Identification of porcine $Lhx3$ and $SF1$ as candidate genes for QTL affecting growth and reproduction traits in swine


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Summary

The distal portion of the long arm of porcine chromosome 1 has been shown to harbour several quantitative trait loci affecting growth and reproductive traits in swine. In order to identify potential candidate genes that might underlie these effects, a comparative mapping analysis was undertaken to define the extent of orthologous segments of human chromosome 9. A microsatellite associated with heat shock protein (HSP) A5 was used to define the proximal boundary of the quantitative trait loci (QTL) region, which suggests the human orthologue of the gene(s) responsible for the observed effects lies between $HSPA5$ and the q arm telomere of human chromosome 9. Examination of this region revealed two candidate genes with known roles in production of hormones essential to growth and reproductive function. The steriodogenic factor 1 and $Lhx3$ LIM homeodomain transcription factor genes were mapped to 123 and 155 cM, respectively, of the Sus scrofa chromosome 1 (SSC1) linkage group, placing both genes within the confidence interval for the observed QTL. To further evaluate $Lhx3$, we examined the expression profile during porcine embryonic development. Low levels were detected at early embryonic stages, when development of the nervous system is proceeding. A transient increase in expression level is observed during the time of pituitary organogenesis and again at the time of differentiation of anterior pituitary cells, with relatively high levels of expression persisting in the adult pituitary gland. This ontology is consistent with $Lhx3$ being a candidate gene for the QTL.

Keywords growth, reproduction, mapping, pituitary, QTL.

Introduction

Development of genetic maps of swine in the past decade has allowed identification of quantitative trait loci (QTL) affecting a variety of traits of economic importance. While these results are promising with respect to potential improvement of pork production through marker-assisted selection procedures, it would be of significant interest to identify the genes and specific allelic polymorphisms underlying the observed variation. This achievement would allow development of specific DNA-based genetic tests to evaluate individual animals for merit and genetic potential, as well as increase understanding of the genetic basis underlying non-disease producing variability among populations. However, identification of genes and DNA sequence differences that contribute to relatively minor (but still of substantial economic importance) variation in phenotype is a daunting task. The most promising avenue is the application of comparative mapping, making use of the wealth of knowledge and resources produced from the human genome project and biomedical research community.

Genomic scans of a Meishan–White Composite reciprocal backcross resource population have identified a number of QTL affecting traits of economic importance (Rohrer et al.
Several QTL were identified on the distal portion of the long arm of SSC1, representing correlated traits of early growth rate (from 8 to 18 weeks), weight at 26 weeks, backfat thickness at 14 and 26 weeks of age, and age at puberty. The peak of probability for these traits was observed at 129–138 cM on SSC1. In order to identify potential candidate genes underlying these QTL effects, we undertook a comparative study of distal SSC1q. Previous mapping data have shown that the comparative map of SSC1q and the human genome appears quite complex, with blocks of conserved synteny to human chromosomes 9, 14, 15, and 18 (Goureau et al. 1996; Fig. 1).

Zoo-fluorescence in-situ hybridization (FISH) experiments indicate that the entire complement to *Homo sapiens* chromosome 9 (HSA9) in the porcine genome is contained in the distal half of SSC1q (Goureau et al. 1996). A number of genes found on HSA9 have been physically assigned to SSC1q, including CDKN2, IFNA1, RPS6, GGTAT, LCN1, RLNI, SF1, ORM1, HSPA5 and HXB (for a complete list of abbreviations and swine map positions, see www.ri.bbsrc.ac.uk/pigmap/pig_genome_mapping.html). However, all of these genes are found on HSA9p (CDKN2, IFNA1, RLNI, RPS6) or in the HSA9q33-q34 region, so there is no gene assignment evidence for the central third of HSA9 on SSC1. Furthermore, the physical assignment of RLNI in swine overlaps with that of LCN1, although they are on opposite ends of HSA9, suggesting that substantial gene order rearrangements may have occurred during evolution. Thus, the telomeric end of the block of conserved synteny appears to be the most likely to carry the gene(s) that map within the QTL interval as indicated by the telomeric position of markers S0056 and SW1301 (Fig. 1; Rohrer et al. 1996; www.marc.usda.gov). In addition, a genetic marker for RLNI has been mapped in swine, approximately 50 cM proximal to the QTL peaks (Rohrer et al. 1996). We concluded that the distal portion of HSA9q is the most likely region to contain the orthologue of the gene(s) underlying the QTL.

