is presented that was performed in the Wageningen Meishan crossbred population to confirm results from Duroc pigs. For practical reasons, we substituted the RFLP polymorphisms in the H-FABP gene as applied in the Duroc population by the linked polymorphic microsatellite described in Chapter 4.

Results from Chapters 5, 6, and 8 demonstrate associations between IMF content and polymorphisms in H-FABP and A-FABP genes. However associations are not necessarily direct relationships but may be due to the effect of closely linked genes. To prove that the H-FABP and A-FABP genes are themselves involved in the genetic variation in IMF content of pigs, we studied the effect of the H-FABP and A-FABP gene polymorphisms on the RNA and protein expression of each gene and the IMF content in Large White fattening pigs (Chapter 9).

Finally, in Chapter 10 the findings of the investigations described in this thesis are discussed and put into perspective with results from literature.

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Chapter 2

Intramuscular fat metabolism and the role of heart and adipocyte fatty acid-binding proteins. A literature survey.

1. Introduction

This chapter will give an overview of the literature concerning heart and adipocyte fatty acid-binding protein, H-FABP and A-FABP, and their role in fat accretion, in particular the intramuscular fat (IMF) accretion.

Different definitions of IMF are commonly used in sport physiology and animal sciences. The first defines IMF as the proportion of fat in the myocytic cells alone whereas for the latter IMF is the proportion of fat in a sample of muscle devoid of fat surrounding muscles i.e. intermuscular fat (Figure 1). Thus, IMF is the chemically extractable fat in a muscle sample originating predominantly from adipocytes and myocytes. This last definition will be applied in this thesis unless indicated otherwise.

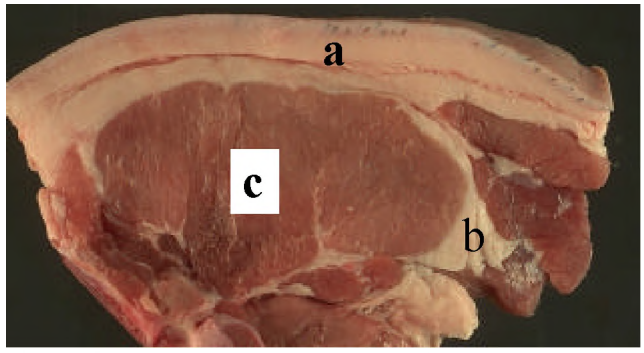

Figure 1. Image of pig meat showing (a) subcutaneous fat, (b) intermuscular fat surrounding the muscle and (c) intramuscular fat between muscle fibres within the muscle.

2. Fat deposition

Generally, four major fat depots are recognized, the subcutaneous, internal, inter- and intramuscular fat depots. Substantial differences in fat distribution are present across and within species. In pigs, the subcutaneous fat depot is the most pronounced depot averaging 60 to 70 % of total fatty tissue at slaughter, whereas the internal and the intermuscular fat depots average about 10 to 15% and 20 to 35% (Kuhn et al., 1997; Kouba et al., 1999). In cattle, the intermuscular fat depot is the most important site of fat deposition at slaughter with 45% of total carcass fat and the internal and subcutaneous fat depot averaging 38% and 17%, respectively (Wegner et al., 1997). Obviously, differences in body fat distribution between species are genetically determined but this is also true within species. This is shown by genetic correlations between IMF content and backfat thickness in pigs (0.37, Hovenier

et al., 1992) and differences in development of intermuscular fat relative to subcutaneous fat between genetically different pigs (Kouba et al., 1999). Interestingly even within major fat depots genetically determined differences can occur. For example, in man the relative fat patterning between different subcutaneous depots was shown to be recessively inherited (Hasstedt et al., 1989).

Besides body fat distribution, fat deposition in each depot itself is also under genetic control. In man, mice but also livestock animals the genetic control of fat deposition is of major interest because of the links between obesity and diabetes, coronary artery disease and atherosclerosis. This has resulted in the identification of various genes involved in fat metabolism, in particular those involved obesity. In this respect, leptin and its receptor have been shown to be important components of fat deposition regulation (reviewed by Friedman and Halaas, 1998).

We will continue this chapter by focussing on the subject of our investigations, the intramuscular fat (IMF) depot.

3. Intramuscular fat

Morphologically, IMF is the total of lipids associated with all cells present in a meat sample, mainly myocytes and adipocytes, but excluding adipocytes from the intermuscular fat depot. Chemically, these lipids can be subdivided in phospholipids, triacylglycerols (TG), mono and diacylglycerols, cholesterol and cholesteryl esters and free fatty acids (FFA). Phospholipids and TG are the major constituents of IMF whereas the contribution of the other lipids is only marginal. However, the contribution of each of these constituents will vary with the method to determine IMF content i.e. on the polarity of the extraction solution and the use of acid pretreatment (Reichardt et al., 1998).

Phospholipids are the main constituents of cellular membranes and their contribution to IMF content in pigs (0.4-0.5%) is nearly constant within similar muscles (Lazo et al., 1994) as was shown for cattle (Ender et al., 1997), rabbits (Gondret et al., 1998) and poultry (Marion et al., 1965). Between muscles, phospholipid content can differ, where the proportion of phospholipids increases from white glycolytic to red oxidative muscle types (Leseigneur-Meynier and Gandemer, 1991). This is possibly due to the smaller fibre diameter and the higher proportion of mitochondrial membranes in oxidative muscles.

Generally, an increase in IMF content is mainly due to an increase in TG content as substantiated in different studies with pigs (Cameron and Enser, 1991; Essen-Gustavsson et al., 1994; Fernandez et al., 1999) and rabbits (Gondret et al., 1998). Consequently, TG

metabolism in muscle tissue should be the subject for identification of candidate genes involved in genetic variation in IMF deposition.

4. Triacylglycerol in muscle

Triacylglycerol contained within muscle is an important and readily mobilized source of energy during excercise. In muscle, TG are stored in adipocytes and myocytes. Proton NMR analysis showed that two fat compartments exist in muscle tissue with similar fatty acid and TG composition (Schick et al., 1993). One compartment corresponded to intramuscular adipocytes whereas the other was suggested to be fat stored in myocytes. Only little information is available regarding the role of each distinct intramuscular fat depot in the variation of IMF content.

Within myocytes, TG are stored in depots, mostly adjacent to mitochondria. The total TG content in human muscles ranges from 0.56 to 3.33% depending on fibre type, nutrition and physical excercise (reviewed by Jeukendrup et al., 1998). Trained individuals have more intramuscular fat which is in accordance with the higher utilization of TG. In contrast, increased TG accumulation within muscles of sedentary individuals is associated with insulin resistance and obesity (reviewed by Goodpaster and Kelley, 1998). However, it is still unclear whether elevated muscle TG content causes insulin resistance or arises as a consequence.

Furthermore, it is well documented that oxidative (type I) muscle fibres contain considerably more intracellular TG than glycolytic (type IIa and IIb) fibres. However, the TG content in muscles is only to a minor extent related to differences in muscle fibre type composition but mainly due to variation in the accumulation of adipocytes between muscle fibres (Kaufmann and Safani, 1967; Gondret et al., 1998). The fact that no correlation was found between TG content and fibre type composition of muscles of different metabolic type in pigs agrees well with this conclusion (Leseigneur-Meynier and Gandemer, 1991; Essen-Gustavsson et al., 1994). With respect to the IMF depot, age-related changes are due to adipocyte hyperplasia and hypertrophy in cattle, pigs and rabbits (Hood and Allen, 1973; Lee et al., 1973; Gondret et al., 1998). Thus both adipocyte hyperplasia and hypertrophy may be involved in the genetic variation in IMF content in pigs. It should be noted that this has no consequences for consumer acceptability of meat due to visibility of fat because in the optimal range of 2.5-3% IMF no fat is visually detectable in pig meat (Van der Wal et al., 1992).

Thus, IMF content can be optimized in three ways: (1) increased number of intramuscular adipocytes (hyperplasia) (2) increased TG content in adipocytes (adipocyte

hypertrophy) and (3) increased TG content in myocytes. In order to propose candidate genes that contribute to IMF content, each of these opportunities will be considered in the next sections.

4.1 Adipocyte hyperplasia

Adipocytes are highly specialized cells serving the crucial function of storage, metabolism and release of lipids. Unfortunately the pattern and regulation of normal adipocyte growth and differentiation are yet incompletely understood. Adipocytes are terminally differentiated cells that develop from multipotent stem cells of mesodermal origin that also give rise to the muscle (myocytes) and cartilage (chondrocytes) lineages. Studies in animals and humans demonstrate that the potential to generate new fat cells continues throughout the lifespan (reviewed by Smas and Sul, 1995; Prins and O'Rahilly, 1997). Moreover, adipocyte number reduction occurs by adipocyte apoptosis and possibly dedifferentiation (reviewed by Prins and O'Rahilly, 1997).

Adipocyte differentiation is influenced by a large number of mitogens and growth factors (reviewed by Hwang et al., 1997). With regard to the transcriptional control of adipocyte differentiation, knowledge has been derived mainly from tissue and cell culture models, especially the mouse 3T3-L1 preadipocyte cell line. In recent years some of the proteins regulating early differentiation of adipocytes have been identified (reviewed by: Smas and Sul, 1995; Hwang et al., 1997; Castillo et al., 1999; Lane et al., 1999; Lazar, 1999). During adipogenesis several transcription factors are involved like c-jun, c-fos, Sp1, CCAAT/enhancer binding protein (C/EBP), adipocyte determination and differentiation-dependent factor 1 (ADD1 or SREBP), C/EBPα undifferentiated protein (CUP or AP-2α) and peroxisome proliferator-activated receptors (PPARs). The C/EBP and PPAR transcription factor families have been studied in most detail because certain members of these protein families are essential for adipocyte determination and differentiation. Namely, ectopic expression of C/EBPα and PPARγ, convert fibroblastic cells into adipocytes (reviewed by Hwang et al., 1997). Interestingly, long-chain fatty acids as well as prostaglandins are potent activators of PPARs and hence induce adipogenesis in heterologous cell systems. The proposed mechanism is that PPARδ can be activated by long-chain fatty acids and induces PPARγ expression which upon specific activation promotes adipocyte differentiation in fibroblasts (Bastie et al., 1999). Interestingly, alleles of PPARγ2 have been associated with human obesity (Ristow et al., 1998).

Besides in adipocytes, fatty acids are also regulating fatty acid metabolism in cultured rat neonatal cardiac myocytes but here PPARα rather than PPARγ is suggested to be involved

(Van der Lee et al., 2000). This observation is in agreement with the observation that the PPARγ2 isoform is expressed almost exclusively in adipose tissue whereas the PPARγ1 isoform, PPARα and PPARδ are expressed in a wider range of tissues and cell types (reviewed by Lazar, 1999). This tissue-dependent expression has also been demonstrated for porcine PPARγ1 and PPARγ2 isoforms (Grindflek et al., 1998; Ding et al., 1999; Kim et al., 2000).

In conclusion, the search for the master regulatory factor in adipocyte differentiation still continues and identification may lead to understanding of those factors that switch on adipocyte determination and differentiation. This may also give insight in the key factors that ultimately regulate adipocyte hyperplasia.

4.2 Adipocyte hypertrophy

During adipocyte differentiation, TG are continually deposited in lipid droplets which coalesce into larger droplets. This proces progresses untill most of the cell volume is occupied by a single large lipid droplet leading to hypertrophy. All tissues increase in mass due to a combination of hyperplasia and hypertrophy but the ratio between both processes varies between tissues and within the same tissue of different species. In cattle, sheep and pigs hypertrophy of adipose cells can be easily 1000-fold in volume as cells grow from 15 to more than 150 μm in diameter (Mersmann, 1991). During the first four weeks, subcutaneous adipocyte volume increases almost ninefold in pigs (Mersmann et al., 1997).

The synthesis of TG in adipocytes and myocytes proceeds from fatty acids synthesized *de novo* or supplied by the diet. The major sites for lipid synthesis are the liver and adipose tissue but differences occur between species with respect to the relative importance of each tissue. In man, the liver is the main site of *de novo* fatty acid synthesis, in pigs adipose tissue is the most important site whereas in rat and mouse both tissues are contributing (reviewed by Mersmann, 1986). In pigs, *de novo* fatty acid synthesis plays a major part in the origin of fatty acids because diets consist predominantly of carbohydrates (Mersmann et al., 1986). Fat metabolism of intramuscular adipocytes is in part identical to myocytes and therefore these will be described jointly in the next section.

4.3 Muscle fatty acid metabolism

Recently, Hocquette et al. (1998) reviewed the role of TG in energy metabolism, its control and the consequences of manipulating muscle energy metabolism in meat-producing

animals. Here we focus on the fatty acid metabolism of the entire muscle tissue, in particular within myocytes and adipocytes.

The mechanism of uptake of fatty acids by intramuscular adipocytes and myocytes is facilitated by the enzyme lipoprotein lipase (LPL) located on the capillary endothelial cells which binds and hydrolyzes lipoprotein TG (see Figure 2). Overexpression of human LPL in mouse myocytes and cardiomyocytes is associated with a small decrease in body fat suggesting that LPL may be a rate limiting step in fatty acid uptake (Levak-Frank et al., 1995). However, manipulation of LPL expression proved to be hazardous. Overexpression of LPL was associated with extensive proliferation of mitochondria and peroxisomes and development of lethal myopathy. Furthermore, amino acid substitutions identified in human LPL are associated with coronary artery disease, atherosclerosis and/or obesity (reviewed by Murthy et al., 1996).

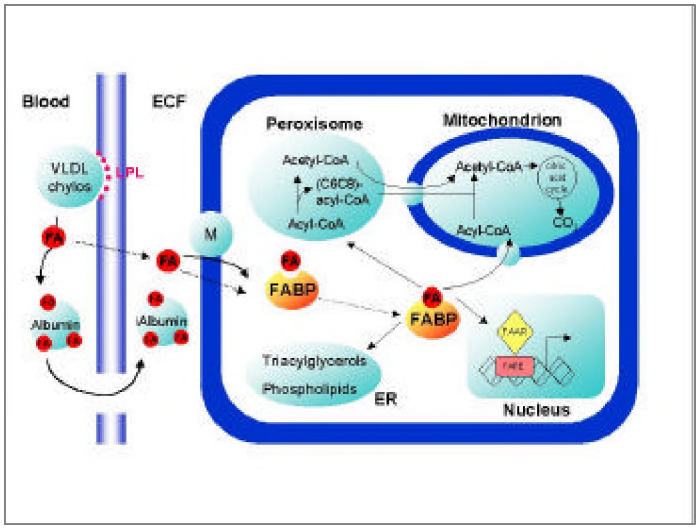

Figure 2. Schematical representation of the role of fatty acid-binding protein (FABP) in fatty acid uptake and intracellular fatty acid trafficking. Abbreviations: FA, fatty acids; VLDL, very low density lipoproteins; Chylos, chylomicrons; LPL, lipoprotein lipase; ECF, extracellular fluid; M, membrane associated fatty acid transport proteins; ER, endoplasmic reticulum; FAAR, fatty acid activated receptor; FARE, fatty acid responsive element.

Fatty acids are translocated across the endomysial and sarcolemmal membranes and taken up intramuscular adipocytes or myocytes. Fatty acids can be translocated across these membranes by a simple diffusion mechanism or facilitated by membrane-associated proteins as plasma membrane fatty acid-binding protein (FABPpm), fatty acid translocase (FAT or

CD36) and fatty acid transporter protein (FATP) (reviewed by Van Nieuwenhoven et al., 1996; Abumrad et al., 1999). The process of fatty acid uptake is not yet fully understood but various parts of this process have been reviewed recently (Luiken et al., 1999). Although, simple diffusion of fatty acids may occur, the major fraction of cellular fatty acid uptake is protein facilitated under physiological conditions (Abumrad et al., 1999). This is supported by FAT (CD36) deficiency in rats and man, and overexpression of FAT which results in reduced peripheral TG and non-esterified fatty acids (Aitman et al., 1999 and references therein).

Intracellularly, fatty acids are bound by fatty acid-binding proteins (FABPs) that are considered to be the important carriers for intracellular fatty acids. FABPs facilitate the transport of fatty acids from the plasma membrane to the sites of fatty acid oxidation or to the sites of fatty acid esterification into TG or phospholipids. Moreover, FABPs may also be involved in bidirectional transport of fatty acids in adipocytes. In this respect the interaction between the hormone-sensitive lipase (HSL) and adipocyte FABP (A-FABP) may be of significance (Shen et al., 1999). Moreover, HSL is also present in skeletal muscle derived myofibres (Langfort et al., 1999).

Other functions of FABPs are thought to be the protection of the cell for the deleterious effect of high concentrations of intracellular free fatty acids, and modulation of the action of (long-chain) fatty acids and other ligands, hence influencing enzymes, membranes, receptors, ion channels or genes (Veerkamp et al., 1993; Graber et al., 1994; Veerkamp en Maatman, 1995; Glatz and Van der Vusse, 1996 and references therein). The role of FABPs in regulating fatty acid-dependent cell signalling has been reviewed by Glatz et al. (1995).

5. Fatty acid-binding proteins

Because of the central role of FABPs in intracellular fatty acid uptake and trafficking we postulate that FABPs are potential candidates accounting for differences in IMF content. First some general aspects of FABPs will be discussed before focussing on the myocyte and adipocyte specific FABPs. Finally some naturally occurring FABP variations will be reviewed.

The FABP family consists of at least nine members which have been named after the first tissue of isolation or identification i.e. liver, intestinal, ileal, heart, adipocyte, epidermal, myelin, testis, and brain FABP. Some FABP members are expressed in the same tissue, either in different cell types or in the same cells whereas other FABP types are expressed in single specified cell types (reviewed by Veerkamp and Maatman, 1995 and

Bernlohr et al., 1997). All FABP family members are small, 14-15 kDa proteins consisting of 127-135 amino acids (see Table 2).

FABPs have been identified in many organisms, from invertebrates (insects, worms) to vertebrates (amphibians, mammals, birds, fish). Evolutionary studies indicate that several subfamilies exist across all members of the FABP family (Matarese et al., 1989; Börchers and Spener, 1994; Hohoff and Spener, 1998; Santome et al., 1998), ultimately relating to a common ancestral gene approximately 700 million years ago (Matarese et al., 1989).

Table 1. *Chromosomal location[a] of genes of members of the FABP family*

			Chromosome (region)		
FABP type	Human gene	Mouse gene	Human	Mouse	Pig
Liver FABP	FABP1	Fabpl	2 (p11)	6	-
Intestinal FABP	FABP2	Fabpi	4 (q28-q31)	3	-
Heart FABP	FABP3	Fabph	1 (p32-p33)	4	6
Adipocyte FABP	FABP4 / AP2	Fabpa / Ap2 / Albp	8 (q21)	3	4
Epidermal FABP	FABP5 / KLBP	Fabpe / Klbp / Mal1	8	3 (3A1-3)	-
Ileal FABP	FABP6 / ILBP	Ilbp	5 (q23-q35)	11	-
Brain FABP	FABP7	Fabpb	6 (q22-q23)	-	-
Myelin FABP	FABP8 / PMP2	Fabpm / Pmp2	8 (q21.3-q22.1)	3	-
Testis FABP	FABP9 / TLBP	Fabpt / Tlbp	-	-	-
CRBP I	CRBP I	Rbp1	3 (q21-q22)	9	-
CRBP II	CRBP II	Rbp2	3	9	-
CRABP I	CRABP I	Crabp1	15 (q24)	9	-
CRABP II	CRABP II	Crabp2	1 (q21.3)	2	-

[a]All human and mouse gene locations have been retrieved from the human genome database (GDB) and mouse genome database (MGD), respectively. The pig gene assignments are described in this thesis.

The chromosomal location of FABP genes for man, mouse and pig is presented in Table 1. It can be seen that the genes are dispersed throughout the genome. However, some evolutionary, closely related FABP genes seem to be clustered. Namely, the A-FABP and myelin (M) FABP genes are mapped in close proximity of each other on human chromosome 8 (Prinsen et al., 1997; Hayasaka et al., 1993) and the murine homologs are both assigned to mouse chromosome 3. In addition, the epidermal (E) FABP gene is also assigned to mouse chromosome 3 (Bleck et al., 1998) whereas an anonymous sequence (EMBL: G36744) related to human E-FABP was assigned close to this region on human chromosome 8 (Hohoff and Spener, 1998). Furthermore, Hohoff and Spener (1998) speculate that the testis (T) FABP gene may also be located in this FABP gene cluster based on close evolutionary distances with A-FABP, M-FABP, and E-FABP.

Table 2. *Overview of identified genes and their structure of members of the FABP family.*

			Gene structure							
FABP type	Gene	Species	Ex 1	Int 1	Ex 2	Int 2	Ex 3	Int 3	Ex 4	Protein length
Liver	FABP1	Rat	22	1454	58	1224	31	610	16	127
Intestinal	FABP2	Human	22	1194	58	1023	36	444	16	132
		Mouse	22	1256	58	1793	36	419	16	132
		Rat	22	1261	58	-	-	-	-	132
Heart	FABP3	Human	24	3386	58	1889	34	1437	17	133
		Mouse	24	3441	58	1490	34	1116	17	133
		Rat	24	3484	58	1475	34	1124	17	133
		Pig	24	4200	58	2079	34	1500	17	133
		Locust	24	12300	58	0	34	2300	17	133
Adipocyte	FABP4	Mouse	24	2315	58	586	34	667	16	132
		Pig	24	2629	58	840	34	471	16	132
Epidermal	FABP5	Mouse	26	2217	58	372	34	546	17	135
Ileal	FABP6	Mouse	22	2725	59	995	30	1294	17	128
		Rabbit	22	1372	59	2293	30	3139	17	128
Brain	FABP7	Mouse	24	424	58	586	34	1757	16	132
Myelin	FABP8	Human	24	-	58	-	34	1200	16	132
		Mouse	24	1189	58	159	34	1318	16	132
Testis	FABP9	Rat	24	2241	58	547	34	164	16	132

The size of exons are represented by the number of amino acids whereas the introns are represented in nucleotides. Spliced codons at exon-intron splice junctions are coded for by the next exon. Protein lengths include the initial methionine residue.

EMBL accesion numbers and references of each gene in respective order: rnlfabp: (M13501) Sweetser et al., 1986; hsifabp: (M18079) Sweetser et al., 1987; mmifabp: (M65033) Green et al., 1991; rnifabp: (M18080) Sweetser et al., 1987; hshfabp: (U57623) Phelan et al., 1996; mmhfabp: (U02884) Treuner et al., 1994; rnhfabp (AF144090) Zhang et al., 1999; sshfabp: (X98558, Y16180/81) Gerbens et al., 1997; lmhfabp: Zhang and Haunerland, 1997; mmafabp: (M13385) Philips et al., 1986; ssafabp: (Y16039) Gerbens et al., 1998b; mmefabp: (AJ223066) Bleck et al., 1998, Hertzel and Bernlohr 1998; mmilbp: (U00938) Crossman et al., 1994; ocilbp: (Z54345) Stengelin et al., 1996; mmbfabp: (U04827) Kurtz et al., 1994; hspmp2: (D16179/80/81) Hayasaka et al., 1993.; mmpmp2: (S39508) Narayanan et al., 1991; rntfabp: (U66878) Pouresmaeili et al., 1997.

The FABP genes that have been cloned and characterized sofar are predominantly of human and mouse origin (Table 2). The structure of these FABP genes is highly conserved, each containing four exons and three introns (Veerkamp and Maatman, 1995; Bernlohr et al., 1997). Moreover, across all FABP genes the number of amino acids represented by each exon and thus the position of introns within each gene are essentially similar. One exception to this rule is the locust H-FABP gene that lacks the second intron (Zhang and Haunerland, 1997). The regulation of FABP gene expression has been discussed in several reviews (Veerkamp and Maatman, 1995; Bernlohr et al., 1997).

The preservation of the gene structure between FABP types is one of the reasons why FABPs are classified as a multigene family. The nucleotide sequences translate to primary protein sequences wich have an identity that ranges from 20-70% (Bernlohr et al., 1997). Santome et al. (1998) compared the primary sequence of over 60 FABP genes from different species and showed that the similarity within each FABP member across species is greater than across FABP members within species. Despite this variability across FABP members, their three-dimensional structure remains highly conserved. X-ray analysis revealed the tertiary structure of A-FABP, H-FABP, E-FABP, liver FABP (L-FABP) and intestinal FABP (I-FABP) showing 10 antiparallel β-strands forming a β-barrel and two α-helices (Zanotti et al., 1992; Banaszak et al., 1994; Lalonde et al., 1994; Thompson et al., 1997; Hohoff et al., 1999). These structural analyses have elucidated the important amino acid residues in fatty acid binding and overall structure (reviewed by Veerkamp and Maatman, 1995, Bernlohr et al., 1997 and Zanotti et al., 1999). For human A-FABP, the amino acid residues Arg 106, Arg 126 and Tyr128 were identified to be important for fatty acid binding by high resolution X-ray diffraction (Xu et al., 1993). The main amino acids involved in fatty acid binding of human H-FABP are Thr 40, Arg106, Arg126 and Tyr128 (Zanotti et al., 1992). Despite the similarity in these residues between H-FABP and A-FABP distinct dynamical differences among corresponding amino acid residues were observed upon ligand binding (Constantine et al., 1998). The role of specific amino acids in fatty acid binding and conformational stability has also been confirmed by site-directed mutagenesis (Prinsen and Veerkamp, 1996; Simpson and Bernlohr, 1998; Zimmerman et al., 1999; Richieri et al., 1998).

Despite the comparable three-dimensional structure of FABP proteins, the ligand specificity and affinity can differ substantially between FABP members and depends also on the fatty acid chain length and its degree of saturation (reviewed by Veerkamp et al., 1993 and Bernlohr et al., 1997; Maatman et al., 1994b; Richieri et al., 1994). Moreover, certain FABP members also have high binding affinities for other hydrophobic ligands like, among

others, retinol, retinoic acid, bile acids and peroxisome proliferators (reviewed by Bernlohr et al., 1997; Ribarik Coe and Bernlohr, 1998; Veerkamp et al., 1999).

