Original Article

Cloning, Characterization, Chromosomal Mapping and Tissue Transcription Analysis of Porcine *CREB2* and *CREB3* Genes

(cloning / *CREB2* / *CREB3* / porcine / tissue transcription)

M. QI[1,2], T. LEI[2], L. ZHOU[2], X. D. CHEN[2], H. LONG[2], Q. Q. LONG[2], R. R. ZHANG[2], Z. Q. YANG[2], L. GAN[1]

[1]College of Plant Science and Technology, [2]Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, College of Life Science and Technology, Huazhong Agricultural University, Wuhan, China

Abstract. CREB2 and CREB3 are two important members of the ATF/CREB family, which negatively and positively regulates CRE-dependent transcription *in vitro*. Here we report the cloning, chromosome mapping and tissue transcription analysis of *CREB2* and *CREB3* in pigs. The full-length coding sequence of *CREB2* and *CREB3* is 1047 bp and 1098 bp, encoding 348 and 365 amino acids, respectively. Porcine *CREB3* comprises nine exons and eight introns, whereas *CREB2* consists of three exons and two introns. *CREB2* and *CREB3* were cytogenetically assigned to porcine chromosome 5p and 1q28, respectively. Tissue transcription analysis revealed that both porcine *CREB2* and *CREB3* mRNA were ubiquitously detected in all examined tissues. Additionally, we cloned the 5' flank genomic sequence of porcine *CREB3* and characterized several putative transcription factor recognition sites including SP1, NF-κB, AP-1 and AP-2 in its promoter region. Our studies provide basic molecular information helpful for further investigation of the function of the two genes in pig models.

Received September 27, 2008. Accepted February 28, 2009.

This study was supported by the grants from Major Projects of National Science and Technology (2009 ZX 08009-151B), 973 (2006CB102100) Programme, 863 Programme (2008AA10 Z134, 2006AA10Z140), High Education Doctoral Subject Research Programme (20060504016), General Programme (30771585, 30600438) and Key Programme of National Natural Science Foundation of China.

Corresponding author: Li Gan, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, 430070, P. R. China. Phone: +86-27-87282669; Fax: +86-27-87287376; e-mail: Ganli@mail.hzau.edu.cn.

Abbreviations: ATF – activating transcription factor, bZIP – basic region and leucine-zipper, CDS – coding sequence, CRE – cAMP response elements, CREB – cAMP responsive element-binding, eIF2α – eukaryotic initiation factor 2α, ER – endoplasmic reticulum, GAPDH – glyceraldehyde-3-phosphate dehydrogenase, HCC – hepatocellular carcinoma, HCF – host cell factor, ISR – integrated stress response, TM – transmembrane, UPRE – unfolded protein response element.

Introduction

The activating transcription factor (ATF)/cAMP responsive element-binding (CREB) family includes transcription factors which bind the cAMP response elements (CRE) *in vitro* through the typical carboxy-terminal basic region and leucine-zipper (bZIP) dimerization domains. CREB2 and CREB3 are two novel important members of the ATF/CREB family (Karpinski et al., 1992; Lu et al., 1997). CREB2, also referred to as ATF4, has firstly been identified as a negative regulator of CRE-dependent transcription (Karpinski et al., 1992), and participates in several stress responses including endoplasmic reticulum (ER) stress, nutrient deprivation and exposure to oxidant, in which the translation activity of *CREB2* is dramatically promoted despite that general translation is inhibited by phosphorylated eukaryotic initiation factor-2α (eIF2α) (Siu et al., 2002; Harding et al., 2003; Lu et al., 2004). Enhanced CREB2 can sequentially induce transcriptional regulators ATF3, C/EBP homologous protein (CHOP) and growth arrest and DNA-damage-inducible 34 (GADD34), which regulate expression of a series of stress genes important for cellular metabolism and apoptosis, and thereby protect cells against a variety of environmental stresses (Jiang et al., 2004). CREB2 is also involved in long-term memory and synaptic plasticity regulation (Chen et al., 2003; Mohamed et al., 2005), and is considered to be critical for cell proliferation and differentiation, due to abnormal lens formation (Tanaka et al., 1998), severe foetal anaemia (Masuoka and Townes, 2002) and delayed bone formation (Yang et al., 2004) in *CREB2*-deficient mice.

Unlike CREB2, CREB3, also designated luman or LZIP, harbours a unique putative transmembrane (TM) domain linked to the bZIP region at their C-termini. As an endoplasmic reticulum (ER) membrane-associated protein, cleavage of the CREB3 transmembrane domain results in nuclear accumulation and regulation of downstream genes. Hitherto, little is known about the cellular stress or stimuli for the activation of CREB3. CREB3 has first been identified as a host cell factor (HCF)-binding factor and can activate transcription from promoters

containing CRE and unfolded protein response element (UPRE) (Lu et al., 1997; Lu and Misra, 2000). It is implied that CREB3 is involved in regulation of cell proliferation through the association with HCF-1, and serves as a cellular tumour repressor in the development of hepatocellular carcinoma (HCC) because CREB3 is a binding target of hepatitis C virus core protein (Jin et al., 2000). Additionally, CREB3 acts as a partner of the transmembrane subunit (TMgp41) of Env (envelope glycoprotein) and Tat, which are two major human immunodeficiency virus type 1 (HIV-1) proteins, and overexpression of the active form of CREB3 down-regulates intracellur expression of Gag and Env and decreases virion release during HIV-1 infection. It is implied that CREB3 may be a cellular target of several viruses, including at least herpes simplex virus (HSV-1), HCC and HIV-1 (Blot et al., 2006). CREB3 also plays an important role in ER stress, being involved in ER stress-associated protein degradation (ERAD) by regulating the target gene *Herp* (Homocysteine-induced endoplasmic reticulum protein) to alleviate ER pressure (Liang et al., 2006), and some ER stress inducers are able to up-regulate *CREB3* and promote release of its active form from ER. Moreover, CREB3 co-localizes with CC chemokine receptor 1 (CCR1) near the membrane region and selectively participates in leukotactin-1 (Lkn-1)-induced cell migration without influencing the chemotactic activities of other CCR1 ligands (Ko et al., 2004), possibly mediated by the nuclear factor-κB (NF-κB) signalling pathway (Jang et al., 2007a,b). In light of the diversity of its function, the exact biological action and more natural targets of *CREB3* are required to be elucidated.