The goal of this study was to further narrow the comparative region containing the QTLs, identify the most likely candidate genes, and begin to characterize these positional candidates for their suitability as functional candidates. We successfully narrowed the search to a small region of HSA9q and identified two candidates for one or more of the

**Figure 1** Comparison of the physical and genetic linkage maps of SSC1 with the map of HSA9. Genes with porcine physical assignments are indicated with approximate assignments along the ideogram. Those genes that have also been placed on the genetic linkage map are joined to the linkage map scale (in centimorgans) in the centre. Genes mapped in swine and found on HSA9 are shown to the right of the linkage map (the size of HSA9 is approximately to scale). Gene symbols shown in bold indicate those mapped in swine for the present study. *ESR* = estrogen receptor alpha; *CGA* = glycoprotein subunit alpha; *MEF2A* = MADS box transcription enhancer factor 2 A; *IGF1R* = insulin-like growth factor 1 receptor; *MC4R* = melanocortin receptor 4; *IFNA* = interferon alpha; *RLN1* = relaxin; *SF1* = steroidogenic factor 1.
QTL. In addition, we provided initial characterization of a promising candidate, Lhx3, and show that its ontogeny of expression is consistent with the possibility that variation in the gene underlies a proportion of observed phenotypic differences in growth and age at puberty.

Materials and methods

Genetic markers and mapping

A yeast artificial chromosome (YAC) clone containing the heat shock protein (HSP) A5 gene was isolated by polymerase chain reaction (PCR) screening of DNA pools of the Meat Animal Research Center (MARC) porcine YAC library as described (Alexander et al. 1996), using primers HSPA5f/HSPA5r (Table 1) developed from the sequence of the porcine HSPA5 cDNA (Accession X92446). Microsatellite screening was obtained by subcloning, hybridization screening of the subclones with radiolabelled (GT)11 oligonucleotide, and sequencing as described (Sonstegard et al. 1997). Primers Grp78f–Grp78r were designed to amplify across the microsatellite and used for genotyping. Allele sizes, genotypes, and map positions were estimated, scored and analysed as described (Rohrer et al. 1994).

Primers to detect single nucleotide polymorphism (SNP) markers (Table 1) were developed from cDNA sequence present in GenBank, either porcine (SF1; accession U84399; primers MARC1432/1433) or an alignment of human, porcine and mouse (Lhx3; accessions AF156888, AF063245 and L38249, respectively; primers I223–I224). Primers were used to generate amplicons from the genomic DNA of the nine parents of the US MARC swine reference population (Rohrer et al. 1994), and the amplicons were directly sequenced using a fluorescent DNA analyser (Applied Biosystems, Foster City, CA, USA). Polymorphisms were detected by visual inspection of the chromatograms and analysed with PHRAP and CONSED programs (Gordon et al. 1998). Genomic DNA amplification with primers I223–I224 was of low reproducibility, so nested amplification primers for genotyping of Lhx3 were developed from the porcine sequence of intron 2 (MARC6871–6872; Table 1). The sequences adjacent to identified polymorphisms in both genes were used to develop primer extension probe oligonucleotides (designated as ‘probe’ in Table 1). Extension products corresponding to specific genotypes were generated from the entire mapping population in the presence of dNTPs, with appropriate ddNTPs to provide termination for assay via MALDI-TOF mass spectrometry (Sequenom, San Diego, CA, USA) as described (Little et al. 1997). The SNP genotypes were scored in the reference population and analysed with all markers previously entered into the genotype database (Rohrer et al. 1994, 1996) using CRIMAP v2.4 (Green et al. 1990) to obtain map position.

