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Search for regulatory DNA variation in genes related to stress response in pigs

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1 Introduction

Stress hormones glucocorticoids and catecholamines play important role in the metabolism of fat, proteins and glucose. Their levels have been shown to correlate with carcass and meat quality traits in pigs and to vary between porcine breeds (Weiler et al. 1998, Foury et al. 2005). Hence genes regulating synthesis/secretion of stress hormones as well as genes mediating and modulating their effects represent candidate genes for carcass and meat quality. We focus on the identification of cis regulatory DNA variation affecting expression of the stress responsive genes assuming, that regulatory DNA variation account for an appreciable proportion of the variation in the activity of the neuroendocrine stress systems. Recent eQTL studies and studies using allele-specific RT-PCR in humans (e.g. Bray et al. 2003, Morley et al. 2004) indicate, that cis regulatory variation is common. Identification of a cis regulatory SNP in the porcine IGF2 gene as the causal QTN underlying a major QTL for muscle growth (van Laere et al. 2003) emphasize the important contribution of cis regulatory variation to natural variation in complex and quantitative traits.
Corticotropin releasing hormone (CRH) acts as a central coordinator of the neuroendocrine and behavioral responses to stress (Deussing and Wurst 2005). Thus the porcine CRH gene was our primary candidate to investigate.

2 Materials and Methods

2.1 Sequencing of pCRH gene and discovery of DNA variation: The published partial sequence of exon 2 (Acc. Nr. : Y15159) was completed by isolation of both UTRs, intron and proximal promoter. To obtain 5´ and 3´ UTR RACE PCR has been performed using the SMARTRACE cDNA Amplification Kit (Clontech) and a T7 tailed oligo $d(T)_{13}$ primer respectively. RNA was isolated from hypothalamus, whole foetal brain, pituitary and uterus using TRI Reagent (Sigma) and subsequently pooled. cDNA for 3´RACE was synthesized using SuperScriptII MMLV RT (Invitrogen). To determine the sequence of the intron opposing primers in exons 1 and 2 were combined. The proximal promoter sequence was amplified by heterologous PCR.
To discover DNA variation the promoter and coding sequence were comparatively sequenced using DNA samples from each 2 animals with high/low post-stress level of cortisol and from a panel of different breeds.The PCR products were gel purified, cloned in pGEM-T (Promega) or pCR2.1 (Invitrogen) vector and sequenced using the SequiTherm EXCEL Sequencing Kit (Epicentre) on a Licor Model 4200 automated sequencer.

2.2 In silico sequence analyses: In order to identify putative cis-regulatory elements and transcription factor binding sites (TFBS) phylogenetic footprinting was performed using Vista (http://genome.lbl.gov/vista/index.shtml) and rVista (http://rvista.dcode.org) tools. Sequences of the *CRH* gene from human, sheep, dog, rat and chicken were retrieved from NCBI and USCS web sites.

2.3 Genotyping: For genotyping of a C>T SNP in the promoter and an $(A)_6$>$(A)_7$ insertion/deletion in 3´ UTR region SSCP assays were developed. A SSCP assay for genotyping of a G>A SNP in the coding sequence was already published (Wimmers et al. 2002a).

2.4 Allele-specific RT-PCR: On the basis of the $(A)_6$>$(A)_7$ indel a simple sequence length polymorphism (SSLP) assay was developed. A 125bp fragment of exon 2 encompassing the $(A)_7$>$(A)_6$ indel was separated on denaturing 7 % LongRanger (41cm) gel run on a Licor 4000 sequencer. The allele ratio was determined using the One-Dscan software (Scanalytics). To assess sensitivity and accuracy of the allele-specific RT-PCR the $(A)_7$:$(A)_6$ ratio was quantified in two series of mixed alternatively homozygous DNA samples with allele ratios ranging form 0,1:0,9 to 0,9:0,1. Hypothalamus RNA was isolated from 13 non-stressed heterozygous DL pigs of 4-weeks age descending from 2 sires and 3 dams and cDNA synthesized using SuperScriptIII MMLV RT (Invitrogen) and oligo $d(T)_{15}$ as well as random primers (Promega). The allelic transcript ratio in cDNA samples was determined along with $(A)_7$:$(A)_6$ ratio from corresponding DNA samples. The mean $(A)_7$:$(A)_6$ ratio from DNA was used to normalize deviations from the expected 1:1 ratio due to the assay.