However, as all the studies on *CREB2* and *CREB3* carried out so far have been performed in mice and humans, the relevance of these findings for domestic animals is unclear. Pigs are not only an economically important livestock, but also a valuable biomedical model organism. In this study, we report for the first time on the molecular cloning and chromosomal assignment of the porcine *CREB2* and *CREB3*, as well as on the transcription profile of both genes in various tissues by the semi-quantitative RT-PCR method. Additionally, to study the transcription regulation mechanism of *CREB3* in pigs, we isolated the 5' flank region of *CREB3* and characterized several putative *cis*-response elements in its promoter region. Our data will contribute to further investigation of the function of *CREB2* and *CREB3* in pig models.

Material and Methods

RNA isolation

Meishan pigs (N = 3, 4-month-old, female), provided by the Laboratory Animal Centre of Huazhong Agricultural University (Wuhan, China), were given *ad libitum* access to a standard growing diet. All procedures and housing of experimental pigs were approved by Hubei Province Committee on Laboratory Animal Care. Ten tissues including heart, liver, spleen, lung, kidney, stomach, white adipose tissues (subcutaneous back fat), skeletal muscle (*longissimus dorsi*), cerebellum and lymph (neck lymph node) were freshly collected and immediately frozen in liquid nitrogen and then stored at –80 °C for RNA extraction. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) referring to the manufacturer's protocol, treated with RNase-free DNase I (Takara, Dalian, China) to remove contaminating genomic DNA, and stored at –80 °C until use. Reverse transcription was carried out with oligo(dT)18, and the obtained cDNA was stored at –20 °C until use.

Cloning of porcine CREB2 and CREB3 coding sequence (CDS)

RT-PCR and 3'RACE technology was carried out to obtain full-length CDS of porcine *CREB2* and *CREB3*. With human *CREB2* (GenBank Accession No. NM_182810) and *CREB3* (GenBank Accession No. NM_006368) as query sequences, we searched against the porcine ESTs at GenBank, and the obtained sequences (BP165177 and DN114576 for *CREB2*, CJ030092 and BX921183 for *CREB3*) were used to design the primers for amplification of the 5' partial sequence of corresponding genes, under conditions as shown in Table 1. For 3'RACE, total RNA was reverse-transcribed using oligo(dT)18-anchor (Table 1); PCR was subsequently performed with the gene-specific primer and anchor (Table 1).

These cloned sequences were evaluated by ORF finder at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to identify the open reading frames, then the translation tool in ExPASy (http://cn.expasy.org/tools/dna.html) was carried out to obtain the inferred amino acid sequences. The physico-chemical parameters of the deduced proteins were analysed using ProtParam (http://cn.expasy.org/tools/protparam.html).

Isolation of CREB2 and CREB3 genomic DNA sequence

Based on the comparison of human *CREB2* (GenBank Accession No. NC_000022) and *CREB3* (GenBank Accession No. NC_000009) genomic sequences with porcine *CREB2* and *CREB3* CDS obtained in our study, primers (Table 1) were designed to amplify the deduced exons from porcine genomic DNA. The obtained fragments were assembled to gain the whole genomic sequences of porcine *CREB2* and *CREB3*.

Isolation of porcine CREB3 promoter

The genomic sequence (GenBank Accession No. CU074285) which is homologous to porcine *CREB3* genomic DNA sequence was retrieved from the porcine HTGS database by BLAST search. We thereby designed primers (Table 1) to isolate the presumed promoter region; due to the high GC content in the targeted sequence, 2×GC buffer (Takara, Tokyo, Japan) was added into the reactions, and PCR conditions were listed in Table 1. We then predicted transcription factor-binding sites using MatInspector (Genomatix, Tokyo, Japan)

Table 1. Primers for the cloning, gene expression and chromosomal mapping of porcine CREB2 and CREB3

