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Assignment of the Leydig insulin-like hormone to porcine Chromosome 2q12-q13 by somatic cell hybrid analysis and fluorescence in situ hybridization

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Leydig insulin-like protein (Ley I-L) belongs to the insulin-like hormone superfamily (Adham et al. 1993), which comprises insulin, relaxin, and insulin-like growth factor I (IGFI) and II (IGFII). The function of the Ley I-L gene is unknown till now. Because the gene is expressed only in prenatal and postnatal Leydig cells (Adham et al. 1993), the Ley I-L peptide is thought to play an important role in testicular function and spermatogenesis. The porcine and human Ley I-L genes consist of two exons and are more similar in structure to the genes for insulin and relaxin than to the insulin-like growth factor I and II. By in situ hybridization, the human gene Ley I-L was assigned to band p13.2-p12 of Chromosome (Chr) 19 (Burkhardt et al. 1993). In this region several oncogenes (JUNB, JUND, RAB3A, and VAV), the genes for insulin receptor (INSR) and low density lipoprotein receptor (LDLR), but also the genes for anti Mullerian hormone (AMH) and complement component 3 (C3) have been mapped (Ropers and Pericak-Vance 1991). The long arm of human Chr 19 is conserved within an extended linkage group on porcine Chr 6 (Archibald and Imlah 1985; Vögeli 1989). From loci assigned to the short arm of Chr 19, only *INSR* has been mapped in pig and was localized on Chr 2 (Gu et al. 1992). In this study the Leydig insulin-like protein, Ley I-L, has been assigned to porcine Chr 2q12-q13 by combined somatic cell hybrid analysis and fluorescence in situ hybridization.

Primers for Leydig insulin-like hormone were created from the "Primer analysis program Oligo 4.0":
primer EB14: 5′GAAGCTGTGCGCCACCACTTT 3′ sequence position sense 105–127, exon 2;
primer EB6: 5′ GCTGTGGGGCCAATACCAGCA 3′ sequence position antisense 1142–1164, exon 3;

primers for microsatellite S0091 have been published by Ellegren and coworkers (1993).

PCR was performed on a Perkin and Elmer thermocycler 480. 300 ng of porcine, rodent, or hybrid DNA as template was amplified in 50-µl reactions. The reaction mix contained 20 pmol of each primer, 40 nmol of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 0.01% gelatin, and 1 U Taq polymerase (Perkin and Elmer Cetus). Template DNA was amplified for 30 cycles with denaturation at 94°C for 1 min 45 s, annealing at 56°C for 1 min, and an extension at 72°C for 2 min. Ten-microliter reactions were run on a 2% agarose gel in 1× TBE.

The primers for Ley I-L amplify an amplification product of about 1050 bp in porcine DNA but not in the parental rodent lines (Fig. 1A).

In Table 1 the PCR findings for the Leydig insulin-like hormone in the 21 hybrid cell lines are shown together with the cytogenetic analysis. Concordance calculations between cytogenetic and biochemical analyses were done according to Chevalet and Corpet (1986). These data point to Chr 2 (Table 1). In two hybrid lines the cytogenetic and biochemical analyses are not concordant (H10 and 18, Table 1). To prove the assignment of the Leydig insulin-like hormone to porcine Chr 2, we have tested the same panel of hybrid cell lines for the presence of the microsatellite S0091, which has been shown to map on Chr 2. This microsatellite has been mapped by linkage analysis in 3.3-cM map distance to INSR (Ellegren et al. 1993). As shown in Fig. 1A and Table 1, the same amplification pattern for S0091 was found in the hybrid lines as determined previously for the Leydig insulin-like hormone. Both sequences show exactly the same amplification pattern in all 21 hybrids. Thus, the somatic cell hybrid analysis assigns *Ley I-L* to pig Chr 2. The positive signal in the hybrid line 10 may be due to small chromosome fragments that we were unable to identify by cytogenetic analysis. The lack of a positive signal in hybrid line 18 remains unexplained.