2.5 Association with stress parameters: The effect of the promoter SNP on the stress response (basal and poststress cortisol, CK and LDH levels at 30 and 80kg) was evaluated in 100 DL animals. The stressors applied represent transport and myostress injection. For detailed description of the material see Wimmers et al. (2002b). The association analysis was performed using a general linear model (PROC GLM; SAS Version 8.02). For all parameters the model included fixed effects of the promoter genotype, sire and dam within sire and for poststress parameters also the fixed effects of treatment and treatment * genotype interaction. Least square means were computed for each genotype and pairwise compared by t-test.

3 Results and Discussion

The complete cDNA sequence of the porcine *CRH* gene along with ca 580bp of promoter and ca 800bp intron sequence was determined. Transcription start was derived from alignment of the porcine with human, ovine and rat sequence. Polyadenylation from two signals was observed. The estimated length of the two cDNA forms is 1210bp and 1274bp respectively. The highest sequence conservation level as revealed by Vista (Avid)- alignment (Fig.1) exhibit ca 350bp of the proximal promoter region. Additional evolutionary conserved regions (ECR) in noncoding sequence, i.e. putative cis regulatory elements, are located in 3´ UTR and the intron. They may represent elements that regulate tissue specific expression of the *CRH* gene proposed by Stenzel-Poore et al. (1992). In 3´ UTR the ECR mostly coincide with polyadenylation signals. In the intron of *pCRH* gene a simple sequence repeat $[CG(GC)_6(GT)_5AT(GT)_5AT(GT)_5AT]$ is located in close proximity to a unique ECR conserved among all mammalian species studied. A simple sequence repeat located at similar position in intron of the human *CRH* gene was shown to be involved in the transcriptional regulation (Wölfl et al. 1996). We found neither length nor sequence variation of the porcine simple sequence repeat (data not shown). In the coding sequence the highest conservation level besides the region coding for the mature hormone retained the region coding for the signal peptide.

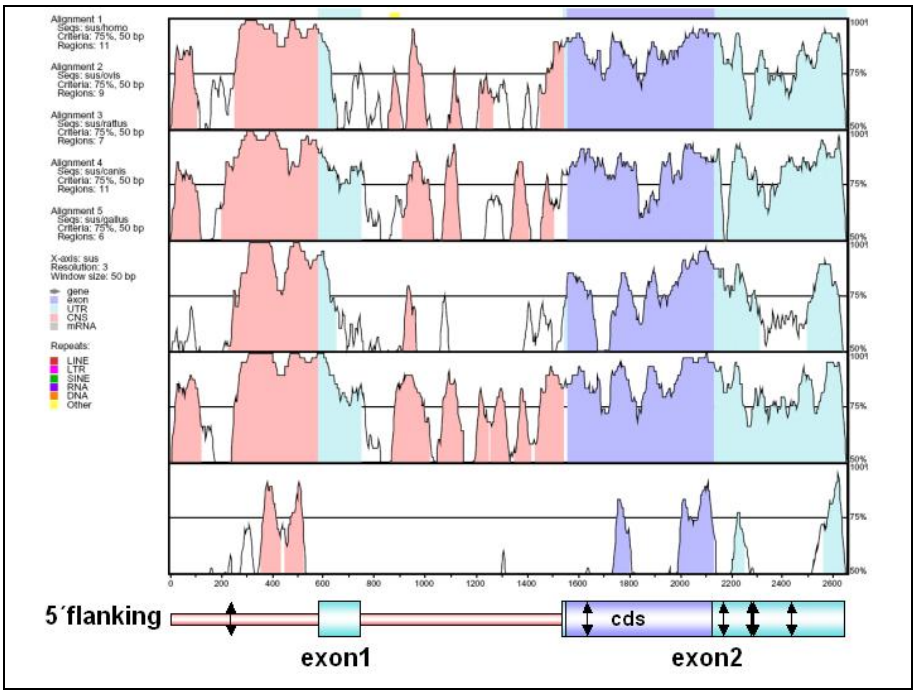

Figure 1: Vista plot of sequence conservation level of the porcine *CRH* gene compared to human, ovine, murine, canine and chicken sequence. Arrows in the gene scheme indicate position of DNA variation.