5.1 Heart fatty acid binding protein.

Heart-FABP is expressed and histochemically identified in various cell types and tissues: myocytes of heart and skeletal muscle, distal tubuli of kidney, endothelial cells of the aorta and cardiac microvessels, adrenal cells, brain, lung, placenta, testes, ovarian interstitial epithelial cells as well as the lactating mammary gland of several species (Watanabe et al., 1991 and 1993; Maatman et al., 1992; Zschiesche et al., 1995 and references therein; Antohe et al., 1998). Intracellularly, the H-FABP protein is mainly localized in the cytoplasm but it was also observed in nuclei (Börchers et al.,1989) and mitochondria (Börchers et al., 1989; Fournier and Rahim 1985; Unterberg et al., 1990) of rat and bovine cardiomyocytes. This occurrence of H-FABP and other FABPs in the nucleus may indicate a role of FABPs in fatty acid-dependent cell signalling (reviewed by Glatz et al., 1995).

The H-FABP amino acid sequence has been determined or deduced from cDNA sequences for man (Peeters et al., 1991), pig (Gerbens et al., 1997), cattle (Billich et al., 1988), mouse (Tweedie et al., 1989), rat (Claffey et al., 1987), locust (Price et al., 1992; Maatman et al., 1994a) and rainbow trout (Ando et al., 1998). The similarity of these proteins, except for locust H-FABP, ranges from 72%-94%. Locust H-FABP is much more distinct, 31%-39% similarity with the other H-FABPs presumably due to evolutionary distinctness of insects (Maatman et al., 1994a). Human, pig and rat H-FABP were purified from cardiac cytosol and revealed similar binding characteristics for myristic, palmitic, oleic and arachidonic acid (Paulussen et al., 1988). Identical binding properties were also found for bovine and rat H-FABP (Richieri et al., 1994),

Heart-FABP genes (FABP3) have been isolated for man (Phelan et al., 1996), mouse (Treuner et al., 1994), rat (Zhang et al., 1999), pig (Gerbens et al., 1997) and locust (Zhang and Haunerland, 1997). The structure of these H-FABP genes is presented in Table 2. Surprisingly, the locust H-FABP gene structure is distinct due to the lack of the second intron compared to the other FABP genes identified (Zhang and Haunerland, 1997).

Comparison of the promoter region of the human and mouse H-FABP gene revealed multiple regions of near identity including a CArG-like element (Qian et al., 1999). This element functions as an atypical myocyte enhancer-binding factor 2 (MEF-2) binding site which in combination with other binding-sites directs tissue-specific expression. Similarly, this binding site, among others, was also identified in the pig H-FABP gene promoter (Gerbens et al., 1997).

The human and mouse H-FABP genes have been localized on chromosome 1(p32-p33) (Peeters et al., 1991; Troxler et al., 1993; Huynh et al., 1995) and 4 (distal of Lck) (Bahary

et al., 1991; Treuner et al., 1994), respectively (Table 1). Besides the genuine H-FABP gene also H-FABP pseudogene regions were reported for man (Prinsen et al., 1998), pig (Gerbens et al., 1997) and mouse (Heuckeroth et al., 1987; Treuner et al., 1994). This phenomenon has also been found for mouse epidermal FABP (Bleck et al., 1998) but so far not for any other FABP member.

5.1.1 Heart fatty acid-binding protein function in fatty acid metabolism

With respect to importance of H-FABP in intracellular fatty acid metabolism, studies report contradictory results. Several studies reported a higher H-FABP content with increasing oxidative capacity of various skeletal muscles (Peeters et al., 1989; Vork et al., 1991; Veerkamp and Van Moerkerk, 1993). Palmitate oxidation, incorporation in TG and citrate synthase activity were all strongly associated with muscle FABP content (Dyck et al., 1997). Moreover, in bovine muscles H-FABP mRNA levels were positively correlated to the fatty acid oxidation capacity (Brandstetter et al., 1998). These results agree with the fact that slow oxidative myofibres use predominantly fatty acids as a source of energy, have a higher fatty acid turnover and hence a higher H-FABP expression than more glycolytic myofibres. Furthermore, mice with a disrupted H-FABP gene showed elevated plasma long-chain fatty acid levels and acute excercise intolerance (Binas et al., 1999). Isolated cardiac myocytes from these knockout mice revealed, despite similar capacity in fatty acid metabolism, a markedly reduced palmitate uptake and oxidation and increased glucose utilization as opposed to wild type mice (Schaap et al., 1999). These findings all support the proposed crucial role of H-FABP in fatty acid metabolism. In contrast, in streptozotocin-induced diabetic rats no correlation was apparent between fatty acid oxidation capacity and H-FABP content in liver, heart and skeletal muscle (Veerkamp et al., 1996). Overexpression of H-FABP in human breast cancer cells in vitro showed only a 67% increase in fatty acid uptake (Buhlman et al., 1999). Similarly, Prinsen and Veerkamp (1998) did not detect differences in fatty acid uptake and/or composition in spite of a changed FABP content due to transfection of rat L6 myoblasts with sense or antisense H-FABP and sense A-FABP cDNA.

5.1.2 Heart fatty acid-binding protein function in growth

Heart-FABP also has been implicated in the modulation of cell growth and differentiation. Although secretion of H-FABP has never been shown, addition of H-FABP induced hypertophy of cardiac myocytes in vitro (Burton et al., 1994). Yeast transformed with bovine H-FABP showded retarded growth (Scholz et al., 1990). Furthermore, mammary-derived growth inhibitor (MDGI), later identified to be a mixture of H-FABP and

A-FABP, was found to inhibit growth of breast carcinoma cells and mammary epithelial cells (reviewed by Hohoff and Spener, 1998). In mice, the antiproliferative effect of isolated MDGI preparation, synthetic MDGI and recombinant bovine H-FABP were demonstrated in primary mouse mammary epithelial cells and mammary gland organ culture (Yang et al., 1994). Similar results were found for mammary epithelial cells from cattle and man (Zavizion et al., 1993; Lehman et al., 1989). A synthetic peptide corresponding to C-terminal amino acids (121-131) of H-FABP mimicked these effects (Wallukat et al., 1991; Yang et al., 1994). Mammary epithelial proliferation and differentiation during early pregnancy, however, are not correlated to the H-FABP mRNA expression level (Binas et al., 1995).

Transfection of human breast cancer cells lacking H-FABP expression with bovine H-FABP resulted in reduced proliferation rate, differentiated morphology (Huynh et al., 1995) and an increase in fatty acid uptake (Buhlmann et al., 1999). In addition, tumor formation in nude mice was reduced at injection with H-FABP cDNA transfected cancer cells relatively to mock transfected and untransfected controls (Huynh et al., 1995). The chromosomal region containing the H-FABP gene is a common region of loss in sporadic human breast cancer (Bieche et al., 1994; Munn et al., 1995). A survey of a panel of 30 breast tumors revealed no inactivation of H-FABP (Phelan et al., 1996). In stead, methylation of the promoter region attributes to the loss of H-FABP expression (Huynh et al., 1996). Interestingly, antiestrogens exhibit protective activity for breast cancers and stabilize H-FABP mRNA transcripts (Huynh and Pollak, 1997).

Recently, Zimmerman and Veerkamp (1998) demonstrated that various recombinant and tissue-derived H-FABP preparations from several species and human M-FABP and I-FABP inhibit cell-free protein synthesis in a rabbit reticulocyte system at physiological concentrations. Surprisingly, tissue-derived porcine H-FABP and rat recombinant H-FABP preparations had no effect. In this respect, the observation that H-FABP inhibits cysteine and serine proteinase activity in bovine muscle extracts (Zabari et al., 1993) may also be of significance.

5.1.3 Experimental H-FABP deficiency.

Conditions in knock-out and wild type animals are essentially different but can give indications about the role of the respective gene. Mice deficient in H-FABP reveal a normal phenotype, had similar total body fat, are fertile and raise their offspring but have a reduced exercise tolerance compared to wild type mice (Binas et al., 1999). Unlike in A-FABP deficient mice (Hotamisligil et al., 1996), no compensatory mRNA expression was observed

for A-FABP, B-FABP, E-FABP or L-FABP in these H-FABP deficient mice (Binas et al., 1999).

Others showed that long-chain fatty acid uptake by cardiac myocytes was markedly reduced in the same H-FABP nullizygous mice whereas oxidation capacity and membrane-associated fatty acid transporter (FAT) expression were similar to wild type mice (Schaap et al., 1999). To meet the cardiac energy demand in H-FABP nullizygous mice, glucose oxidation capacity of cardiomyocytes was maximized and cardiac glycogen content was elevated (Schaap et al., 1999). Histologically, in H-FABP nullizygous mice, mitochondria appear to accumulate near the sarcolemma of cardiomyocytes suggesting that close contact of sarcolemmal and mitochondrial membranes is essential to transfer fatty acids and other hydrophobic ligands in the absence of H-FABP (Binas et al., 1999).

In conclusion, H-FABP is not essential for mice under normal conditions but it is essential in high energy demanding circumstances possibly due to the lack of proper compensation by other FABP types.

5.2 Adipocyte fatty acid binding protein.

Adipocyte-FABP is expressed in adipocytes and also in human monocytes (Pelton et al., 1999). Amino acid sequences of A-FABP have been determined or deduced for man (Baxa et al., 1989), cattle (Specht et al., 1996), pig (Gerbens et al., 1998b), mouse (Phillips et al., 1986) and rat (Prinsen and Veerkamp, 1998). The similarity of these proteins is very high and ranges from 82%-93%. A-FABP genes (FABP4) were identified only for mouse (Hunt et al., 1986; Phillips et al., 1986) and pig (Gerbens et al., 1998b). These genes are localized to chromosome 3 (Heuckeroth et al., 1987) and 4 (Gerbens et al., 1998b), respectively, whereas human FABP4 was assigned to chromosome 8q21 (Prinsen et al., 1997) (Table 1). As mentioned previously, this chromosomal region encompasses a cluster of closely related genes encoding A-FABP, M-FABP and most likely E-FABP and presumably T-FABP (Hohoff and Spener, 1998).

5.2.1 Adipocyte fatty acid-binding protein and adipogenesis

Adipocyte-FABP expression increases during differentiation from precursor cells via preadipocytes to mature adipocytes in vitro (Amri et al., 1991; Kim and Spiegelman, 1996; Ding et al., 1999). Therefore the regulation of the A-FABP gene has been studied extensively in order to isolate the factors that trigger and/or regulate adipocyte differentiation (reviewed by Vasseur-Cogent and Lane, 1993; Smas and Sul, 1995).

Several cis-regulatory elements were identified in the promoter of the A-FABP gene and other genes that activate and suppress expression during adipocyte differentiation (reviewed by Vasseur-Cognet and Lane, 1993). Studies with transgenic mice revealed adipogenic enhancer elements (AREs) 5.4 kb upstream of the transcription start site of the A-FABP gene which are necessary and sufficient to direct adipocyte specific expression of a reporter gene (Ross et al., 1990). Similarly, Kopecky et al. (1995) used this entire 5.4 kb A-FABP upstream region to direct the brown fat specific uncoupling protein (UCP) gene expression also in white adipose tissue. This adipose specific ARE enhancer sequences were found to be bound by a heterodimer of peroxisome proliferator-activated receptor $\gamma 2$ (PPAR$\gamma 2$) and retinoic X receptor α (RXRα) (Kliewer et al., 1992). As already mentioned previously, fatty acids activate PPAR expression and hence activate adipocyte differentiation (reviewed by Schoonjans et al., 1996ab; Bastie et al., 1999). Fatty acids, in particular long-chain fatty acids can dramatically activate A-FABP mRNA expression in preadipocytes (Amri et al., 1991; Distel et al., 1992). These findings indicate that A-FABP may regulate its own expression and that of other genes by means of its ligand. For instance, A-FABP deficient mice (see further) did not express TNFα in adipocytes (Hotamisligil et al., 1996).

In this respect, Nielsen and Spener (1993) speculated that phosphorylation of H-FABP may be involved in targeting the protein/ligand complex to the nucleus where the ligand can excert its function in regulating the transcription of genes. A-FABP also contains a putative phosphorylation site which can be phosphorylated *in vitro* by the tyrosine kinase activity of the insulin receptor (Bernier et al., 1987; Hresko et al., 1988). Phosphorylation at Tyr19 of murine A-FABP was increased by the presence of bound fatty acids (Buelt et al., 1991) but phosphorylation reduced ligand binding affinity without dramatic structural changes to the protein (Buelt et al., 1992). On the other hand, the extent of A-FABP phosphorylation *in vivo* is only about 1% (Bernier et al., 1987; Hresko et al., 1988). In vitro phosphorylation of H-FABP was estimated to be less than 0.1% whereas in isolated rat soleus muscle no phosphorylation was detected with or without insulin stimulation (Prinsen et al., 1994). Moreover, phosphorylation might be an aspecific event, especially considering the major conformational change in the protein required to access the Tyr19 residue (Banaszak et al., 1994).

5.2.2 Adipocyte fatty acid-binding protein function in growth

Adipocyte-FABP has also been implicated in growth. For instance, loss of A-FABP expression has been associated with the progression of human bladder transitional cell carcinomas (Celis et al., 1996; Gromova et al., 1998). Expression of rat A-FABP in rat L6 myoblasts did not interfere with the endogenous H-FABP expression but increased the proliferation rate of these cells twofold, compared to control cells. These cells were unable to fuse after induction of differentiation (Prinsen and Veerkamp, 1998). Interestingly, no differences were identified in fatty acid uptake but A-FABP-transfected myotubes exhibited a higher fatty acid oxidation rate and an altered phospholipid composition in differentiation medium (Prinsen and Veerkamp, 1998).

5.2.3 Experimental adipocyte fatty acid-binding protein deficiency

Mice deficient in A-FABP reveal no obvious morphological or metabolic phenotype when fed a standard diet but, on a high fat diet these mice failed to develop obesity-related non-insulin-dependent diabetes mellitus (NIDDM) (Hotamisligil et al., 1996). In other words, obese nullizygous mice maintained low circulating levels of insulin and glucose and responded well in insulin and glucose tolerance tests. In these A-FABP deficient mice the expresssion of E-FABP was significantly upregulated although the total FABP content was decreased 8-fold (Ribarik Coe et al., 1999). Moreover, murine A-FABP and E-FABP have distinct biochemical and biophysical features (Simpson et al., 1999). This inefficient compensation may explain the striking differences in fatty acid metabolism. Despite the 8-fold decrease in total FABP content in adipocytes, no difference in fatty acid influx was observed whereas the efflux was significantly reduced (Ribarik Coe et al., 1999).

With respect to the absence of obesity-related NIDDM in A-FABP deficient mice, further examination revealed that obese nullizygous mice and lean wild type mice did not express TNFα in adipose tissue in contrast to obese wild type mice (Hotamisligil et al., 1996). Evidence from mice deficient in TNFα function, either by absence of TNFα or both TNFα-receptors, showed that A-FABP is a factor contributing to insulin resistance upon obesity (Uysal et al., 1997). Thus A-FABP links obesity to insulin resistance possibly by linking fatty acid metabolism to the expression of TNFα.

Another explanation for the role of A-FABP in obesity-induced insulin resistance may be through its involvement in lipolysis. Upon stimulation of lipolysis, A-FABP nullizygous mice have (1) impaired fatty acid efflux from adipocytes, (2) elevated free fatty acid levels in adipocytes and (3) significant differences in specific plasma free fatty acid levels (Scheja et al., 1999; Ribarik Coe et al., 1999). Therefore the existence of an adipo-pancreatic axis with an essential role for A-FABP is suggested (Scheja et al., 1999). Further evidence for an

important role of A-FABP in lipolysis is given by Shen et al. (1999). They reported the existence of a physical interaction between A-FABP and hormone sensitive lipase (HSL), the key mediator of lipolysis.

Table 3. *Natural occurring polymorphisms identified in FABP genes*

FABP type	Gene	Species	Polymorphism	Position	Reference
I-FABP	FABP2	Man	$[TTA]_n$ repeat	Intron 2	Polymeropoulos et al., 1990
			Ala54 Thr	Exon 2	Baier et al., 1995
			A ↔ C	Exon 4	Pihlajamaki et al., 1997
			G ↔ A	3' UTR	
		Cattle	*Taq*I RFLP	Unknown	Barendse et al., 1991
H-FABP	FABP3	Pig	*Hae*III RFLP	Intron 2	Gerbens et al., 1997
			*Hin*fI RFLP	5' UTR	
			*Msp*I RFLP	Intron 2	
			$[G[T]_6]$ duplication	Intron 2	Gerbens et al., 1998a
		Cattle	Asn98Asp	Exon 3	Bartetzko et al., 1993
		Man	Lys53Arg	Exon 2	Phelan et al., 1996
			$[CA]_n$ repeat	Intron 3	Arlt et al., 1996
A-FABP	FABP4	Pig	$[CA]_n$ repeat	Intron 1	Gerbens et al., 1998b
M-FABP	FABP8	Mouse	$[CA]_n$ repeat	3' UTR	Marker D3Mit130

5.3 Genetic variation and deficiencies of FABPs.

Polymorphisms of FABP genes have been reported for several species (Table 3). Some polymorphisms have been shown to exert physiological consequences. For human I-FABP an Ala54Thr substitution was reported in the Pima Indian population, an ethnic group with an pronounced disposition for obesity and non-insulin-dependent diabetes mellitus (NIDDM) (Baier et al., 1995). The threonine-containing protein had a twofold greater affinity for long-chain fatty acids and the Thr/Thr and Thr/Ala individuals had increased fat oxidation rates *in vivo* and showed insulin resistance compared to Ala/Ala individuals (Baier et al., 1995). Transfected CaCo-2 cells expressing Thr^{54} I-FABP showed higher long-chain fatty acid transport and triacylglycerol secretion than cells expressing Ala^{54} I-FABP (Baier et al., 1996). This polymorphism was also tested for consequences in a number of ethnic populations and, in general, shown to exert an effect on energy metabolism (reviewed by Hegele, 1998). In Canadian Oji Cree indians the Ala54Thr substitution was associated with

differences in body mass index, percent body fat and fasting plasma triacylglycerol concentration (Hegele et al., 1996). In both Pima and Oji Cree indians, no association of the Thr allele with NIDDM was found (Baier et al., 1995; Hegele et al., 1996). Mitchell et al. (1995) found strong association of the I-FABP locus with insulin concentration and body mass index in Mexican-Americans whereas in a Finnish population no significant differences in frequencies of each variant were detected between groups of patients with coronary heart disease and NIDDM and nondiabetic controls (Saarinen et al., 1998). Finally, significant differences were found in plasma lipoprotein content in response to dietary fiber and in fecal bile acid secretion between Thr/Thr and Ala/Ala individuals (Hegele et al., 1997). Obesity is not associated with variation in the human I-FABP gene in Finnish and Japanese subjects (Sipilainen et al., 1997; Hayakawa et al., 1999)

Phelan et al. (1996) reported a Lys53Arg substitution in human H-FABP which had a low frequency in a Swedish population and showed no association with breast cancer incidence. In cattle, Asp^{98} and Asn^{98} isoforms of H-FABP were detected (Unterberg et al., 1990) which were coded by distinct mRNA transcripts (Bartetzko et al., 1993). Only the Asp^{98} isoform was found in mitochondria of bovine heart (Unterberg et al., 1990).

Three charged isoforms were reported for rat liver FABP which could be solely attributed to modifications or mutations of Cys^{69} (Thumser and Wilton, 1997). Besides single amino acid substitutions, these different isoforms may also be explained by bound ligands, protein conformation or post-translational S-thiolation and/or acetylation (reviewed by Jolly et al., 1998). Two rat L-FABP isoforms resolved by ion-exchange chromatography but with the same isoelectric point, are most likely due to alternative folding of these proteins (Murphy et al., 1999). Interestingly, two charge isoforms of rat L-FABP have different effects on micrososmal phospholipid metabolism (Jolly et al., 1998).

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Chapter 3

Characterization, chromosomal localization and genetic variation of the porcine heart fatty acid-binding protein gene.

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Summary

The purpose of this study was to detect genetic variation in the porcine H-FABP gene, a candidate gene for meat quality traits in pigs. Lambda phages containing the porcine H-FABP gene were isolated by plaque hybridization with human H-FABP cDNA. The coding and flanking intronic sequences of the porcine H-FABP gene were determined as well as 1.6 kb of the 5' upstream region. The various potential regulatory sequences in this region are in accordance with the function and expression of the protein in muscle and mammary tissue. Furthermore, comparison with the homolog region of the mouse identified a highly conserved 13-bp element (CTTCCT[A/C]TTTCGG) that may be involved in regulation of expression. The porcine H-FABP gene was localized on Chromosome (Chr) 6 by porcine sequence-specific PCR on DNA from a pig-rodent cell hybrid panel. In addition, part of the H-FABP gene was screened for genetic variation by PCR-RFLP analysis. Three PCR-RFLPs were detected, one in the upstream region (*Hin*fI) and two in the second intron (*Hae*III and *Msp*I). In most pig breeds the corresponding alleles have a variable distribution, possibly a consequence of selective breeding. This genetic variation will enable us to investigate the role of the H-FABP locus in porcine production and meat quality traits.

Introduction

One objective in pig breeding programs is the reduction of fat in the carcass to meet the consumers' demands for lean meat. Generally, fat reduction is surveyed as a decrease in backfat thickness. However, other fat depots such as the intramuscular fat (IMF) are reduced as well. Further reduction of this IMF would be undesirable because it is the main fat depot in meat and is positively correlated with meat quality traits (Wood et al., 1988). Hovenier et al. (1992) showed that IMF reduction is not completely correlated with backfat reduction so both traits can be treated separately. Since IMF is hardly measurable in living animals, a (genetic) marker for this trait is necessary to exclude this depot from further reduction. Recently, it has been statistically shown that a major gene for IMF deposition is present in pigs (Janss et al., 1994), but the location and mode of action of this gene is still unknown. A candidate for this major gene may be the gene that encodes for heart fatty acid-binding protein (H-FABP).

Fatty acid binding proteins (FABPs) are small intracellular proteins involved in fatty acid transport from the plasma membrane to the sites of ß-oxidation and/or triacylglycerol or phospholipid synthesis (Veerkamp and Maatman, 1995). Furthermore, FABPs may modulate

the intracellular fatty acid concentration (Veerkamp et al., 1993) and in this manner regulate various cellular processes and lipid metabolism in particular. FABPs are members of a family of intracellular lipid binding proteins of at least eight structurally distinct types: adipocyte, brain, epidermal, heart, intestinal, ileal, liver and myelin.

The heart type FABP (H-FABP) is a 15 kDa protein present in several tissues with a high demand for fatty acids such as cardiac and skeletal muscle and lactating mammary gland. Recently it became clear that mammary derived growth inhibitor (MDGI), a protein that inhibits tumor cell growth (Bohmer et al., 1987), is in fact a mixture of H-FABP and adipocyte-type FABP (Specht et al., 1996). The murine H-FABP gene has been isolated and contains four exons encoding 24, 58, 34 and 17 amino acids, respectively (Treuner et al., 1994).

The aim of our studies is to look for a possible relationship between genetic heterogeneity in the H-FABP gene and variation in IMF deposition in pigs. To initiate this, we isolated and characterized the porcine H-FABP gene and determined its chromosomal localization. In particular, genetic variation of this gene was identified which can be used for future association studies or linkage analysis with production and meat quality traits.

Materials and methods

Isolation of H-FABP containing phage clones

A porcine genomic DNA EMBL3/SP6/T7 lambda library (Clontech Laboratories Inc. Palo Alto, CA) was screened by plaque hybridization (Sambrook et al., 1989) to human H-FABP cDNA in the pSP65 vector (Peeters et al., 1991) labeled with [α-^{32}P]dCTP by nick translation (Sambrook et al., 1989). Briefly, 500,000 plaques were transferred to replica nitrocellulose filters and incubated in denaturation buffer (1.5 M NaCl/.5 M NaOH) for 2 min, neutralization buffer (1.5 M NaCl/.5 M Tris-HCl pH 8.0) for 5 min and fixation buffer (.2 M Tris-HCl pH 7.5/2X SSC (.3 M NaCl, .03 M sodium citrate)) for 30 s. The filters were air-dried and baked at 80°C for 2 h. The filters were prehybridized (6 X SSC/.5% (wt/vol) SDS/5 X Denhardt's and 100 μg/ml NaOH-treated salmon sperm DNA) for two h at 67°C and hybridized at 67°C overnight in the same buffer containing the radioactive probe. The filters were washed four times with 2 X SSC, .1% (wt/vol) SDS for 30 min at room temperature. Twenty plaques that showed positive signals on both replica filters were purified by two additional rounds of low density plaque hybridization. Phage DNA was isolated by the plate lysate method (Sambrook et al., 1989).

Polymerase chain reactions

PCR amplifications were performed on 1 μl of a 1:1000 dilution of phage DNA preparations or 50 ng of genomic DNA in 50 μl containing .2 units Super Tth polymerase (SphaeroQ, Leiden, NL) in 10 mM Tris-HCl (pH 9.0)/50 mM KCl/1.5 mM $MgCl_2$/.1% (wt/vol) gelatin/1% Triton X-100/.5 μM of each primer (Pharmacia Biotechnologies, Uppsala, Sweden) and .2 mM of each dNTP (Boehringer Mannheim, Mannheim, Germany). After 3 min of denaturation at 94°C, 33 cycles of amplification were carried out: 94°C for 1 min, the indicated annealing temperature (Tables 1 and 2) for 1 min and 72°C for the time considering the length of the expected fragment (ca 1 min for every kb).

Table 1. *The conditions for porcine H-FABP gene specific PCR reactions.*

PCR	Site[a]	Primer sequence	T_{ann} (°C)	Size (kb)
#1	exon 1	5'gccagcatcactatggtggacgctttc	57	4.4
	exon 2	5'cttaaagctgatctctgtgttc		
#2	exon 3	5'ggaggcaaacttgttcacctgc	57	1.6
	exon 4	5'tctttctcgtaagtgcgagtgc		
#3	exon 3	5'ggaggcaaacttgttcacctgc	62	1.5
	intron 3	5'gtactgggagcactcttcactc		

[a] Exon primers are based on human and mouse H-FABP cDNA sequences and the intron 3 primer on porcine H-FABP genomic DNA sequence.