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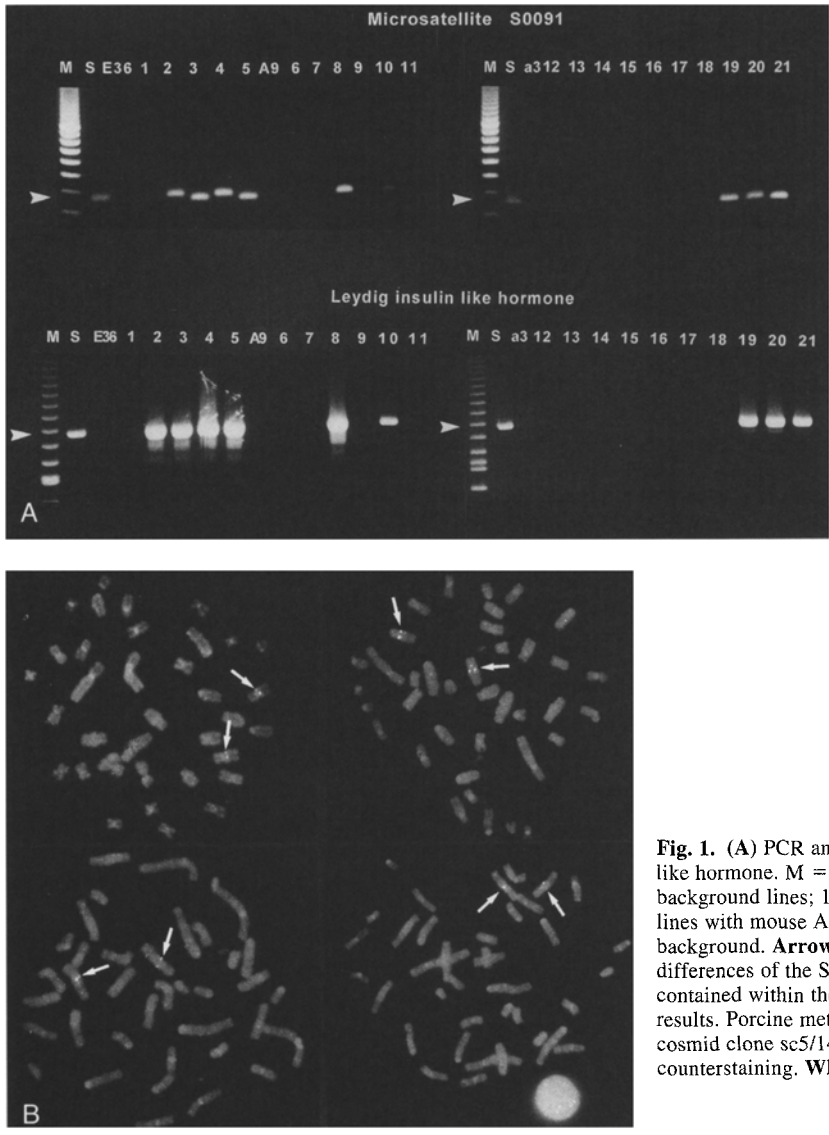

Fig. 1. (**A**) PCR analysis for the Chr 2 marker S0091 and Ley insulin-like hormone. M = 100 bp markers; S = pig; E36, A9, a3 = rodent background lines; 1–5, hybrid lines with E36 background; 6–11, hybrid lines with mouse A9 background; 12–21, hybrid lines with a3 background. **Arrows** indicate the specific PCR products. The size differences of the S0091 microsatellite are due to different chromosomes contained within the hybrids. (**B**) Fluorescence in situ hybridization results. Porcine metaphase spreads after CISS hybridization with the cosmid clone sc5/14. Green FITC signals with propidium iodide counterstaining. **White arrows** point to signals on Chr 2q12-q13.

To corroborate the localization as achieved by somatic cell hybrid mapping and to further refine the localization, we have performed CISS hybridization with the cosmid sc5/14 containing the complete *Ley I-L* gene (Adham et al. 1993). Lymphocytes were prepared according to standard techniques. R-banding cultures were treated 5 h before harvest with 20 µg/ml BrdUrd. 100 ng cosmid DNA was labeled with bio 16-dUTP in a nick translation reaction (Boehringer Mannheim).

Labeled DNA was dissolved in 50% formamide, 10% dextran sulfate, 2× SSC, 500 µg/ml salmon sperm DNA, and 150 µg/ml sonicated genomic porcine DNA as competitor. Slides were denatured for 2 min in 50% formamide/2× SSC at 70°C. Probe was denatured at 80°C for 10 min, and preannealing of the whole mix was performed for 40 min at 37°C. Hybridization with 100 ng labeled DNA in 20 µl mix per slide was performed at 37°C for 16 h. After hybridization, the slides were washed three times for 15 min at 45°C in 50% formamide/2× SSC followed by five washing steps for 2 min in 2× SSC. For detection of the signal, the slides were blocked in 5% BSA/4× SSC/0.1% Tween 20 for 30 min and incubated with avidin DCS-FITC (Vector Laboratories, Burlingame) at a dilution of 1:100 in 1% BSA/4× SSC/0.1% Tween 20. Amplification of the signal was carried out by incubation at 37°C with biotinylated anti-avidin from goat (Vector Laboratories, Burlingame), 1:200, and the detection step with DCS-FITC was repeated. Slides were counterstained in propidium iodide (0.3 µg/ml) and DAPI (1 µg/ml) for 10 min and embedded in antifading solution (233 mg DABCO, in 10 ml 1× PBS). Metaphases were photographed on a Zeiss axiophot with Kodak Ectachrome 400 films and the filter set for FITC. Chromosomes were identified in a DAPI filter set according to the standardized karyotype of the domestic pig (Gustavsson 1988).

