Functional study and regional mapping of 44 hormono-regulated genes isolated from a porcine granulosa cell library

Gwenola Tosser-Klopp, Agnès Bonnet, Martine M. Yerle, François Hatey

Genet. Sel. Evol. 33 (2001) 69–87

Original article

Functional study and regional mapping of 44 hormono-regulated genes isolated from a porcine granulosa cell library

Gwenola TOSSER-KLOPP*, Agnès BONNET, Martine YERLE, François HATEY

Laboratoire de génétique cellulaire, Institut national de la recherche agronomique, BP 27, 31326 Castanet-Tolosan Cedex, France

(Received 12 May 2000; accepted 31 August 2000)

Abstract – cDNA clones from a pig granulosa cell cDNA library were isolated by differential hybridisation for follicle stimulating hormone (FSH) regulation in granulosa cells in a previous study. The clones that did not match any known sequence were studied for their expression in granulosa cells (treated or not by FSH) and in fresh isolated ovarian follicles mainly by comparative RT-PCR analysis. These results give functional data on genes that may be implicated in follicular growing. These ESTs have been localised on the porcine genome, using a somatic cell hybrid panel, providing new type I markers on the porcine map and information on the comparative map between humans and pigs.

pig / ovarian follicle / cDNA mapping / comparative RT-PCR / comparative map

1. INTRODUCTION

Folliculogenesis is defined as the development of ovarian follicles, leading either to ovulation or, more frequently, to atresia. Follicular development is regulated by hormonal networks where the pituitary hormones play a central role, locally modulated at least by intraovarian peptides. Since granulosa cells play a major role in follicular function and can be easily isolated from follicles, they have been extensively studied in several species including pigs and are used as an *in vitro* model for understanding follicular development [4,16,23]. The effects of follicle stimulating hormone (FSH) treatment on granulosa cells in culture have been studied in depth: FSH induces the stimulation of various key genes in folliculogenesis and steroidogenesis, *e.g.* P450 scc [10].

This cell culture model was used to construct a cDNA library and to isolate FSH-regulated genes. A hundred and thirty-six expressed sequence tags (ESTs) were isolated, 82 of which could not be identified by comparing them with

* Correspondence and reprints
E-mail: tosser@toulouse.inra.fr

sequences stored in the databases in 1996 [24]. In 2000, only seven of them had a putative identification. These ESTs are of interest since they represent a source of new genes. Moreover, the reason they have not been isolated in humans may be that the process of reproduction is difficult to study with functional ovaries.

A functional analysis of these genes was therefore carried out, by Northern blot or comparative RT-PCR to demonstrate the regulation of the corresponding mRNA in either cultured granulosa cells (different conditions) or in well-characterised isolated follicles.

In pigs, genetic and cytogenetic maps have been developed mainly by mapping anonymous polymorphic markers like microsatellites. Several strategies were then developed to obtain information on the correspondences between the genomes of pigs and other mammals.

The conservation of large segments of the mammalian genome between humans and pigs has been demonstrated through heterologous hybridisation approaches [7,9,21]. However, within these segments, the order of the genes is not always maintained, some very small segments may not be detected and the limits of the regions are not very precisely determined.

Consequently, expressed genes are needed as markers, since they are generally well conserved between species, especially between pigs and humans, and are a source of candidate genes for the zootechnical functions studied. cDNAs thus represent a powerful tool since a great number of cDNAs have been isolated and located on the human genome and the information is available through databases. Lahbib-Mansais *et al.* [14] demonstrated that human sequences are useful (even if the success rate of heterologous amplification is low) for mapping cDNAs onto the pig genome. In pigs, 532 genes have been localised on the cytogenetic map (http://www.toulouse.inra.fr/lgc/pig/cyto/genes.htm).

In the last few years, several tissue-specific pig libraries have been constructed and used in regional localisation with the somatic-cell hybrid made available by M. Yerle *et al.* [28]. A library of the small intestine [26] enabled the localisation of 89 new porcine genes [6,13,27]. A project is under way to localise 700 cDNAs on the porcine genome by using this panel [8].

Since several regions of the pig genome are involved in production traits [1, 25], it would be interesting to localise the genes isolated from our cDNA library on the porcine genome. These genes may become candidate genes for reproduction traits. In this study, 44 cDNAs were localised on the porcine genome. This also provides new data for comparing pig and human genomes when a human sequence is found.

2. MATERIALS AND METHODS

2.1. Clones and primers

The eighty-two potentially hormono-regulated clones that had already been sequenced but not identified [24] were studied. The Codonpreference program of the Wisconsin Genetic Computer Group software package (GCG Package, Wisconsin Package, Version 9.1, September 1997, Genetics Computer Group,

Madison, Wisconsin 53711, USA) was used to predict ORFs (Open Reading Frame) in the ESTs, in order to select the primers in the 3'UTR, if possible. The primers were selected by the use of the Prime program (GCG Package) in order to amplify 85 to 286 bp products. The primers are listed in Table I.

2.2. Obtainment of granulosa cells or follicles and RNA extraction

The culture conditions and RNA extraction of granulosa cultured cells have been described previously [24].

For the study of fresh calibrated follicles, follicles were isolated from swine 24 or 96 h after the end of altrenogest (a progesterone agonist) treatment, (REGUMATE; Hoechst-Roussel Agri-Vet Company, Somerville, NJ, 20 mg per day for 18 days). The diameter of the follicles was measured and they were classified as follows: small (1 to 2 mm in diameter), medium (3 to 4 mm), and large (5 to 8 mm) follicles. The granulosa cells were collected as with cell culture experiments and a few cells were examined as described by Monget *et al.* [18] to determine the physiological status of the follicles.

The RNA was extracted from the granulosa cells of healthy follicles with the Rneasy kit (Qiagen) using pools of follicles of the same class (8 to 20 samples, depending on the follicle diameter) and treated by DNase I for 30 min at 37 °C (1 U/mg of RNA, Boehringer). Then RNA was reextracted using the same purification kit.