Expression analysis of Lhx3

First strand cDNA was generated from embryonic and adult porcine RNA samples using reverse transcriptase (RT) (Life Technologies, Rockville, MD, USA) and oligo d(T) as a primer. Hot-start PCR reactions for Lhx3 were performed using RT–PCR primers with β-actin primers as control (Table 1) as described (Sloop et al. 2000b). Thermal cycling parameters were one cycle at 94 °C for 2 min, 30 cycles of 94 °C for 30 s/59 °C for 30 s/72 °C for 30 s, and one cycle at 72 °C for 10 min. The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. For each developmental stage, the experiment was performed at least in triplicate to provide semiquantitative estimation of RNA expression.

Results

The first goal of the study was to reduce the amount of HSA9 to scan for potential candidate genes. This was accomplished by developing a microsatellite marker from a YAC clone containing the HSPA5 gene, which had previously been physically assigned to SSC1q2.12-q2.13 (Yasue et al. 1994). Primers designated GRP78 (a synonym locus name for HSPA5) amplified a sequence length polymorph-
nuclear receptor 5A1, NR5A1 to as fushi tazaru factor 1 homologue, associated with the QTL. These genes are that could impact all of the phenotypic characteristics with known roles in development and hormone expression blood cells. Most interesting was the presence of two genes these proteins are related to maturation and activation of TNFSF8 candidate genes in swine. The swine cDNA sequence of their position and determine if they represent positional TRAF1 factor (TNF) associated protein genes (TRAF1, TRAF2 and TNFSF8) lie in this area. A group of zinc finger-containing transcription factors, homeobox proteins, and oncogenes lie in this region. A group number of genes encoding proteins affecting the QTL, but within the 95% confidence interval (Fig. 1). Because HSPA5 maps to HSA9q34, mapping of this gene in swine reduced the likely region carrying the orthologue of the QTL gene(s) to the telomeric region of HSA9q.

Examination of the distal end of HSA9q using the Mapviewer resources at the National Center for Biolog-ical Information (http://www.ncbi.nlm.nih.gov) indicated numerous conceivable candidate genes, as this region of HSA9 has a relatively high gene density. Genes encoding a number of zinc finger-containing transcription factors, homeobox proteins, and oncogenes lie in this area. A group of five members of the lipocalin family of proteins are found in this region, and may conceivably play roles in processes affected by the QTL. In addition, a group of tumour necrosis factor (TNF) associated protein genes (TRAF1, TRAF2 and TRAF5) lie in this region, although the primary roles for these proteins are related to maturation and activation of blood cells. Most interesting was the presence of two genes with known roles in development and hormone expression in the pituitary gland, an organ with far-reaching effects that could impact all of the phenotypic characteristics associated with the QTL. These genes are SF1 (also referred to as fushi tazaru factor 1 homologue, FTZF1, AD4BP or nuclear receptor 5A1, NR5A1) and Lhx3.

The SF1 and Lhx3 genes were mapped in order to verify their position and determine if they represent positional candidate genes in swine. The swine cDNA sequence of SF1 in GenBank (U84399) was compared with the human genome draft sequence using BLASTN, which identified the position of a predicted intron at position 969 of the swine sequence. Primers MARC1432/1433 (Table 1) corresponding to positions 935–955 and 1079–1097 of the cDNA sequence produced a 305-bp product (accession number AF356174) that included a 142-bp intron. This intron displayed two C/T polymorphisms in the MARC reference mapping parents at positions 80 and 138 of the amplicon sequence. Genotyping of the offspring produced 44 informative meioses, and allowed the SF1 locus to be mapped at 123 cM on SSC1 (maximum LOD of 11.44). This result suggests that SF1 lies within the 95% confidence interval for the QTLs (Rohrer et al. 1999).