DNA sequence analysis

PCR#1 (Table 1) was performed on DNA of the purified phage clones to identify the clones containing the H-FABP gene. Two positive phage clones were used to subclone the H-FABP gene. Therefore, the *Sac*I and *Kpn*I (Boehringer Mannheim) restriction digestion fragments of the phage DNA, containing the 5'upstream region and exon 1 through exon 3 were subcloned in pBS. Intron 3 and exon 4 were amplified using porcine genomic DNA as template and primers from PCR#2 (Table 1). The 3' untranslated region was amplified with porcine muscle cDNA as template and specific primers for porcine H-FABP exon 1 or 3 (Table 1) in combination with the anchor primer from the 5'/3' RACE-PCR kit (Boehringer Mannheim). PCR products were cloned in the pT7Blue vector (Novagen Inc., Madison, Wis., USA). Products of two independent PCR reactions were cloned to identify errors by the Super Tth polymerase upon sequence analysis.

Recombinant plasmid DNA from H-FABP clones was purified with the Wizard Maxiprep kit (Promega). The nucleotide sequence was determined by dideoxy sequencing, either by cycle sequencing (Perkin Elmer, Foster City, Calif., USA) or autoread sequencing (Pharmacia Biotechnologies, Uppsala, Sweden) and the analysis was performed on a ABI 373 (Perkin Elmer) or ALF DNA sequenator (Pharmacia Biotechnologies), respectively. The DNA sequence was analyzed by the Genetics Computer Group (University of Wisconsin, Madison, Wis., USA) software packages.

Table 2. *The PCR conditions for the PCR-RFLP detection assays.*

RFLP	Primer	T_{ann} (°C)	Size (bp)
*Hae*III/*Msp*I	5'attgcttcggtgtgtttgag	57	850
	5'tcaggaatgggagttattgg		
*Hin*fI	5'ggacccaagatgcctacgccg	57	700
	5'ctgcatctttgaccaagagg		

Chromosomal localization

Two independently established pig/rodent somatic cell hybrid panels (Panel A: Rettenberger et al., 1996 and Panel B: Zijlstra et al., 1994) were used to map the H-FABP gene to a specific chromosome by PCR. DNA (100 ng) from each cell hybrid containing porcine chromosomes in various combinations was used in the PCR#3 (Table 1) reaction, which unambiguously amplified porcine H-FABP intron 3 sequences.

Concordancy and correlation were statistically evaluated as described by Chevalet and Corpet (1986). The distribution patterns of the PCR signals for porcine H-FABP were compared with the distribution patterns of the pig chromosomes and of reference loci of individual pig chromosomes. A marker is syntenic with a chromosome or reference locus with a probability of 97.5% if φ is >.74 for 20 hybrid lines. Synteny can be excluded if φ is <.59.

RFLP screening

Porcine genomic DNA was isolated as described (Sambrook et al., 1989) from EDTA-treated blood stored at -80°C. Genomic DNA (100 ng) was used for PCR amplification in 50 μl reaction mixture as described before. The primer sequences and its corresponding product size and annealing temperature for each combination are given in Table 2. Fifteen μl of the PCR reaction was used for restriction digestion with 2 units of *Hae*III, *Hin*fI or *Msp*I

(Boehringer Mannheim, Mannheim, Germany) in a total volume of 20 μl. *Msp*I digestion was carried out directly in the PCR buffer, *Hae*III and *Hin*fI digestions were carried out upon addition of the recommended concentrated reaction buffer. Restriction digestion fragments were loaded on a 2% (*Msp*I) or 3% (*Hae*III and *Hin*fI) agarose (Sigma, St Louis, MO, USA) gel. After electrophoresis the RFLP patterns were scored by two persons, independently. Allele frequencies between breeds were compared using a binomial model with a significance treshold of 95%. Genotype distributions within breeds were tested for Hardy-Weinberg equilibrium as described by Falconer and Mackay (1996).

Table 3. *Interspecies identity of the H-FABP encoding DNA sequence and the predicted amino acid sequence.*

	Percentage identity with porcine H-FABP	
Species	DNA	Protein
Cattle[a]	92	92
Human[b]	91	90
Mouse[c]	84	87
Rat[d]	85	86

[a] Billich et al., 1988; [b] Peeters et al., 1991; [c] Binas et al., 1992; [d] Claffey et al., 1987.

Results

Porcine H-FABP gene sequence analysis

The H-FABP gene sequence was determined including 1.6 kb of the upstream regulatory region and .2 kb of the 3' untranslated region (Fig. 1). To do this, three phage clones that contained the H-FABP gene were isolated from the porcine genomic DNA library by plaque hybridisation. Subsequently, DNA from these phage clones was cloned and subjected to sequence analysis. The region downstream of exon 3 was isolated as PCR fragments amplified on porcine genomic DNA and muscle cDNA.

The exons were identified in the porcine H-FABP gene sequence (Fig. 1) according to homology with known H-FABP sequen-ces of cattle and mouse. The percentage of identity of the coding part of the porcine H-FABP gene with the homolo-gous genes of other species is very high (Table 3) both for the DNA sequence as well as for the predicted amino acid sequence. The resulting four exons encoded in respective order 24, 58, 34 and 17 amino acids, and the sizes of the introns were about 4.2, 2.5 and 1.5 kb, respectively.

Sequence analysis of the 5' regulatory region revealed a potential TATA box 92 bp upstream the ATG start codon (Fig. 1) and potential consensus binding sites for various transcription factors such as MYOD and hormone receptors (Table 4). In the 3' untranslated region the consensus poly-A signal sequence was identified (Fig. 1).

Table 4. *Position of transcription factor binding sites in the porcine H-FABP gene upstream region.*

Factor	Consensus sequence	Position
Activator protein-1 (AP-1)	stgactma	-875
Activator protein-1 (AP-1)	ccscrggc	-408
Activator protein-1 (AP-1)	tgtgwww	-1545, -714
CCAAT-EBP (C/EBP)	tknngyaak	-1608, -1504, -1072, -665, -628
E-box	canntg	-1593, -1328, -1111, -1308, -768, -110
Growth hormone (GH-cse2)	aataaat	-1441
Glucocortocoids (GRE)	tgttct	-1000
Krox-24	gcgsgggcg	-134
Mammary activ factor (MAF)	grrgsaagk	-1134
Stat-5 (MGF)	ttcnnngaa	-1178

Screening of the porcine genomic DNA library also yielded H-FABP pseudogene-like sequences as identified by the absence of intronic sequences. Two of these pseudogenes were analyzed by sequencing the PCR#2 (Table 1) amplification products. Furthermore, a pseudogene product amplified on porcine genomic DNA was also analyzed. Various nucleotide substitutions as well as frameshifts were detected between the H-FABP gene coding region and pseudogene sequences. All three pseudogene sequences were distinct from one another, and one contained an 27 bp internal duplication (data not shown, EMBL X98555, X98556 and X98557).

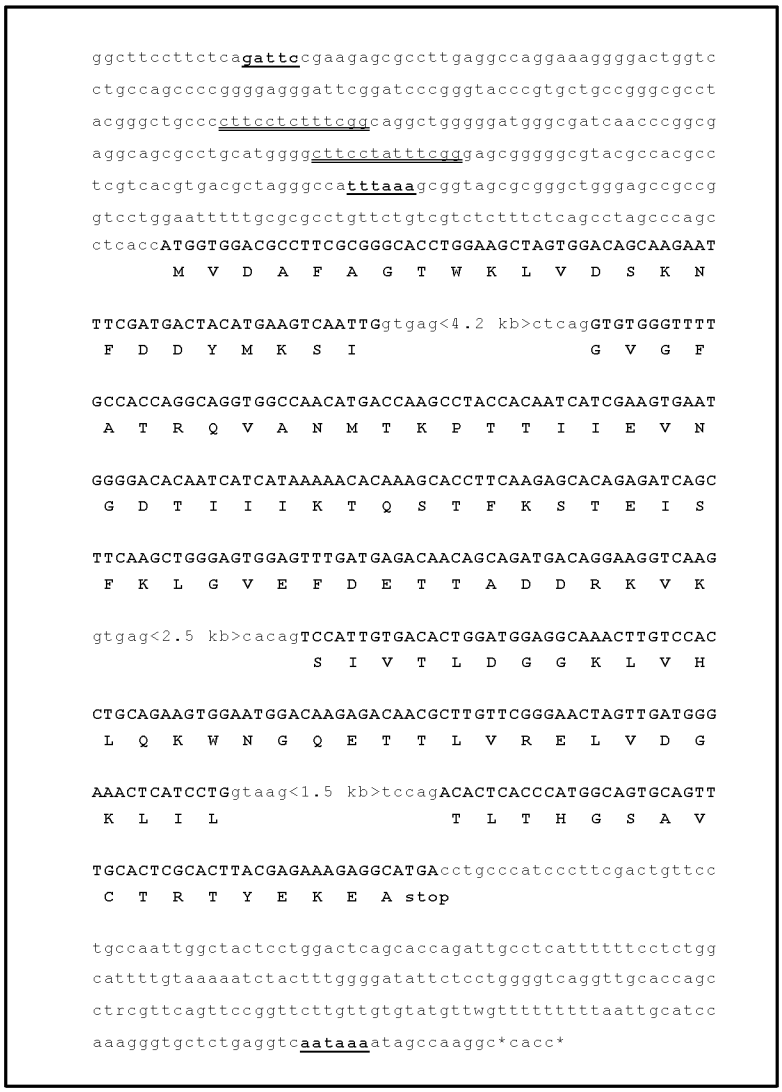

Figure 1. The porcine H-FABP gene sequence including 330 bp of the 5' upstream region and the 200 bp of the 3' untranslated region. Exons are represented by bold capital letters, and the deduced amino acid sequence is shown underneath. The putative TATA-box, the polyadenylation signal in the 3' UTR, and the polymorphic *Hin*fI site (GATTC) are depicted bold and underlined. The 13-nucleotide element is depicted double underlined. The size of the nondepicted intron sequences is shown between arrowheads. The two poly-A tail starting positions are indicated by asterisks

Genomic location of the porcine H-FABP gene

The presence of the porcine H-FABP gene in two independently established pig/rodent cell hybrid panels, A and B, was tested by the porcine specific PCR#3 (Table 1). The obtained data were compared with the known cytogenetic data of both panels and data from already mapped loci for each chromosome, the so-called reference loci, for panel A. The H-FABP gene could be assigned to pig chromosome 6 because a single significant correlation (φ) was detected for both panels of .89 and .83, respectively. All other chromosomes were asyntenic for both panels because the correlation did not exceed .54.

Sites of genetic variation in the porcine H-FABP gene

Digestion of PCR (Table 2) products with the restriction digestion enzymes *Hae*III, *Hin*fI and *Msp*I revealed three fragment length polymorphisms (RFLP). The *Hin*fI site is located in the 5' upstream region whereas the *Hae*III and *Msp*I sites are about 300 bp apart in intron 2 of the H-FABP gene.

Table 5. *Allele frequency and number of heterozygotes for the H-FABP RFLPs in various pig breeds.*

		Allele frequency and number of heterozygotes per pig breed[a]						
RFLP	Allele[b]	DL	DU	GY	HS	ME	PI	WP
*Msp*I	A	.98[c]	.40[d]	.81[e]	1.0	1.0[c]	.90	.70
	Aa	1	4	11	0	0	1	3
*Hae*III	D	.32[c]	.40[c]	.31[c]	1.0	1.0[d]	.50	.10
	Dd	11	4	17	0	0	1	1
*Hin*fI	H	.70[c]	.70[c,d]	.97[d]	.33	.45[c]	.70	.90
	Hh	10	2	2	2	6	1	1
n[f]		20	10	34	6	11	5	5

[a] DL, Dutch Landrace; DU, Duroc; GY, Great Yorkshire; HS, Hampshire; ME, Meishan; PI, Pietrain; WP, Wild Pig.
[b] Alleles represented by letters: A, 750 + 100 bp; a, 850 bp; D, 850 bp; d, 450 + 400 bp; H, 197 + 59 bp; h, 256 bp (besides other fragments). [c,d,e] Data within a row lacking the same superscript letter differ ($P<.05$).[f] The number of unrelated animals tested per breed.

The frequencies of the different RFLP genotypes for various pig breeds were determined in unrelated animals (Table 5). The three polymorphisms are present in all breeds tested, except for the *Hae*III and *Msp*I RFLPs in the Hampshire and Meishan breed. The allele frequencies estimated for pig breeds represented by more than 9 animals revealed significant differences between them (Table 5). Furthermore, in the Duroc breed the *Hin*fI allele frequency distribution is at disequilibrium.

Discussion

Although the fatty acid binding and antigenic crossreactivity of porcine H-FABP have been studied extensively (Paulussen et al., 1988) its amino acid sequence has not been known until now. We show that 86% to 92% of the predicted amino acid sequence of porcine H-FABP is identical to the H-FABP sequences of other mammalian species (Table 3). The higher similarity of porcine and human H-FABP, compared with rat H-FABP, is reflected also in the antigenic crossreactivity patterns (Paulussen et al., 1990).

The porcine H-FABP gene resembles the murine H-FABP gene (Treuner et al., 1994) in overall structure although the porcine introns are larger.

Furthermore, a potential TATA box (TTTAAA) and poly-A signal (AATAAA) are present. Analysis of the porcine H-FABP cDNA sequences revealed two poly-A tail start sites 4 nucleotides apart (Fig. 1). In fact, an alternative poly-A start site with a spacing of 4 nucleotides was previously reported as one of the differences between bovine H-FABP and MDGI cDNA sequences (Spener et al., 1990) but also corresponds with the differences between the murine H-FABP gene and a pseudogene-like sequence (Treuner et al., 1994).

H-FABP pseudogene-like sequences have been localized to murine Chrs 8, 10 and 17 (Heuckeroth et al., 1987; Treuner et al., 1994) and human chromosome 13 (Veerkamp and Maatman, 1995), respectively. Several clones that contained pseudogenes were isolated from the porcine genomic DNA library. On the basis of restriction digestion patterns of the clones we estimated that the porcine genome contains at least 3 different H-FABP pseudogenes (data not shown). Three porcine pseudogenes from two different pigs were analyzed by sequencing a PCR product containing a part of the H-FABP pseudogene. Alignment of these sequences with the H-FABP gene sequence showed that all three had frameshift mutations, and one had an internal 27 bp duplication (data not shown). Moreover, we detected various nucleotide substitutions in all three porcine pseudogene fragments whereas the murine H-FABP pseudogene only differed by 3 nucleotides from the complete murine H-FABP coding sequence. In mice no expression of the H-FABP pseudogene was detected in an array of tissues (Treuner et al., 1994). In conclusion, it is unlikely that the porcine pseudogenes are expressed because of the additional high degeneracy of these sequences.

Potential transcription factor binding sites have been found in the 5' upstream region of the murine H-FABP gene (Treuner et al., 1994). These binding sites can also be found in the porcine H-FABP gene upstream region (Table 4) and are consistent with the reported tissue-specific expression or function of H-FABP. For instance, the presence of potential E-boxes, which direct expression to skeletal muscle cells upon binding of the myogenic MyoD

protein family, could direct the H-FABP expression. On the other hand, H-FABP is highly expressed in cardiac tissue, but this expression is not regulated by these myogenic factors which are not present in cardiac myocytes (Olson et al., 1995).

General metabolic regulation of the H-FABP gene could be explained by the presence of potential binding sites for activator proteins (AP) 1, 2 and 3, Krox 24 and the glucocorticoid-responsive element which also renders responsiveness for the estrogen and progesteron receptors. The significance of the potential C/EBP (CAAT enhancer binding protein) binding sites is unclear since this protein is thought to be primarily expressed in adipocytes (MacDougald and Lane, 1995).

Interestingly, a 13 nucleotides (CTTCCT[A/C]TTTCGG) long element found twice in 250 base pairs preceding the ATG start codon of the porcine H-FABP gene (Fig. 1) was also found once in the murine H-FABP gene upstream region. This element has no homology to any potential binding site in a transcription factor database as well as in the GENBANK/EMBL nucleotide databases and probably represents a new element involved in transcriptional regulation.

The presence of potential Stat 5 binding sites would explain the pregnancy- and lactation-dependent expression of the H-FABP gene in the mammary gland (Binas et al., 1992). Namely, Stat 5 is a member of the Jak/Stat signal transduction pathway and is activated by the pregnancy dependent hormone prolactin in the mouse mammary gland (Liu et al., 1995). This Stat 5 binding site is also detected in the promoters of various milk protein genes (Watson et al., 1991) which are expressed in late pregnancy and during lactation.

Two independent cell hybrid panels (Rettenberger et al., 1996 and Zijlstra et al., 1994) have been used for mapping . The porcine H-FABP gene was localized on Chr 6. The corresponding human and mouse H-FABP genes are localized on Chr 1p32-p33 (Peeters et al., 1991; Troxler et al., 1993) and Chr 4 (distal to Lck; Bahary et al., 1991), respectively, in a region which is highly conserved in evolution (Paszek et al., 1995). Several other genes that are mapped in this conserved region in mice and human, like ENO-1 and PGD (Yerle et al., 1995), are also found on porcine Chr 6. Indeed, heterologous chromosome painting of human chromosomes to the pig genome indicated that porcine Chr 6q21-26 is homologous to parts of human Chr 1 (Rettenberger et al., 1995).

In our search for genetic variation in the H-FABP gene, RFLPs were detected in the upstream region and intron 2. The subsequent genotypes show a diverse distribution in the pig breeds tested except in the Hampshire and Meishan breeds for the intron 2 specific RFLPs (Table 5). Interestingly, also wild pigs show heterogeneity for each RFLP.

Although for some breeds the sample size is very small, the observed difference in the

genotype frequency distribution between breeds may be the result of selective breeding. However, a small number of founder animals for a population either by changed selection criteria or import, as for the western Meishan population, also changes the allele frequency.

The *Hin*fI allele frequency distribution is at disequilibrium in the Duroc breed which may suggest selection pressure in favor of the H allele. Interestingly, the Duroc breed has a different *Msp*I allele frequency distribution than the Great Yorkshire and the Dutch Landrace breeds. The Duroc breed has a higher percentage of IMF and a lower growth rate compared to the Great Yorkshire breed (Hovenier et al., 1992) which, upon selection for growth rate, may have changed the allelic distribution of the H-FABP RFLPs. Currently we are investigating possible relations of the H-FABP genotypes with meat quality traits like IMF and the more general traits as fat deposition and lean growth.

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Chapter 4

A dimorphic microsatellite in the porcine H-FABP gene at chromosome 6.

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Source/description

Sequence analysis of the porcine H-FABP gene sequence (EMBL: Y16180) revealed a $G[T]_3G[T]_6G[T]_7GC[T]_5$ nucleotide stretch in the second intron. To investigate genetic variation in this nucleotide stretch this region was amplified by PCR.

Primer sequence

[TET]- 5'ggctcacatgatatggaccaag

5'cggcagatgcagctcaaattag

PCR conditions

PCR was performed in a 15 µl reaction containing: 50 ng genomic DNA, 3.75 pmoles of each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, .5 U of ampliTaq DNA polymerase in 10 mM Tris-HCl pH 9.0/ 50 mM KCl/.1% (wt/vol) gelatin/1% Triton X-100. Thirtyfour thermal cycles were performed as follows: 40 s at 94°C, 40 s at 57°C and 40 s at 72°C followed by a final extension step for 10 min at 72°C. PCR products were electrophoresed on a 8% polyacryl amide gel in an Applied Biosystems 373A Sequencer. Allele sizes were estimated according to a commercial fluorescently labeled marker using Genescan 2.0.2/Genotyper 1.1.1 software (Perkin Elmer).

Table 1. *The frequency of H-FABP microsatellite alleles in several pig breeds represented by unrelated animals.*

		Allele frequency	
Pig breed	n	169	176
Meishan	11	.45	.55
Hampshire	9	.06	.94
Duroc	10	.05	.95
Dutch Landrace	20	.03	.97
Great Yorkshire	37	.30	.70
Pietrain	5	.20	.80

Frequency

Two alleles of 169 and 176 nucleotides were detected in DNA from 6 different breeds each represented by unrelated pigs. This variation was due to a duplication of a $G[T]_6$ as confirmed by sequencing (data not shown). The frequency of this dimorphism in the different breeds is shown in Table 1.

Mendelian inheritance

Codominant segregation of both H-FABP microsatellite alleles was observed in animals from the Wageningen Meishan x White resource family.

Chromosomal localisation

The H-FABP gene was assigned to porcine chromosome 6 by analysis of two independent porcine/rodent cell hybrid panels (Gerbens et al., 1997).

Reference

Gerbens F, Rettenberger G, Lenstra JA, Veerkamp JH, Te Pas MFW (1997) Characterization, chromosomal localization and genetic variation of the porcine heart fatty acid-binding protein gene. Mamm Genome 8, 328-332

Chapter 5

Effect of genetic variants of the heart fatty acid-binding protein gene on intramuscular fat and performance traits in pigs.

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Summary

In order to find genetic markers to improve the meat quality of pigs by breeding we studied the relationship between variation in the heart fatty acid-binding protein (H-FABP) gene (*FABP3*) and intramuscular fat (IMF) content.

To estimate the effect of H-FABP, pigs from two Duroc populations were selectively mated in such a way that at least two genotypes were present in each litter. In total, data from 983 pigs and pedigree information from three preceding generations were analyzed. Offspring were tested for IMF content as well as backfat thickness (BFT), BW, and drip loss of the meat (DRIP). All pigs were assigned to H-FABP RFLP genotype classes either by the assessed genotype (75%) or based on a probability score determined according to genotypic information of their relatives (25%).

Contrasts were detected between homozygous H-FABP RFLP genotype classes for IMF content (.4%, $P<.05$), BFT (.6 mm, $P<.01$), and BW (2.4 kg, $P<.10$). No significant contrasts were detected for DRIP. Results for IMF content, BFT, and BW were confirmed when only genotyped animals were analyzed. Variation in BFT partially explained the effect on IMF content.

Although other closely linked genes on porcine chromosome 6 might be responsible for the observed effect, interference of the halothane gene was excluded because all parental animals were noncarriers. In conclusion, H-FABP RFLP can be used as markers to select for increased IMF content and growth in breeding programs.

Introduction

Meat quality is attracting increasingly more attention in pig breeding. One of the main factors affecting sensory meat quality is intramuscular fat (IMF) content, which has been positively correlated with meat tenderness, juiciness, and taste (Bejerholm and Barton-Gade, 1986; De Vol et al., 1988; Wood et al., 1988). The optimum range of IMF content for meat acceptability was suggested to be 2 to 3% (Bejerholm and Barton-Gade, 1986; De Vol et al., 1988). However, in recent decades pigs have been selected for high lean content by selection for decreased backfat thickness (BFT) and high growth capacity. Because, BFT and IMF content are genetically correlated (approximately .37; Hovenier et al.,1992), IMF content of pig meat has decreased below the suggested level for meat acceptability.

Selection for improved IMF content is based on recording the trait on slaughtered

littermates. To increase the rate of selection response, additional marker information could be very useful for carcass traits (Meuwissen and Goddard, 1996).

To identify an informative marker or gene for IMF deposition, the candidate gene approach was applied. Heart fatty acid-binding protein (H-FABP) was postulated to be a candidate that explains part of the variation in IMF content in pigs. This protein is mainly expressed in cardiac and skeletal muscle cells and it is a member of the fatty acid binding protein (FABP) family. The currently identified eight members of the FABP family are all involved in fatty acid transport from the cell membrane to the intracellular sites of fatty acid utilization (Veerkamp and Maatman, 1995). In addition, FABP may regulate lipid metabolism and other cellular processes such as gene transcription, cellular signaling, growth, and differentiation.

The objective of this study was to explore the relationship between recently detected polymorphisms of the H-FABP gene (*FABP3*) (Gerbens et al., 1997) and variation in IMF content in Duroc pigs.

Materials and methods

Animals and data collection

Pigs from two purebred Duroc populations from Stamboek and Dumeco Breeding, each housed at separate test stations, were used. Both Duroc populations were distinct for at least five generations (i.e., the last 10 yr). Duroc pigs were known to segregate for three RFLP located in the H-FABP gene (Gerbens et al., 1997).

Boars and sows were selected and mated, according to their genotype, in such a way that at least two, and preferably three, genotype classes were present in each litter. Matings were performed within each population. With respect to the *Msp*I and *Hae*III H-FABP PCR-RFLP, three types of matings were performed: AaDd × AaDd, AaDd × AADD and AaDd × aadd. For the *Hin*fI H-FABP PCR-RFLP these were Hh × Hh, Hh × HH, Hh × hh and HH × HH (alleles described in section below). Although this last mating type was not informative, it involved only a small number of matings. Besides data from these selected matings, data from matings with genotype information from either one or none of the parents were also included in the analysis.

Offspring were housed in groups and grown with ad libitum feed access until the slaughter weight of about 110 kg was reached. For each animal, body weight (BW), age at slaughter, and ultrasonically measured backfat thickness (BFT) were recorded. After slaughter, drip loss

(DRIP) and IMF content were measured in littermates belonging to different H-FABP RFLP genotype classes. Furthermore, existing DRIP and IMF content records from animals within the pedigree were also included in the analysis.

Data from 553 gilts and 430 boars were included in the analysis. The total number of animals analyzed for each trait is shown in Table 1 and the distribution of the animals for each H-FABP RFLP genotype class is shown in Table 2.

Table 1. *The number of animals per trait (N), means, phenotypic standard deviation (SD) and the heritability (h^2) for each trait.*

Trait[a]	N	Mean	SD	h^{2} [b]
ADG, g/d	983	611.2	48.8	-
AGE, d	983	175.5	7.0	-
BFT, mm	980	12.0	1.6	.5
BW, kg	983	110.7	9.1	.3
DRIP, %	403	4.2	2.1	.3
IMF, %	440	2.7	1.0	.6

[a]ADG: average daily gain; AGE: age at slaughter; BFT: backfat thickness; BW: body weight; DRIP: drip loss of the meat; IMF: intramuscular fat content of the meat.