Application	Sequence (5'-3')	Size (bp)	PCR parameters
5' part of *CREB2* CDS	P1: TTCCTCTCCGCTGTAGATGTAGTCC (+) P2: TGCCTTGCGGACCTCTTCTATC (–)	1298	37 cycles, 94°C 45 s, 58°C 40 s, 72°C 1 min
3'-RACE of *CREB2*	P3: AGCAGAACAAGACAGCAGCCACTCGG (+)		
tissue distribution of *CREB2*	P4: CGTTGCTGTAACCGACAAAGAC (+) P5: CTTCCGAGATTTCCACTTCACC (–)	627	35 cycles, 94°C 45 s, 55°C 40 s, 72°C 25 s
intron 2 of *CREB2*	P4: CGTTGCTGTAACCGACAAAGAC' (+) P5: CTTCCGAGATTTCCACTTCACC' (–)	723	37 cycles, 94°C 45 s, 56°C 40 s, 72°C 30 s
intron 1 of *CREB2*	P1: TTCCTCTCCGCTGTAGATGTAGTCC (+) P6: GGAACCCAGAATCAGATGCAC (–)	1148	37 cycles, 94°C 45 s, 56°C 40 s, 72°C 1 min
mapping of *CREB2*	P4: CGTTGCTGTAACCGACAAAGAC (+) P6: GGAACCCAGAATCAGATGCAC' (–)	368	37 cycles, 94°C 45 s, 56°C 40 s, 72°C 20 s
5' part of *CREB3* CDS	P7: GTTGACTCCGGGTGTACTTC (+) P8: AAGCCATGTGGTGGAACAG (–)	987	37 cycles, 94°C 45 s, 58°C 40 s, 72°C 1 min
3'-RACE of *CREB3*	P9: GGTACCACAGGACAGCTCAACCCACG (+)		
tissue distribution of *CREB3*	P10: AAGTTCTGAGACGAGTGCGAAGG (+) P11: GGGCTAGAAGACCTTGAGAAGC (–)	550	32 cycles, 94°C 45 s, 55°C 40 s, 72°C 25 s
mapping of *CREB3*	P10: AAGTTCTGAGACGAGTGCGAAGG (+) P12: CCCATTGCTGTGAACTTCTTGG (–)	438	37 cycles, 94°C 45 s, 57°C 40 s, 72°C 20 s
intron 1,2,3 of *CREB3*	P13: TTGGACTCGGGTGACGATGAC (+) P14: CGGGCAGAGTAAGCCCCTC (–)	701	37 cycles, 94°C 45 s, 57°C 40 s, 72°C 30 s
intron 4 of *CREB3*	P13: TTGGTGAGCTGATACTGACGGAG (+) P14: TTCCTTCGCACTCGTCTCAG (–)	2491	37 cycles, 94°C 45 s, 53°C 40 s, 72°C 2 min
intron 5,6 of *CREB3*	P10: AAGTTCTGAGACGAGTGCGAAGG (+) P15: TTTGTTTGCCGTCTGAATCACC (–)	1112	37 cycles, 94°C 45 s, 58°C 40 s, 72°C 1 min
intron 7,8 of *CREB3*	P16: TTTGGATCAGTTGAGGAGGCTG (+) P17: GGTGGAACAGGCAGCAAGAGT (–)	483	37 cycles, 94°C 45 s, 57°C 40 s, 72°C 20 s
promoter of *CREB3*	P18: AAAAAGAGCACCTGCCAATCTC P19:TGATCATGGTGGACAAGATAGGG	1926	16 cycles, 94°C 45 s, 63°C →56°C -0.5°C/cycle 40 s, 72°C 2 min 20 cycles, 94°C 45 s, 58°C 40 s, 72°C 2 min
GAPDH	P20: GGTGAAGGTCGGAGTGAACG (+) P21: CAGCAGAAGGGGCAGAGATG (–)	371	28 cycles 94°C 45 s, 58°C 40 s, 72°C 20 s
The reverse transcription TTTTTTTTTTTTTTTT (–) of mRNA			

(http://www.genomatix.de/products/MatInspector/index.html).

Chromosome localization by radiation hybrid mapping

The Minnesota porcine Radiation Hybrid (IMpRH) panels (Yerle et al., 1996) containing 118 hybrid clones were used to map porcine *CREB2* and *CREB3*. Primers P6 and P12 (Table 1) were designed from intron 2 of *CREB2* and intron 6 of *CREB3*, respectively; the positive (with porcine genomic DNA as template) and two negative (with hamster DNA or with water as template) controls were carried out in parallel. The amplifying results from the 118 DNA clones were analysed by the IMpRH mapping tool (http://imprh.toulouse.inra.fr), the chromosomal position was estimated by their close-linked markers on the porcine cytogenetic map; the results with a logarithm of the odds (LOD) greater than 5.0 were considered to be reliable.

Semi-quantitative RT-PCR

Tissue distribution of porcine *CREB2* and *CREB3* was measured by the semi-quantitative RT-PCR method as described previously (Marone et al., 2001). Porcine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal standard, primer sequences and PCR parameters were listed in Table 1. The PCR cycle number for each gene was adjusted within their corresponding exponential phase to avoid the saturation effect. The mRNA level of the target gene was determined relative to the endogenous *GAPDH* mRNA level and was expressed as the target gene/*GAPDH* ratio. The semi-quantitative RT-PCR procedure was repeated three times. Finally, the PCR products were resolved in 1% agarose gel containing ethidium bromide and quantitated using GelScan software (Ver.5.1, BioSciTec Science Group, Marburg, Germany).

Results

Cloning and characterization of porcine CREB2 and CREB3

The obtained 1400-bp porcine *CREB2* cDNA (GenBank accession No. EF062580) contained a 5'-UTR of 275 bp, a 3'-UTR of 78 bp including poly(A) signal (AATAAA), and a coding region of 1047 bp which encoded 348 amino acids with a calculated molecular weight of 37.9 kDa and a theoretical isoelectric point of 4.67. The coding sequence of porcine *CREB2* shared 87.9% and 83.1% nucleotide identity with human (GenBank accession no. NM_182810) and mouse (GenBank accession No. NM_009716) *CREB2*, respectively. The deduced amino acid sequence of porcine CREB2 shared