A similar approach was used to map Lhx3. Because porcine sequence of this gene was not available at the start of this study, an alignment of human and murine sequences was used to develop primer pair I223/I224. This primer pair amplified portions of exons 1b and 2, and the entire 817 bp intron 2 (accession number AF345446). For sequencing of the MARC reference mapping parents, a pair of nested primers (MARC6871/6872) were used to obtain higher quality amplification for direct sequencing of PCR products. The nucleotide sequence of the amplicon produced, with polymorphic positions indicated in the submission via standard nomenclature, has been deposited in GenBank (accession number AF356175). The observed set of SNPs formed two haplotypes, one of which was found only in the six Chinese cross sows of the reference population. A single SNP sufficient to distinguish the haplotypes (an A/G polymorphism detected at position 312 of the amplicon) was used to genotype the offspring, providing 69 informative meioses. The SNP showed significant linkage with markers in the SSC1 linkage group (maximum LOD of 8.62 with SW1301; recombination fraction 0.10) that allowed the Lhx3 locus to be mapped at 155 cM of the linkage group. This result placed the gene 8.4 cM in the telomeric direction from marker SW2572, previously the most telomeric marker of the linkage group. Because this placed the marker outside the current linkage group, which the CRIMAP program may do when genotyping errors are present, the individual genotype spectra were reinspected manually to ensure integrity. No errors could be detected, so we conclude that the Lhx3 marker extends the SSC1 linkage group and appears to represent the telomeric end of this group. In addition, this position placed the locus within the 95% confidence interval for the observed QTLs.

To examine the ontogeny of Lhx3 gene expression in swine, we performed RT–PCR analysis during embryogene-sis. Porcine Lhx3 gene transcripts were weakly detected on embryonic day 13 (e13; Fig. 2). At this time, neurulation occurs and development of nervous system structures rapidly follows (Fig. 2; Patten 1948). The Lhx3 expression continues throughout embryogenesis and was readily detected in the adult pituitary (Fig. 2). Although these assays are semiquantitative, we observed intriguing pulses of increased Lhx3 gene transcription in samples from embry-onic days e18 and e31 (Fig. 2). These time points mark the early structural events that consign the cells of Rathke’s pouch, the pituitary primordium, to a pituitary fate and the onset of differentiation of porcine hormone-secreting anterior pituitary cells, respectively. This observation correlates with the prediction that Lhx3 is required for both the early development of the pituitary gland and later for expression of pituitary trophic hormone-encoding genes, based on gene ablation experiments in mice (Sheng et al. 1996) and observation of human patients with Lhx3 gene mutations (Netchine et al. 2000). By contrast, using a similar approach, we have reported that expression of the porcine
negative controls (Fig. 2). were not detected in non-pituitary tissues such as liver or in pituitary develop from Rathke’s pouch, a region of early oral products. The anterior and intermediate lobes of the gland are specialized to release these characteristic hormone lactin (PRL). Differentiated cells within the mature pituitary luteinizing hormone (LH), growth hormone (GH) and pro-hormone (TSH), follicle-stimulating hormone (FSH), many other physiological functions. These hormones are production, the stress response, metabolic homeostasis and ary gland mediate endocrine regulation of growth, repro-

Figure 2 Lhx3 gene expression during development. (a) Timeline of porcine pituitary ontogeny. The times of relevant stages in development are given in days post coitus (eN). Following neurulation and opening of the stomodaeum, an invagination of oral ectoderm (Rathke’s pouch) is observed. This structure gives rise to the anterior and intermediate lobes of the pituitary gland. Later, regulated cellular proliferation and differentiation events occur that result in the first release of hormones at the indicated times (Ma et al. 1994, 1996; Granz et al. 1997). ACTH = adrenocorticotrophic hormone; GH = growth hormone; TSH = thyroid-stimulating hormone; FSH = follicle-stimulating hormone; LH = luteinizing hormone; PRL = prolactin. The expression of the Prop-1 pituitary transcription factor gene (Sloop et al. 2000b) is also depicted. (b) Analysis of Lhx3 gene expression by RT–PCR. RNA was isolated from pig embryos of the indicated stages, adult female pituitaries (Pit), or liver (Liv), CDNA was synthesized, and Lhx3 cDNA was amplified by PCR. ß-actin was amplified in parallel reactions as a positive control. Negative control reactions (Neg) with no input CDNA also are shown. The assays for each stage were performed at least three times; a representative gel is shown.

Prop-1 gene is detected at consistent levels during the time of pituitary organogenesis and is found at lower levels in mature pituitary tissue (Sloop et al. 2000b). The Lhx3 RNAs were not detected in non-pituitary tissues such as liver or in negative controls (Fig. 2).