2.3. cDNA synthesis, comparative RT-PCR, and tissue analysis

Total DNase I treated RNA (2 μg) was reverse transcribed in a 20 μL reaction by using 100 pmol of a dT15 oligonucleotide with Superscript II kit (Life Technologies), and 0.5 μCi of α^{32}P labeled dCTP (specific activity $> 3\,000$ Ci $\cdot$ mmol^{-1}, Dupont de Nemours) was added to monitor the transcription. After one hour of incubation at 37 °C, the RNA was hydrolyzed by adding 10 μL of 0.9 N NaOH and incubating 15 min at 65 °C. The reaction was neutralized by adding 15 μL of 0.9 N acetic acid and 53 μL of distilled water. The first strand cDNAs were purified by G50 chromatography and quantified by measuring the radioactivity incorporated. From each cDNA sample (control and FSH-treated cells, small, medium, or large healthy follicles) the same dilutions of cDNA were made: 2 pg $\cdot$ μL^{-1}, 10 pg $\cdot$ μL^{-1}, 50 pg $\cdot$ μL^{-1}, and 200 pg$\cdot\mu$L^{-1}. The number of cycles in the PCR was selected so as to fall within the exponential phase of the amplification reaction, for at least two dilutions. Master mixes were prepared and each PCR amplification (30 μL final reaction volume) was carried out with 5 μL of each dilution of first strand cDNA, 1 μM of each specific primer, 200 μM of each dNTP, 1.5 or 2.5 mM $MgCl_2$ and 2 units of Taq DNA polymerase (Life Technologies), in the buffer provided by the manufacturer. PCR amplifications were performed on a PHC 3 thermocycler (Techne) as follows: denaturation 5 min at 94 °C, followed by at least 25 cycles of (1 min at 94 °C, 30 s at the specific hybridisation temperature of each primer pair, 30 s at 72 °C). The number of cycles of amplification depends

Table I. PCR conditions and localisation results. *(continued on the next pages)*

Clone	Accession number	Primer pairs (upper/lower)	Size (bp) [1]	PCR conditions [2]	Localisation	Type [3]	Statistics [4] ρ,P, ER, D+/D-
D59	X93233	GAA CCA TGT AAA GAA GTT CC CAG CTC CAA TGA CTA GAA C	119	56, 1.5, 30	1q23-q27	S	1, 0.86, 0.5, 0/0
XIIIF2	Z72412	TTC AGG ATG GTG GAA TAT C AAA ATG ACT GAC CAA GGC	140	TD 67/57, 1.5, 50	1q23-q27	A	0.89, 0.86, 0.5, 1/0
K85	X91314	TGA ATG ATA AGT AAA GGC AGA A CAT GAG TTC ATC AGT AGT AAG G	85	57, 1.5, 30	2 p14-p17	A	1, 0.79, 0.5, 0/0
D62	X93231	AAC TCT GGC TTG GTG CTT G GCC GAA TCA CTT AAA ACG C	174	59, 1.5, 30	2 1/2q21-1/2q22	S	1, 0.69; 0.5; 0/0
IIID3	X91704	AAT ACC TGA TTC CAC CCC AC GGT TTT ATT TCC TTT GGT CCA C	140	TD 67/57, 2.5, 50	2 1/2q21-1/2q22	A	1, 0.68, 0.5, 0/0
VIIA12	X91713	ACT CTT GGC TGG TAG TTT C CCC ACT TCC AAC CAT TAT C	115	54, 1.5, 35	3p16-p17 or 3q12	A	0.78, 2 X 0.4, 0.5, 2/1 or 3/0
E*9	X93242	GGG GAG CTA GGA ACA AAA A CCA AAT AAA ACT CAG TAA CTA A	120	55, 1.5, 30	3 p16-17 or 16 q14 or 16 q22-q24	A A	0.85, 0.72, 0.1, 1/1 0.78, 2 X 0.08, 0.1, 2/1
IF4	X91689	TCC CCA TAA GAC CCA CTG AG CAT AAA CCC CCA TCA AAG AAA C	108	TD 67/57, 1.5, 50	3 p11	A	0.93, 0.97, 0.1, 1/0
C134	X89989	TAA TTC CCC AAT CCC TGC C ACA CCA CAC CAC AAC CCA TC	139	61, 1.5, 30	3 q11	A	1, 0.88, 0.1, 0/0
D47	X89992	TGA ACC GTA GCA TCA GAG GAA AAG ACA ACC AAG CAG	140	53, 2.5, 30	3 q14	A	1, 0.81, 0.1, 0/0
IF5	X91690	CAA GTC CCT CTC TCC CTC CCT TTK GCT GTG TTC TCT TA	222	65, 1.5, 35	3 q14	A	0.77, 0.81, 0.5, 2/1
H*37	X91318	CAA CCA ATC CAC CAA ACC GCC AGC AAA TAC CAC AAG	117	56, 1.5, 30	3 q21-q27	A	1, 0.80, 0.1, 0/0
XIIB9	Z72403	ACC TAT GTA AGA AGG GGG TGT AAA TGG GGA GTT ATC AG	137	55, 1.5, 30	3 q21-q27	A	1, 0.81, 0.1, 0/0
J31	X91321	TCC TTC CGT GAC ACC TCT TC GGG ATT GAA TCT GCC TCC TC	108	62, 1.5, 30	4 p14-p15 or 4 p13	A	0.93, 2 x 0.47, 0.1, 0/1 or1/0
IIE9	X91693	TGA AAG CAG GTG TGA CAG GTT AAA ATC GGG ACC AAG	133	54, 1.5, 30	4 q21-q23	A	1, 0.89,0.1, 0/0
N*9	X91330	CAG CTA ATT CAG CTA AGT ACC CCA GAG TAT TAC ACC CTA CAA C	116	62, 2.5, 30	5 p11-p15	A	1, 1, 0.1, 0/0

Table I. Continued.