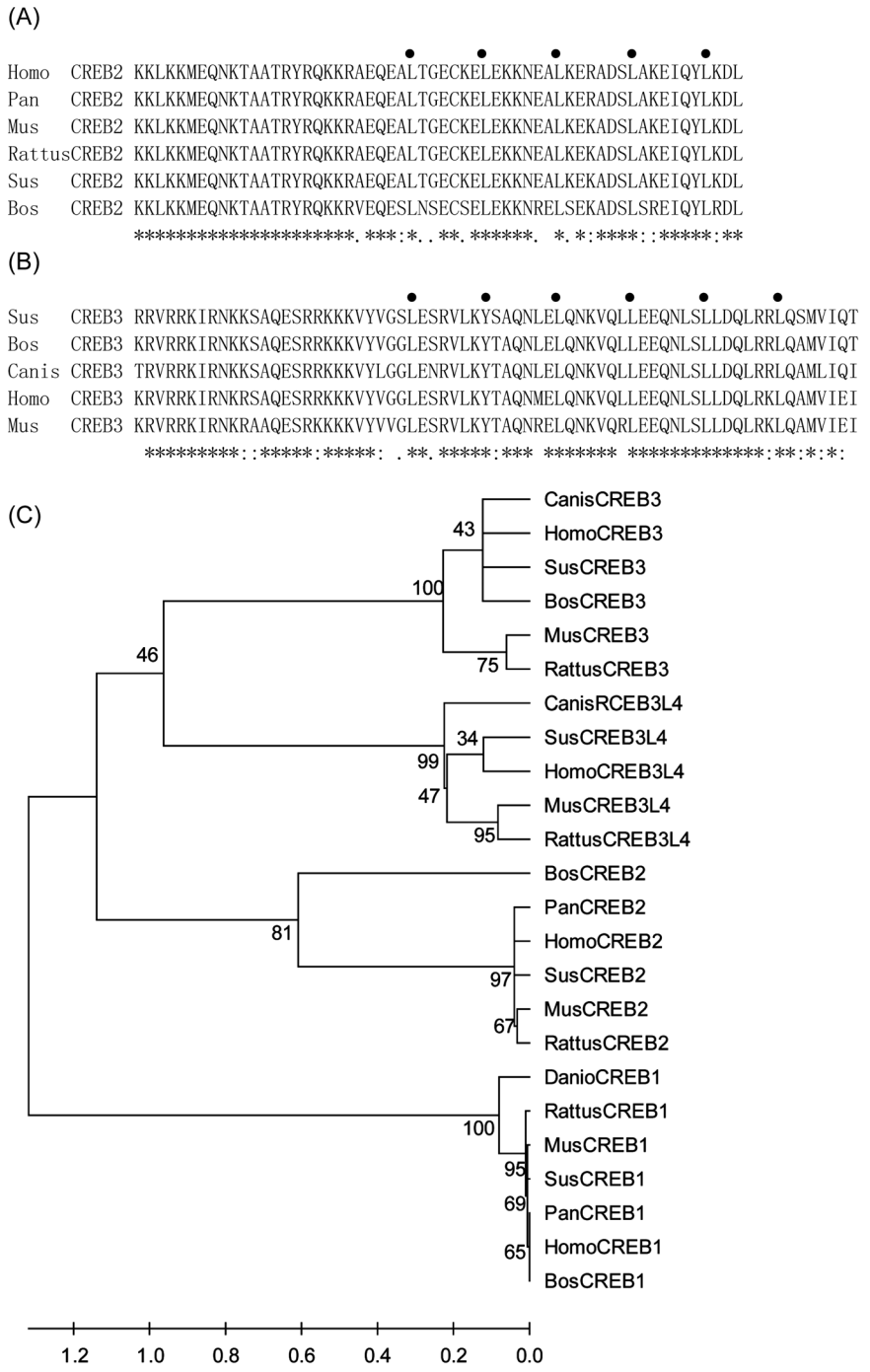

Fig. 1. Sequence alignment of the basic region and leucine zipper (bZIP) motif of porcine *CREB2* (**A**) and *CREB3* (**B**) with their corresponding homologues in other species. '*'represents conserved amino acids, ':'represents high similarity, '.' represents low similarity, solid cycles indicate the conserved repeated leucine in leucine zipper motif. (**C**) Phylogenetic relation of *CREB1, 2, 3, 3L4* in several species. Phylogenetic trees constructed by the neighbour-joining method using MEGA4.0 (http://www.megasoftware.net/) base on the multiple sequence alignments at the amino acid level by use of ClustalW (http://www.ebi.ac.uk/clustalw/). Numbers represent bootstrap confidence probabilities (%), scale bar represents the evolutionary distance. Sequence data are from GenBank:

CREB1: Sus (AY862387), Homo (NP_604391), Mus (NP_034082), Rattus (NP_112279), Bos (NP_776710), Pan (XP_001139927), Danio (NP_957203)

CREB2: Sus (EF062580), Homo (NP_877962), Mus (NP_033846), Rattus (NP_077379), Bos (NP_001029514), Pan (XP_001166016), Gallus (NP_990211), Danio (NP_998398) Canis (XP_859677)

CREB3: Sus (ABY76170), Homo (NP_006359), Mus (NP_038525), Rattus (NP_001013110), Bos (NP_776711), Canis (XP_854750), Gallus (XP_424990)

CREB3L4: Sus (EU427542), Homo (NP_570968), Mus (NP_084356), Rattus (NP_001007094), Canis (XP_855139), Pan (XP_001148367)