Chromosome 2 could be identified on BrdUrd-substituted and propidium iodide-stained chromosomes; this treatment induces a faint R-banding pattern (Fig. 1B). The identification of Chr 2 was subsequently confirmed on DAPI-stained metaphases (data not shown). Twenty metaphases were analyzed with a hybridization signal on both chromatids. In 19 of all scored metaphases, symmet-

Table 1. Cytogenetic analysis of the somatic cell hybrid panel and PCR results for the microsatellite S0091 and the Ley insulin-like hormone. Some Hybrids show unidentified pig/rodent translocations or small unknown porcine fragments indicated in a seperate column. The statistical analysis is given at the bottom. Both loci were tested against each chromosome for statistically significant values for synteny. If φ is <0.74, synteny is sure (error rate Q = 0.025, and probability for correct decision P = 0.91). If (0.58 < φ < 0.75), synteny cannot be excluded (Chevalet and Corpet, 1986).

	Cytogenetics																				PCR	
Chromosome hybrid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	X	Y	Additional fragments	S0091	Leydig insulin-like hormone
1	x		x	x	x		x	x	x	x	x	x					x		x	x	x		
2	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x		x	x
3	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x		x	x	x
4	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x
5	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x		x	x	x
6								x			x		x	x	x	x		x	x				
7					x			x			x		x	x	x	x	x		x				
8		x		x		x	x	x	x	x			x		x	x	x	x	x			x	x
9								x			x		x	x	x	x	x	x	x		x		
10				x				x				x	x	x	x	x	x	x	x			x	x
11					x								x		x	x			x				
12										x	x	x									x		
13						x	x	x	x	x		x					x	x	x		x		
14												x									x		
15	x		x		x				x	x	x	x							x				
16			x		x		x	x	x	x		x									x		
17	x			x		x	x	x		x	x	x									x		
18	x	x	x			x	x	x	x	x		x											
19	x	x	x	x	x		x	x	x	x		x					x	x		x	x	x	x
20		x		x		x	x	x	x	x	x	x									x	x	x
21		x	x		x	x	x	x		x		x									x	x	x
concordance in %	62	90	67	86	62	76	67	62	67	62	43	57	67	67	67	67	71	71	52	67			
correlation φ	0.22	0.80	0.33	0.71	0.25	0.52	0.36	0.42	0.36	0.33	−0.14	0.26	0.33	0.31	0.33	0.33	0.44	0.42	0.08	0.32			

ric double fluorescence signals on both chromatids were obtained exclusively on the proximal long arm of Chr 2 in the region 2q12-q13 (Fig. 1B). The combined analysis assigns the porcine Leydig insulin-like hormone to Chr 2q12-q13. The human gene for Leydig insulin-like hormone has been mapped to Chr 19p13.3-13.2 (Burkhardt et al. 1993). An extended linkage group, including the halothane loci CRC, GPI, TGFB, APOE, and LIPE on human Chr 19q maps to porcine Chr 6 (Archibald and Imlah 1985). The insulin receptor INSR, located on the short arm of human Chr 19 (Yang-Feng et al. 1985), maps to porcine Chr 2 (Gu et al. 1992). This locus is designated to be an anchor locus for comparative mapping (O'Brien et al. 1993). By somatic cell hybrid analysis, *Ley I-L* most likely maps on Chr 2. Because of inconsistencies in two hybrid lines, we have included the analysis for the microsatellite S0091, which has been mapped by linkage analysis to Chr 2 (Ellegren et al. 1993). The results for *Ley I-L* and *S0091* are completely identical (Fig. 1A), which means a first confirmation. A second, unequivocal confirmation is provided by FISH analysis. Double fluorescence signals were observed only on Chr 2q12-q13. This is exactly the same region in which *INSR* has been mapped by in situ hybridization (Gu et al. 1992). Both *INSR* and *Ley I-L* define a new common linkage group conserved on the long arm of porcine Chr 2. The human short-arm loci are found on several different chromosomes in the mouse genome. Preliminary results in the cattle show one conserved linkage group for the short arm and another for the long arm of human Chr 19, a situation very similar to that now described for pig.

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