[b]Heritability estimates from Hovenier et al. (1992).

Meat quality measurements

After slaughter, a sample of the longissimus muscle was isolated at the third lumbar vertebra and divided into three slices. Intramuscular fat content was determined in one slice using Soxleth petroleum-ether extraction and expressed as the weight percentage of wet muscle tissue. In the remaining slices, DRIP was measured in duplicate essentially according to Honikel (1985). Meat samples were weighed before and after incubation at 4°C for 48 h hanging in glass bottles, and DRIP was expressed as the percentage of the total weight lost.

RFLP screening

Porcine genomic DNA was isolated essentially as described by Sambrook et al. (1989) from EDTA-treated blood or hair root cells. For each H-FABP RFLP test, the conditions of PCR and restriction digestions with *Hae*III, *Hin*fI, and *Msp*I have been described by Gerbens et al. (1997). The RFLP alleles are represented by letters: *Msp*I, A allele, which has two fragments (one with approximately 700 bp and another with 100 bp), and a allele (816bp); *Hae*III, D allele (683bp), and d allele (one fragment with 405 bp and another with 278 bp); *Hin*fI, H allele

(one fragment with 173 bp and another with 59 bp), and h allele (232bp).

To test pigs for halothane susceptibility, the halothane 1843 PCR-RFLP test was performed as described by Fuji et al. (1991).

Table 2. *Distribution of the predicted and actually genotyped number of animals[a] for each H-FABP RFLP genotype class for each trait.*

	*Msp*I			*Hae*III			*Hin*fI		
Trait[b]	AA	Aa	Aa	DD	Dd	dd	HH	Hh	Hh
BFT	189.8	497.5	292.7	180.3	492.5	307.2	435.0	426.4	118.5
	(142)	(378)	(230)	(133)	(372)	(236)	(330)	(313)	(90)
BW	191.3	497.0	294.8	181.3	492.5	309.2	438.0	424.9	120.0
	(142)	(378)	(230)	(133)	(372)	(236)	(330)	(313)	(90)
IMF	116.6	211.3	112.1	113.1	208.5	118.4	163.3	194.1	82.7
	(73)	(105)	(59)	(73)	(102)	(60)	(75)	(96)	(58)
DRIP	110.5	192.3	100.2	107.5	189.5	106.0	144.0	181.9	77.1
	(68)	(90)	(49)	(68)	(87)	(50)	(60)	(88)	(54)

[a]The number of actually genotyped animals is presented in parentheses.

[b]BFT: backfat thickness; BW: body weight; IMF: intramuscular fat content of the meat; DRIP: drip loss of the meat.

Statistical analysis

The statistical analysis was performed with the PEST program (Groeneveld et al., 1990), which uses pedigree information to estimate best linear unbiased estimates of the effect of each H-FABP RFLP genotype class on DRIP, IMF, BFT, and BW. Pedigree information was included from three preceding generations. Because data were not sufficient to estimate heritabilities, the heritability estimates (h^2) for each trait in Duroc pigs (Table 1) were taken from Hovenier et al. (1992). For each animal, BFT was adjusted to a weight of 110 kg, and BW was adjusted to a weight at 180 d of age, before statistical analysis. This regression was performed with recently estimated factors from a within-animal analysis for both sexes in this Duroc line.

For the animals with missing H-FABP RFLP genotypes, the GENEPROB program (Kerr and Kinghorn, 1996) was used to estimate the probabilities of belonging to each genotype class. These genotype class probabilities were estimated using the genotypic information of relatives and were included in the statistical analysis. The number of animals with predicted and actual genotypes is shown in Table 2.

Data were first analyzed with SAS (1990) using the GLM procedure. For each trait, fixed effects and covariables that were significant were included in the model. The following linear model was used to estimate the effect of each genotype class, for each H-FABP RFLP separately, on the traits of interest: $y = X\beta + Pg + Zu + e$; where y is the vector of observations for all traits in the analysis, β, g, u, and e are vectors for fixed, genotype, animal, and residual effects, and X and Z are known design matrices. The P is a matrix of genotype probabilities, where P_{ij} is the probability that animal i has genotype j as estimated by the GENEPROB program (Kerr and Kinghorn, 1996). In the case of genotyped animals, this probability is 1 for the assessed genotype class and 0 for the other genotype classes. Genotype effects g were estimated as a fixed effect. The fixed effects in β were sex (two classes), litter (237 classes), and a station $\times$ year $\times$ month of test effect (42 classes), and for IMF content the covariate age at slaughter was also included. Furthermore, in a separate analysis BFT was also included in β to estimate the effect of the H-FABP genotype classes on IMF content at a standard BFT. This latter analysis was performed because IMF and BFT have a significant genetic correlation in pigs (Hovenier et al., 1992). De Vries et al. (1994) showed that, unlike the carcass trait IMF, DRIP and other meat quality traits were significantly influenced by environmental changes prior to and on the day of slaughter. Including slaughter date (40 classes) in the analysis of DRIP resulted in nonsignificance of the station $\times$ year $\times$ month of test effect, and this latter effect was therefore omitted from the final model analyzing DRIP. The animal effect was random, with a relationship matrix that followed from the pedigree of the animals.

The contrast between the homozygous genotype classes, g(XX) – g(xx), and the dominance effect, g(Xx) – .5(g(XX) + g(xx)), were estimated, where XX and xx are the homozygous and Xx is the heterozygous genotype class for each H-FABP RFLP. Dominance was defined as the deviation of the heterozygous genotype class from the mean of the homozygous genotype classes. Error variances of these contrasts were estimated as follows: $Var(g(XX) - g(xx)) = (c_{kk} - 2c_{kl} + c_{ll})\ \sigma^2_e$, where c_{kl} is the kl^{th} element of the inverse of the coefficient matrix of the mixed-model equations (Henderson, 1984), with k and l refering to the equation numbers of the genotype classes XX and xx, respectively, and σ^2_e is the estimate of the error variance. Similarly, Var(g(Xx) – .5(g(XX) + g(xx))) was calculated.

Results

Experimental population

For each trait the number of pigs tested, mean value, and respective phenotypic variation are presented in Table 1. It should be noted that the coefficient of variation for DRIP and IMF was high.

The GENEPROB algorithm (Kerr and Kinghorn, 1996) enabled approximately 25, 25, 45, and 45% more animals to be included in the analysis for BFT, BW, DRIP and IMF, respectively. For each trait, the number of estimated and actually genotyped animals belonging to each H-FABP RFLP genotype class is presented in Table 2.

From the segregation of the parental alleles in the offspring, the linkage phase was deduced to be ADh, adH, and in some cases ADH. Thus, the *Msp*I and *Hae*III RFLP alleles were completely linked in the parental animals. Numerical differences between the *Msp*I and *Hae*III RFLP genotype classes (Table 2), however, were due to missing genotype information for either RFLP. This resulted in an estimated probability of belonging to this particular genotype class.

Because some parental animals had the ADH linkage phase, the *Hin*fI RFLP genotype classes had a different frequency distribution in the data set (Table 2) in comparison with the *Msp*I and *Hae*III RFLP classes.

Intramuscular fat content

For each H-FABP RFLP a significant contrast of approximately .4% was detected in IMF content between the homozygous genotype classes (Table 3), with the aaddHH genotype having the highest IMF content. Interestingly, the analysis with the model including actually genotyped animals (IMF2) resulted in a more pronounced and significant effect between H-FABP RFLP genotype classes.

Because responses of different fat depots in pigs are correlated, it was of interest to determine the effect of H-FABP genotype classes on IMF accretion corrected for BFT. Due to the inclusion of BFT as a covariate in the model (IMF3), the contrasts in IMF content decreased consistently with approximately .1% for each H-FABP RFLP (Table 3). These contrasts approached significance ($P < .10$) for the *Hin*fI H-FABP RFLP homozygous genotype classes, whereas all other contrasts were not significant (Table 3).

Obviously, contrasts for the *Msp*I and *Hae*III RFLP genotype classes were almost identical as would be expected from the complete linkage of the respective alleles in this experimental population.

The inheritance of the alleles associated with increased IMF content for each H-FABP RFLP, a, d, and H, showed no significant dominance effects, although for the *Hin*fI H-FABP RFLP the dominance effect of .21% IMF approached significance.

Table 3. *Contrast and dominance effects and standard errors between H-FABP RFLP genotype classes for intramuscular fat (IMF) content for three different analyses*[ab].

RFLP Contrast	IMF, %[a]	IMF2, %[a]	IMF3, %[b]
*Msp*I			
AA–aa	–.37 ± .20†	–.44 ± .19*	–.23 ± .19
Dominance	.06 ± .13	.14 ± .13	.07 ± .12
*Hae*III			
DD–dd	–.38 ± .19*	–.44 ± .19*	–.23 ± .18
Dominance	.08 ± .13	.16 ± .13	.09 ± .12
*Hin*fI			
HH–hh	.40 ± .19*	.42 ± .19*	.32 ± .18†
Dominance.	.15 ± .12	.21 ± .12†	.16 ± .11

[a]IMF and IMF2: all animals or actually genotyped animals are included in the analysis, respectively.
[b]IMF3: analysis with all animals and backfat thickness as a covariate in the model.
† P < .1; * P < .05

Backfat thickness

Results for BFT (Table 4) were similar to those for IMF content. For the *Msp*I and *Hae*III H-FABP RFLP, genotype classes differed significantly. A highly significant contrast was observed between the homozygous *Hae*III RFLP genotype classes, in which the DD genotype class had .6 mm less backfat (P < .01) at a standard weight of 110 kg. Moreover, for the *Msp*I and *Hae*III RFLP a significant contrast between the heterozygous and homozygous (aa or dd) genotype classes was also observed (data not shown). Contrasts approached significance (P < .10) for the *Hin*fI H-FABP RFLP homozygous genotype classes.

These contrasts were smaller but with the same level of significance when analysis was performed with only the actually genotyped animals (BFT2; Table 4) except for the *Hin*fI H-FABP RFLP.

The alleles associated with increased BFT (a, d, and H) for the *Msp*I and *Hae*III, and *Hin*fI H-FABP RFLP showed no significant dominance effects.

Body weight

With respect to BW, a significant contrast was detected for the *Hae*III H-FABP RFLP when only actually genotyped animals were analyzed (Table 4). This contrast approached significance when all animals were included. For the *Msp*I H-FABP RFLP, the contrast

approached significance when actually genotyped animals were analyzed. Animals from the homozygous aa and dd genotype classes had gained approximately 2.3 kg more at 180 d of age.

The alleles associated with the higher body weight at 180 d of age (a and d) showed no significant dominance effects.

Drip loss

For DRIP, large but nonsignificant contrasts were detected between all H-FABP RFLP genotype classes regardless of the analysis with all or actually genotyped animals (Table 4).

Table 4. *Contrast and dominance effects and standard errors between H-FABP RFLP genotype classes for BFT[a], BW[b], and DRIP analyzed for all and actually genotyped animals[c].*

RFLP Contrast	BFT (mm)	BFT2 (mm)	BW (kg)	BW2 (kg)	DRIP (%)	DRIP2 (%)
*Msp*I						
AA–aa	–.56 ± .21*	–.44 ± .19*	–1.95 ± 1.27	–2.20 ± 1.19†	–.21 ± .50	–.21 ± .49
Dominance	.08 ± .12	.05 ± .12	.68 ± .75	.65 ± .74	.38 ± .35	.44 ± .35
*Hae*III						
DD–dd	–.60 ± .21**	–.53 ± .19**	–2.35 ± 1.25†	–2.43 ± 1.20*	–.17 ± .49	–.19 ± .49
Dominance	.07 ± .12	.04 ± .12	.57 ± .74	.50 ± .73	.30 ± .34	.32 ± .34
*Hin*fI						
HH–hh	.38 ± .21†	.25 ± .20	.46 ± 1.29	.48 ± 1.26	.15 ± .50	.13 ± .50
Dominance	.12 ± .13	.12 ± .13	.77 ± .78	.90 ± .78	.44 ± .33	.45 ± .33

[a]BFT: backfat thickness defined as the difference from 10 mm at 110 kg. [b]BW: body weight defined as the difference from 110 kg at 180 d of age. [c]BFT, BW, and drip loss (DRIP) results of analysis with all animals; BFT2, BW2 and DRIP2 results of analysis with actually genotyped animals.

† P < .1; * P < .05; ** P < .01.

Discussion

The GENEPROB program

The GENEPROB program that calculates for each animal estimated probabilities of belonging to each genotype class (Kerr and Kinghorn, 1996) was useful in increasing the number of animals in the analysis. The GENEPROB program led to the inclusion of more genotyped animals and should provide better estimates of the genotype class effects. However, in this study, the analysis of each trait with actually genotyped animals vs all animals did not lower the standard errors of contrasts between genotypes. This may be due to genotyping of animals from a breeding population selected for increased growth rate and reduced backfat

thickness. Because of this selection, error variance would be underestimated when nongenotyped animals are excluded from the analysis. An additional explanation for the unchanged standard error of the contrasts is that the uncertainty of the estimated genotype probabilities adds to the error variance of a record (Xu, 1995).

Association analysis

Results show that the H-FABP RFLP polymorphisms had a significant association with IMF content as well as with BFT and BW. This H-FABP gene was assigned to porcine chromosome 6 (Gerbens et al., 1997), and comparative mapping data suggest that it is located near the phosphogluconate dehydrogenase (*PGD*) gene. Genes in this chromosomal region have been implicated in the regulation of several traits in pigs. Genetic variation in the PGD gene is associated with meat firmness score in Duroc pigs (Clamp et al., 1992) and with average daily gain and feed conversion in another Duroc population (Tagliaro et al., 1995). Other nearby genes such as the glucose phosphate isomerase gene and the S-system are associated with average daily gain and age at 103.4 kg in Duroc pigs (Clamp et al., 1992) and average daily gain in Swiss Landrace pigs (Vogeli et al., 1984). However, in all these association studies, the effects could well be the result of the segregation of the malignant hyperthermia gene (*Ryr-1* or *CRC*). In our study, the effect of this halothane gene could be excluded because all parental animals tested halothane-negative (data not shown). So far, no genes or markers on chromosome 6 have been reported to affect IMF content or BFT. However, the leptin receptor gene (*LEPR*) is also located on chromosome 6 (Ernst et al., 1997), and this gene might also be a potential candidate for variation in fatness traits.

However, the observed effects are most likely due to the H-FABP gene itself for two reasons. First, the genetic distance between the polymorphic site and the actual mutation must be very small because the analysis included different families from two distinct populations. In this kind of analysis, effects can only be detected when recombination frequency is virtually zero. Second, as we proposed, the H-FABP gene is a candidate gene for IMF accretion because of its central role in intracellular fatty acid trafficking.

The effect of H-FABP polymorphisms on IMF accretion might be due to differences in intracellular fatty acid transport by the H-FABP protein. Different alleles of the H-FABP gene might enable IMF accretion by a more efficient influx of fatty acids in skeletal muscle cells and(or) regulate intracellular fatty acid trafficking differently. In contrast, the effect of H-FABP on backfat accretion is presumably indirect because subcutaneous adipocytes do not express H-FABP but an adipocyte-specific FABP (A-FABP) (reviewed by Veerkamp and Maatman, 1995). Moreover, H-FABP is not secreted by muscle cells, and, thus, H-FABP has no direct

interaction with subcutaneous adipocytes. Despite this indirect effect of H-FABP polymorphisms on BFT, the effect on IMF accretion is partially explained by variation in BFT. However, the effect on IMF is more pronounced, indicating that selection for increased IMF content while maintaining BFT might still be possible. The mechanisms responsible for these effects remain unclear; however, an influence of daily feed intake with additional differences in fat partitioning might be suggested. This possible explanation would also be supported by the effect of H-FABP polymorphisms on growth (BW). Unfortunately, no feed intake data were available from these experimental animals.

With respect to the effect of H-FABP on growth (BW), several members of the FABP family have been implicated in modulation of cell growth and proliferation. A mixture of H-FABP and A-FABP also known as mammary-derived growth inhibitor (MDGI; Specht et al., 1996) and H-FABP itself are inhibitors of growth and proliferation of bovine, mouse, and human mammary epithelial cells in cell and organ culture when added extracellularly (Lehmann et al., 1989; Zavizion et al., 1993; Yang et al., 1994). Overexpression of H-FABP in transfected rat L6 myoblasts did not change the growth and proliferation characteristics whereas ectopic expression of A-FABP increased proliferation rate and blocked differentiation (Prinsen and Veerkamp, 1998).

In conclusion, the H-FABP gene or a closely linked gene on chromosome 6 affects IMF content, BFT, and growth in this halothane-negative Duroc population. The role of H-FABP in the differentiation of these traits in Duroc pigs needs to be investigated further. Moreover, it is of interest to study these associations also in other pig breeds that have a polymorphic H-FABP gene (Gerbens et al., 1997).

Implications

Increased backfat thickness, growth, and intramuscular fat content are associated with identical heart fatty acid-binding protein (H-FABP) restriction fragment length polymorphism genotypes. Thus, the H-FABP polymorphisms can be used as markers in breeding programs to simultaneously improve intramuscular fat content and daily gain. However, backfat thickness will increase, and conventional selection methods or another marker should be used to control backfat deposition. The existence of H-FABP polymorphisms in various other pig breeds may make marker assisted selection feasible for several breeds provided that the effect of the gene is segregating.

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Chapter 6

The adipocyte fatty acid-binding protein locus: characterization and association with intramuscular fat content in pigs.

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Summary

The porcine A-FABP gene (FABP4) was isolated and sequenced to study the role of A-FABP in the differentiation of intramuscular fat (IMF) accretion in pigs. The coding sequence of the porcine A-FABP gene is highly conserved across human, mouse, and rat. Moreover, all the functionally important amino acids are conserved. This high similarity extends into the first 270 bp of the 5' upstream region. Within this region, a 56-bp nucleotide sequence was completely identical with the corresponding sequence in the mouse A-FABP gene which, contains the transcription factor binding sites for C/EBP and AP-1, and is implicated in the differentiation-dependent regulation of A-FABP.

The A-FABP gene was assigned to porcine chromosome (Chr) 4 by a porcine sequence-specific PCR on a cell hybrid panel, fully consistent with comparative mapping data with human and mouse.

In the first intron of the porcine A-FABP gene, a microsatellite sequence was detected that was polymorphic for all six pig breeds tested. This genetic variation within the A-FABP gene was associated with differences in IMF content and possibly growth in a Duroc population, whereas no effect on backfat thickness and drip loss of the meat were detected. A considerable and significant contrast of approximately 1% IMF was observed between certain genotype classes. We conclude that the A-FABP locus is involved in the regulation of intramuscular fat accretion in Duroc pigs.

Introduction

Intramuscular fat (IMF) content is related to organoleptic characteristics of pig meat (Wood et al., 1988) and a IMF content of 2-3% was suggested to be optimal for eating quality (Bejerholm and Barton-Gade, 1986; DeVol et al., 1988). However, the average IMF content of pig meat has decreased below this optimum because of the selection for lean meat content.

Genetic improvement of IMF content is possible because it is highly heritable (0.6; Hovenier et al., 1992), and genetic correlations with other selection parameters like backfat thickness (BFT), although unfavorable, are moderate to low. Of all methods to improve IMF content, marker-assisted selection (MAS) is the most cost-effective method. Especially for a carcass trait like IMF content, a high genetic gain can be obtained when marker information is included in breeding programs (Meuwissen and Goddard, 1996).

To enhance IMF content, the accretion of triacylglycerols in the muscle either in the myocytes or adipocytes, is the main target (Cameron and Enser, 1991). In adipocytes, a candidate that might be involved in differentiation of triacylglycerol accretion is the adipocyte-specific fatty acid-binding protein (A-FABP, also known as aP2 or ALBP). A-FABP is exclusively expressed in adipocytes and is one of the eight identified members of the fatty acid-binding protein (FABP) family. Each FABP type is expressed in distinct tissues and cells, although coexpression of more types has been detected (reviewed by Veerkamp et al., 1993; Veerkamp and Maatman, 1995). FABPs are presumed to be intracellular fatty acid transporters. Fatty acids are bound by FABP at the cell membrane and transported to the sites of fatty acid oxidation, acylglycerol, or phospholipid synthesis (Veerkamp et al., 1993). In addition, in adipocytes A-FABP can transfer fatty acids back to the plasma membrane after lipolysis. The A-FABP gene (FABP4) sequence has been determined only for the mouse (Hunt et al., 1986 ; Phillips et al., 1986). This A-FABP gene has been studied extensively as a model system to identify transcriptional regulators of adipocyte-specific expression and to study its involvement in terminal adipocyte differentiation (reviewed by Veerkamp and Maatman, 1995).

The role of A-FABP in fat accretion can be at different levels. For instance, differences in A-FABP activity or expression might simply disturb fatty acid uptake or trafficking. Moreover, A-FABP may also modulate physiological actions of fatty acids (Hotamisligil et al., 1996). Furthermore, since adipocytes from different fat depots respond differently to lipolytic or lipogenic stimuli (Pond and Mattacks, 1991), regional distribution of fat might change because A-FABP is influenced differently by adipogenic stimuli.

To investigate the role of A-FABP in fat accretion in pigs, first the porcine A-FABP gene (FABP4) was cloned, sequenced and chromosomally localized. Subsequently, genetic variation within this gene was identified and its association with back fat and intramuscular fat content was studied.

Materials and methods

Isolation of A-FABP containing phage clones.

A porcine genomic DNA EMBL3/SP6/T7 lambda library (Clontech Laboratories Inc., Palo Alto, Calif., USA) was screened by plaque hybridization (Sambrook et al., 1989) with mouse A-FABP (ALBP) cDNA (Bernlohr et al., 1984) as a probe. This probe was cloned in the

pGEM vector and was radioactively labeled by nick translation (Sambrook et al., 1989). A single plaque that showed positive signals on replicate filters was purified by two additional rounds of low density plaque hybridization as described by Gerbens and coauthors (1997). Phage DNA was isolated using the plate lysate method (Sam-brook et al., 1989).

DNA sequence analysis

DNA from the positive phage clone was used to subclone the A-FABP gene. Therefore, the *Bam*HI, *Hin*dIII and *Sac*I restriction digestion fragments of the phage DNA were subcloned in pBS. Recombinant plasmid DNA from A-FABP clones was purified with the Wizard Maxiprep kit (Promega, Madison, Wis., USA). The nucleotide sequence was determined by cycle sequencing (Perkin Elmer, Foster City, Calif., USA), and the analysis was performed on an ABI 373 sequencer (Perkin Elmer). The DNA sequence was analyzed by the Genetics Computer Group (University of Wisconsin) software packages.

Polymerase chain reactions

PCR amplifications were performed on 1 μl of a 1:1000 dilution of phage DNA preparations or 50 ng of genomic DNA in 15 μl containing .2 units ampliTaq DNA polymerase (Perkin Elmer) in 10 mM Tris-HCl (pH 8.3)/50 mM KCl/1.5 mM MgCl2/.5 μM of each primer and .2 mM of each dNTP. After 3 min of denaturation at 94°C, 33 cycles of amplification were carried out at 94°C for 1 min, the indicated annealing temperature for 1 min, and 72°C for 1 min. Finally, an extension step at 72°C for 10 min was performed.

Microsatellite analysis

According to the sequence flanking the CA dinucleotide repeat in the A-FABP gene, primers (forward: 5'ggtactttctgatctaatggtg and reverse: 5'gggaactcttgaagtctttctc) were designed to amplify the corresponding region at an annealing temperature of 56°C. The forward primer was fluorescently labeled and the PCR product was analyzed on a denaturing polyacrylamide gel on an ABI 373 sequencer. The length of the PCR product was estimated according to standard marker fragments (Perkin Elmer) with the GENESCAN software package (Perkin Elmer).

Chromosomal localization

A pig/rodent somatic cell hybrid panel (Rettenberger et al., 1996) was used to assign the A-FABP gene to a specific chromosome by PCR. DNA (100 ng) from each cell hybrid containing porcine chromosomes in various combinations was used in a PCR reaction which unambiguously amplified porcine A-FABP gene exon 3 through exon 4 (forward:

5'agcaccataagcttagatgg and reverse: 5'cttggcttatgctctctcata). The distribution patterns of the PCR signals for porcine A-FABP were compared with the distribution patterns of the reference loci of individual pig chromosomes as described earlier (Rettenberger et al., 1996). Concordance and correlation (φ) were statistically evaluated as described by Chevalet and Corpet (1986). A marker is syntenic with a chromosome or reference locus with a probability of 97.5% if φ is > .74 for 20 hybrid lines. Synteny can be excluded if φ is < .59.

Animals and statistical analysis

The Duroc pigs used in this experiment were originally selected for another association experiment described by Gerbens and coauthors (1998). Experimental animals were housed in groups until slaughter weight (110 kg) and fed ad libitum. At slaughter, age, body weight (BW), and ultrasonically measured backfat thickness (BFT) were recorded. After slaughter, drip loss (DRIP) and IMF content were measured in a sample of the Longissimus dorsi muscle essentially according to Honikel (1985).

Statistical analysis was performed with the program PEST (Groeneveld et al., 1990) that uses pedigree information to estimate best linear unbiased estimates of the effect of each A-FABP genotype class on each trait. Pedigree data were included from three preceding generations. Heritability estimates for each trait in Duroc pigs were taken from Hovenier et al. (1992). Prior to statistical analysis, the traits BW and BFT were adjusted to BW at 180 days of age and BFT at a weight of 110 kg for each animal.

Data were first analyzed with the Statistical Analysis System (SAS Institute Inc., 1990) by use of the GLM procedure. Fixed effects and covariates that were significant for each trait were included in the model. For the analysis of each trait, sex, litter and genotype class were included as fixed effects in the model while for the traits BW, IMF content, and BFT the fixed effect herd x year x month of test was added. Furthermore, for the analysis of the traits IMF content and DRIP, age at slaughter and day of slaughter were included as fixed effects, respectively. In a separate analysis of IMF content, BFT was also included as a covariate to correct for the significant genetic correlation (.37) between these traits in pigs (Hovenier et al., 1992). All analyses included the random animal and residual effects. A more detailed description of the analysis, the estimation of contrasts between the genotype classes and respective error variances are described by Gerbens et al. (1998).