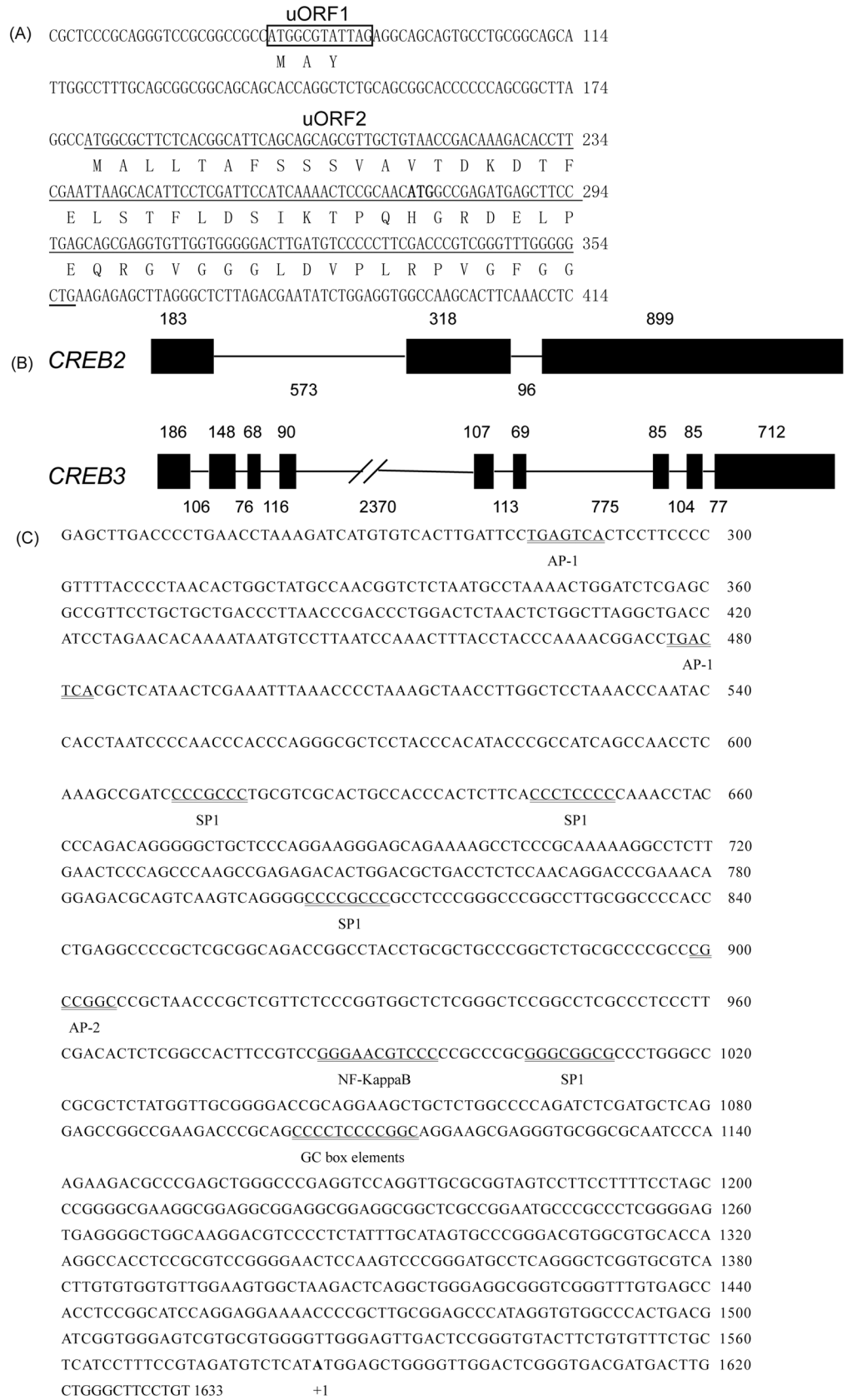

Fig. 2. (**A**) The two putative uORF of porcine *CREB2*. The first uORF (uORF1) is boxed, and the second uORF (uORF2) is underlined, the normal initiation translation code (ATG) is indicated with boldface. (**B**) Gene structure of porcine *CREB2* and *CREB3*. Black rectangles represent the exons and connecting lines represent the introns, exon and intron lengths in bp are also shown. (**C**) Nucleotide sequences of the 5'-flanking region of the porcine *CREB3* gene. Sequence analysis of the region reveals several putative transcription factor binding sites underlined. The first nucleotide (boldface) of the ATG codon designated +1.

85.8%, 85.2%, 71.7%, 83.0% and 33.8% homology with its human, chimpanzee, mouse, rat and zebrafish counterparts, respectively. The porcine *CREB3* mRNA (GenBank accession No. EU588585, 1550 bp) contained 57 bp of 5'-UTR, 395 bp of 3'-UTR, and 1,098 bp of the coding region which encoded a 365-amino acid polypeptide with a calculated molecular weight of 40.5 kDa and a theoretical isoelectric point of 4.89. The coding sequence of porcine *CREB3* shared 79.9% and 71.0% nucleotide identity with human (GenBank accession No. NM_006368) and mouse (GenBank accession No. NM_013497) *CREB3*, and the predicted amino acid sequence of porcine *CREB3* shared 72.6%, 65.0%, 20.5% and 65.7% similarity with its homologues in human, mouse, rat and cattle, respectively.

Porcine CREB2 and CREB3 both possessed a typical stucture of the CREB/ATF family, including basic region and leucine-zipper (bZIP) DNA binding motif, and the bZIP domains of the two transcription factors were evolutionarily conserved (Fig. 1A and Fig. 1B). However, CREB3 also contained a putative transmembrane region (TM) with a stretch of 17 hydrophobic amino acids (residues 231 to 247), implying a possible subcellular localization of porcine CREB3 to the endoplasmic reticulum (ER) membrane. The Ser residue 215 in DSGIXXS (residues 214–220) of porcine CREB2 was extremely conserved among various species, which was pivotal to CREB2 ubiquitination and degradation in humans (Lassot et al., 2001). Porcine CREB3 contained a conserved HCF-binding motif (DHTY, residues 78–81) at its N terminus, as reported for human CREB3, which was responsible for protein-protein interaction (Luciano and Wilson, 2000). In phylogenetic trees (Fig. 1C), *CREB1* and *CREB2* diverged early into a cluster; then *CREB3* and *CREB3L4* separated into another cluster, consistent with the structural differences between CREB2 and CREB3 proteins.