Seven DNA polymorphism: one in promoter, one in coding sequence and five in 3´ UTR were identified. The coding G>A SNP alters the amino acid sequence at a residue in close proximity to signal peptide, which is conserved among mammals (R28Q). Majewski and Ott (2003) predicted, that R (arginine) represents one of the least mutable residues. In addition the R>Q exchange occurrence significantly deviates from expected (Majewski and Ott, 2003). Thus the R28Q exchange may affect secretion of the hormone. The mutant variant originates from Berlin mini pigs. No segregation of this variant in commercial breeds was observed (Wimmers et al. 2002a). The DNA variation in 3´ UTR occurs in less well-conserved sequence.

In the promoter a C>T SNP on the distal border of the ECR (Fig. 1) was found. A 10 bp "motif" encompassing the SNP position is perfectly conserved among pig, sheep and rat. The C variant is conserved among all mammalian species analysed (human, sheep, cattle, dog, rat). rVista analysis revealed a CDX1 and a GR half site overlapping the SNP position. No experimentally derived TFBS were reported at this position. However the studies of the *CRH* gene promoter focused mainly on human sequence, where the motif is not conserved.

To study the effect of the promoter SNP on the *pCRH* gene transcription in vivo we established an allele-specific RT-PCR assay on the basis of a $(A)_6$>$(A)_7$ indel in 3´ UTR. In allele-specific RT-PCR alleles of a polymorphism are quantified in cDNA samples of heterozygous individuals. A significant deviation of the allelic transcript ratio from the expected 1:1 ratio is considered to be an indication of cis regulatory variation. Since in the German Landrace material we used the promoter SNP and the 3´ UTR indel are in complete linkage disequlibrium, the allele-specific RT-PCR was a direct test of the effect of the promoter SNP on the *pCRH* gene transcription. An important advantage of the allele-specific RT-PCR over quantification of the *CRH* gene transcription in alternatively homozygous individuals is that the transcription of the two promoter alleles was compared within individuals and so potential confounding due to experimental variation was eliminated. The regression line in Fig. 2 indicates, that the established allele-specific RT-PCR assay was highly sensitive and accurate. The ratios obtained from cDNA and DNA differ significantly (two sided ttest, p=0.017), however the shift towards higher expression of the $(A)_7$ i.e. C allele

was only slight and inconsistent. This indicates that either the promoter SNP has no effect on transcription, other regulatory SNPs confound the results or that the affected TFBS is inactive at the physiological state (nonstressed), age or in the tissue used.

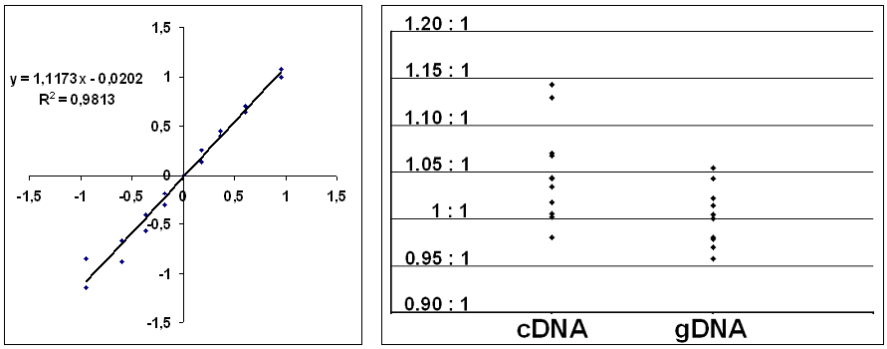

Figure 2: Allele-specific RT-PCR: Left panel) Sensitivity and accuracy assayed in series of mixed alternatively homozygous DNAs with allele ratios ranging from 10:90 to 90:10 Right panel) $(A)_7 : (A)_6$ (corresponding to C:T) allelic ratios obtained with cDNA and DNA

The association analysis revealed no significant effect of the promoter SNP on stress parameters. The stress response parameters tended to be higher by animals bearing the T allele however the association did not reach significance.

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