Clone	Accession number	Primer pairs (upper/lower)	Size (bp) [1]	PCR conditions[2]	Localisation	Type [3]	Statistics [4] ρ,P, ER, D+/D-
VIIIE1	X91721	AAG AAA ATG CTA AAT TGC TG GGA TCT ATT TCT ATC CAA AC	113	53, 1.5, 30	5 1/2q21-q24	A	0.93, 0.89, 0.1, 0/1
IF10	X91688	TGT TAA GTC GTC GTT AGC AG ATC ATA TCC CTT CCA GCC	124	58, 1.5, 35	5 1/2q21-q24	A	1, 0.89, 0.1, 0/0
M19	X93248	AGC TGC TTA TTC ACC AAG AAA C ACT GGG AGA TAC GGC TTC AC	301	62, 1.5, 30	5 q25	A	1, 0.75, 0.1, 1/1
XIIA1	Z72401	TGT TAT TTG GGG GGT GGA G GGC ACA GGT ACA TGG AAA AA	104	TD 63/53,1.5, 50	6 1/2p14-p15	A	0.88, 0.87, 0.5, 0/1
D46	X89991	TGG AGC AGA GAT ATG AGG CAG TAT GTA AAA GGC AGT GG	124	TD 63/53,1.5, 50	6 1/2q32	A	1, 0.95, 0.5, 0/0
XIIB1	Z72420	ACA GCA ACA TGG GAC CTG AG TTA AGG ACC CAA CGT AGT CTC C	127	64, 1.5, 30	6 1/2q31 or 6 1/2q32 or 6q33-q34 or 6 1/2q35	A	0.87, 4 X 0.23,0.5, 1/0 or 0/1 or 0/1 or 0/1
D*9	X91674	GAA ATT GGG AGT GAG AGG G ACA TTG TTA CAG TCA GGA TAC G	90	58, 1.5, 30	7 1/2p11-p12	A	1, 0.82, 0.1, 0/0
I36	X91309	TTT CTA AAC AGC CCC AAG ATA AAG GAC AAA CAG AGC AG	270	TD 63/53,1.5, 50	7q12-q23 or 7q26	S	1, 2 X 0.45, 0.1, 0/0
VIIIH4	X91722	AAG TGC TCT CTG GAT GTG TGC TAC AGC AAA ACA AGG	140	55, 1.5, 30	7q12-q23 or 7q26	A	1, 2 X 0.45, 0.1, 0/0
M21	X91325	TAT TGA GTT GAA TAT TTG CC GST TTA TGT AAT TGG GTG GA	286	57, 3, 35	9 1/3p21-p24	A	0.86, 0.98, 0.1, 2/0
J20	X93244	TCC CAC ATT TTC CTC TTT C ACA TTC CAG CAA GCA CAC	142	55.5, 2.5, 30	9q12-1/3q21	S	1, 0.98, 0.1, 0/0
IVF11	X91725	ACC AGG CAC ATG AGA TAC GCT TAT CAC TTT CCA ACA AC	146	55, 1.5, 30	9q11 or 9 2/3q21-q26	A	1, 2 X 0.50, 0.1, 0/0
IVA5	X91699	CCA CAG CCT CCT ATT AAC CC AGC AAA GTC CCT CCT TCT CC	102	62, 1.5, 35	10q17	A	0.78, 0.88, 0.1, 1/2
IVF1	X91708	AAC TGA GCT TTT CCA TCG ATT TTC CCA GGA CCT TAT AC	135	TD 63/53,1.5, 50	12 p11-2/3 p13	A	1, 0.89, 0.1, 0/0
XID7	Z72398	AAG GAC AGA ACC ATC CAC TTA AGG ACA CGC CTT CAG	252	56, 1.5, 30	12q11-q15	A	1, 0.99, 0.1, 0/0
M*5	X91328	CTG AAG TCA TCC TCA TAA CC CAG AAA GCA AGA ACA GAA AG	113	59, 2.5, 30	12q11-q15	A	1, 0.99, 0.1, 0/0

Table I. Continued.

Clone	Accession number	Primer pairs (upper/lower)	Size (bp) [1]	PCR conditions[2]	Localisation	Type [3]	Statistics [4] ρ,P, ER, D+/D-
XIVF3	Z72417	TTA GGG CAG AAA GAG GAC AGA ACC ATA GCA ATG CAG	255	TD 63/53,1.5, 50	13q21-22 or 13q23-1/2q41	S	0.80, 2 X 0.42, 5, 0/1
IVF4	X91709	GAA AAC CCA GGA CAA AAT TC GTT TCT CAA GCT TTT CAT GC	152	56, 1.5, 30	13q42-1/2q46	A	1, 0.81, 0.5, 0/0
VIIE12	X91717	AAC TGG CTA CCT GGA CAT C ACA AAC ATA CAC GGG ACA C	110	58, 1.5, 30	14B2	A	0.82, 0.73, 0.5, 0/2
K27	X91312	AGA TGA GAA AGG AAA GCA G ATC CAC AGG AAT CAC CAG	142	55, 2.5, 30	14 C or 14G	S	0.86, 2 X 0.46, 0.5, 0/1 or 1/0
XIIE3	Z72407	GAG AAA GGA GGA AAG AAA AG TTG GTG TAA AGA CAG GAA G	259	54, 1.5, 30	14E	A	1, 0.80, 0.5, 0/0
M*4	X91327	TGT ACA TAT TGG AAG GGT C ACT TCA TAA GGG TAA GTT GG	110	55, 1.5, 30	14G	A	0.85, 0.85, 1, 1/0
XIIID3	Z72411	AGA CTC CAT TCT TCC TCC C CCA CTG ACC AAT AAA CAG AAC	139	TD 67/57, 1.5, 50	16q11-q13	A	0.91, 0.99, 0.1, 0/1
VB5	X93272	AAG TTA GCG AGG AGG AAG AAA GGA GAG GGT AGA AAA G	125	55.5, 1.5, 30	16 q14 or 16q22-q23	A	0.93, 2 x 0.47, 0.1, 0/1
IIC12	X91681	GCA CAA CAC CAC AGC AAT AAG CAG TAA ATA ATG GGT AGC CTG G	86	TD 67/57, 2.5, 50	16 q14 or 16q22-q23	A	0.85, 2 x 0.45, 0.1, 0/2
XIIIA10	Z72410	AGT TCC CGT TGT GGG TTA C ACA GCA ACA GCA ACA CAG G	149	TD 67/57, 1.5, 50	17 1/2q21-q23	A	1, 0.88, 0.5, 0/0
H*147	X91320	AGC ACC CCC TTA GAA CAG AC TCC CCT ATT TCC ACC CTT C	109	TD 67/57, 1.5, 50	18q13-q21	A	0.90, 0.80, 0.1, 1/0
IVE5	X91703	CAA ACT GAA AAG GAA GGA AC CAT TCT AAC AGA CAG CTC AAC	163	TD 63/53,1.5, 50	Xq13	A	0.85, 0.73, 0.1, 2/0

[1] Size of the amplified product on genomic DNA with the primers described in the previous column. [2] PCR conditions: annealing temperature or Touch down (TD) maximum temperature/minimum temperature experiment, MgCl2 concentration (mM), number of cycles. [3] Type of analysis : S (SSCP) or A (agarose). [4] Statistical scores obtained in the data analysis : ρ (correlation coefficient), P (probability), E (error risk), D+ (number of positive discordants), D- (number of negative discordants).

on the abundance of the cDNA (27 to 36 cycles). Fifteen μL of PCR products were analyzed by electrophoresis on 1% agarose gel. cDNA quantification and dilutions were tested using 2 internal standards: the P450scc, the Insulin-like Growth Factor I (IGF I) and as an external standard a plant messenger RNA: I11a [2]. P450scc and IGF1 which are up regulated by FSH [10,11] were analyzed using the same cDNA dilutions. Analysis of the data indicated that FSH increased the mRNA levels by a factor of at least 5. I11a mRNA was added to the RNA (200 fg for 2 μg RNA sample) in order to check that the specific amplification of the corresponding cDNA gave an equal signal in control and FSH-treated cells. The primers are described in Table I and the PCR conditions in Table II. The experiments were performed at least twice.