Additionally, two deduced upstream ORF (uORF) were found in the 5' uncoding region of porcine *CREB2*. The first uORF (uORF1) encoded a polypeptide of three amino acids, the second uORF (uORF2) encoded 59 amino acids and overlapped the first 82 nucleotides of the coding region; the space between these two uORF was 87 bp (Fig. 2A). Similar uORFs had also been identified in murine and human *CREB2* (Harding et al., 2000; Vattem and Wek, 2004).

Genomic characterization of the porcine CREB2 and CREB3 genes

The genomic region of porcine *CREB2* (GenBank Accession No. EU588584) covering 2069 bp consisted of three exons and two introns, while porcine *CREB3* (GenBank Accession No. EU338533) spanned approximately 5.3 kb of the genomic DNA and comprised nine exons and eight introns (Fig. 2B); the splice pattern of each intron conformed to the GT-AG rule. Comparison of porcine *CREB2* genomic DNA with its human and murine homologues (GenBank Accession No. NC_000081) showed conserved organization of *CREB2* among various species. The genomic structure of porcine *CREB3* was also similar to that of human, mouse (GenBank Accession No. NC_000070) and cattle (GenBank Accession No. NC_007306), with the exception of rat *CREB3* (GenBank Accession No. NC_005104), which contained seven exons and six introns and was obviously distinct from that of pig and other species, implying that rat *CREB3* may have experienced sequence gain and loss during evolution.

Additionally, the 5′ flanking region of porcine *CREB3* was cloned and sequenced to investigate its transcriptional regulation. We identified four consensus sequences for SP1 (stimulating protein 1), a canonical NF-κB binding site (GGGAACGTCCC), two consensus recognition sequences for activator protein-1 (AP-1) and an activator protein-2 (AP-2) recognition site in the predicted promoter (Fig. 2C). All these putative transcription recognition sites need to be further investigated and may be critical for the regulation of porcine CREB3 biological functions.

Chromosomal mapping of porcine CREB2 and CREB3

In our study, pig/rodent somatic cell hybrid panels containing 118 cell lines were used to cytogenetically map the porcine *CREB2* and *CREB3*. Porcine *CREB2* was assigned to 5p with a distance of 43 cR from the most signicantly linked marker AC02 (LOD = 9.31), a region syntenic to human chromosome 22q13.1 where *CREB2* was located. The locus of porcine *CREB3,* chromosome 1q28 with a distance of 38 cR from the most signicantly linked marker SW2551 (LOD = 9.63), was also syntenic to human chromosome 9p22.1 where

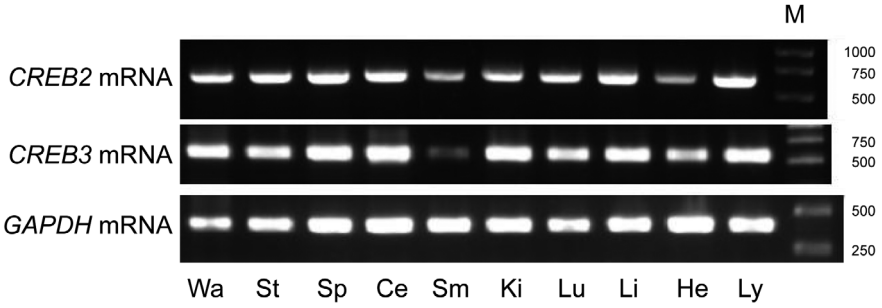

Fig. 3. Representative semi-quantitative RT-PCR results for *CREB2*, *CREB3* and *GAPDH* (internal standard) mRNA in different porcine tissues: white adipose (Wa), stomach (St), spleen (Sp), cerebellum (Ce), skeletal muscle (Sm), kidney (Ki), lung (Lu), liver (Li), heart (He) and lymph (Ly). M: Marker DL2000.

CREB3 was found. The mapping results further confirm the comparative mapping between humans and pigs, and will be useful for further insight into the evolution of this CREB/ATF family in various species.

Tissue distribution of porcine CREB2 and CREB3

Semi-quantitative RT-PCR was employed to examine the transcription pattern of porcine *CREB2* and *CREB3* in various tissues. The results (Fig. 3) indicated that both porcine *CREB2* and *CREB3* were ubiquitously transcribed in all examined tissues, including white adipose tissue, stomach, lung, cerebellum, liver, lymph, spleen, kidney, heart and skeletal muscle. However, porcine *CREB3* was present at an extremely low level in skeletal muscle.

Discussion

In the present study, we report the molecular cloning and characterization of the *CREB2* and *CREB3* genes in pigs. Similar primary structure of the deduced proteins and conserved essential motif and residues of the two genes between pig and human indicate that the two genes cloned by us are indeed homologues of the human genes *CREB2* and *CREB3*, respectively.

The two upstream ORFs (uORFs) in the 5' uncoding region have been reported to be involved in the regulation of *CREB2* expression during integrated stress response (ISR). uORF1 is considered to be a positive-acting element, while uORF2 is an inhibitory element which blocks CREB2 expression (Lu et al., 2004; Vattem and Wek, 2004). Moreover, a similar role of uORFs had been elucidated in great detail in yeast *GCN4* (General Control Nondepressible) gene (Hinnebusch and Natarajan, 2002). Hence, the two uORFs found in porcine *CREB2* may play a role in the regulation of *CREB2* expression to adapt to stress and non-stress conditions, which may further imply conservation of the regulation mechanism involving uORFs from yeast to mammals.