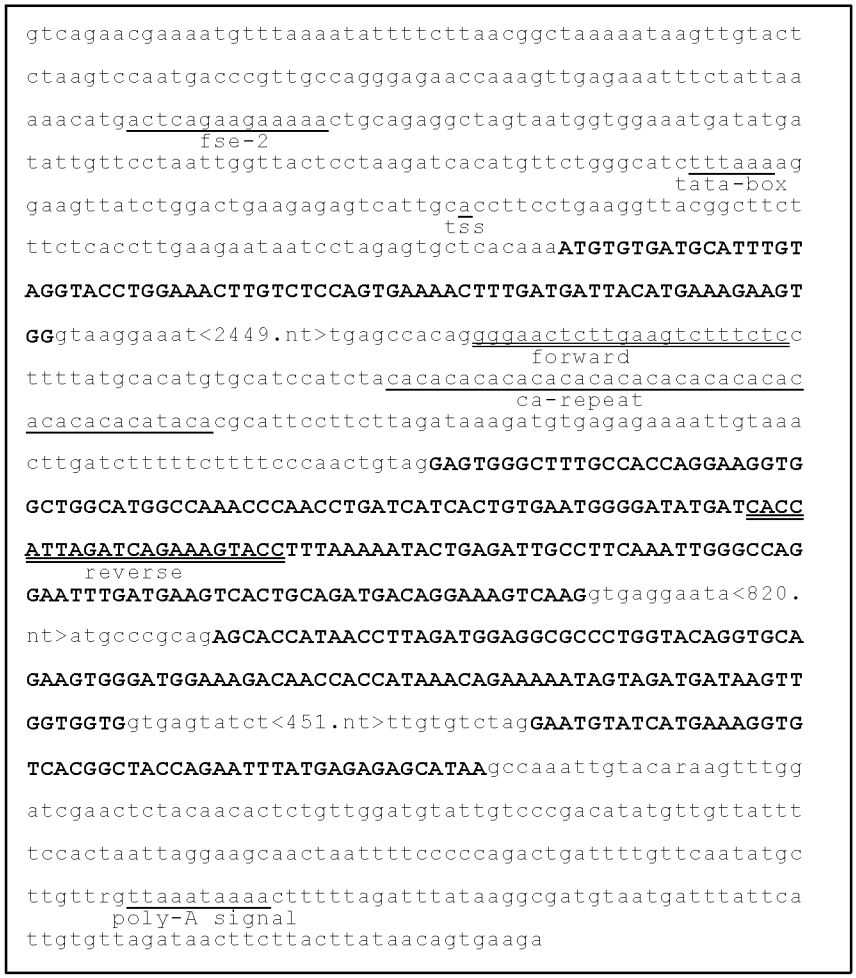

Figure 1. The porcine A-FABP gene sequence including 307 bp of the 5' upstream and 220 bp of the 3' downstream region. Each of the four exons is shown in bold capitals, and omitted intronic sequences are given between arrow heads. A putative TATA box and a poly-adenylation signal are underlined in the 5'upstream and 3' downstream region, respectively. The CA repeat is underlined, and the respective forward and reverse primers are double underlined.

Results

Sequence analysis

The porcine A-FABP gene (*FABP4*) was isolated according to homology with murine A-FABP cDNA (Bernlohr et al., 1984) and sequenced. The 8144-nucleotide sequence encompasses the entire porcine A-FABP gene including 2370 bp of the 5' upstream region and 1435 bp of the 3' downstream region. The A-FABP gene exhibits the four-exon/ three-intron structure common to all known FABP genes. The four exons encode 24, 58, 34, and 16 amino acids, and the size of the corresponding introns is 2629 bp, 840 bp, and 471 bp, respectively. The overall structure and parts of the porcine A-FABP gene sequence are shown in Figure 1.

The entire coding region of the porcine A-FABP gene shows 90, 83, and 81% similarity with corresponding human, mouse, and rat A-FABP nucleotide sequences, respectively (Baxa et al., 1989; Bernlohr et al., 1984; Prinsen and Veerkamp 1998). A high identity between the porcine and murine (Hunt et al., 1986; Phillips et al., 1986) A-FABP gene sequences was also observed in the first 270 bp of the 5' flanking sequence. Within this region, the putative TATA box and CCAAT box (Fig. 1) as well as the thymidine residue which was determined as the transcription start site in mice (Hunt et al., 1986), are conserved (Fig. 1). In addition, a completely identical 56 bp region was present containing the transcription factor binding site for CCAAT/enhancer-binding protein (C/EBP) and the activator protein (AP-1).

Furthermore, the A-FABP gene sequence contains five regions (denoted SINE-1 to 5) which have high similarity with the porcine short interspersed repeat element (SINE) consensus sequence, SSPRE (Lenstra et al., 1993) (Fig. 2). SINE-1 and 2 are present in the 5' regulatory region, SINE-3 in the second intron and SINE-4 and 5 in the 3' downstream region of the gene. SINE-2 and 3 encompasses the complete repeat sequence (233 nt) and the degenerate 3' poly-A tract. Moreover, SINE-2 is orientated head-to-tail with the incomplete SINE-1 element.

Genetic variation

Within the first intron of the A-FABP gene sequence, a $[CA]_{21}$ repeat was detected 69 bp upstream of the start of the second exon (Fig. 1). This potential polymorphic site was amplified by PCR and analyzed. In a panel containing unrelated pigs from six different breeds, 9 alleles were revealed (Table 1). These alleles correspond with 19, 20, 21, 22, 23, 27, 28, 29, and 33 consecutive repeat units, respectively. The allele frequency between breeds showed high diversity. Moreover, the A-FABP alleles segregated in a mendelian manner in a three-generation family of Yorkshire pigs (data not shown).

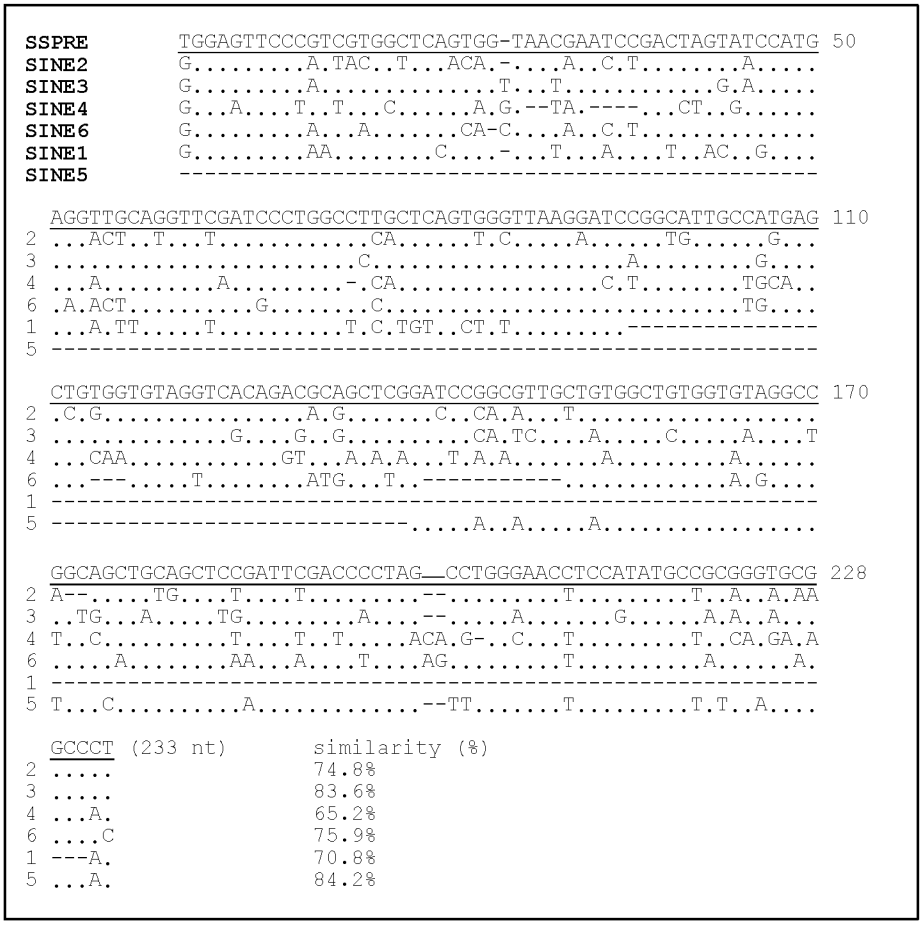

Figure 2. Alignment of SINE sequences present in the porcine A-FABP and H-FABP genes and comparison with the known consensus SINE sequence, SSPRE. SINEs 1-5 are detected in the A-FABP gene and 6 in the porcine H-FABP gene (EMBL: X98555). SINE 1 is a partial sequence and SINE 5 was abrogated because it is located at the end of the phage clone. Identical nucleotides are indicated with dots (.), and gaps (-) have been introduced to maximize alignment. Following each sequence the percentage similarity with SSPRE is shown.

Table 1. *The frequency of A-FABP microsatellite alleles in several pig breeds represented by unrelated animals*

		Number of CA repeat units								
Pig Breed	n	19	20	21	22	23	27	28	29	33
Meishan	8			.56	.44					
Hampshire	9				.06			.94		
Duroc	7				.79					.21
Dutch Landrace	19	.21	.32		.13	.13	.08		.13	
Great Yorkshire	37	.05	.37		.20				.38	
Pietrain	5	.30	.10		.10			.10	.40	

Chromosomal localization

Analysis of the presence of the porcine A-FABP gene in each cell hybrid showed a single significant correlation (φ) of .81 with the presence of the chromosome 4 specific S0001 microsatellite marker. All other reference loci were asyntenic (data not shown). Hence, the porcine A-FABP gene was assigned to porcine Chr 4.

Table 2. *The number of animals, phenotypic means, and standard deviation (SD) for each trait.*

Trait	N	Mean	SD
BFT, mm	992	12.0	1.6
BW, kg	992	110.7	9.1
DRIP, %	419	4.2	2.1
IMF, %	440	2.7	1.0

Association analysis

In total, data from 992 animals were included. For each trait, the total number of animals, the population mean, and respective standard deviation are presented in Table 2. Of 747 animals, the A-FABP genotype was determined. Three A-FABP alleles were segregating in both Duroc populations, corresponding to repeat lengths of 22 (A1), 33 (A2), and 19 (A3) CA repeat units, respectively. These alleles resulted in six different genotype classes (Table 3). However, the number of animals belonging to the A2A3 and A3A3 genotype classes was too small to be included in the analysis of IMF content and DRIP.

Results of the statistical analysis for each trait are presented in Table 4. The effect of each genotype class is shown as the contrast with the A1A1 genotype class because this genotype class revealed the smallest effect for each trait, except for DRIP.

The A1A3 genotype class had a significantly higher IMF content than the A1A1 genotype

class. Including BFT as a covariate in the analysis of IMF content (IMF2) resulted in a smaller contrast that still approached significance. The size of this contrast of about 1% IMF was considerable compared with the phenotypical average IMF content of 2.7% (±1%) of the experimental population (Table 2). However, the A1A3 genotype class consisted of only six animals originating from three different families.

With respect to BW, the weight of the A1A1 genotype class was smaller than the A1A2 ($P< .05$), A2A3 ($P< .10$) and A3A3 ($P< .10$) genotype classes. For the A3A3 genotype class the effect was quite large (9.5 kg at 180 days of age) but this genotype class contained only four animals (Table 3). For backfat thickness and drip loss no significant contrasts were observed between any of the genotype classes.

Table 3. *Distribution of animals for each A-FABP genotype class and the remaining nongenotyped animals for each trait.*

	IMF	BW	BFT	DRIP
A1A1	47	154	154	40
A1A2	126	344	344	110
A1A3	6	33	33	3
A2A2	46	181	181	41
A2A3	2	31	31	2
A3A3	1	4	4	1
REST	212	245	245	222
Total	440	992	992	419

Discussion

The present study reports the isolation, characterization and chromosomal localization of the porcine A-FABP gene and its association with performance and meat quality traits in pigs. The nucleotide sequence of the coding sequence as well as the 5' untranslated region show high similarity to the corresponding regions of the A-FABP gene from other species. Furthermore, the sequence of the porcine A-FABP peptide reported by Armstrong and coauthors (1990), encompassing amino acid positions 36 to 48, was completely identical with the same region of the amino acid sequence deduced from the A-FABP gene. Generally, the majority (17 of 25) of all amino acid substitutions are conservative among the A-FABP sequences from mouse (Bernlohr et al., 1984), rat (Prinsen and Veerkamp, 1998), human (Baxa et al., 1989) and pig.

Among all substitutions, 10 amino acid substitutions are unique to the porcine A-FABP protein. However, the amino acids that are presumed to be essential to FABP function (Veerkamp et al., 1993) like Arg106, Arg126, Tyr128, and Cys1 and 117, as well as the possible tyrosine phosphorylation site, are fully conserved.

Table 4. *Contrasts and standard errors of A-FABP genotype classes for IMF, IMF2, BFT, BW, and DRIP.*

Genotype classes	IMF (%)	IMF2 (%)[a]	BW (kg)[a]	BFT (mm)[a]	DRIP (%)
A1A2-A1A1	.36 ± .39	.30 ± .37	3.34 ± 1.67*	.14 ± .27	-1.21 ± 1.53
A1A3-A1A1	1.17 ± .58*	1.01 ± .55†	1.92 ± 2.73	.69 ± .45	-.55 ± 2.07
A2A2-A1A1	.25 ± .41	.19 ± .39	2.32 ± 1.77	.10 ± .29	-.75 ± 1.55
A2A3-A1A1	- -	- -	5.71 ± 3.10†	.18 ± .51	- -
A3A3-A1A1	- -	- -	9.41 ± 4.95†	.37 ± .81	- -
REST-A1A1	.13 ± .29	.16 ± .27	2.61 ± 1.30*	.51 ± .21*	-1.21 ± 1.11

[a]IMF2: IMF analysis with BFT included as covariate in the model; BW: body weight defined as the difference from 110 kg at 180 days of age; BFT: backfat thickness defined as the difference from 10 mm at 110 kg. † $P < .1$; * $P < .05$

The first 270 nucleotides of the 5' upstream region are very similar to the corresponding region in the murine A-FABP gene. This region contains several regulatory elements like the FSE2/AP-1 and C/EBP binding sites and a cAMP-dependent site, which are all involved in the regulation of the expression of A-FABP and other fat genes (reviewed by Smas and Sul, 1995; MacDougald and Lane, 1995). The high similarity of this region indicates its importance for the regulation of the A-FABP expression.

The porcine A-FABP gene is assigned to Chr 4, which is consistent with the localization of the human and mouse A-FABP gene on Chrs 8q21 (Prinsen et al., 1997) and 3 (Heuckeroth et al., 1987), respectively. Heterologous chromosome painting demonstrated that the short arm and centromeric end of the long arm of porcine Chr 4 (4pter-q14) have conserved synteny with human Chr 8q (Goureau et al., 1996). Several attempts to assign the A-FABP gene to Chr 4 by FISH analysis have failed, most likely because of the relatively high number of SINE sequences within the phage clone.

To examine whether A-FABP is involved in the differentiation of fatness in pigs, we used the polymorphic $[CA]_n$ repeat (Table 1) within this gene. The Duroc population under investigation, was previously used to examine association between variation in the H-FABP gene and performance and meat quality traits (Gerbens et al., 1998).

Statistical analysis revealed that the A1A3 genotype class of the A-FABP gene had approximately 1% more IMF than the A1A1 genotype class. Because the A3 allele seems to be

beneficial, it was of interest to determine the effect of the A2A3 and A3A3 genotype classes on IMF content. Unfortunately, these genotype classes contained data from only two and one animal, respectively.

Interestingly, the A3A3 genotype class had a considerably higher BW at 180 days of age than the A1A1 genotype class, whereas, on the other hand, the A1A3 genotype class showed no difference. Therefore, as judged from the effect of the A1A3 genotype class, effects on IMF content and BW are unrelated. Furthermore, a significant difference in BW at 180 days of age was observed between the A1A2 and A1A1 genotype classes (Table 4). Despite this effect, no significant difference was observed for the A2A2 genotype class.

These results indicate that the A-FABP locus on chromosome 4 has a considerable effect on IMF content and possibly on growth in pigs. Andersson and coauthors (1994) demonstrated the presence of QTL on porcine Chr 4 affecting fatness, both BFT and abdominal fat, and growth rate in a F2 population of a wild pig/Large White cross. Similar results were obtained in a F2 population of a Meishan/Large White cross (Walling et al., 1998). Furthermore, in mice a QTL for relative abdominal fat content was identified in a region on Chr 3 syntenic with pig Chr 4 (Brockmann et al., 1996). Unfortunately, none of these studies have analyzed IMF content. On the other hand, genetic variants of the A-FABP gene were not involved in differences in backfat thickness in our study. However, taking all data into account, the effects on fatness traits in pigs might be controlled by the same gene affecting fat distribution in pigs. Differences between studies might reflect only the effect of this gene in different pig breeds or populations.

Because A-FABP is expressed exclusively in adipocytes and its gene, FABP4, resides within the region containing a QTL for fatness, the role of the A-FABP gene in fat deposition in pigs needs further study. In the case of the, previously mentioned, abdominal fat content QTL identified in mice, the role of A-FABP could not be confirmed by differences in mRNA expression (Brockmann et al., 1996). However, A-FABP protein expression and or functionallity have not been studied.

In conclusion, we isolated and sequenced the porcine A-FABP gene and localized it to porcine Chr 4. Furthermore, we provided evidence that this gene might be involved in the differentiation of IMF content and possibly growth in pigs.

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Chapter 7

An RFLP within the microsatellite of the porcine adipocyte fatty acid-binding protein gene.

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Submitted for publication.

Source/description

For the porcine A-FABP gene (GenBank: Y16039) a polymorphic $[CA]_n$ nucleotide stretch was identified in the second intron (Gerbens et al., 1998). PCR amplification of this region with a primer set distinct from the one published before (Gerbens et al., 1998), revealed incompatible inheritance profiles. Subsequent sequence analysis of this region showed within the start of the CA repeat region two separate mutations responsible for differential amplification of this region by our experimental primer set. One of these mutations eliminated a *Bsm*I restriction endonuclease recognition site (gaatgcnn) resulting in two alleles.

Allele F: aagaaggaatg*t*gtgtg*t*gtgtgtgtgt
Allele f: aagaaggaatg*c*gtgt*a*tgtgtgtgtgt

Therefore an A-FABP *Bsm*I PCR-RFLP test was generated to investigate genetic variation in this specific region. PCR conditions were as decribed before for the PCR amplification of A-FABP microsatellite region (Gerbens et al., 1998). Restriction digestions with *Bsm*I were performed by addition of the recommended restriction digestion buffer to the PCR reaction.

Primer sequence

5′gggaactcttgaagtctttctc
5′ggtactttctgatctaatggtg

Table 1: *Frequency of A-FABP BsmI PCR-RFLP genotypes in several pig breeds represented by unrelated animals.*

		Genotype frequency		
Pig Breed	n	FF	Ff	ff
Meishan	10	-	-	1.00
Hampshire	9	-	-	1.00
Duroc	10	-	.50	.50
Dutch Landrace	20	.15	.35	.50
Large White	34	-	.24	.76

Frequency

Since this PCR fragment also encompasses the microsatellite region undigested fragments (allele F) can vary between about 253 and 283 bp. Upon successful BsmI restriction digestion (allele f) two fragments are observed, a fragment of 165 bp and a fragment varying in length

between 88 and 118 bp. The frequency of this PCR-RFLP polymorphism in different pig breeds is shown in Table 1.

Mendelian inheritance

Codominant segregation of A-FABP BsmI PCR-RFLP alleles was observed in animals from a Large White family.

Chromosomal localisation

The A-FABP gene was assigned to porcine chromosome 4 by porcine/rodent cell hybrid panel analysis (Gerbens et al., 1998) and linkage analysis (Gerbens et al., 2000).

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Chapter 8

The effect of adipocyte and heart fatty acid-binding protein genes on intramuscular fat and backfat content in Meishan crossbred pigs.

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Summary

Effects of genetic variation in porcine adipocyte and heart fatty acid-binding protein genes, A-FABP and H-FABP, respectively, on intramuscular fat (IMF) content and backfat thickness (BFT) were examined in F_2 crossbreds of Meishan and Western pigs. The involvement of each FABP gene in IMF accretion was studied to confirm previous results for Duroc pigs.

The F2 crossbred pigs were genotyped for various markers including microsatellite sequences situated within both FABP genes. Linkage analysis assigned the A-FABP and H-FABP genes to marker intervals S0001-S0217 (20 cM) on SSC4 and Sw316-S0003 (16.6 cM) on SSC6, respectively, refining previous chromosomal assignments.

Next, the role of both chromosome regions/genes on genetic variation in IMF content and BFT was studied by 1) screening SSC4 and SSC6 for QTL affecting both traits by performing a line-cross analysis and 2) estimation of the effect of individual A-FABP and H-FABP alleles on both traits.

In the first analysis, suggestive and chromosome-wise significant evidence for a QTL affecting IMF was detected on SSC6. The H-FABP gene is a candidate gene for this effect because it resides within the large region containing this putative QTL.

The second analysis showed a considerable but nonsignificant effect of H-FABP microsatellite alleles on IMF content. Suggestive evidence for a QTL affecting BFT was found on SSC6, but H-FABP was excluded as a candidate gene.

In conclusion, present and previous results support involvement of H-FABP gene polymorphisms in IMF accretion independently from BFT in pigs. Therefore, implementation of these polymorphisms in marker-assisted selection to control IMF content independently from BFT may be considered. In contrast to previous findings for Duroc pigs, no evidence was found for an effect of the A-FABP gene on IMF or BFT in this population.

Introduction

Intramuscular fat (IMF) content is a major determinant of meat quality. In particular, eating quality traits are influenced by the amount of intramuscular fat (reviewed by Hovenier et al., 1993). Intramuscular fat content is highly heritable (h^2=.5; Hovenier et al., 1993) but genetic correlations with other production traits are unfavorable but moderate (Hovenier et al., 1992). However, improving IMF content by selective breeding is difficult because this trait is measured on the carcass. Marker or gene-assisted selection is a promising strategy for genetic

improvement of such carcass traits (Meuwissen and Goddard, 1996).

Previously, genetic variants of both heart (H) and adipocyte (A) fatty acid-binding protein (FABP) genes (*FABP3* and *FABP4*) were shown to be associated with IMF content, backfat thickness (BFT), and growth (Gerbens et al., 1998b and 1999). These findings are consistent with the function and tissue-specific expression of these FABPs (Veerkamp and Van Moerkerk, 1993; Veerkamp and Maatman, 1995). Functionally, FABP are intracellular proteins that transport fatty acids from the cell membrane to sites of fatty acid oxidation or phospholipid or triacylglycerol synthesis. The H-FABP is expressed predominantly in muscle cells whereas A-FABP is expressed almost exclusively in adipocytes. However, whether the observed effects of the genetic variants are due to the FABP genes themselves or closely linked genes is still inconclusive. Analysis of data from other pig breeds, lines or populations might provide additional insight into FABP's role in these traits.

Therefore, our objective was to examine the effect of genetic variants of both FABP genes in F_2 pigs from a cross between Meishan boars and Western breed sows that has been shown to segregate for QTL and a major gene affecting IMF content (Janss et al., 1997; De Koning et al., 1999). Furthermore, these data allowed us to estimate the chromosomal position of FABP3 and FABP4 more accurately by linkage analysis.

Materials and methods

Animal data

Pigs from the F_2 generation of crosses of the Chinese Meishan breed and western pig lines were available from an experiment involving five Dutch pig breeding companies. Nineteen purebred Meishan boars were crossed with 126 purebred sows either from the Large White or the Dutch Landrace breed to produce F_1 crossbreds. Randomly selected F_1 crossbreds, 39 boars and 264 sows were intercrossed, except that no full sibs were mated. Of the F_2 crossbreds, 1200 pigs were performance tested and 844 randomly selected pigs were slaughtered at 90 kg live weight to assess meat quality traits. Detailed information about the breeding strategy and the setup of the experiment is given by Janss et al. (1997).

Animals were housed at five test stations. To avoid confounding of the background effects of the various western pig lines with test-station effects, semen of F_1 boars was distributed across test stations. Furthermore, pigs were slaughtered at a single slaughter house on 26 slaughter days, and pigs from the different test stations were slaughtered on the same day to avoid confounding the effects of test station with slaughter day.

Pigs from 19 paternal half-sib families had informative segregation of alleles for a major gene affecting IMF content according to Janss et al. (1997) and were used for subsequent statistical analyses. In total, 619 F_2 crossbred pigs were included of which meat quality traits were recorded from 418, randomly selected, pigs.

Performance and meat quality trait data

Pigs included in this analysis were all performance tested. At approximately 90 kg, age, live weight, and backfat thickness (BFT) were recorded. For pigs selected for meat quality evaluation, carcass weight and BFT were also recorded after slaughter. Backfat thickness was measured with the Hennesy Grading Probe between the third and fourth ribs, 6 cm from the spine of the carcass.

Twenty-four hours after slaughter, samples of the longissimus muscle were obtained for IMF content evaluation according to Hovenier et al. (1992). Intramuscular fat content was determined using the Soxleth petroleum-ether extraction method and expressed as the weight percentage of wet muscle tissue.

Genotype data

In total, 619 F_2 crossbred pigs, their F_1 parents, and Meishan grandparents were genotyped for the described microsatellite sequences present in the porcine H-FABP (Gerbens et al., 1998a) and A-FABP gene (Gerbens et al.,1998b). For the H-FABP microsatellite, only two alleles were segregating. For the A-FABP microsatellite eight alleles were segregating in this pig population. Genotypic data from nine and seven additional microsatellites, covering SSC4 and SSC6, respectively, had been evaluated previously (De Koning et al., 1999) and were included in the present analysis. The linkage groups of SSC4 and SSC6 span 111 and 152 cM with an average marker interval of 10 and 20 cM, respectively.