2.4. Regional assignment on somatic cell hybrid panel

The optimization of the PCR conditions was performed on 50 ng of pig, hamster, mouse, and human DNA or 1 ng of plasmid DNA in 25 μL using 0.5 μM of each primer, 100 μM of each dNTP, 0.5 U of Life Technologies Taq polymerase, in the buffer provided by the manufacturer. The first experiments were carried out with different concentrations of $MgCl_2$ (1 to 3 mM) and annealing temperatures, using thirty to thirty-five cycles (annealing temperature 30 s - 72 °C 30 s - 94 °C 30 s) with an initial 5-min denaturation step and a final 10-min elongation step. If no amplification was observed for pig DNA in these conditions, touch down (TD) conditions were used: 5 × 3 cycles were effected with an annealing temperature decreasing from 67 °C or 63 °C down to 59 °C or 55 °C in two-degree steps and then thirty-five cycles were performed at either 57 °C or 53 °C. This optimization step enabled a product of amplification with pig DNA and no amplification with rodent DNA, or products that could be differentiated by either agarose or SSCP analysis.

The panel used for the regional assignments consisted of 19 pig × hamster and 8 pig × mouse hybrid cell lines (Yerle *et al.* [28]). The PCR conditions determined above were used on 50 ng of each hybrid using pig and rodent DNA as controls. 10 μL of the PCR reactions were analysed on 2% agarose gel or, when necessary, in SSCP with silver staining [15].

Each hybrid was then scored for the presence of a pig specific DNA fragment and the assignments were performed, using software that calculates the probability of the localisation of a marker among the 115 regions of the porcine genome determined by this panel
(http://www.toulouse.inra.fr/lgc/pig/pcr/pcr.htm, [3,22]).

2.5. Sequence comparison and comparative map with humans

For each clone, the sequence data were analysed with the FASTA program [19] for similarities with known sequences in the entire Genbank (release 110, 12/98) and EMBL (release 56, 09/98) databases. The most significant similarities (more than 80% identity using the 5′ end sequence of a clone and more than 75% identity using the 3′ end) were further used to collect data on human mapping. Different sources of information were used:
the TIGR database (http://www.tigr.org/index.html),

Unigene (http://www.ncbi.nlm.nih.gov/UniGene/Hs.Home.html) and the Gene Map 98 (http://www.ncbi.nlm.nih.gov/genemap).

3. RESULTS

Eleven of the 82 clones studied did not permit the selection of appropriate primers, because either inserts were too short or the melting point of the sequence was too low. Of the 71 primer pairs tested on porcine genomic DNA, 24 either failed to amplify or produced several non-specific bands. The other 47 (66%) amplified pig DNA and were used for regulation and localisation studies.

3.1. Regulation studies

The regulation studies using Northern blot analysis proved to be difficult, since only 8 out of the 24 genes analysed gave an interpretable result (data not shown). The other 16 probes either gave too weak a signal, no signal or a signal that was not interpretable (Tab. II). Consequently a study of the regulation of these genes by RT-PCR analysis was undertaken.

Forty genes were tested by this method, using control or FSH-stimulated granulosa cells (Tab. II). Fourteen clones were stimulated by FSH (among them, three that had already proven to be stimulated by Northern blot analysis), one was down-regulated by FSH (confirmed by Northern blot analysis). The level of expression of the remaining 25 was not changed by FSH.

FSH stimulation is illustrated in Figure 1. Figure 1A shows that the signals of amplification of an exogenous control mRNA were identical for FSH-treated cells (250 pg of cDNA in lane c and 1 ng in lane d) or control cells (250 pg of cDNA in lane g and 1 ng in lane h). Figure 1B shows that for the P450scc gene, a signal was obtained in a 27-cycle PCR reaction on 50 pg (lane b), 250 pg (lane c) or 1 ng (lane d) of cDNA from FSH-treated cells and only in reactions containing 250 pg (lane g) or 1 ng (lane h) of cDNA from control cells. For the IGF1 gene, similar results, showing an up-regulation of the gene by FSH were obtained (data not shown). Using the same cDNA preparations for the VIIIH4 gene (Fig. 1C), we obtained a signal in a 31-cycle PCR reaction on 250 pg (lane c) or 1 ng (lane d) of cDNA from FSH-treated cells and only in reactions containing 1 ng (lane h) of cDNA from control cells, indicating an approximately 4-fold stimulation by FSH.

Twelve of the 15 regulated clones were tested by RT-PCR analysis on the granulosa cells of freshly isolated porcine follicles. Six of these gave a differential signal, depending on the state of development of the follicle. Figure 1D shows that VIIIH4 was expressed at a higher level in large follicles (lanes a to d) and in small follicles harvested 96 h post Regumate treatment (lanes i to l) than in medium-size follicles (lanes e to h) or in small follicles harvested 24 h post Regumate treatment (lanes m to p).

3.2. Regional assignment using the somatic hybrid panel

Of the 47 primer pairs that amplified the pig DNA, one (M19) resulted in the amplification of a longer fragment than the fragment obtained with the plasmid

