INTRODUCTION

Quantitative trait loci (QTL) mapping and candidate gene approaches have been used to find the genes responsible for genetic variation in the traits of interest in farm animals (Rothschild, 1998). Several QTL mapping have been performed for production and meat quality traits using reference family produced by crosses between phenotypically divergent breeds (Andersson et al., 1994; Knott et al., 1998; Rohrer and Keele, 1998; Paszek et al., 1999; Bidanel et al., 2001; Malek et al., 2001). As a result, several research groups identified the same QTL affecting growth and fat deposition traits found in the long arm of SSC6 (de Koning et al., 1999; 2000; Gerbens et al., 2000; Grindflek et al., 2001; Malek et al., 2001).

Using candidate gene analysis, several major genes such as melanocortin 4 receptor (MC4R) (Kim et al., 2000) for growth, ryanodine receptor gene (RYR1) (Fujii, 1991), and heart fatty acid binding protein (hFABP) (Gerbens et al., 1999) for meat quality have been identified for their association with economic traits. Recently, Van Laere et al. (2003) revealed that single nucleotide polymorphism within intron 3 of insulin-like growth factor 2 (IGF2) controls a paternally expressed QTL previously mapped on SSC 2. However, until recently, only a small number of the major genes controlling economically important traits have been identified. Candidate gene analysis also is supplemented by comparative gene analysis that allows researchers to find positional candidate genes in the regions associated with QTL (Rothschild, 1999).

The AMPK (AMP-activated protein kinase) gene family has been assumed to act as a metabolic mediator for glucose and lipid metabolism. The AMPK-activated protein kinase alpha 2 (PRKAA2) specially plays a major role as fuel sensor by modulating the activity of the autonomous nervous system (Viollet et al., 2003). Minokoshi et al. (2002) demonstrated that leptin stimulates fatty acid metabolism. This study investigated the porcine PRKAA2 gene as a positional candidate for intramuscular fat and backfat thickness traits in pig chromosome 6. A partial fragment of the porcine PRKAA2 gene, amplified by PCR, contained a putative intron 3 including a part of exon 3 and 4, comparable with that of human PRKAA2 gene. Within the fragment, several single nucleotide polymorphisms were identified using multiple sequence alignments. Of these, TaqI restriction enzyme polymorphism was used for genotyping various pig breeds including Korean reference family. Using linkage and physical mapping, the porcine PRKAA2 gene was mapped in the region between microsatellite markers SW1881 and SW1680 on chromosome 6. Allele frequencies were quite different among pig breeds. The full length cDNA of the porcine PRKAA2 (2,145 bp) obtained by RACE containing 1,656 bp open reading frame of deduced 552 amino acids, had sequence identities with PRKAA2 of human (98.2%), rat (97.8%), and mouse (97.5%). These results suggested that the porcine PRKAA2 is a positional candidate gene for fat deposition trait at near telomeric region of the long arm of SSC 6. (Key Words : PRKAA2, Mapping, Candidate Gene, Pig)
oxidation in muscle through inhibition of ACC (acetyl-CoA carboxylase) by activation of PRKAA2.

The AMPK has been known to exist as heterotrimeric complexes comprising a catalytic subunit (α) and two regulatory subunits (β and γ). In mammals, each subunit is encoded by two or three genes (α1, α2, β1, β2, γ1, γ2 and γ3), and at least 12 heterotrimeric combinations are reported (Hardie and Hawley, 2001). Human AMPK gene encodes 552 amino acids and is highly conserved with rat AMPK with identities of 97.3 and 90% at the amino acid and nucleotide levels, respectively (Aguan et al., 1994).

The PRKAA2 gene was mapped in human chromosome 1p31 (Beri et al., 1994), which is equivalent to the long arm of pig chromosome 6, according to comparative map information between human and pig (Goureau et al., 1996; http://www.toulouse.inra.fr/lgc/pig/comparSSCHTML/SSC6HTM). Therefore, based on its position and biological role, the PRKAA2 gene can be considered a potential positional candidate gene for QTL affecting fat metabolism and growth traits.

As a first step towards evaluation of the porcine PRKAA2 gene as a positional candidate controlling QTL for growth and fat deposition traits on SSC 6 in pigs, we report here the molecular cloning, characterization and chromosomal localization of the porcine PRKAA2 gene.

MATERIALS AND METHODS

Animals

Three-generation reference family was developed from crosses between Korean native pig (five boars) and Landrace (nine sows). The F1 animals, with 10 sires and 36 dams, were used to produce approximately 550 F2 animals. All animals were raised under same feeding condition at National Livestock Research Institute (NLRI) in Korea.