All microsatellite sequences were amplified using PCR as described by Groenen et al. (1996). Fragment lengths were determined upon electrophoresis on a 8% denaturing polyacryl-amide gel in a ABI377 automatic sequencer (ABI, Perkin Elmer, Foster City, CA). Genotyping results were independently evaluated by two examiners and by segregation analysis using the pedigree file.

Marker linkage analysis

Previously, the A-FABP and H-FABP genes were assigned to SSC 4 and 6, respectively, using cell hybrid analysis (Gerbens et al., 1997, 1998b). In the present analysis, A-FABP

and H-FABP microsatellites were assigned to chromosomes on the basis of two-point and multipoint linkage analysis (CRIMAP 2.4; Green et al., 1990) using genotyping data from all individuals of the 19 half sib families. Two-point linkage assignments were considered significant when LOD score exceeded 3.0. The most likely multipoint linkage map was based on the highest LOD score value.

Quantitative trait loci analysis

Data were statistically analyzed in two ways. First, the effect of the positions of both FABP genes was evaluated by including microsatellite data of both FABP genes in a chromosome scan for QTL as developed by Haley et al. (1994) for semi-inbred line crosses. This approach assumes that both founder populations are fixed for different alleles of QTL affecting the trait of interest, but this does not exclude breeds having marker alleles in common.

At a given location along the genome, for each F_2 animal, the probabilities were estimated whether it inherited two Meishan alleles, two Western alleles, or one of each founder line based on its marker genotypes. These probabilities can be used in a least squares model to investigate the role of a genomic region on the trait of interest. This type of analysis has been applied to several crossbred pig populations (Andersson et al., 1994; Knott et al., 1998; De Koning et al., 1999).

The assumption of QTL allele fixation in both founder populations allows direct estimation of dominance and additive effects of a putative QTL at any position. Additive effects are defined as half the phenotypic difference between pigs homozygous for the QTL alleles originating from Meishan and the Western breeds. These additive effects are estimated for the Meishan QTL alleles; that is, a positive value indicates an increase of the trait of interest due to the respective Meishan QTL allele. Dominance effects are estimated as the deviation of pigs heterozygous for the QTL alleles from the mean of the two types of homozygous pigs. If the heterozygous animals are closer to the homozygotes with the Meishan alleles, the dominance is defined as positive.

Interval mapping is done with multimarker analysis (Knott et al., 1998) using 1-cM interval lengths. Test statistics were evaluated along both chromosomes, with the highest value appearing at the most likely position of the QTL. Phenotypic data were preadjusted for non-genetic effects of day of slaughter, breeding company, sex, and carcass weight and analyzed in a model assuming polygenic inheritance as described previously by De Koning et al. (1999).

For the QTL analysis, the following model was fitted:

$$y_i = \mu + c_{ai}a + c_{di}d + e_i$$

where y_i is the adjusted trait observation of individual i, μ is the population mean, a and d are the estimated additive and dominant effect of a putative QTL at the given location, c_{ai} and c_{di} are the coefficients for the additive and dominance component for individual i denoting the probability of individual i carrying two Meishan alleles or being heterozygous at the given location, respectively, and e_i is the residual error.

Significance of the chromosome-scan approach was evaluated according to Lander and Kruglyak (1995). Suggestive evidence was defined as the F-ratio threshold value that resulted in one expected false positive in this experiment. Although Lander and Kruglyak (1995) suggest that a genome-wide significance threshold should always be applied, in this case the effect of a specific part of the genome (i.e., two chromosomes) was evaluated to validate earlier findings. Therefore, chromosome-wise significance thresholds were applied. These significance levels do not account for testing multiple traits. Suggestive and chromosome-wise significance thresholds were obtained empirically by permutation tests of the data (Churchill and Doerge, 1994). Ten thousand permutations resulted in suggestive and chromosome-wise significance thresholds of 5.0 and 5.2 for SSC4 and 4.5 and 5.1 for SSC6, respectively, for both IMF content and BFT data.

Candidate gene analysis

The second approach to analyze the data was a straightforward candidate gene analysis for comparison with previous studies in Duroc pigs (Gerbens et al., 1998b, 1999). Here, genetic variation at both FABP genes was assumed to affect the trait of interest directly. Analysis was performed within litters using SAS (1990). Because of complete confounding between litter and company, company could be excluded from all models. For the evaluation of IMF content, prior analysis with SAS indicated that sex and carcass weight should be included in the model. In a separate evaluation of IMF content, BFT was included as a covariate in the model to account for the correlation between these traits (Hovenier et al., 1992) (i.e., to estimate the effect of IMF for the same level of BFT).

For the evaluation of BFT, sex and carcass weight were included in the model. Moreover, an additional analysis included also growth from start of test until day of

slaughter to account for differences in growth and fat accretion characteristics between both founder populations.

Because eight A-FABP alleles are segregating in the Meishan population, analysis of the individual genotype classes would be inefficient. Hence substitution effects of individual alleles were estimated according to Ostergard et al. (1989). Using this procedure, it is possible to estimate all allelic effects simultaneously, provided that the sum of all estimated allelic regression coefficients is constrained. For A-FABP, this constraint was applied by conditioning all other allele classes on the A1 allele class. The A1 allele class was chosen because this was the most abundant allele, and this class was demonstrated to be the less favorable allele in previous analyses for Duroc pigs (Gerbens et al., 1998b). For H-FABP, a similar analysis was applied. Here, the constraint was applied by regressing on allele H1 corrected for H2. In the latter case, H1H1, H1H2, and H2H2 genotypic animals were assigned the score 2, 0, and -2, respectively.

Significant effects of allele classes were determined according to the F statistic of the analysis.

Results

Marker linkage analysis

Two-point linkage analysis revealed that the A-FABP gene was significantly associated with markers on SSC 4. According to multipoint linkage analysis, the most likely order was (sex averaged; Kosambi centimorgans within brackets): *S0227* - [19.9] - *S0301*- [18.1] - *S0001* - [9.9] - ***A-FABP*** – [10.1] – *S0217* – [6.4] – *S0073* –[3.7] - *Sw589* – [2.7] – *S0214* – [20.6] - *Sw445* – [19.8] – *S0097*.

The H-FABP gene was significantly associated with markers on SSC 6. Multipoint linkage analysis revealed the most likely order to be (sex averaged; Kosambi centimorgans within brackets): *S0035* – [11.7] – *Sw2406* – [29.9] – *Sw1057* – [29.6] – *S0220* – [12.7] – *Sw316* – [5.1] – ***H-FABP*** - [11.5] – *S0003* – [51.3] – *Sw2419*.

Quantitative trait loci analysis

Information Content. A QTL analysis was performed with and without microsatellite information from both FABP genes to detect QTL on SSC4 and SSC6 contributing to genetic variation in IMF content and BFT (Figure 1). According to the Haldane map used in this analysis, the A-FABP gene was positioned at 54 cM on SSC4 and the H-FABP gene at 106 cM on SSC6 (Figure 1).

Local information content (Figure 1ab) increased by inclusion of FABP microsatellite information, especially in the case of SSC4/A-FABP. This increase in information content is due to the high number of A-FABP microsatellite alleles segregating in this population and the increased coverage of the chromosome in this region. Inclusion of additional A-FABP microsatellite information not only increased the information content in the specific interval but also affected flanking intervals due to increased information content in previously uninformative families.

For SSC6/H-FABP, hardly any increase was seen due to its position in a relative densely covered area of SSC6, and the existence of only two alleles for the H-FABP microsatellite in this Meishan crossbred population.

Table 1. *Additive and dominance effects of most significant QTL loci on SSC4 and SSC6 for intramuscular fat content(IMF) and backfat thickness (BFT)*

Trait	SSC	Position, cM	F-ratio	Additive	SD	Dominance	SD
IMF	4	65	4.67	.22	.07	-.05	.10
	6	149	6.61 sc	-.44	.12	.10	.33
BFT	4	103	4.50	-.22	.41	-2.01	.68
	6	189	5.18 s	-.61	.40	-1.75	.63

s indicates significance at the suggestive level; c at the chromosome-wise level. For reference, A-FABP and H-FABP genes are positioned at 54 cM on SSC4 and 106 cM on SSC6, respectively.

SSC4/A-FABP. For IMF, the most probable position of a QTL on SSC4 was found around 65 cM (Figure 1c). However this test statistic did not exceed suggestive and chromosome-wise significance levels. Strikingly, the F-statistic profile was lowered when including A-FABP microsatellite information. Although this QTL did not significantly contribute to the variation in IMF content in this population, the effect of this locus was still considerable (Table 1). Individuals homozygous for the Meishan alleles have on average

.44% more IMF than those homozygous for the western breed alleles, and this effect appears to be additive in nature.

There was no evidence of any region on SSC 4 significantly affecting BFT (Figure 1e). The highest F-statistic became more pronounced after inclusion of A-FABP microsatellite information but remained nonsignificant. In particular around the position of the A-FABP gene, the test statistic was very low.

SSC6/H-FABP. Suggestive evidence for a QTL affecting IMF content was detected on SSC6 around 149 cM, which was significant at the chromosome-wise level. The maximum test statistic for this QTL is found in the marker interval adjacent to the H-FABP gene. This marker interval covers a very wide range of approximately 70 cM of SSC6 (Figure 1d). This QTL appears to be additive in action, and Meishan alleles are causing a decrease in IMF content (Table 1). Individuals carrying both Meishan alleles have on average .88% less IMF than those homozygous for the western pig alleles.

Furthermore, there was also suggestive evidence for a QTL on the end of the linkage group of SSC6 affecting BFT that nearly approached the chromosome-wise (P = .05) significance threshold (Figure 1f). At the position of the H-FABP gene, the test statistic showed no evidence of any contribution to variation in BFT.

Candidate gene analysis

SSC4/A-FABP. The effect of each individual allele of A-FABP was estimated simultaneously, both for IMF content and BFT. For both traits, inclusion of A-FABP microsatellite information did not provide a significantly better explanation of the data. No significant effects of separate A-FABP alleles on IMF content were detected in this analysis (Table 2).

In contrast, for BFT, the effects of the A-FABP A5 and A9 alleles differed significantly from zero (Table 2). These effects were even more pronounced when fitting growth data in this model (BFT2; Table 2). Both alleles had unfavorable regression coefficients (i.e., BFT increased with increasing number of either the A5 or A9 allele in the individuals). The A5 allele originated almost exclusively from the Meishan population whereas the A9 allele was present in several subpopulations of the western pigs.

SSC6/H-FABP. Inclusion of H-FABP genetic variation data in the model did not provide a significantly better explanation of the data for either IMF content or BFT. For the individual alleles of H-FABP, the effects on IMF content approached significance (Table 2). Because in this analysis only two H-FABP alleles were present, the regression coefficient

expresses the difference between alleles. Therefore, the contrast between both homozygous H-FABP genotype classes H1H1 and H2H2 is .36% IMF where the H2 allele increased the IMF content. Because H1 and H2 alleles both segregate in the Meishan and western pig populations, no conclusions as to the origin of this effect can be drawn.

No evidence for differences in BFT between the H-FABP alleles was found (Table 2).

Table 2. *Regression coefficients (se) of each individual A-FABP or H-FABP allele for backfat thickness (BFT) and intramuscular fat content (IMF).*

Gene	Allele	BFT[a]	BFT2[a]	IMF[a]	IMF2[a]
A-FABP	A1	1.02 (.79)	1.08 (.78)	.19 (.15)	.12 (.15)
	A3	.16 (1.13)	.13 (1.12)	-.10 (.21)	-.11 (.21)
	A4	.74 (.91)	.65 (.90)	-.07 (.17)	-.11 (.17)
	A5	1.49 (.67)*	1.64 (.66)*	.08 (.13)	-.01 (.12)
	A6	.82 (1.88)	.73 (1.86)	-.03 (.37)	-.04 (.35)
	A9	1.91 (.92)*	2.00 (.92)*	-.03 (.17)	-.15 (.17)
	A10	-4.19 (3.10)	-3.89 (3.07)	.04 (.59)	.28 (.57)
	A11	-1.96 (1.65)	-2.35 (1.64)	-.09 (.32)	.02 (.30)
H-FABP	H1	-.06 (.27)	-.05 (.27)	-.09 (.05)†	-.09 (.05)†

[a] BFT: model includes sex and carcass weight; BFT2: model includes sex, carcass weight and growth from start of test till slaughter; IMF: model includes sex and carcass weight; IMF2: model includes sex, carcass weight and BFT.

† $.05 < P < .10$; * $P < .05$

Discussion

Marker linkage analysis

According to genetic linkage analysis the A-FABP and H-FABP genes are located on SSC 4 and 6, respectively. These findings confirm previous results from cell hybrid analysis (Gerbens et al., 1997, 1998b) and are supported by comparative mapping data. Recently, the human A-FABP gene (*FABP4*) was assigned to human chromosome 8q21 (Prinsen et al., 1997), a region syntenic with porcine chromosome 4pter-4q14 as observed by ZOO-FISH analysis (Goureau et al., 1996). The porcine A-FABP gene was located between the markers *S0001* and *S0073* that have been assigned to SSC 4p12-p13 (Marklund et al., 1993) and SSC 4q15-q16 (Robic et al., 1996), respectively. Thus, the A-FABP gene resides around the centromere of SSC 4. So far, the flanking *S0217* marker has not been physically assigned.

The A-FABP microsatellite sequence resides in the middle of the region between *S0001* and *S0217*, the marker is highly polymorphic, and because it is located within a gene it is also a good reference in comparative genome analysis with other species.

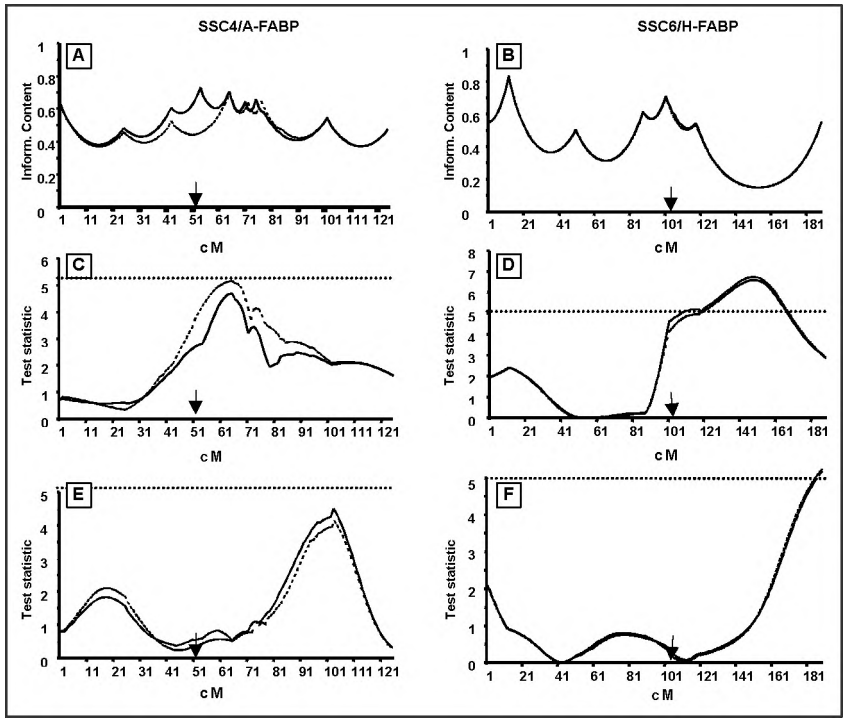

Figure 1. Information content and test statistics for SSC4 (left) and SSC6 (right). In each panel results are shown without (dashed line) and with (solid line) information of both FABP microsatellites included in the analysis. Panels A and B show the information content along the chromosome. Panels C and D represent the F-ratio plot along the chromosome for intramuscular fat content (IMF). Similarly, Panels E and F for backfat thickness (BFT). Haldane map positions of the A-FABP and H-FABP genes on SSC4 (54 cM) and 6 (106 cM), respectively, are indicated with arrows. Horizontal dotted lines represent the chromosome-wise (P = .05) significance threshold.

The human H-FABP gene (*FABP3*) is located on chromosome 1p32-35 (Troxler et al., 1993; Hung et al., 1995) a region syntenic with porcine SSC 6q21-q26 and 6q32-qter, as established by comparative mapping analysis and ZOO-FISH analysis (Goureau et al., 1996). To our knowledge, none of the flanking markers has been physically localized on

SSC 6. Therefore, no distinction can be made whether H-FABP is located in the centromeric or telomeric region of chromosome 6 syntenic with human chromosome 1. Moreover, the rearrangements of the gene order between humans and pigs in this region, presumably by intrachromosomal inversion (Johansson et al., 1995), indicates that physical localization of the H-FABP gene should be considered.

QTL and candidate gene analysis

SSC4/ A-FABP: In the present study, no significant evidence was detected for QTL affecting either IMF content or BFT on SSC4. In contrast, De Koning et al. (1999) found suggestive linkage between SSC4 and IMF content in the same population using two statistical models. This study adds the A-FABP microsatellite information to the line-cross analysis. The overall reduction of the test statistic due to this addition is probably an effect of the increased information content and consequently an increased power to estimate the chromosomal contribution to IMF content. The fact that, in contrast to earlier findings (Gerbens et al., 1998b), no evidence for an effect of A-FABP on IMF content was found, may be due to absence of variation in this population, epistatic interaction of genes or the magnitude of the background gene effects. In the QTL analysis, fixation of alleles in the two founder populations was assumed. However, when the QTL alleles are not fixed the expected contrast decreases and makes the QTL harder to detect.

Previously, a significant contrast between A-FABP A1A1 and A1A3 genotype classes was found of approximately 1% IMF in a commercial Duroc population (Gerbens et al., 1998b). In the Meishan crossbred population, the candidate gene analysis showed no significant effect of the A-FABP alleles, including the A3 allele, on IMF content. This failure to detect an association between A-FABP alleles and IMF content in the present study might be due to the presence of eight distinct A-FABP microsatellite alleles in the Meishan crossbred pig population. Most likely, presuming a single mutation event, only two alleles are affecting a trait, in this case IMF content. Therefore, the high number of A-FABP alleles is unrealistic and reduces statistical power of the candidate gene approach. On the other hand, the use of more marker alleles or multiple marker haplotypes might avoid the possibility of linkage equilibrium between the marker site and the causative mutation (Templeton et al., 1987).

For BFT, no overall significant effect of A-FABP allele classes could be detected in the candidate gene analysis. However, the A-FABP A5 and A9 alleles were significantly associated with higher BFT. In contrast, no evidence for a QTL affecting BFT was found on SSC4 in the vicinity of the A-FABP gene in the QTL analysis. This difference between

methods might be due to a false assumption that QTL alleles were fixed in each population in the QTL analysis. Namely, both A-FABP alleles increase BFT with similar magnitude, but the A5 allele originates from the Meishan founder boars whereas the A9 allele from the western founder pigs. However, within-family QTL analysis (De Koning et al., 1999) instead of line-cross QTL analysis showed also no evidence for the presence of a QTL affecting BFT around the A-FABP gene on SSC4 in the Meishan crossbred population.

In contrast, others reported and substantiated QTL affecting BFT on SSC4 in Wild pig and Meishan crossbred populations (Andersson et al., 1994; Knott et al., 1998; Walling et al., 1998). According to the reported position of these QTL, A-FABP might be a candidate gene involved in genetic variation of BFT in these breeds or populations. The lack of evidence for a clear effect of A-FABP on BFT in the candidate gene analysis and QTL analysis in the Meishan crossbred population and also in the Duroc population (Gerbens et al., 1998b) might be due to differences in control of BFT between breeds and(or) that the QTL was not segregating in the selected breeds.

SSC6/ H-FABP: In general, screening for QTL on SSC6 should be given special attention because a specific mutation of the ryanodine receptor gene (Ryr-1) that resides on SSC6 might be segregating in the population under investigation. This mutation renders pigs halothane-susceptible, a condition that also affects performance and meat quality of pigs (Zhang et al., 1992). However, this mutation was not segregating in the Meishan crossbred population (Janss et al., 1997).

Results from the QTL analysis indicated a suggestive and chromosome-wise significant QTL affecting IMF content on SSC6. Although the highest peak is about 50 cM telomeric of the H-FABP gene, the test statistic at the H-FABP gene was still seen to exceed the suggestive threshold level. The wide region suggested to contain the QTL is very poorly covered with markers, indicating that the position of the QTL peak can still change considerably with additional data. In this respect, the H-FABP gene can still be responsible for the additive QTL effect of about one phenotypic standard deviation (.88% IMF) between the homozygotes for the Meishan and western pig alleles. However, the width of the QTL region also leaves the possibility of two or more QTL on SSC6.

Previously, De Koning et al. (1999) found evidence suggestive of QTL on SSC2, SSC4, SSC6, and SSC7, whereas only the QTL on SSC 6 reached chromosome-wise (P = .05) and even approached genome-wide (P = .13) significance. This result was confirmed by our study that contributed an additional marker and candidate gene to this interesting chromosomal region.

In the candidate gene analysis, also effects of the H-FABP alleles on IMF content were detected that approached significance. This effect of .36% IMF between either homozygous genotype class is high considering the overall mean of 1.84% IMF (SD .87%) in the Meishan crossbred population and is similar to a previously reported effect in a Duroc population (Gerbens et al., 1999). However, two essential differences are present between both studies. First, the candidate gene approach relies on the presence of localised linkage disequilibrium within a population at the level of the gene (Haley, 1999). In contrast to the Duroc outbred population, this linkage disequilibrium is expected to be much higher in the Meishan F_2 crossbred population; thus, associations may be due to loci some distance away from the H-FABP gene on SSC6 and(or) differences between founder breeds. Second, the significant association between genetic variation in the H-FABP gene and IMF content in a Duroc population was detected using H-FABP *Msp*I, *Hae*III, and *Hin*fI RFLP alleles as a source of genetic variation (Gerbens et al., 1999), whereas in the present study a dimorphic microsatellite within the H-FABP gene was used for practical reasons. These polymorphic sites are all located within a 10-kb region of the H-FABP gene and thus genetically closely linked. However, the observed effects with the microsatellite alleles in this study can not be ascribed to specific H-FABP RFLP alleles because linkage phases between these polymorphic alleles are not similar in both founder populations of the Meishan crossbreds (data not shown).

For BFT, results from both the QTL and candidate gene analysis in the Meishan crossbred population did not confirm the involvement of H-FABP in backfat accretion as found in Duroc pigs (Gerbens et al., 1999). The suggestive QTL affecting BFT at the end of SSC6, however, could be influencing the significant QTL affecting IMF found in the same region. The difference in both QTL peak positions suggests that the QTL affecting IMF is in fact two QTL of which the most telomeric is affecting both BFT and IMF and the other specifically IMF. Future analyses, by including BFT information in the QTL analysis of IMF content and additional genotyping information of the rest of the Meishan crossbred pig population may confirm this hypothesis.

In conclusion, results of the H-FABP gene in the Meishan crossbred population confirm previous results in Duroc pigs for IMF content but not for BFT (Gerbens et al., 1999). The fact that the effects on IMF are detected in two very distinct pig breeds/populations supports the hypothesis that the H-FABP gene rather than a closely linked gene will be responsible for these effects. Therefore, it will be of interest to evaluate whether these allelic variants are associated with differences in H-FABP gene expression and(or) H-FABP protein functionality. Two of the three RFLP together with the microsatellite sequence are located in

the second intron of the porcine H-FABP gene whereas the other RFLP site is located in the 5' untranslated region (Gerbens et al., 1997, 1998a). Both intronic sequences and 5' untranslated regions have been reported to affect gene expression. Therefore, one of the mutations that cause these H-FABP allelic variations might be responsible for the observed effects on IMF itself, although linkage with other sites of genetic variation in or near the H-FABP gene cannot be excluded and needs further investigation.

Implications

Findings from this study with Meishan crossbred pigs support the association of heart fatty acid-binding protein (H-FABP) genetic variation with intramuscular fat content, but not with backfat thickness, as found in a previous study with Duroc pigs. The evidence that the H-FABP gene is responsible for part of the genetic variation in intramuscular fat content in pigs can initiate the search for the causative mutation in this gene. Furthermore, presently known polymorphisms in the H-FABP gene can be implemented in marker-assisted selection to improve meat quality of pigs. However, before this implementation 1) the presence of the association, 2) the linkage phase between quatitative trait locus allele and H-FABP gene polymorphism, and 3) the linkage with the halothane allele, when present, should be established for the pig population under investigation.

In contrast, no evidence was found to support the role of the A-FABP gene in intramuscular fat content.

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Chapter 9

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

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Summary

Intramuscular fat (IMF) content is a major determinant of meat quality in pigs. Previously, polymorphisms in the adipocyte and heart fatty acid-binding protein, A-FABP and H-FABP, genes have been significantly associated with genetic variation of IMF 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 IMF 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 IMF content in longissimus lumborum muscle.

For H-FABP, statistical analyses showed significant differences in mRNA but not protein expression levels between H-FABP *Hae*III PCR-RFLP genotype classes. Between these genotype classes, significant differences in IMF content were found within barrows but not in gilts. Moreover, H-FABP mRNA but not protein expression levels are significantly related to IMF content.

For A-FABP genotype classes, no significant differences in mRNA and protein expression levels were found, however, a significant difference in IMF content was found within barrows but not in gilts. In addition, a significant relationship between A-FABP mRNA but not protein expression levels and IMF content was found.

In conclusion, variation of IMF 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

Introduction

Intramuscular fat (IMF) content is a major determinant of eating quality of pork and is highly heritable (reviewed by Hovenier et al., 1993). Significant associations between genetic variation at the A-FABP and H-FABP gene loci, FABP4 and FABP3, and IMF content in purebred Duroc pigs have been identified (Gerbens et al., 1998, 1999). Further analysis of pigs from a crossbred Meishan x Dutch White pig population supports the

involvement of H-FABP but not A-FABP in IMF accretion (Gerbens et al., 2000). However, despite these associations the effect of closely linked genes in both analyses can not be excluded.