To investigate the regulation mechanism of *CREB3* transcription, the promoter region of porcine *CREB3* was isolated and characterized. One interesting transcription factor recognition site is the NF-κB binding site, which has been indicated in human *CREB3* promoter to mediate Lkn-1-induced chemotaxis in monocytes (Jang et al., 2007a). Besides, NF-κB signalling was considered to enlarge a cascade of inflammation signals and contributed to the different stages of atherosclerosis (de Winther et al., 2005), and Lkn-1 was involved in development of inflammation and human atherosclerosis (Lee et al., 2002). A certain relationship, therefore, may exist among *CREB3* and inflammation and atherosclerosis.

Radiation hybrid mapping is a precise method for chromosome localization; we demonstrated that porcine *CREB2* cytogenetically maps to 5p with a distance of 43 cR from the most signicantly linked marker AC02 using this method. By further search of the pig quantitative trait loci (QTL) database (http://www.animalgenome.org/QTLdb/pig.html), we showed that the porcine *CREB2* gene locates in the vicinity of QTLs for backfat at the tenth rib and feed intake, which is similar to the report on the porcine *PPARα* gene (Szczerbal et al., 2007), encoding a transcription factor involving lipid catabolism (Li et al., 2005). Additionally, activation of CREB1 has been implied to induce adipogenesis (Reusch et al., 2000), and Aplysia CREB2, similar to vertebrate CREB2 (ATF4), represses activation of CREB1 in neurons (Mohamed et al., 2005). We thereby presume that CREB2 may be involved in lipid oxidation by inhibiting CREB1 activity.

The ubiquitous transcription pattern of porcine *CREB2* and *CREB3* implies fundamental importance of these genes in the regulation of cellular events, such as the involvement in ER stress response (Harding et al., 2003; Liang et al., 2006). Porcine *CREB2* mRNA is detected in several tissues including white adipose tissue, stomach, lung, cerebellum, liver and lymph, which is similar to the transcription pattern of *CREB2* in mice (Yang and Karsenty, 2004). Moreover, the transcription pattern of porcine *CREB3* is also similar to that of its human homologue (Lu et al., 1997). The detection of *CREB3* mRNA in lymph implies its involvement in immunity, which is consistent with its possible role during HIV-1 infection (Blot et al., 2006). However, the extremely low transcription of *CREB3* in skeletal muscle implies that it may undertake a less important role in this tissue. Based on the previous report on the diversity of CREB3 functions, the ubiquitous transcription pattern of CREB3 further supports the involvement of CREB3 in the regulation of various cellular actions.

In conclusion, we report for the first time on the molecular cloning of porcine *CREB2* and *CREB3.* The resemblance of the protein primary structure, genomic structure, chromosome assignment and tissue transcription profile of the two genes between pig and human implies their conserved biological function across species.

Acknowledgments

We are grateful to Dr. Martine Yerle and Denis Milan (INRA, Castanet-Tolosan, France) for kindly providing the RH panel.

References

Blot, G., Lopez-Verges, S., Treand, C., Kubat, N. J., Delcroix-Genete, D., Emiliani, S., Benarous, R., Berlioz-Torrent, C. (2006) Luman, a new partner of HIV-1 TMgp41, interferes with Tat-mediated transcription of the HIV-1 LTR. *J. Mol. Biol.* **364,** 1034-1047.

Chen, A., Muzzio, I. A., Malleret, G., Bartsch, D., Verbitsky, M., Pavlidis, P., Yonan, A. L., Vronskaya, S., Grody, M. B., Cepeda, I., Gilliam, T. C., Kandel, E. R. (2003) Inducible enhancement of memory storage and synaptic plasticity in transgenic mice expressing an inhibitor of ATF4 (CREB-2) and C/EBP proteins. *Neuron* **39,** 655-669.

de Winther, M. P., Kanters, E., Kraal, G., Hofker, M. H. (2005) Nuclear factor κB signaling in atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **25,** 904-914.

Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., Ron, D. (2000) Regulated translation

initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* **6,** 1099-1108.

Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D. F., Bell, J. C., Hettmann, T., Leiden, J. M., Ron, D. (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell* **11,** 619-633.

Hinnebusch, A. G., Natarajan, K. (2002) Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. *Eukaryotic Cell* **1,** 22-32.

Jang, S. W., Kim, Y. S., Kim, Y. R., Sung, H. J., Ko, J. (2007a) Regulation of human LZIP expression by NF-κB and its involvement in monocyte cell migration induced by Lkn-1. *J. Biol. Chem.* **282,** 11092-11100.

Jang, S. W., Kim, Y. S., Lee, Y. H., Ko, J. (2007b) Role of human LZIP in differential activation of the NF-κB pathway that is induced by CCR1-dependent chemokines. *J. Cell. Physiol.* **211,** 630-637.

Jiang, H. Y., Wek, S. A., McGrath, B. C., Lu, D., Hai, T., Harding, H. P., Wang, X., Ron, D., Cavener, D. R., Wek, R. C. (2004) Activating transcription factor 3 is integral to the eukaryotic initiation factor 2 kinase stress response. *Mol. Cell. Biol.* **24,** 1365-1377.

Jin, D. Y., Wang, H. L., Zhou, Y., Chun, A. C., Kibler, K. V., Hou, Y. D., Kung, H., Jeang, K. T. (2000) Hepatitis C virus core protein-induced loss of LZIP function correlates with cellular transformation. *EMBO J.* **19,** 729-740.