To examine allele frequency of polymorphisms, 204 pigs of nine different pig breeds (Berkshire, Duroc, Korean native pig, Korean wild boar, Landrace, Min pig, Wuzhishan pig, Xiang pig, and Yorkshire) were investigated in the study.

Amplification of porcine PRKAA2 gene fragment and mutation detection

Primers for amplification of a fragment of pig PRKAA2 gene were designed from a published partial sequence of the pig PRKAA2 gene (GenBank accession no. U12148). The primer sequences were selected, as follows: forward primer: 5'- TGG TAA TGG AAT ATG TGT CTG G-3'; reverse primer: 5'- ATC CAC GGC AGA GAG AAT CT -3'.

Polymerase chain reaction (PCR) was performed in 25-μl reactions with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 μM each dNTP, 10 pmol each primer, 1.0 units Taq DNA polymerase (TaKaRa, Japan), and 50 ng genomic DNA. Thermal cycling conditions included an initial denaturation for 5 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 62°C and 1 min at 72°C, and a final extension of 10 min at 72°C in GeneAmp PCR System 9600 (Applied Biosystems, USA).

For detection of nucleotide differences, direct sequencing of the PCR products was performed using Big Dye Terminator Cycle Sequencing Ready Reaction kit Ver 3.0 (Applied Biosystems, USA) and the ABI 377 DNA sequencer (Applied Biosystems, USA). The sequences were compared to find single nucleotide polymorphisms from five different breeds, namely: Korean native pig, Berkshire, Duroc, Landrace, and Large White.

Physical and genetic linkage mapping

Using the primer set to amplify a porcine PRKAA2 gene fragment, chromosomal localization of the PRKAA2 gene was conducted by PCR analysis of a porcine+rodent somatic cell hybrid panel (Yerle et al., 1996) as well as a porcine whole genome radiation hybrid panel (Yerle et al., 1998). PCR results were analyzed using the interpreting web pages of the National Institute for Agricultural Research (INRA) (http://www.toulouse.inra.fr/lgc/pig/pcr.htm, and http://imnph.toulouse.inra.fr/).

Furthermore, two-point and multipoint linkage analyses were performed using the genotypes of the three-generation Korean reference family and the CRI-MAP software version 2.4 (Green et al., 1990).

Cloning of full length cDNA by rapid amplification of cDNA ends (RACE)

The porcine mRNA for RACE experiment was extracted from the porcine longissimus dorsi muscle using FastTrack™ 2.0 kit (Invitogen Co., CA, USA). To amplify the 3' and 5' cDNA ends of the PRKAA2 gene, SMART™ RACE cDNA Amplification kit (CLONTECH Laboratories Inc., CA, USA) was used according to the manufacturer’s instruction.

The porcine PRKAA2 specific internal primer sequences for 5'RACE were used, as follows: 5' - 5'-CAT CAA CTG ACA GGC CAT AAA GTG GCA-3' for the first round and 5' - ATA CCA GGT GAT CAG CAC TCC GAC AGA -3' for the second round.

For 3' RACE, internal primers used for the first and the second round amplifications were 5'- CCA TAT GCC TGT GAC AGT AAT CCA CGG -3' and 5' - GCC TGG CTT CCA TCT CTT CAA CCC G -3', respectively.

RESULTS

The amplification of porcine PRKAA2 gene fragment and mutation detection

The primers for amplification of a fragment from a
porcine PRKAA2 gene were designed based on the published sequence at GenBank (GenBank accession no. U12148). Approximately 1,200 bp of the porcine PRKAA2 gene fragment was amplified by PCR and sequenced. The sequence of the PCR product was verified by comparison with PRKAA2 gene sequences of the pig (GenBank accession no. U12148), and the human (GenBank accession no. NM006252). The fragment contained a putative intron 3 including a part of exon 3 and 4 according to the comparison with that of human PRKAA2 gene (GenBank accession no. AY877365).

Several single nucleotide polymorphisms within the PCR fragment were found using multiple sequence alignments, including a TaqI restriction enzyme recognition site. There were two nucleotide substitution sites that can be recognized by TaqI restriction endonuclease; these were 506th and 969th bp of the sequence. Thus, the “A” allele has 505, 324, 156, 138, and 61 bp bands, and the “a” allele has 643, 384, and 156 bp bands (Figure 1).