In order to control IMF, 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 IMF accretion in pigs through RNA 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 x Dutch Landrace crossbred sows, were used. These boars and sows were selected based on heterozygosity for the H-FABP *Hae*III PCR RFLP (Gerbens et al., 1997) to produce litters containing all respective genotype classes. Offspring were genotyped for the H-FABP *Hae*III PCR RFLP. From each litter, at least three, randomly selected pigs belonging to different H-FABP *Hae*III PCR RFLP genotype classes were included in the analysis. None of the matings or individual pigs were selected based on their H-FABP *Msp*I 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 (BW) were recorded for each pig at slaughter. Furthermore, backfat thickness (BFT) 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 IMF content. A muscle sample was taken from this slice carefully avoiding intermuscular fat depots surrounding the muscle. The IMF 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 *Msp*I and *Hae*III PCR-RFLPs (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 was taken from the longissimus lumborum muscle using a shot biopsy method as described by Geverink et al. (1999). Biopsies were taken 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.

Total RNA extraction

From each biopsy sample, about 100 mg 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 two days 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 to its respective equivalent of cDNA.

PCR amplification and mRNA level quantification

Messenger RNA expression levels were quantified using RT-PCR-ELISA. An equivalent of one 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'ctgagattgccttcaaattg) and reverse (5'cttggcttatgctctctcata) primers were designed to amplify a 225-bp cDNA fragment that hybridizes to a biotin-labeled capture probe (5'ctggtacaggtgcagaagtg). Similarly, for H-FABP a forward (5'gagtttgatgagacaacagcag) and a reverse (5'tctttctcgtaagtgcgagtgc) primer were designed to amplify a 188-bp fragment that hybridizes to the capture probe (5'cttgtccacctgcagaagtg). For β-actin, forward (5'ggacttcgagcaggagatgg) and reverse (5'gcaccgtgttggcgtagagg) primers were described by Baarsch (1994) and the respective capture probe (5'gagtcctgcggcatccacgag) was designed according to the porcine β-actin mRNA sequence (GENBANK: U07786).

PCR amplifications were performed on 10 μL of cDNA template, i.e., equivalent to .05 μg total RNA, in a total volume of 50 μL containing .2 units AmpliTaq DNA polymerase (PE Biosystems, Foster City, CA) in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, .5 μM of each primer, .2 mM of dATP, dCTP and dGTP, .19 mM dTTP, and .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 since 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 (.01 units/mL) and its substrate ABTS allowed detection and colorimetrical 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 like PCR reactions without sample cDNA as well as H_2O and ABTS to account for background and standard controls to correct data from different microtitreplates. 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 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 three days later as for the A-FABP gene (n = 46). The repeatability estimate (see statistical analysis) of the entire PCR-ELISA procedure for H-FABP was .97 which addresses the intra-assay variability whereas for A-FABP the repeatability estimate of .87 addresses the inter-assay variability. These repeatability estimates agree well with the estimates from literature that range from .80 to .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 antiserum 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 muscle tissue was isolated from each muscle biopsy and homogenized in phosphate buffered saline (5%, wt/vol) using a Potter-Elvejhem 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 .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- 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 rat A-FABP or porcine H-FABP) and expressed in ng FABP per μg cytosolic protein. To determine repeatability (see next section) 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 .95 and addresses both intra and inter-assay variability whereas the repeatability estimate of the A-FABP ELISA assay (n = 159) was .82 which addresses inter-assay variability.

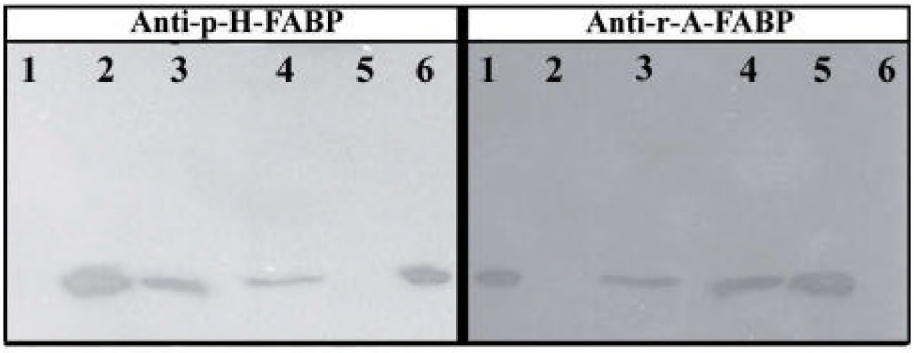

Figure 1: Specificity of affinity-purified anti-porcine H-FABP (p-H-FABP) and anti-rat A-FABP (r-A-FABP) antisera by Western blot analysis. Lanes 1 and 2 contain 50 (20 in right panel) ng rat A-FABP and porcine H-FABP, respectively; Lanes 3 and 4 contain 50 μg porcine muscle cytosolic proteins, Lane 5 and 6 contain 20 (50 in right panel) ng rat A-FABP and porcine H-FABP, respectively.

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

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 by 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, since diagnostic plots suggested that the variance increased with the mean. In 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.

IMF 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 BFT. Backfat thickness was included to account for its considerable genetic correlation with IMF content in pigs.

The relationship between IMF content and H-FABP or A-FABP mRNA and protein expression levels was studied with a mixed linear model, with IMF 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.

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.*

RFLP	Class	H-FABP mRNA[a]				H-FABP protein[a]				IMF content			
		N	Barrows	N	Gilts	N	Barrows	N	Gilts	N	Barrows	N	Gilts
HaeIII	DD	12	.25 (.02)^{b}	25	.26 (.02)^{b}	13	4.28 (.38)	25	4.08 (.36)	13	1.07 (.44)^{b}	24	.76 (.39)
	Dd	27	.24 (.01)^{c}	27	.25 (.01)^{c}	27	4.75 (.38)	30	4.53 (.37)	27	.90 (.33)^{b}	27	.80 (.28)
	Dd	9	.21 (.02)^{c}	18	.22 (.02)^{bc}	10	4.38 (.42)	18	4.18 (.39)	9	1.31 (.88)^{c}	16	.78 (.19)
MspI	AA	27	.24 (.01)	40	.25 (.01)	28	4.54 (.37)	42	4.30 (.35)	28	.87 (.07)^{b}	40	.85 (.06)
	Aa	18	.23 (.01)	25	.24 (.01)	19	4.54 (.40)	26	4.30 (.38)	19	.92 (.08)^{b}	23	.74 (.07)
	aa	3	.24 (.03)	5	.24 (.03)	3	4.77 (.69)	5	4.52 (.65)	2	2.60 (.24)^{c}	4	.94 (.17)

[a]mRNA expression levels are expressed in relative ELISA values per .05 μg total RNA and protein expression levels are expressed in ng H-FABP per μg cytosolic protein.

[bc]Within a column, for each H-FABP RFLP, means without a common superscript letter differ ($P < .05$).

Results

H-FABP analyses

Three H-FABP PCR-RFLPs (*Hae*III, *Hin*fI, and *Msp*I) 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 *Hae*III and *Msp*I PCR-RFLP alleles were segregating. Designed matings were used based on the *Hae*III PCR-RFLP to obtain expected balanced sample sizes and to estimate genotype effects within litters. The *Msp*I PCR-RFLP yielded unbalanced sample sizes because the linkage phase between the alleles of the *Hae*III and *Msp*I PCR-RFLP differs between experimental pigs.

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

Significantly lower H-FABP mRNA expression levels were found for the *Hae*III PCR-RFLP dd genotype class compared to the other genotype classes. This effect was irrespective of the gender of the animals. For comparison with the IMF 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 of the *Msp*I PCR-RFLPs (Table 1). In addition, for H-FABP protein expression levels no significant differences were observed between genotype classes of both H-FABP PCR-RFLPs (Table 1).

In an overall analysis a significant relationship between H-FABP mRNA expression levels and IMF content was found whereas no relationship was detectable between H-FABP protein expression levels and IMF content. The correlation between H-FABP mRNA and protein expression levels within the muscle sample was extremely low (.05).

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.*

Class	A-FABP mRNA				A-FABP protein				IMF content			
	N	Barrows	N	Gilts	N	Barrows	N	Gilts	N	Barrows	N	Gilts
A1A4	2	.26 (.04)	4	.25 (.04)	2	2.34 (.28)	4	2.18 (.26)	2	1.32 (.24)^{b}	5	.74 (.15)
A1A9	9	.21 (.02)	5	.20 (.03)	9	2.14 (.19)	5	1.99 (.19)	10	1.08 (.10)^{b}	5	.70 (.15)
A4A9	20	.23 (.02)	25	.23 (.02)	20	2.29 (.18)	26	2.13 (.17)	23	.77 (.07)^{b}	25	.86 (.07)
A6A9	3	.24 (.04)	8	.24 (.03)	3	2.56 (.27)	8	2.39 (.24)	2	2.60 (.24)^{c}	8	.95 (.12)
A9A9	9	.22 (.02)	22	.22 (.02)	9	2.38 (.21)	22	2.21 (.18)	7	.91 (.12)^{b}	18	.81 (.08)

[a]mRNA expression levels are expressed in relative ELISA values per .05 μg total RNA and protein expression levels are expressed in ng A-FABP per μg cytosolic protein.

[bc]Within a column, means without a common superscript letter differ ($P < .05$)

A-FABP analyses

For A-FABP, six microsatellite alleles were detected in the experimental population resulting in ten different genotype classes. Because mating of parental animals was based on H-FABP *Hae*III 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 IMF 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 *Msp*I PCR-RFLP aa genotype class, one of which has the highest IMF 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 IMF 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 (.09).

Discussion

Previous studies have demonstrated associations between polymorphisms in the A-FABP and H-FABP genes and IMF content in pigs (Gerbens et al, 1998, 1999). However, these analyses do not exclude other closely linked genes to be responsible for these effects on IMF 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, relationships were investigated between A-FABP and H-FABP polymorphisms, mRNA and protein expression levels and IMF 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 IMF 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 sexe effect. Interestingly, the dd and aa genotype classes of the H-FABP *Hae*III and *Msp*I PCR-RFLPs, respectively, also had the highest IMF content in these previous studies (Gerbens et al., 1999; Grindflek et al., 2000). With respect to the H-FABP *Hae*III PCR-RFLP, the dd genotype class has a significantly lower H-FABP mRNA expression level and a significantly higher IMF content in the longissimus lumborum muscle of barrows as compared to the DD genotype class. Although these findings may suggest a causal relationship between H-FABP mRNA expression level and IMF 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 IMF content was found. Therefore these results do not explain the association between H-FABP and IMF content in pigs. However, several reasons may be responsible for these inconclusive results which will be described in a following paragraph.

With respect to the A-FABP analyses, the A6A9 genotype class has a significantly higher IMF 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 are small and

unbalanced. Therefore these genotype classes may not represent the population as a whole. Previously, a considerable and significant contrast in IMF 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 IMF 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 IMF 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 IMF content was detectable, these results suggest no role for A-FABP in IMF 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 IMF content was demonstrated. Because no further evidence was found for the involvement of these FABP genes in IMF content, this relationship is most likely a consequence of higher fatty acid metabolism in the longissimus lumborum muscle. Probably, genes involved in fatty acid metabolism like FABPs are up-regulated in cells or tissue containing a higher concentration of fatty acids (i.e., IMF content).

Results from the current study do not support the involvement of either A-FABP or H-FABP mRNA or protein expression level in IMF 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 IMF 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. The absence of significant differences in protein expression levels therefore suggests no role for mRNA transcript quality as a possible explanation for genetic variation in IMF content.

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, IMF content is a trait that results from fat accretion during 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 lifetime of a growing pig is responsible for genetic variation in IMF 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 IMF 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 (Veerkamp 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 IMF 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 gene expression are translationally rather than transcriptionally regulated. In this respect, the fact that FABPs 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 was presented (Binas et al., 1999). 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 IMF 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 IMF 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 IMF 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.

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Chapter 10

General discussion

General discussion

The fields of animal genomics and genetics are ever evolving faster and faster. Recent technological advances are improving, especially the accessibility of the genome in identifying genes, fast and efficient screening of genetic variation present in the genome, gene expression profiling, and statistical methods to identify and use genomic regions of particular interest for breeding purposes. The study described in this thesis contributes to these fields but also to a better understanding of the role of FABPs in a broader perspective. In this chapter results that have been achieved in this study will be evaluated and discussed in retrospect with the original objectives and future directions will be given. First the methodological aspects of this study will be discussed followed by a discussion of the main findings. Finally, the main conclusions and implications are presented.

Methodological aspects

Meat quality

The rationale of the study described in this thesis is that the intramuscular fat (IMF) content is one of the most important traits that influences meat quality in pigs. In particular, eating quality traits like meat tenderness, juiciness and taste, as assessed by consumer or trained panels, are correlated with IMF content of pig meat (reviewed by Hovenier et al., 1993 and Verbeke et al., 1999). Other studies have confirmed this relationship as well (Kirchheim et al., 1997; Fernandez et al., 1999). The need for improving IMF content in pigs is evident when the optimal range of IMF content is 2.5-3% (Bejerholm and Barton-Gade, 1986; Kirchheim et al., 1997) while the IMF content of meat produced in Europe nowadays is generally well below this threshold (Casteels et al., 1995, Cameron et al., 1999b; Von Rohr et al., 1999 and references therein). Various strategies to improve IMF content and other meat quality traits in pigs have been reviewed by Verbeke and coworkers (1999). This thesis adds another possible strategy to their list by means of the genetic tests for H-FABP and A-FABP.

Ultimately, the implementation of genetic tests to improve meat quality depends on the willingness of consumers to purchase and pay more for meat of better quality. To market meat with better eating quality, consumers need to be able to discriminate between different categories of eating quality, thus favoring meat grading, branding and labeling. It was concluded from a survey among Dutch consumers that the willingness-to-pay extra for meat with improved eating quality depends on the kind of meat (e.g. chops or steaks), the attitude

towards quality and the age of consumers (Steenkamp and Van Trijp, 1988). Recently, a survey among Swiss meat quality experts from slaughter and retail industry reported a willingness-to-pay different prices for carcasses from different quality classes for several meat quality traits among which IMF content (Von Rohr et al., 1999).

Intramuscular fat content

With respect to the candidate gene approach, prior information is the most essential part of the selection of candidate genes. In the case of IMF content, a lot of information exists on the associations with meat quality traits (Verbeke et al., 1999), however closer examination into the factors that determine these associations is surely appreciated. For example, the relationship between IMF content and tenderness in cattle can be explained by the development of adipocytes between muscle fibres and hence disorganizing the structure of intramuscular connective tissue (Nishimura et al., 1999). This phenomenon depends on the muscle and the age of the animal which may explain the contradictory results for associations between IMF content and tenderness between studies in pig (reviewed by Hovenier et al., 1993).

Regarding the association of IMF content with flavor and taste, this is believed to be largely due to the amount and chemical composition of IMF. However, identification of the specific fatty acids or fatty acid analogs would surely reduce the number of biochemical pathways and hence the number of candidate genes. Cameron and coworkers (1999a) showed that fatty acid composition of IMF and eating quality differ in pigs due to the different selection strategies for lean growth rate. Unfortunately, they did not examine the relationship between fatty acid composition of IMF and eating quality of the meat but they showed that these responses are of genetic and environmental origin. In addition, (positional) candidate genes can be identified by genome scans. Perez-Encisco and coworkers (2000) report QTL affecting the proportion of linoleic acid in backfat, BFT and other traits on SSC4 near the FAT1 locus (Andersson et al., 1994). However, it is unclear whether the proportion of linoleic acid in backfat is elevated due to the increased BFT. For cattle, fatty acid proportions in TG of subcutaneous fat were shown to be heritable but unfortunately relationships with sensory traits were not reported (Malau-Aduli et al., 1998).

The candidate gene approach.

The success of the candidate gene approach largely depends on the amount of prior information, in particular information and similarities from studies in other, evolutionary closely related species. Until now, a number of candidate gene approaches in livestock have been successful in identifying linkage association (ESR, A-FABP, H-FABP, FUT1, MC4R,

MYOG, PRLR) or even causative relations (GDF-8, Ryr-1, KIT, MSHR) (reviewed by Rothschild, 1998). In these latter cases the prior evidence that these proteins would be excellent candidates in pigs came predominantly from studies in other species. The myostatin gene (GDF-8) was identified in mice as a member of the transforming growth factor-beta (TGF-beta) superfamily that, when disrupted, showed a marked increase in skeletal muscle mass (MacPherron et al., 1997). This phenomenon resulted in the identification of five mutations that disrupt the bovine myostatin gene and thus cause the "double muscling" phenotype in cattle (Grobet et al., 1997 and 1998; Kambadur et al., 1997).

The most extensively studied and discussed major gene in pigs is the Halothane gene (Hal) which causes stress susceptibility and concomitantly inferior meat quality. This malignant hyperthermia syndrome (MHS) was identified as an autosomal recessive inherited trait in man and pigs. The responsible C to T point mutation at nucleotide 1843 in the ryanodine receptor (Ryr-1) gene of the pig was discovered (Fuji et al, 1991). This allowed the identification of all three genotypes and subsequently demonstrated the pleiotropic effects of this allele on lean meat content and meat quality (reviewed by Sellier, 1998). These studies have led to recommendations to eradicate the recessive allele (NPPC, 1995) whereas others propose breeding strategies to use the beneficial effects of this allele (De Vries and Plastow, 1998).

The hypothesis that A-FABP and H-FABP may be involved in the genetic variation in intramuscular fat (IMF) content in pigs has been argued for in Chapter 2. Since differences in IMF content can be explained by variation in the amount of TG (see Chapter 2), fatty acid uptake and trafficking are the most promising processes to study. However, a lot of proteins are involved in these processes which makes the selection of candidate genes a hazardous task in case no additional information is available. Moreover, the list of potential candidate genes will increase substantially as more and more genes are being identified. A considerable number of proteins that are physically involved in fatty acid uptake and trafficking have already been elucidated and in some cases shown to be rate-limiting (reviewed by Abumrad et al., 1999; Luiken et al., 1999). On the other hand, information on the factors that regulate these genes like the PPAR family and long-chain fatty acids is just emerging (Bastie et al., 1999). Interestingly, the PPARγ gene was shown to be associated with several meat quality traits but not IMF content in pigs (Emnett et al., 2000). Others have described a considerable number of genes involved in human adipocyte differentiation and obesity (Hwang et al., 1997; Bray and Bouchard, 1997). Furthermore, the lamin A/C gene (LMNA) which causes partial lipodystrophy syndromes in man (Shackleton et al., 2000) may also give insights in the factors regulating site specific fat deposition.

Insulin resistance may also be important because this is associated with an increased amount of TG content in skeletal muscle. Some proteins have been shown to be involved in the mechanisms that lead to insulin resistance like TNF-α and both its receptors, A-FABP and possibly free fatty acids and leptin (reviewed by Hotamisligil, 1999; Scheja et al., 1999). Indeed, a TNF-α promoter polymorphism was associated with obesity in women but not in men (Hoffsted et al., 2000) and the region containing the TNF-α gene on SSC7 is implicated in the variation of BFT in various pig populations (Wang et al., 1998 and references therein). However, there is evidence that insulin resistance is a consequence of elevated TG content and not the cause (reviewed by Goodpaster and Kelley, 1998).

Methodological aspects of the candidate gene approach

There are three practical considerations as to association studies: (1) the stringency of the significance thresholds applied, (2) the presence of distinct subpopulations within the experimental population and (3) the extent of linkage disequilibrium in the population under investigation (Lander and Schork, 1994; Haley, 1999). As to the first, nominal significance thresholds are often applied in candidate gene studies whereas often several or many candidate gene are studied simultaneously. Therefore, Lander and Kruglyak (1995) argued that significance thresholds should be adjusted for multiple testing as for a genome scan. The studies presented in this thesis all have used the nominal significance threshold and did not account for the testing of multiple polymorphic sites within two genes, H-FABP and A-FABP, and several traits. Based on the amount of tests performed (20), the applied nominal significance threshold of 5% and assuming independence of the tests, at least one significant result may be a false positive. However, this is an overestimate because, in reality, tests were not independent and we estimate that there is a non-negligible chance for at maximum one false positive in this entire study. This, however, does not undermine the general findings of the association studies described in this thesis.

Secondly, population admixture may also result in false positive associations. This problem can be circumvented by performing association analysis within families that are segregating for the polymorphism as was done for the Duroc and Meishan crossbred populations (Chapters 5, 6 and 8).

Another problem in candidate gene studies may be the prevalence of linkage disequilibrium. The extent of linkage disequilibrium determines the size of the region around the causative mutation that will still give significant associations. Linkage disequilibrium is caused by forces like selection, small populations and hybridization which all are common to livestock breeding (Haley, 1999). Additional genotyping with closely linked markers would allow the extent of linkage disequilibrium to be estimated. This kind of analysis has not been performed for the H-FABP and A-FABP genes in the association

studies described in this thesis. However, such an approach should be part of future candidate gene studies in case linkage disequilibria in a population are unknown or expected to extend over relatively large regions.

The future of the candidate gene approach

Completion of the genome sequencing projects for man, mice and other organisms in the next years in conjunction with technological breakthroughs like high throughput RNA transcript and protein expression analysis will have a major impact on candidate gene identification. The complete genome sequences will reveal all genes that add to an organisms genotype and phenotype. This will enormously improve the identification of positional candidate genes for chromosomal regions that are involved in particular traits. Moreover, thorough investigations will reveal the functional properties and the contribution of each gene to particular physiological pathways. This development will boost the identification of candidate genes provided that the trait has been characterized sufficiently.

Finally, the development of single nucleotide polymorphisms (SNPs) detection methods together with the powerful DNA chip technology will cause rapid generation of large amounts of genetic data. The big challenge in biomolecular sciences and genetics will be the development of highly sophisticated bioinformatic tools to compile, manipulate and analyse this SNP and high throughput gene expression data to discover the genes that contribute to a specific trait.

Main findings and perspectives in relation to other studies

Characterization of heart and adipocyte fatty acid-binding protein genes.

In Chapter 2 the role of H-FABP and A-FABP proteins in fatty acid transport in myocytes and adipocytes has been described. It has been argued that one or both genes encoding for H-FABP (*FABP3*) or A-FABP (*FABP4*) may be involved in the genetically determined differences in IMF content in pigs. Therefore, the *FABP3* and *FABP4* candidate genes were first isolated and characterized as described in Chapter 3 and 6, respectively. The structural organization of both genes, e.g., four exons and three introns, is similar to all FABP genes currently known except for the locust H-FABP gene (see Chapter 2, table 2). The size of each exon is also conserved across orthologous genes whereas between paralogous genes minor differences exist in exon length (see Chapter 2). Furthermore, the chromosomal localizations of the porcine *FABP3* and *FABP4* genes to SSC6 and SSC4 by radiation hybrid analysis (Chapter 3 and 6) and later, more accurately, by linkage analysis

(Chapter 7) are also in good agreement with comparative mapping data from man and mice (Rettenberger et al, 1995; Goureau et al. 1996).

To perform association studies it is essential to identify polymorphisms within or near these FABP genes. No effort was taken to specifically identify polymorphisms in the coding or regulatory regions of both genes. Although such polymorphisms may have possible biological relevance, for association analyses closely linked polymorphisms are sufficient.

Differences in the phase between polymorphic alleles and the causal mutation may, however, affect the result of association analyses. Hence, several polymorphisms were identified in the H-FABP and A-FABP gene to improve chances to identify associations. Three RFLPs and a dimorphic microsatellite marker were identified in and near the H-FABP gene (Chapters 3 and 4). For A-FABP, an RFLP and a microsatellite marker were identified (Chapters 6 and 7). In the candidate gene analyses with the FABP genes (Chapters 5, 6 and 8) we used each polymorphism separately. Soller and Genzini (1998), on the other hand, indicate that a haplotype-based analysis would improve the power of candidate gene analyses. However, the design of our experiments, i.e. selection of heterozygous boars and sows, excludes the identification of recombinant haplotypes and hence a re-analysis of our data. The A-FABP microsatellite marker has revealed more than 12 alleles already and therefore is highly useful for linkage analysis studies. On the other hand such a highly polymorphic marker reduces the power of an association analysis because of the increased number of classes and hence the reduced number of individuals per class.

The relation of H-FABP to intramuscular fat content.

Heart-FABP was significantly associated with genetic variation in IMF content, backfat thickness and growth in purebred Duroc pigs (Chapter 5). Others identified a QTL for IMF content on SSC6 in a Duroc/Norwegian Landrace backcross population. This QTL region contains the H-FABP gene and subsequent analysis showed that H-FABP genotypes have a significant effect on IMF content corresponding with our results (Grindflek et al., 2000). Similarly, in Meishan/Large White crossbred pigs a suggestive QTL for IMF content was detected on SSC6 for the line-cross analysis (De Koning et al., 1999). Jointly, we showed that the H-FABP gene mapped to this QTL region and found a considerable difference in IMF content between H-FABP microsatellite genotypes that approached significance (Chapter 8). Recently, De Koning and coworkers (2000) found evidence for a maternally as well as a paternally imprinted QTL for IMF content on different parts of SSC6. Interestingly, the position of the paternally imprinted QTL was relatively close to the H-FABP gene. In another Meishan/Large White crossbred pig population, a QTL affecting IMF content was reported for SSC7 but not for SSC6 (Bidanel et al., 1998). However,

results from QTL studies should be interpreted cautiously because they may vary according to the prior assumptions in the analyses (De Koning et al., 1999) as well as due to the genetic background of the pigs under investigation.

Taking all data into account, we can conclude that the H-FABP gene or a closely linked gene may be responsible for part of the genetic variation in IMF content in pig breeds or populations. The existence of significant associations in two independent pig populations strongly favors H-FABP as opposed to closely linked genes.

Obviously, a number of genes have been shown to be closely linked to the H-FABP gene in humans and mice and others will be identified in the near future. One particular gene should be highlighted, the leptin receptor gene (LEPR) which mediates the effects of leptin, the major hormonal controller of long-term energy balance (Friedman and Halaas, 1998). The porcine leptin receptor (LEPR) has been mapped to the distal part of the long arm of SSC6 (Ernst et al., 1997) and like the H-FABP gene, has its human counterpart on chromosome 1 (HSA1). However, so far it is unknown whether the H-FABP gene resides on the distal or the proximal region of the long arm of SSC6 which both are syntenic with HSA1. To exclude the LEPR gene as being responsible for the effects observed for H-FABP, this latter gene should be fine mapped relatively to the LEPR gene.