Table II. Regulation studies. *(continued on the next page)*

Clone	Putative identification	PCR conditions (1)	Northern (2)	RT-PCR results (3)	
				cells	follicles
D59	UBA3	56, 1.5, 30	NT	FSH > T	SF24=SF96=MF24=LF96
XIIIF2	rab1 1a GTPase	53, 1.5, 29	NT	FSH=T	
K85	FGFR4	60, 1.5, 30	NT	FSH > T	SF24=SF96=MF24=LF96
D62		59, 1.5, 30	NT	FSH > T	SF24=SF96=MF24=LF96
IIID3		62, 1.5, 28	T>FSH	T>FSH	
VIIA12		56, 1.5, 30	T>FSH	FSH=T	
E*9		56, 1.5, 36	FSH>T	FSH > T	
IF4	KIAA0183 gene	ND	FSH=T		
C134		61, 1.5, 30	NT	FSH=T	
D47		54, 1.5, 30	NT	FSH=T	
IF5		57, 1.5, 27	FSH>T	FSH>T	
H*37		56, 1.5, 28	NC	FSH>T	SF24=SF96=MF24=LF96
XIIB9		55, 1.5, 31	NT	FSH=T	
J31		ND	NC		
IIE9		54, 1.5, 29	NC	FSH> T	SF24=SF96=MF24=LF96
N*9		62, 2.5, 33	NC	FSH>T	SF96>SF24=MF24=LF96
VIIIE1		55, 1.5, 35	NT	FSH> T	SF96>SF24=MF24=LF96
IF10	Methionine aminopeptidase	56, 1.5, 31	FSH>T	FSH>T	
M19	DRIM protein	62, 2.5, 31	NC	FSH=T	
XIIA1		58, 1.5, 33	NT	FSH=T	
D46		56, 1.5, 30	NC	FSH=T	LF96>>SF96>MF24>SF24
XIIB1		64, 1.5, 31	NT	FSH>T	SF96=MF24=LF96>SF24
D*9		ND	NT		
I36		54, 1.5, 31	T>FSH	FSH=T	
VIIIH4		55, 1.5, 31	NT	FSH>T	SF96=LF96>MF24>=SF24
M21		53, 1.5, 29	FSH=T	FSH=T	
J20		55.5, 2.5, 32	NT	FSH=T	
IVF11		55, 1.5, 32	NC	FSH=T	
IVA5	D123 protein	62, 1.5, 30	NC	FSH > T	SF24=SF96=MF24=LF96
IVF1		55, 1.5, 30	NC	FSH=T	
XID7	rabaptin-5	56, 1.5, 31	NT	FSH=T	
M*5		ND	NT		
XIVF3		53, 1.5, 27	NT	FSH=T	
IVF4		56, 1.5, 27	NC	FSH=T	
VIIE12		58, 1.5, 28	NC	FSH=T	
K27	KIAA0262 gene	55, 1.5, 36	NT	FSH=T	
XIIE3		55, 1.5, 30	NT	FSH=T	

Table II. Continued.

Clone	Putative identification	PCR conditions (1)	Northern (2)	RT-PCR results (3)	
				cells	follicles
M*4		55, 1.5, 33	NC	FSH>T	SF24=MF24<SF96=LF96
XIIID3	Highly similar to elastin like protein	59, 1.5, 26	NC	FSH=T	
VB5		55.5, 1.5, 35	NC	FSH=T	
IIC12		63, 1.5,35	NC	FSH=T	
XIIIA10		58, 1.5, 29	NT	FSH=T	
H*147		60, 1.5, 35	NT	FSH=T	
IVE5		58, 1.5, 30	NC	FSH=T	

(1) PCR conditions: annealing temperature, MgCl2 concentration (mM), number of cycles or the experiment was not done (ND). (2) Northern blot results: the gene was up-regulated (FSH>T), down-regulated (FSH<T), not regulated (FSH=T) by FSH, not conclusive (NC) or the experiment was not done (NT). (3) For RT-PCR results on cells, the same notation has been used as for Northern blot analysis.
Small follicles (SF), medium size follicles (MF) or large follicles (LF) were collected either 24 h (24) or 96 h (96) after the end of the treatment by Regumate.

control. This product was sequenced and was found to contain a small intron (about 200 bp) in addition to the expected sequence. For all other primer pairs, the product obtained had the expected size, ranging from 85 to 286 bp. For the seven primer pairs that allowed the amplification of the rodent DNA, the pig specific product was identified with the SSCP technique.

Forty-four clones were assigned with a probability > 0.72 and a correlation coefficient > 0.75. The other three were assigned with a correlation coefficient between 0.7 and 0.75, this was considered insufficiently significant (data not shown). However, one of them, E*9 was localised by the use of a porcine irradiated panel [29] on Sscr16 (data not shown) although the probability of this localisation was only 0.16 with the somatic cell hybrid panel. The results are listed in Table I, along with the correlation coefficient, the error risk, the probability of the localisation, and the number of discordant results (positive or negative). Among the 44 localisations presented here, 42 showed an error risk of less than 0.5%, including 27 with an error risk $< 0.1\%$. Two markers (XIVF3 , error risk $< 5\%$ and M*4, error risk $< 1\%$) had a higher error risk, due to a low number of positive hybrids in the region of localisation.

4. DISCUSSION

4.1. Regulation studies

This paper describes the successful use of comparative RT-PCR analysis. Forty-four ESTs were studied using this method. This approach was particularly useful since, in our experiments, Northern experiments have often failed (a failure rate of 80%). This could be due to the low level of expression of many of the mRNAs, since they need a considerable number of cycles of amplification (about 30). In contrast to Northern blot analysis, RT-PCR does not give any information on the size of the mRNA corresponding to the EST, but it enables