Physical mapping and genetic linkage

Physical map location of PRKAA2 gene was assigned to SSC6 using somatic cell hybrid (SCH) and INRA-University of Minnesota porcine Radiation Hybrid (ImpRH) panels (Yerle et al., 1996; Yerle et al., 1998).

Initial screening with SCH panel showed that the hybrid clones 9, 10, 18, and 26 were positive harboring the region of chromosome 6, as follows: 6(1/2)q31 (correlation = 0.85), 6(1/2)q32 (correlation = 0.88), 6q33-q34 (correlation = 0.88), and 6(1/2)q35 (correlation = 0.88), respectively. The PRKAA2 gene was mapped clearly in these regions based on the mapping statistics (error risk, <0.5%, maximal correlation, 0.87).

The porcine PRKAA2 gene was further assigned with 118 clones of ImpRH panel to find out the more precise map location. Multipoint analysis revealed that significantly linked markers adjacent to the PRKAA2 gene were SW322, SW1069, and SW1680 on SSC6 with LOD scores of 12.25, 8.49, and 8.48, respectively (Figure 2) (Hawken et al., 1999).

Linkage mapping by two- and multipoint analysis showed that the most significant linkages between PRKAA2 and markers were obtained from SW1881 (recombination fraction = 0.20; LOD = 7.65) and SW1680 (recombination fraction = 0.08; LOD = 15.39) on chromosome 6 (Figure 2).

Genotype frequency of various pig breeds and Korean reference family

The frequency of TaqI-RFLP genotype was determined in 204 unrelated animals from nine breeds, namely: three Chinese breeds (Min pig, Wuzhishan pig, and Xiang pig),...
Table 1. Allele frequencies of a TaqI PCR-RFLP genotypes in different pig breeds

<table>
<thead>
<tr>
<th>Breeds</th>
<th>n</th>
<th>Genotypes</th>
<th></th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>Aa</td>
<td>aa</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n %</td>
<td>n %</td>
</tr>
<tr>
<td>Berkshire</td>
<td>32</td>
<td>29</td>
<td>90.6%</td>
<td>2</td>
</tr>
<tr>
<td>Duroc</td>
<td>64</td>
<td>62</td>
<td>96.9%</td>
<td>2</td>
</tr>
<tr>
<td>Korean native pig</td>
<td>30</td>
<td>23</td>
<td>76.7%</td>
<td>6</td>
</tr>
<tr>
<td>Korean wild boar</td>
<td>6</td>
<td>0</td>
<td>0.0%</td>
<td>0</td>
</tr>
<tr>
<td>Landrace</td>
<td>38</td>
<td>27</td>
<td>71.1%</td>
<td>10</td>
</tr>
<tr>
<td>Min pig</td>
<td>8</td>
<td>4</td>
<td>50.0%</td>
<td>3</td>
</tr>
<tr>
<td>Wuzhishan</td>
<td>18</td>
<td>0</td>
<td>0.0%</td>
<td>1</td>
</tr>
<tr>
<td>Xiang pig</td>
<td>27</td>
<td>0</td>
<td>0.0%</td>
<td>2</td>
</tr>
<tr>
<td>Yorkshire</td>
<td>99</td>
<td>56</td>
<td>56.6%</td>
<td>39</td>
</tr>
<tr>
<td>German Pietrain</td>
<td>20</td>
<td>5</td>
<td>25.0%</td>
<td>13</td>
</tr>
<tr>
<td>Hampshire</td>
<td>29</td>
<td>28</td>
<td>96.6%</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. Interspecies identities of the PRKAA2 coding sequence and the predicted amino acid sequence

<table>
<thead>
<tr>
<th>Species</th>
<th>Identity with the porcine PRKAA2 gene (%)</th>
<th>DNA</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>94</td>
<td>98.2</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>89</td>
<td>97.8</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>89</td>
<td>97.5</td>
<td></td>
</tr>
</tbody>
</table>

a Beri et al., 1994; b Gao et al., 1995; c GenBank accession number XM_131633.

The porcine PRKAA2 amino acid sequence was almost identical with that of the PRKAA2 gene of human (98.2%), rat (97.8%), and mouse (97.5%). The coding sequence of the porcine PRKAA2 gene also had some identity with that of human (94%), rat (89%), and mouse (89%) (Table 1 and Figure 2).

The PRKAA2 gene was highly conserved among the species except for variations of c-terminal. By comparing with human PRKAA2 genomic sequences, the porcine PRKAA2 gene was predicted to have nine exons (http://www.ncbi.nlm.nih.gov/genome/seq/page.cgi?F=Hs&ORG=Hs).