Karpinski, B. A., Morle, G. D., Huggenvik, J., Uhler, M. D., Leiden, J. M. (1992) Molecular cloning of human CREB-2: an ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response element. *Proc. Natl. Acad. Sci. USA* **89,** 4820-4824.

Ko, J., Jang, S. W., Kim, Y. S., Kim, I. S., Sung, H. J., Kim, H. H., Park, J. Y., Lee, Y. H., Kim, J., Na, D. S. (2004) Human LZIP binds to CCR1 and differentially affects the chemotactic activities of CCR1-dependent chemokines. *FASEB J.* **18,** 890-892.

Lassot, I., Segeral, E., Berlioz-Torrent, C., Durand, H., Groussin, L., Hai, T., Benarous, R., Margottin-Goguet, F. (2001) ATF4 degradation relies on a phosphorylation-dependent interaction with the SCF(βTrCP) ubiquitin ligase. *Mol. Cell. Biol.* **21,** 2192-2202.

Lee, W. H., Kim, S. H., Jeong, E. M., Choi, Y. H., Kim, D. I., Lee, B. B., Cho, Y. S., Kwon, B. S., Park, J. E. (2002) A novel chemokine, Leukotactin-1, induces chemotaxis, pro-atherogenic cytokines, and tissue factor expression in atherosclerosis. *Atherosclerosis* **161,** 255-260.

Li, P., Zhu, Z., Lu, Y., Granneman, J. G. (2005) Metabolic and cellular plasticity in white adipose tissue II: role of peroxisome proliferator-activated receptor-α. *Am. J. Physiol. Endocrinol. Metab.* **289,** E617-626.

Liang, G., Audas, T. E., Li, Y., Cockram, G. P., Dean, J. D., Martyn, A. C., Kokame, K., Lu, R. (2006) Luman/CREB3 induces transcription of the endoplasmic reticulum (ER) stress response protein Herp through an ER stress response element. *Mol. Cell. Biol.* **26,** 7999-8010.

Lu, P. D., Harding, H. P., Ron, D. (2004) Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. *J. Cell. Biol.* **167,** 27-33.

Lu, R., Yang, P., O'Hare, P., Misra, V. (1997) Luman, a new member of the CREB/ATF family, binds to herpes simplex virus VP16-associated host cellular factor. *Mol. Cell. Biol.* **17,** 5117-5126.

Lu, R., Misra, V. (2000) Potential role for luman, the cellular homologue of herpes simplex virus VP16 (α gene trans-inducing factor), in herpesvirus latency. *J. Virol.* **74,** 934-943.

Luciano, R. L., Wilson, A. C. (2000) N-terminal transcriptional activation domain of LZIP comprises two LxxLL motifs and the host cell factor-1 binding motif. *Proc. Natl. Acad. Sci. USA* **97,** 10757-10762.

Marone, M., Mozzetti, S., De Ritis, D., Pierelli, L., Scambia, G. (2001) Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. *Biol. Proced. Online* **3,** 19-25.

Masuoka, H. C., Townes, T. M. (2002) Targeted disruption of the activating transcription factor 4 gene results in severe fetal anemia in mice. *Blood* **99,** 736-745.

Mohamed, H. A., Yao, W., Fioravante, D., Smolen, P. D., Byrne, J. H. (2005) cAMP-response elements in Aplysia creb1, creb2, and Ap-uch promoters: implications for feedback loops modulating long term memory. *J. Biol. Chem.* **280,** 27035-27043.

Reusch, J. E., Colton, L. A., Klemm, D. J. (2000) CREB activation induces adipogenesis in 3T3-L1 cells. *Mol. Cell. Biol.* **20,** 1008-1020.

Siu, F., Bain, P. J., LeBlanc-Chaffin, R., Chen, H., Kilberg, M. S. (2002) ATF4 is a mediator of the nutrient-sensing response pathway that activates the human asparagine synthetase gene. *J. Biol. Chem.* **277,** 24120-24127.

Szczerbal, I., Lin, L., Stachowiak, M., Chmurzynska, A., Mackowski, M., Winter, A., Flisikowski, K., Fries, R., Switonski, M. (2007) Cytogenetic mapping of DGAT1, PPARA, ADIPOR1 and CREB genes in the pig. *J. Appl. Genet.* **48,** 73-76.

Tanaka, T., Tsujimura, T., Takeda, K., Sugihara, A., Maekawa, A., Terada, N., Yoshida, N., Akira, S. (1998) Targeted disruption of ATF4 discloses its essential role in the formation of eye lens fibres. *Genes Cells* **3,** 801-810.

Vattem, K. M., Wek, R. C. (2004) Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc. Natl. Acad. Sci. USA* **101,** 11269-11274.

Yang, X., Karsenty, G. (2004) ATF4, the osteoblast accumulation of which is determined post-translationally, can induce osteoblast-specific gene expression in non-osteoblastic cells. *J. Biol. Chem.* **279,** 47109-47114.

Yang, X., Matsuda, K., Bialek, P., Jacquot, S., Masuoka, H. C., Schinke, T., Li, L., Brancorsini, S., Sassone-Corsi, P., Townes, T. M., Hanauer, A., Karsenty, G. (2004) ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell* **117,** 387-398.

Yerle, M., Echard, G., Robic, A., Mairal, A., Dubut-Fontana, C., Riquet, J., Pinton, P., Milan, D., Lahbib-Mansais, Y., Gellin, J. (1996) A somatic cell hybrid panel for pig regional gene mapping characterized by molecular cytogenetics. *Cytogenet. Cell Genet.* **73,** 194-202.