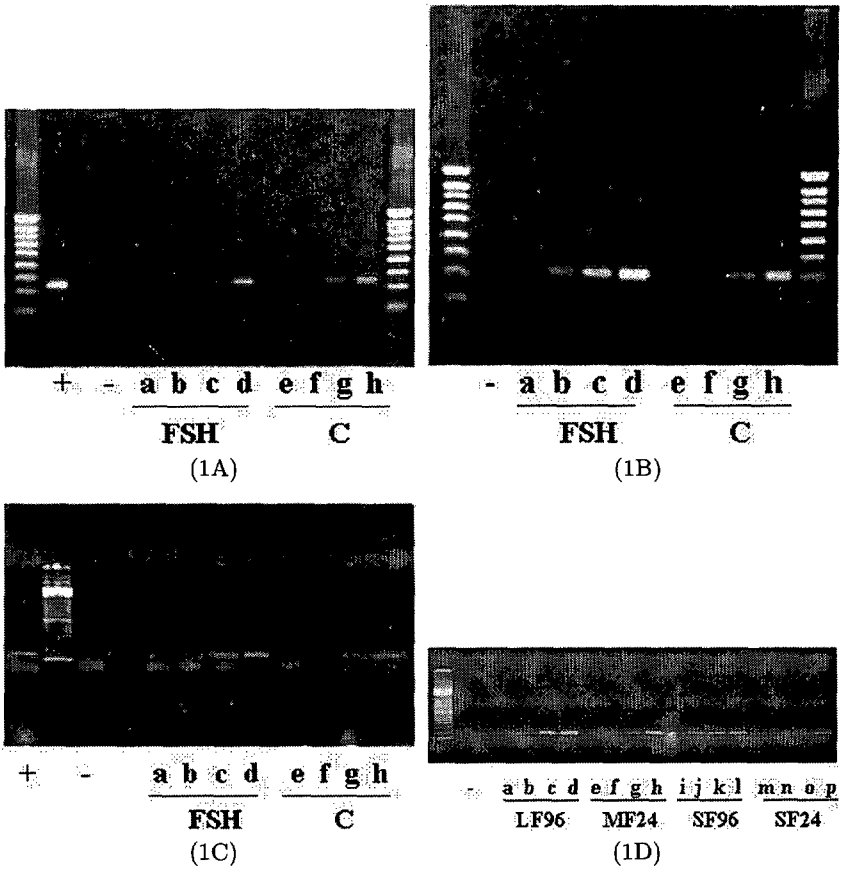

Figure 1. Comparative RT-PCR analysis study.
Total RNA extracted from control granulosa cells or FSH-treated cells (1A, 1B, 1C) or from small (SF), medium size (MF) or large (LF) ovarian follicles harvested 24 or 96 h post-Regumate treatment (1D) were reverse transcribed and four different quantities (10 pg, 50 pg, 250 pg, 1 ng from left to right) of cDNA were used for a PCR amplification using specific primers.
Figure 1A shows the amplification of I11a gene, an external control mRNA.
Figure 1B shows the amplification of the P450scc gene, which is up regulated in porcine granulosa cells.
Figure 1C and 1D show the amplification of VIIIH4, which is up regulated in FSH-treated cells, in comparison with control cells (1C) and in large and small size follicles harvested 96 h post Regumate treatment, in comparison with medium size and small size follicles harvested 24 h post Regumate treatment (1D).

faster analysis using less mRNA, with no radioactivity. Moreover, the PCR technique enables the study of many genes simultaneously by the use of 96-well PCR plates. It allows the study of fresh follicles, which would not be possible by Northern blot experiments since very little mRNA can be extracted from this material.

Since the development of the follicles is probably under the control of genes whose expression varies according to the size and the hormonal environment of the follicle, the genes that have been shown to be regulated according to the size of the follicle are of potential interest. Similarly, the study of follicles undergoing atresia would provide further data. However, so far, it has been difficult to obtain good quality mRNA from this material (data not shown).

More experiments should be carried out, in order to localise these transcripts more precisely, *e.g.* by *in situ* hybridisation of ovaries at different physiological stages. Moreover, this should give more accurate data on the time and localisation of the expression of these genes.

4.2. Localisation of cDNA and comparative mapping

This paper also describes forty-four regional localisations of previously unknown porcine genes on the pig genome, using a somatic cell hybrid panel. Whenever possible, primers were designed in a region predicted to be a 3′ UTR, a region that contains few introns [17] and that is highly polymorphic, in order to reduce the possibility of amplifying rodent DNA. Sixty-two percent of the sequences were localised, which is a standard rate: different teams using this somatic cell hybrid panel, mostly on identified genes, have described a success rate between 50% and 80% [6,13,27]. Moreover, most of the localisations were obtained using agarose analysis and the PCR fragment had the expected size, with one exception.

Comparative data for the human genome were available for 22 of the 44 localisations reported in this paper (Tab. III). Fifteen localisations were in accordance with the data derived from the three experiments of heterologous painting approaches [7,9,21], two were in accordance with the data derived from the first two experiments and five suggest new correspondences between pig and human genomes.

Localisations on Sscr4, Sscr6, Sscr7, Sscr10, Sscr12, Sscr13, and Sscr14 were in accordance with heterologous painting experiments and will not be discussed.

4.3. Sscr1

Sscr1q is in correspondence with Hsap 18, 15, 14, and 9.

The correspondence with Hsap 15 concerns the zones Sscr1 q13-q14 [21], q15-q18 [7,21], and q12-q22 [9]. The localisation of XIIIF2 on Sscr1 suggests an extension of the correspondence between Hsap 15 and Sscr1 to the Sscr 1 q23-q27 zone, and, probably to the top of this region (1 q23), since two other markers (MGAT2 [20] and H74180 [14]) located in the same pig region are located on Hsap14.

The localisation of D59 gave a new correspondence between Sscr1 and Hsap3, although available comparative data suggest a localisation on either Hsap9 or Hsap14. Our cDNA clone was 2 000 bp-long and its sequence was very similar to the human one (more than 90% identity on 1 300 bp on the entire coding region and more than 80% on the 700 bp 3′UTR).

Table III. Comparative mapping of porcine ESTs in pigs and humans. *(continued on the next pages)*

Clone	Significatively homologous human sequence	% identity	bp	Putative identification	Localisation (on human genome)	Markers [1]		Localisation (on porcine genome)	Comparative map [2]
						top	bottom		
D59	AF046024	93	301	UBA3	3 p13-p12.3	D3S1261	D3S1604	1q23-q27	HSA 9 or 14 expected
XIIIF2	AA504142	90	376	rab1 1a GTPase	15 q23-q24.1	D15S125	D15S216	1q23-q27	OK, extension of the conserved segment
K85	AF001542	83	241	FGFR4	11 p11.12-q12.	D11S1357	D11S913	2 p14-p17	OK
D62	D54233	93	349		5 q21.1	D5S618	D5S644	2 1/2q21-1/2q22	OK
IIID3								2 1/2q21-1/2q22	
VIIA12	D11610	90	286		2 p16-p14	D2S119	D2S391	3p16-p17 or 3q12	OK [7, 21]
E*9	R76127	83	354		5 p13.3-q11.2	D5S651	D5S634	3 p16-17 or 16 q14 or 16 q22-q24	OK, if SSC16
IF4	AA428023	79	466		16 p13.3-p13.2	D16S21	D16S418	3 p11	OK
C134	D80005	93	295	KIAA0183 gene	3, 9	D9S1842	D9S196	3 q11	non painted zone by Goureau [9]
D47	AA494354	94	300		2 p22	D2S165	D2S352	3 q14	OK
IF5	N24480	76	334		2 p22	D2S165	D2S352	3 q14	OK
H*37	AA377312	77	258		2 p25.1-p22.1	D2S359	D2S375	3 q21-q27	OK
XIIB9								3 q21-q27	
J31								4 p14-p15 or 4 p13	
IIE9	W19691	74	303		1 q12-q21.2	D1S418	D1S514	4 q21-q23	OK
N*9	AA582194	89	104					5 p11-p15	
VIIIE1								5 1/2q21-q24	
IF10	N33173	81	466	methionine aminopeptidase	12 q22	D12S327	D12S657	5 1/2q21-q24	OK
M19	AA358888	73	283	DRIM protein	12 q22-q24.11	D12S346	D12S78	5 q25	OK [7, 21]
XIIA1	N52770	76	369		16 q21-q22.2	D16S3031	D16S3139	6 1/2p14-p15	OK

Table III. Continued.