**DISCUSSION**

The AMPK plays a major role in the regulation of fatty acid and cholesterol metabolism. Using linkage analysis, the porcine PRKAA2 gene was mapped in the region between SW1881 and SW1680 on SSC6. The human and bovine PRKAA2 genes were mapped in chromosome 1p31 (Beri et al., 1994; Aguan et al., 1994) and chromosome 3 (Mckay et al., 2003), respectively.

Several QTL affecting growth and fat deposition were identified near the PRKAA2 locus of SSC6 (Grindflek et al., 2001; de Koning et al., 1999; 2000; Gerbens et al., 2000; Ovilo et al., 2000; Malek et al., 2001). A significant QTL affecting BFT, IMF content and eye muscle area was detected in the intervals between S0228 and SW1881 on SSC6 in an F2 cross between Iberian and Landrace pigs (Ovilo et al., 2000).

Suggestive QTL for tenth rib back fat was mapped in the region between SW322 and SW2052 (Malek et al., 2001); for fatness, in the intervals between S0121 and SW322 (Bidanel et al., 2001); and for IMF content and BFT, in the intervals between S0003 and SW2419 (Gerbens et al., 2000) on SSC 6. It is assumed that those QTL were located closely in the same region where the PRKAA2 was located.

Grindflek et al. (2001) reported that a significant QTL...
for IMF content was found in the region between SW1823 and S0003 on SSC6 in a commercial pigs. Interestingly, the paternally expressed QTL affecting IMF content on SSC6 had been detected in the region between SW316 and S0003 with a high significance level (De Koning et al., 2000). This region was a suggestive evidence of Mendelian QTL for IMF content, without the imprinting effect in the model (De Koning et al., 1999). Those QTL also are likely to be closely linked with the PRKAA2 locus identified in this study.

Furthermore, leptin, a hormone secreted by adipocytes, plays a major role in the regulation of food intake and energy expenditure (Friedman and Halaas, 1998). Leptin stimulates fatty acid oxidation and glucose uptake in skeletal muscles (Muoio et al., 1997; Minokoshi et al., 2002).

On the other hand, AMPK stimulates the oxidation of fatty acids in skeletal muscle by inhibiting the activity of

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**Figure 3.** Amino acid sequence alignment of the AMP-activated protein kinase alpha2 of pig, human, rat and mouse. Human (Genbank accession no. MN006252; Beri et al., 1994), rat (accession no. Q09137; Gao et al., 1995), and mouse (accession no. XM131633; unpublished data). Dash (-) indicates the homology of the amino acid sequences.
acetyl coenzyme A carboxylase (ACC). Thus, AMPK plays as a principal mediator of effects of leptin on fatty acids metabolism in muscle (Hardie et al., 1998; Minokoshi et al., 2002). AMPK is highly conserved in mammals, yeast (SNF1) and plants (RRK1) suggesting its significant biological role (Carling et al., 1994).

PRKAA2 gene was mapped in more distal region away from the leptin receptor (LEPR) approximately 21.7 cM; and from the human heart-type cytoplasmic fatty acid-binding protein (H-FABP), 61.9 cM.

H-FABP and LEPR are considered as candidate genes associated with growth and fatness in the long arm of SSC 6. The polymorphism in H-FABP gene was associated with IMF as well as BFT in Duroc (Gerbens et al., 1999), and it was mapped in the region between markers SW316 and S0003 on SSC6 (Gerbens et al., 2000).

Although there has been no reported polymorphism in LEPR associated with other traits in pig, LEPR also is considered as one of candidate genes associated with growth and fat traits because of its position and biological function.

In addition, several other genes including ACADM, PRKACG, PGM1, SCP2, C8A, and MCAD were mapped in this region (http://www.toulouse.inra.fr/lgc/pig/cyto/gen mar/ht6/6GM. HTM).

Other candidate genes on other chromosomes for growth and fatness, found to be associated with economically important traits in pigs, include MC4R (Kim et al., 2000; Milan et al., 2000); and IGF2 (Van Laere et al., 2003) reported the relationship between single nucleotide polymorphism (SNP) in intron 3 of IGF2 and a QTL effect.

These results are suggested that the porcine PRKAA2 is a potential positional candidate gene controlling QTL for fat deposition trait near telomeric region of the long arm of SSC 6. However, in order to develop a marker for marker-assisted selection, it needs to discovery and evaluate the single nucleotide polymorphisms within exons or regulatory region in the porcine PRKAA2.

ACKNOWLEDGMENTS

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