In order to find the responsible mutation either the level of expression or functionality of H-FABP should be analyzed. In chapter 9 we conclude that the H-FABP mRNA and protein expression were not involved in IMF content either directly or by means of different H-FABP genotypes in the pig population under investigation. However, it is unclear whether (1) the association between H-FABP and genetic variation in IMF content exists in this pig population and (2) this conclusion also is applicable to other pig populations. Ideally, the Duroc pig population that was segregating for the IMF related H-FABP alleles should have been subject for the investigation of FABP expression. However, unfortunately this pig population was eradicated due to the 1997 classical swine fever outbreak in The Netherlands. The sensitivity of the assays used may also have been another reason for not detecting functional differences in mRNA or protein expression levels. On the other hand, the sensitivity of the assays may have been sufficient but possibly functional differences in mRNA or protein expression levels were very small and would need a larger number of pigs to be detected. Another problem with the study described in Chapter 9 is the timing during pig growth that H-FABP expression was assessed. The moment of measuring H-FABP expression may be crucial to the outcome and this moment obviously depends on the moment genetic variation in IMF content is induced and the time this is prolonged during the development and growth of pigs. However, this has never been studied and therefore the most appropriate time for assessing H-FABP mRNA or protein expression, or both is not known.

With respect to H-FABP functionality, screening for nucleotide substitutions, deletions, or insertions causing naturally occurring H-FABP variants in pigs has been disregarded due to limitations of time. Such H-FABP variants may however exist in pigs because naturally occurring protein variants were identified for human I-FABP and human and cattle H-FABP (see Table 3, Chapter 2). So far, only I-FABP variation has been shown to exert an effect. In general I-FABP polymorphisms affect energy metabolism in several ethnic populations (reviewed by Hegele et al., 1998).

The relation of A-FABP to intramuscular fat content.

In the Duroc population A-FABP genotype classes differed significantly in IMF content (Chapter 6). Although the estimated difference of 1% IMF equals one phenotypic standard deviation, the favorable genotype class was represented by only six animals. It was argued that this association with IMF content was independent from backfat thickness and body weight of the animals. In the Meishan/Large White pig population a suggestive QTL for IMF content were detected in both the line-cross and half-sib analysis, among others, on SSC4 but at different parts of the chromosome (De Koning et al., 1999). This line-cross QTL and the A-FABP gene were identified in the same marker interval, however A-FABP is not the gene responsible for this QTL as shown in Chapter 8.

So far, other QTL mapping studies did not report significant QTL affecting IMF content on SSC4. However, the involvement of SSC4 in fat deposition has been recognized in several studies. Andersson and coworkers (1994) reported QTL for fatness, both BFT and abdominal fat, on SSC4 in Wild pig/Large White crossbred pigs that were confirmed in a more complete analysis (Knott et al., 1998). However, subsequent analysis in this pig population revealed that A-FABP resides in a flanking marker interval and hence is less likely to be a candidate gene for this backfat QTL (Andersson, personal communication). Furthermore, this QTL on SSC4 was also identified in Meishan/Large White crossbred pigs (Walling et al., 1998). In mice, several QTL affecting fatness traits (West et al., 1994; Brockmann et al., 1996) have their porcine counterparts most likely also on SSC4. In a Large White selection line a QTL affecting growth performance was found near the A-FABP gene (Bink et al., 2000).

In conclusion, the involvement of A-FABP in the genetic variation of IMF content in Duroc pigs is not substantiated in other pig populations. Therefore we can not discriminate between A-FABP and closely linked genes to account for the effect on IMF content in the Duroc pig population. The effect of A-FABP on IMF content in the Duroc pig population was independent from BFT as shown in Chapter 6. Therefore this effect is less likely to be due to genetic correlations with other fatness traits despite the fact that the A-FABP region

on SSC4 is involved in variation of fatness traits in pigs and other species (Andersson et al., 1994; West et al., 1994; Brockmann et al., 1996; Walling et al., 1998). Finally, the absence of a significant relationship between A-FABP mRNA or protein expression levels and IMF content (Chapter 9) is of little relevance to this discussion for similar reasons as described for H-FABP in the previous section.

Marker-assisted selection.

Upon identification, polymorphic genes or genomic regions may be applied in breeding by eliminating the detrimental allele(s) from the population, by marker-assisted selection (see Meuwissen and Goddard, 1996; Spelman and Bovenhuis, 1998) or by introgression of the beneficial allele(s) in populations or breeds lacking these alleles (Visscher et al., 1996). Marker-assisted selection can considerably increase the selection response in particular for traits with a low heritability and/or carcass and meat quality traits, like IMF content (Meuwissen and Goddard, 1996). Earlier in this discussion it was concluded that the H-FABP locus on SSC6 is involved in the variation of IMF content in pigs. Therefore, the H-FABP polymorphisms can be implemented in marker-assisted selection to improve IMF content and growth. In this respect the following points should be determined: 1) the presence of association in the respective pig population, 2) the absence of unfavorable associations with important performance traits, 3) the linkage phase between the mutation responsible for the genetic variation in IMF content and H-FABP alleles, and 4) the linkage with the halothane allele, when present.

The rationale for the first point is that the association between H-FABP polymorphisms and IMF content is unlikely to be present in every pig population. This opinion is based on the results from various QTL mapping studies in pigs. These studies map QTL affecting the same trait at different genomic positions in different but also similar pig populations. Therefore, for each population the presence of the association has to be established before marker-assisted selection can be initiated.

Besides this association, effects of H-FABP polymorphisms on performance traits, most notably BFT, should also be evaluated. For example, H-FABP polymorphisms are significantly associated with IMF content, BFT and growth in Duroc pigs (Chapter 5). Nevertheless, here, the effects on IMF content and BFT are largely independent and the favorable effect on growth coincides with the favorable effect on IMF content.

As to the third, H-FABP polymorphisms merely function as a marker because it is unlikely that these polymorphic sites are responsible for the genetic variation in IMF content. For this reason, recombination between these polymorphic sites and the responsible

mutation can occur in pigs and result in a switch from favorable to unfavorable selection responses. To avoid this, it is recommended to regularly evaluate the association.

Similarly, the linkage phase with the halothane allele, when present, should be established because both genes reside on SSC6. Although the halothane allele has been eliminated from most commercial breeding populations due to its deleterious effect on meat quality in homozygous pigs, some breeding organizations have implemented strategies to make use of the beneficial effects of this allele on lean growth by means of heterozygous pigs (De Vries and Plastow, 1998).

Finally, the following should be considered as well. Heart-FABP is predominantly expressed in skeletal and cardiac muscle cells but to a lesser extent also in various cell types of other tissues like kidney, stomach, mammary gland, ovary, testis, lung and brain tissue (reviewed by Veerkamp and Maatman, 1995 and Hohoff and Spener, 1998). Selection for a higher IMF content in pigs by means of the H-FABP polymorphisms may also affect fatty acid metabolism in these non-muscle tissues. In this respect, the involvement of FABP in fatty acid-mediated regulation of gene expression, and other metabolic and signalling processes (Veerkamp et al., 1993; Graber et al., 1994; Glatz et al., 1995; Veerkamp en Maatman, 1995; Glatz and Van der Vusse, 1996 and references therein) may be of significance. However, these genetic variants are segregating in pigs already and therefore changes in fatty acid metabolism due to selection are merely part of the normal genetic variation in each tissue or cell type.

Conclusions and implications

Heart fatty acid-binding protein

We conclude that H-FABP is responsible for part of the genetic variation in IMF content in pigs. However, we can not fully exclude the effects of closely linked genes. Hence, further examination of this chromosomal region and the identification of the causal mutation is recommended.

Despite the absence of a causal relationship, this finding allows the use of the H-FABP polymorphisms in pig breeding programs through marker-assisted selection to improve meat quality and possibly growth. This marker-assisted selection by means of H-FABP polymorphisms requires (1) the presence of association in the respective pig population, (2) the absence of unfavorable associations with important performance traits, (3) a continuous estimation of the linkage phase between the mutation responsible for the genetic variation in IMF content and the H-FABP alleles, and (4) the linkage with the halothane allele, when present.

Adipocyte fatty acid-binding protein

With respect to A-FABP, an association with IMF content exists in Duroc pigs. Nevertheless, no additional evidence for a role of A-FABP has been found, so far. Further investigation into this relationship in other pig populations is needed to validate these results and exclude closely linked genes.

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Summary

The intramuscular fat (IMF) content of pig muscles is important for meat quality traits like tenderness, juiciness and taste (Chapter 1). Currently, the IMF content has dropped below the optimal threshold for tenderness. Despite the high heritability of IMF content in pigs, conventional breeding strategies to improve IMF content in pigs are not very effective (Chapter 1). Novel strategies that exploit the genetic control of this trait directly are a suitable alternative. The aim of the studies described in this thesis is to investigate the contribution of the genes encoding for heart and adipocyte fatty acid-binding proteins, H-FABP and A-FABP, to the genetic variation in IMF content in pigs. The role of H-FABP and A-FABP in IMF deposition has been reviewed in Chapter 2. The candidate gene approach has been applied to establish the contribution of both genes to the genetic variation in IMF content.

H-FABP

In chapter 3, we describe the isolation of the H-FABP gene (*FABP3*) of the pig. The coding region and the deduced amino acid sequence of the H-FABP gene of the pig reveals high homology with orthologous genes from human, cattle, mouse and rat. The assignment of the H-FABP gene to chromosome 6 of the pig was in agreement with comparative mapping data from human and mouse. This assignment was also confirmed by linkage analysis with other chromosome 6 markers (Chapter 8). The H-FABP gene is mapped in the region flanked by the markers Sw316 and S0003.

Further DNA-sequence analysis showed that three RFLPs and a dimorphic microsatellite sequence are present in the H-FABP gene of the pig (Chapter 3 and 4). These polymorphisms are essential to study the association between the H-FABP gene and IMF content. It is assumed that the functional polymorphism (which contributes to the genetic variation in IMF content) is tightly linked to and in linkage disequilibrium with the RFLPs and microsatellite marker in the H-FABP gene.

These polymorphisms were used in two association studies involving an outbred Duroc pig population and a Meishan × Large White crossbred population. In the Duroc pigs significant differences were found between genotype classes for IMF content, backfat thickness (BFT) and growth but not for drip loss of the meat (Chapter 5). The favorable genotypes had higher IMF content, grew more rapidly but also had an increased BFT. Further analysis showed that the effect on IMF was not attributable to differences in BFT.

In the Meishan × Large White crossbred population two types of analysis were performed: (1) the microsatellite polymorphism in the H-FABP gene was included in the

chromosome scan analysis and (2) the effect of both H-FABP alleles was analyzed directly (Chapter 8). The chromosome scan revealed a suggestive and chromosome-wise significant quantitative trait locus (QTL) for IMF content on chromosome 6 of the pig. The effect of this QTL may be due to H-FABP because the H-FABP gene is part of this QTL. The second approach showed a considerable but nonsignificant effect of the H-FABP microsatellite alleles on IMF content. Furthermore, suggestive evidence for a QTL affecting backfat thickness (BFT) was found on chromosome 6, but H-FABP was excluded as a candidate gene based on the distinct positions of these loci.

Thus, these findings indicate a role for H-FABP or a closely linked gene in the genetic variation of IMF content in pigs. To exclude the role of closely linked genes we investigated the relationship between H-FABP polymorphisms, H-FABP gene expression and IMF content in pigs. Ideally, the H-FABP polymorphisms would affect the H-FABP gene in a physiologically meaningful way. Because of the positions of these polymorphisms in intron and promoter sequences, H-FABP mRNA and protein expression was studied in stead of functional differences of H-FABP. Differences in H-FABP mRNA transcript levels but not protein levels in pig muscle tissue were identified between RFLP genotype classes (Chapter 9). Similarly, IMF content was associated with H-FABP mRNA transcript levels but not with H-FABP protein expression levels. Because, in general, protein molecules rather than mRNA molecules affect the phenotype it was concluded that results from this study do not support the role of H-FABP expression level in IMF content. This may be due to the selection of an inappropriate time for assessing H-FABP mRNA transcript and protein levels and(or) limitations as to the sensitivity of the assays used. Moreover, this study was performed in crossbred pigs different from the Duroc population that initially revealed the association between H-FABP and IMF content. Since then others have also found evidence for the involvement of H-FABP in IMF content in a Duroc × Norwegian Landrace pig population.

Based on these findings, we conclude that the H-FABP locus is responsible for part of the genetic variation in IMF content in pigs. However, we can not fully exclude the effects of closely linked genes. Further analysis of this region on chromosome 6 of the pig may lead to the identification of the responsible polymorphism(s), most likely in or very close to the H-FABP gene. These findings support the implementation of the H-FABP polymorphisms to improve meat quality in pigs by marker-assisted selection. However, before this implementation the presence of the association and the linkage phase between QTL and H-FABP alleles should be established in the pig populations under investigation.

A-FABP

In chapter 6, the isolation of the A-FABP gene has been described. The gene encodes A-FABP based on the high homology of the coding region and deduced amino acid sequence with orthologous genes from human, mouse and rat. The A-FABP gene maps to the S0001-S0217 marker interval on chromosome 4 of the pig (Chapter 8) which is in agreement with comparative mapping data from human and mouse.

A highly polymorphic microsatellite sequence was identified in the first intron of the pig A-FABP gene (Chapter 6). Moreover, within this microsatellite sequence an RFLP was found which is decribed in Chapter 7.

The microsatellite polymorphism was used for association studies using the same pig populations as described for the H-FABP gene. In the Duroc pig population, significant differences in IMF content and possibly growth were found between A-FABP genotype classes. No differences in BFT and drip loss of the meat were detected. In the Meishan × Large White crossbred pig population no evidence was found for an effect of the A-FABP gene on IMF content and BFT.

Different genotype classes according to this intronic microsatellite showed no differences in A-FABP mRNA transcript and protein level (Chapter 9). However, the difference in pig populations under investigation, the moment to assess the A-FABP mRNA transcript and protein level and the sensitivity of the assays used, may have been crucial to the outcome.

In conclusion, the A-FABP locus and IMF content are associated in Duroc pigs. Nevertheless no additional support for the role of A-FABP has been found. Hence, further investigations into this relationship in other pig populations are needed to validate these results and exclude closely linked genes.

Samenvatting

Varkens die geproduceerd worden in Nederland voor de vleesconsumptie zijn kruisingen van verschillende selectielijnen afkomstig van varkensfokkerij organisaties. Deze fokkerij organisaties leveren enerzijds de moederdieren en anderzijds sperma van beren aan vermeerderingsbedrijven die op hun beurt biggen produceren voor de afmestbedrijven. De moeder- en vaderdieren zelf zijn in veel gevallen ook kruisingen van zuivere selectielijnen. Binnen deze selectielijnen wordt selectie op verschillende kenmerken toegepast.

Zodra een kenmerk overgedragen kan worden van ouder op nakomeling oftewel erfelijk is, kan een dergelijk kenmerk in selectie of fokprogramma's meegenomen worden. De fokkerij beoogt die dieren te selecteren die de beste genen hebben, waarbij in het algemeen de erfelijke aanleg van een dier voor een bepaald kenmerk bijvoorbeeld, rugspekdikte of aantal biggen per worp, door middel van kwantitatieve genetische analyse geschat wordt.

De fokkerij is zeer succesvol geweest met gemiddelde verbeteringspercentages van 1 à 2% per jaar voor kwantitatieve kenmerken als groei en spekdikte en karkas parameters. Deze successen werden behaald zonder enige kennis van de genen die verantwoordelijk zijn voor een bepaald kenmerk. In vele gevallen, met name voor gemakkelijk meetbare kwantitatieve kenmerken is deze kennis van generlei noodzaak. Echter in bepaalde gevallen kan het voordelig zijn om de genen te kennen die verantwoordelijk zijn voor de erfelijk bepaalde verschillen tussen dieren. Dit geldt bijvoorbeeld voor kenmerken die maar door één of een beperkt aantal genen bepaald worden zoals respectievelijk stressgevoeligheid en huidkleur. Bovendien geldt dit voor kenmerken die lastig te meten zijn of die pas na een lange tijd gemeten kunnen worden zoals respectievelijk, intramusculair vetgehalte en worpgrootte.

Het onderzoek dat in dit proefschrift beschreven is, is uitgevoerd in het kader van het identificeren van de genen die verantwoordelijk zijn voor de verschillen in erfelijke aanleg van het intramusculair vetgehalte bij varkens. Het intramusculaire vetgehalte oftewel de hoeveelheid vet binnen de spier is één van de belangrijke kenmerken die betrokken zijn bij vleeskwaliteit. Het is aangetoond dat het intramusculaire vetgehalte de malsheid en sappigheid maar ook de smaak van varkensvlees kan beïnvloeden. Bovendien is het intramusculaire vetgehalte van varkensvlees in Europese landen beduidend lager dan het optimale gehalte dat benodigd is voor een aantrekkelijk en mals lapje varkensvlees. Het verbeteren van het intramusculaire vetgehalte van varkens door middel van fokkerij is mogelijk aangezien dit kenmerk een hoge mate van erfelijke aanleg heeft. Echter het intramusculaire vetgehalte van varkens kan pas bepaald worden na het slachten en dit betekent dat het desbetreffende dier niet meer voor selectie in aanmerking komt. Dus zal

selectie van naaste familieleden moeten plaatsvinden hetwelk de effectiviteit van de selectie nadelig beïnvloedt. In dit geval zou het handig zijn wanneer bekend was welke genen verantwoordelijk zijn voor de verschillen in de erfelijke aanleg voor het intramusculaire vetgehalte zodat dieren vlak na hun geboorte geselecteerd kunnen worden op de aanwezigheid van de gewenste genen.

Het intramusculaire vetgehalte is een kenmerk dat hoogstwaarschijnlijk door een groot aantal genen beïnvloed wordt waarvan een klein aantal een relatief groot effect heeft in vergelijking met de rest. Deze genen met een relatief groot effect zijn natuurlijk het meest interessant om toegepast te worden in de varkensfokkerij en kunnen op twee manieren ontdekt worden. De eerste manier maakt gebruik van merkers, dit zijn over het algemeen anonieme stukjes DNA die over alle chromosomen verdeeld liggen. Simpel gezegd, door de overerving van deze merkers te vergelijken met de overerving van het kenmerk intramusculair vetgehalte kunnen redelijkerwijs die delen van de chromosomen bepaald worden waar verantwoordelijke genen liggen. Dit wordt de "genoom scan" benadering genoemd.

De andere benadering is de kandidaatgen benadering. Hierbij worden belangrijke genen voor het kenmerk geïdentificeerd op basis van bestaande wetenschappelijke kennis op, onder andere, het gebied van de fysiologie en biochemie. Vervolgens wordt het effect van deze genen op het kenmerk bepaald. Deze methode is toegepast bij de studies die in dit proefschrift beschreven zijn.

Voor het intramusculaire vetgehalte werden twee genen als kandidaten beschouwd namelijk de genen die respectievelijk coderen voor hart (spier) en vetcel vetzuurbindend eiwit, H-FABP en A-FABP. De familie van cytosolische vetzuurbindende eiwitten bestaat momenteel uit negen geïdentificeerde eiwitten. Van al deze vetzuurbindende eiwitten zijn juist H-FABP en A-FABP interessant vanwege hun aanwezigheid in respectievelijk spier- en vetcellen, de voornaamste celtypen van spierweefsel. Het proces van selectie en de verantwoording voor de kandidaatstelling van deze genen is uitvoerig beschreven in Hoofdstuk 2 aan de hand van de wetenschappelijke literatuur. Vetzuurbindende eiwitten bevinden zich in het cytosol van de cel en zijn betrokken bij het transport van vetzuren van de celmembraan naar plaatsen in de cel waar de vetzuren gebruikt kunnen worden voor de energie winning (mitochondriën) of naar plaatsen in de cel waar vetzuren ingebouwd worden in triacylglycerolen (vetreserve) of fosfolipiden (membraancomponenten). Bovendien zijn sinds kort, ook andere functies van vetzuurbindende eiwitten bekend zoals het reguleren van de effecten van vetzuren en andere liganden op enzymen, membranen, receptoren, ionkanalen en/of genen. De vetzuurbindende eiwitten spelen een centrale rol in het vetzuurmetabolisme in de cel en kunnen daardoor betrokken zijn bij de erfelijke verschillen in het intramusculaire vetgehalte in varkens.

De eerste stap van de kandidaatgen benadering was de opheldering van de DNA sequentie van de H-FABP en A-FABP genen van het varken (Hoofdstuk 3 en 6). De DNA sequentie van beide genen vertoonde zeer grote overeenkomst met dezelfde genen van mens, rund, muis, en rat. Verder werd de plaats van beide genen op de chromosomen van het varken bepaald. Het H-FABP gen bevindt zich in het gebied tussen de merkers Sw316 en S0003 op chromosoom 6 terwijl het A-FABP gen tussen de merkers S0001 en S0217 op chromosoom 4 ligt. Deze gebieden komen overeen met vergelijkbare delen van chromosomen van mens en muis.

In beide genen werden mutaties (polymorfiën) ontdekt (Hoofdstukken 3, 4, 6 en 7) waarvan vervolgens de associatie met het intramusculaire vetgehalte bepaald werd in varkens van een Duroc populatie en een Meishan × Large White kruisingspopulatie.

In de Duroc populatie tonen de resultaten een significante associatie van het H-FABP gen met intramusculair vetgehalte, groei en spekdikte maar niet met het waterbindend vermogen van het vlees (Hoofdstuk 5). Het gunstige genotype had een hoger intramusculair vetgehalte, een betere groei maar ook een dikkere laag spek. Aanvullende analyses toonden aan dat het effect op het intramusculaire vetgehalte onafhankelijk was van de spekdikte.

Voor A-FABP werden eveneens significante associaties met intramusculair vetgehalte en groei aangetoond in de Duroc populatie terwijl geen associaties met spekdikte en waterbindend vermogen werden gevonden (Hoofdstuk 6).

In de Meishan × Large White populatie werden op twee verschillende manieren aanwijzingen gevonden dat H-FABP betrokken is bij intramusculair vetgehalte in deze varkens. Voor A-FABP werden daarentegen geen aanwijzingen gevonden voor een dergelijke associatie (Hoofdstuk 8).

De gevonden polymorfiën in de H-FABP and A-FABP genen bleken niet gerelateerd te zijn met de hoeveelheid H-FABP en A-FABP in spierweefsel van Large White mestvarkens (Hoofdstuk 9). Daarentegen werd wel een significant verschil in H-FABP mRNA expressie gevonden tussen deze varkens met verschillende genotypen. Een bezwaar van deze studie is dat een andere varkenspopulatie gebruikt werd dan de populatie waarin in eerste instantie de associatie met het intramusculaire vetgehalte werd aangetoond. Bovendien kan het tijdstip waarop de expressie van de eiwitten werd bepaald mogelijk van invloed zijn op de resultaten.

Tot slot is het vermeldenswaardig dat een andere onderzoeksgroep in een onafhankelijke studie van een Duroc × Noors Landras kruisingspopulatie een vergelijkbaar effect van het H-FABP gen heeft gevonden als wij met onze Duroc populatie.

Uit de voorgaande resultaten concluderen wij dat het H-FABP gen of een gen wat zeer dicht in de buurt ligt verantwoordelijk is voor een deel van de erfelijke verschillen in het intramusculaire vetgehalte tussen varkens. Ondanks het ontbreken van een definitief bewijs

voor een relatie met het intramusculaire vetgehalte, is de toepassing van de H-FABP polymorfiën in fokprogramma's voor varkens mogelijk. Wel moet de associatie tussen polymorfiën in het H-FABP gen en het intramusculaire vetgehalte aangetoond worden in de populatie. Hierbij kan ook de gunstige vorm van de polymorfie in het H-FABP gen bepaald worden waarop selectie plaats kan vinden. Aangezien onduidelijk is of de gevonden polymorfiën in het H-FABP gen verantwoordelijk zijn voor de effecten op het intramusculaire vetgehalte in varkens, is het aan te bevelen om regelmatig te bepalen welke vorm van de polymorfie het gunstigst is. Immers, een overkruising tussen de verantwoordelijke mutatie en de polymorfiën in het H-FABP gen kan het effect van selectie van gunstig in ongunstig veranderen.

In het geval van het A-FABP gen ligt het gecompliceerder aangezien het effect op het intramusculaire vetgehalte in de Duroc varkens niet bevestigd werd in de Meishan × Large White populatie. Het verdient daarom aanbeveling om het effect van het A-FABP gen op het intramusculaire vetgehalte eerst in andere varkenspopulaties te onderzoeken alvorens het toe te passen in fokprogramma's van varkens ter verbetering van de vleeskwaliteit.

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About the author

Frans Gerbens was born on november 6, 1967, in Lieve Vrouwenparochie, The Netherlands. In 1987, he passed secondary school at the Rijks Scholengemeenschap (RSG) in Leeuwarden. In the same year he started his study Molecular Sciences at the Wageningen Agricultural University (WAU). In 1993, he graduated with majors in Molecular Biology, Cell Biology and Immunology (including a traineeship at the Department of Zoology at the University of Aberdeen, UK), and Genetics (conducted at the Department of Medical Genetics at the University of Groningen). During his academic study, he also obtained the national certification (article 9) for designing and performing experiments involving animals.

In 1993, he started as a PhD-research-fellow at the Department of Biochemistry at the University of Nijmegen. The studies of his PhD-project entitled "Molecular regulation of intramuscular fat metabolism of the pig" were conducted at the Department of Breeding and Genetics, of the DLO-Research Institute for Animal Production (IVO-DLO) in Zeist, that later merged to the Department of Genetics & Reproduction of the Institute for Animal Science and Health (ID-Lelystad) in Lelystad. From january 1, 1999, he has been appointed as a molecular genetic research scientist at the Department of Genetics & Reproduction of ID-Lelystad.

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