Discussion

In vertebrates, hormones secreted from the anterior pituitary gland mediate endocrine regulation of growth, reproduction, the stress response, metabolic homeostasis and many other physiological functions. These hormones are adrenocorticotrophic hormone (ACTH), thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), growth hormone (GH) and prolactin (PRL). Differentiated cells within the mature pituitary gland are specialized to release these characteristic hormone products. The anterior and intermediate lobes of the pituitary develop from Rathke’s pouch, a region of early oral ectoderm near the anterior neural ridge. As embryogenesis proceeds, Rathke’s pouch associates with neural tissue destined to form the posterior pituitary. During this time, cells within the developing anterior lobe are determined to specific fates, and the hormone-secreting cells populate the gland by the proliferation and differentiation of these precursor cells. This developmental process is regulated by the coordinated actions of cell-specific and tissue-specific gene regulatory proteins (Burrows et al. 1999).

Among the potential candidate genes that lie on HSA9q34, we find Lhx3 and SF-1 to be the most attractive, based on the known functions of the proteins and their influence on the growth and reproduction parameters identified with the QTLs. Lhx3 is a LIM homeodomain transcription factor that is critical for pituitary development and function in rodents and humans. The mouse and human Lhx3 genes have seven protein-coding exons and produce two distinct protein isoforms with different gene activation properties (Zhadanov et al. 1995; Sloop et al. 1999, 2000a). Lhx3 is expressed in the developing nervous system but is later restricted to the developing and mature pituitary gland (Bach et al. 1995). Mice with null alleles of Lhx3 lack the anterior pituitary, exhibit improper assignment of specific motor neuron subtypes, and die shortly after birth (Sheng et al. 1996; Sharma et al. 1998). In addition, these mice lack a number of hormones that play important roles in numerous processes. Human patients with mutations of the Lhx3 gene have a compound pituitary hormone deficiency disease with loss of the same hormones as the Lhx3 knockout mice (Netchine et al. 2000). The absence of these hormones results in symptoms including short stature, failure to enter puberty and hypothyroidism. Because the hormones lost include GH, PRL, TSH, LH and FSH, all of which have significant roles in porcine development, growth, and reproduction, it is possible that allelic variation at the Lhx3 locus may influence growth and reproduction traits in swine. We previously demonstrated that the Lhx3 gene is conserved in swine, and that porcine Lhx3 can activate transcription from pituitary hormone genes (Meier et al. 1999). In this manuscript, we demonstrated that the ontology of expression of Lhx3 is consistent with the hypothesis that the same influence on growth and reproduction traits will occur in swine as seen in mice and humans.

The SF-1 gene represents an attractive candidate for influencing age at puberty, growth rate and nutrient partitioning, but may act through the adrenal axis rather than through its activity in the pituitary. Mice lacking the SF-1 gene die postnatally and exhibit gonadal and adrenal agenesis, reduced expression of pituitary gonadotropins and loss of the ventromedial hypothalamus (reviewed in Hanley et al. 2000). Targeted conditional knockout of the SF-1 gene in pituitary cells results in mice with hypogo-
nadotropic hypogonadism (Zhao et al. 2001), confirming that SF-1 activity is essential for the development and function of pituitary gonadotrope cells. In human patients, heterozygous mutations in SF-1 are associated with a range of phenotypes ranging from adrenal insufficiency (Biaison-Lauber & Schoenle 2000) to sex reversal and adrenal failure (Achermann et al. 1999). A mild phenotype, marked by poor adrenal development and an impaired stress response, is observed in heterozygous mice (Bland et al. 2000).

The mapping and analysis of pituitary regulatory genes such as SF-1 and Lhx3 provides potential positional candidate genes for genetic analysis of growth, reproductive and metabolic traits in meat species. Application of genetic markers developed in this study will help to refine the position of the QTL and assist in evaluating this hypothesis. Further characterization of the genes, specifically to look for potential functional allelic variants, will also help to test the idea. Finally, characterization of the biochemical mechanisms of the encoded transcription factors may allow the development of genetic protocols to improve productivity in the meat industry.

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