Clone	Significatively homologous human sequence	% identity	bp	Putative identification	Localisation (on human genome)	Markers [1]		Localisation (on porcine genome)	Comparative map [2]
						top	bottom		
D46	AA233880	81	357					6 1/2q32	
XIIB1								6 1/2q31 or 61/2q32 or 6q33-q34 or 6 1/2q35	
D*9								7 1/2p11-p12	
I36	AA523364	88	353					7q12-q23 or 7q26	
VIIIH4	AA173981	87	195		6 p12.1-q12	D6S1616	D6S427	7q12-q23 or 7q26	OK (SSC 7q12-q23)
M21	AA424687	83	433		1 p31.3-p22.3	D1S417	D1S2846	9 1/3p21-p24	HSA11 expected
J20								9q12-1/3q21	
IVF11								9q11 or 9 2/3q21-q26	
IVA5	AA448289	75	389	D123 protein	10 p15.2-p13	D10S189	D10S191	10q17	OK
IVF1								12 p11-2/3 p13	
XID7	AA040801	76	370	rabaptin-5	17 p13.1-p12	D17S1828	D17S786	12q11-q15	OK
M*5								12q11-q15	
XIVF3	D31425	81	143		3 p13-p12.2	D3S1261	D3S1604	13q21-22 or 13q23-1/2q41	OK
IVF4	N75819	89	269		3 q13.12-q13.32	D3S1302	D3S1610	13q42-1/2q46	OK
VIIIE12								14B2	
K27								14 C or 14G	
XIIE3	D53868	87	230	KIAA0262 gene	12 p24.21-p24.32	D12S366	D12S340	14 q22-1/2q24	OK
M*4						D8S549	D8S258	14 1/2q25-q29	

Table III. Continued.

Clone	Significatively homologous human sequence	% identity	bp	Putative identification	Localisation (on human genome)	Markers [1]		Localisation (on *porcine genome*)	Comparative map [2]
						top	bottom		
XIIID3	D51179	91	340	Highly similar to elastin like protein				16q11-q13	
VB5								16 q14 or 16q22-q23	
IIC12	R66533	90	215					16 q14 or 16q22-q23	
XIIIA10								17 1/2q21-q23	
H*147	H48677							18q13-q21	
IVE5	N41995	87	287					Xq13	

[1] The linkage markers have been found in http://www.ncbi.nlm.nih.gov/genemap/ [2] OK : data in accordance with comparative mapping publications.

The sequences of our PCR primers were found on the human sequence (5′ coding region, sequence AF046024). But the three primer pairs (Gene Map 98 data) that permitted the mapping of AF046024 onto the human irradiated panel on Hsap3 are situated at the 3′-end of the human sequence and were not found in our pig 3′-end sequence without a mismatch. So, since the 5′ sequence of a gene can be found in several genes of the same family (with a specific 3′ end), we may have localised another member of the family, sharing part of its 5′-end sequence with the human AF046024 sequence.

4.4. Sscr3

VIIA12 was localised on Sscr3 p16-p17 or 3q12 and the corresponding human sequence was localised on Hsap2. This was in accordance with heterologous painting experiments [7,21] which demonstrate a correspondence between Sscr3 q11-q12 and Hsap2. For Sscr3p15-p17, only one team gives a correspondence with Hsap16 [7] and the localisation of two other markers on Sscr3p15-p17 (EPO, ZP3) suggests a correspondence with Hsap7. This suggests that VIIA12 is most probably localised on Sscr3 q12 and confirms the correspondence between Sscr3 q12 and Hsap2.

The localisation of C134 suggests a correspondence of Sscr3 q11 with Hsap3 or 9, since the D80005 human sequence which is similar to C134 has been mapped onto these chromosomes. Moreover, the sequence of our primers was found (with only one mismatch, in the middle of the primers) on the D80005 mRNA sequence of the *KIAA0183* gene (position 4 710 and 4 828) mapped onto Hsap3 or 9. Other neighboring markers should be mapped to confirm this new correspondence, since two of the three painting experiments [7,21] have found this zone to be in correspondence with Hsap2 [7]; Goureau *et al.* did not obtain any painting signal in this zone [9].

4.5. Sscr5, Sscr9 and Sscr16

The localisation of M19 on Sscr5 q25 suggests that this zone, unpainted according to Goureau *et al.* [9] is in correspondence with Hsap12, as suggested by Rettenberger *et al.* [21] and Frönicke *et al.* [7]. Lahbib-Mansais *et al.* [14] found the same result for one human EST (D29485).

The localisation of M21 on Sscr9 1/3p21-p24 suggests a correspondence between Sscr9 and Hsap1. This has also been suggested by the localisation of MYOG [5]. This result should be confirmed by the localisation of neighbouring ESTs.

E*9 was mapped on Sscr3 with a 0.72 probability (2 discordant results). Its localisation on Sscr16 however, had a 0.16 probability (3 discordant results), with a 0.78 correlation coefficient, but was in agreement with the heterologous painting experiments. This was due to the very similar representation of these regions of Sscr3 and Sscr16 in the somatic cell hybrid panel.

Altogether, these results improved the transcriptional porcine map of most chromosomes. This data will generate new results since the human map is still in progress and new homologies between porcine and human genomes will be found.

For the results that show discrepancies with the human map, more data is required. These markers could be mapped on the porcine irradiated hybrid panel characterised by Hawken *et al.* [12]. Furthermore, other markers should be mapped in the same region to confirm new correspondences between the porcine and human genomes. Alternatively, genetic mapping could be used, if a polymorphism is found in the porcine genes to be mapped.

These localisations of coding sequences will be useful when Quantitative Trait Loci (QTL) are identified more precisely, since some candidates will have been localised in the region of interest. Moreover, since our cDNAs were isolated from ovarian tissue and some of them are regulated by FSH, they will be good candidates for reproductive traits.

ACKNOWLEDGEMENTS

We would like to thank Janine Rallières and Francis Benne for technical assistance and Elsa Bonnafé, Laure Caussanel, Virginie Gindraud, and Sylvie Perret for regional mapping. We also thank Hélène Quesnel (Unité mixte de recherche sur le veau et le porc, INRA) who provided the ovaries of cycled sows.

REFERENCES

[1] Andersson L., Haley C.S., Ellegren H., Knott S.A., Johansson M., Andersson K., Andersson-Eklund L., Edfors-Lilja I., Fredholm M., Hansson I. *et al.*, Genetic mapping of quantitative trait loci for growth and fatness in pigs, Science 263 (1994) 1771–1774.

[2] Callard D., Mazzolini L., Identification of proliferation-induced genes in Arabidopsis thaliana. Characterization of a new member of the highly evolutionarily conserved histone H2A.F/Z variant subfamily, Plant Physiol. 115 (1997) 1385–1395.

[3] Chevalet C., Gouzy J., San Cristobal-Gaudy M., Regional assignment of genetic markers using a somatic cell hybrid panel: a WWW interactive program available for the pig genome, Comput. Appl. Biosci. 13 (1997) 69–73.

[4] Erickson G.F., Primary cultures of ovarian cells in serum-free medium as models of hormone-dependent differentiation, Mol. Cell. Endocr. 29 (1983) 21–49.

[5] Ernst C.W., Mendez E.A., Robic A., Rothschild M.F., Rapid communication: myogenin (MYOG) physically maps to porcine chromosome 9q2.1-q2.6, J. Anim. Sci. 76 (1998) 328.

[6] Fridolfsson A.K., Hori T., Winterø A.K., Fredholm M., Yerle M., Robic A., Andersson L., Ellegren H., Expansion of the pig comparative map by expressed sequence tags (EST) mapping, Mamm. Genome 8 (1997) 907–912.

[7] Frönicke L., Chowdhary B.P., Scherthan H., Gustavsson I., A comparative map of the porcine and human genomes demonstrates ZOO- FISH and gene mapping-based chromosomal homologies, Mamm. Genome 7 (1996) 285–290.

[8] Gellin J., Barillot E., Chowdhary B.P., Davoli R., Fredholm M., Hatey F., Wimmer K., Mapping and identification of 700 genes in the pig genome, a European initiative, Anim. Genet. 29 (1998) 37.

[9] Goureau A., Yerle M., Schmitz A., Riquet J., Milan D., Pinton P., Frelat G., Gellin J., Human and porcine correspondence of chromosome segments using bidirectional chromosome painting, Genomics 36 (1996) 252–262.

[10] Hatey F., Gasparoux J.P., Mulsant P., Bonnet A., Gasser F., P450scc regulation in pig granulosa cells: investigation into the mechanism of induction, J. Steroid Biochem. Mol. Biol. 43 (1992) 869–874.

[11] Hatey F., Langlois I., Mulsant P., Bonnet A., Benne F., Gasser F., Gonadotropins induce accumulation of insulin-like growth factor I mRNA in pig granulosa cells *in vitro*, Mol. Cell. Endocr. 86 (1992) 205–211.

[12] Hawken R.J., Murtaugh J., Flickinger G.H., Yerle M., Robic A., Milan D., Gellin J., Beattie C.W., Schook L.B., Alexander L.J., A first-generation porcine whole-genome radiation hybrid map, Mamm. Genome 10 (1999) 824–830.

[13] Jørgensen C.B., Winterø A.K., Yerle M., Fredholm M., Mapping of 22 expressed sequence tags isolated from a porcine small intestine cDNA library, Mamm. Genome 8 (1997) 423–427.

[14] Lahbib-Mansais Y., Dalias G., Milan D., Yerle M., Robic A., Gyapay G., Gellin J., A successful strategy for comparative mapping with human ESTs: 65 new regional assignments in the pig, Mamm. Genome 10 (1999) 145–153.

[15] Lanneluc I., Mulsant P., Saidi-Mehtar N., Elsen J.M., Synteny conservation between parts of human chromosome 4q and bovine and ovine chromosomes 6, Cytogenet. Cell Genet. 72 (1996) 212–214.

[16] Lino J., Baranao S., Hammond J.M., Multihormone regulation of steroidogenesis in cultured porcine granulosa cells: studies in serum-free medium, Endocrinology 116 (1985) 2143–2151.

[17] Makalowski W., Zhang J., Boguski M.S., Comparative analysis of 1196 orthologous mouse and human full-length mRNA and protein sequences, Genome Res. 6 (1996) 846–857.

[18] Monget P., Monniaux D., Pisselet C., Durand P., Changes in insulin-like growth factor-I (IGF-I), IGF-II, and their binding proteins during growth and atresia of ovine ovarian follicles, Endocrinology 132 (1993) 1438–1446.

[19] Pearson W.R., Lipman D.J., Improved tools for biological sequence comparison, Proc. Natl. Acad. Sci., USA 85 (1988) 2444–2448.

[20] Pinton P., Schibler L., Cribiu E., Gellin J., Yerle M., Localisation of 113 anchor loci in pigs: improvement of the comparative map for humans, pigs and goats, Mamm. Genome 11 (2000) 306–315.

[21] Rettenberger G., Klett C., Zechner U., Kunz J., Vogel W., Hameister H., Visualization of the conservation of synteny between humans and pigs by heterologous chromosomal painting, Genomics 26 (1995) 372–378.

[22] Robic A., Riquet J., Yerle M., Milan D., Lahbib-Mansais Y., Dubut-Fontana C., Gellin J., Porcine linkage and cytogenetic maps integrated by regional mapping of 100 microsatellites on somatic cell hybrid panel, Mamm. Genome 7 (1996) 438–445.

[23] Saumande J., Culture of bovine granulosa cells in a chemically defined serum-free medium: the effect of insulin and fibronectin on the response to FSH, J. Steroid Biochem. Mol. Biol. 38 (1991) 189–196.

[24] Tosser-Klopp G., Benne F., Bonnet A., Mulsant P., Gasser F., Hatey F., A first catalog of genes involved in pig ovarian follicular differentiation, Mamm. Genome 8 (1997) 250–254.

[25] Wilkie P.J., Paszek A.A., Beattie C.W., Alexander L.J., Wheeler M.B., Schook L.B., A genomic scan of porcine reproductive traits reveals possible quantitative

trait loci (QTLs) for number of corpora lutea, Mamm. Genome 10 (1999) 573–578.

[26] Winterø A.K., Fredholm M., Davies W., Evaluation and characterization of a porcine small intestine cDNA library: analysis of 839 clones, Mamm. Genome 7 (1996) 509–517.

[27] Winterø A.K., Jørgensen C.B., Robic A., Yerle M., Fredholm M., Improvement of the porcine transcription map: localization of 33 genes, of which 24 are orthologous, Mamm. Genome 9 (1998) 366–372.

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

[29] Yerle M., Pinton P., Robic A., Alfonso A., Palvadeau Y., Delcros C., Hawken R., Alexander L., Beattie C., Schook L., Milan D., Gellin J., Construction of a whole-genome radiation hybrid panel for high-resolution gene mapping in pigs, Cytogenet. Cell Genet. 82 (1998) 182–188.