The *INSL3* gene is a direct target for the orphan nuclear receptor, COUP-TFII, in Leydig cells

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Abstract

Insulin-like 3 (INSL3), a hormone produced by Leydig cells, regulates testicular descent during foetal life and bone metabolism in adults. Despite its importance, little is known about the molecular mechanisms controlling *INSL3* expression. Reduced *Insl3* mRNA levels were reported in the testis of mice deficient for chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), an orphan nuclear receptor known to play critical roles in cell differentiation and lineage determination in several tissues. Although COUP-TFII-deficient mice had Leydig cell dysfunction and impaired fertility, it remained unknown whether *Insl3* expression was directly regulated by COUP-TFII. In this study, we observed a significant decrease in *Insl3* mRNA levels in MA-10 Leydig cells depleted of COUP-TFII. Furthermore, a — 1087 bp mouse *Insl3* promoter was activated fourfold by COUP-TFII in MA-10 Leydig cells. Using 5⁰ progressive deletions, the COUP-TFII-responsive element was located between — 186 and — 79 bp, a region containing previously uncharacterised direct repeat 0-like (DR0-like) and DR3 elements. The recruitment and direct binding of COUP-TFII to the DR0-like element were confirmed by chromatin immunoprecipitation and DNA precipitation assay respectively. Mutation of the DR0-like element, which prevented COUP-TFII binding, significantly decreased COUP-TFII-mediated activation of the — 1087 bp *Insl3* reporter in CV-1 fibroblast cells but not in MA-10 Leydig cells. Finally, we found that COUP-TFII cooperates with the nuclear receptor steroidogenic factor 1 (SF1) to further enhance *Insl3* promoter activity. Our results identify *Insl3* as a target for COUP-TFII in Leydig cells and revealed that COUP-TFII acts through protein–protein interactions with other DNA-bound transcription factors, including SF1, to activate *Insl3* transcription in these cells.

Key Words

- insulin-like 3
- NR2F2
- nuclear receptor
- Leydig cells
- cooperation

Introduction

Insulin-like 3 (INSL3), also known as relaxin-like factor, belongs to the insulin–insulin-like growth factor–relaxin family of growth factors and hormones that was originally discovered by screening a boar testicular cDNA library (Adham et al. 1993). The corresponding sequences were later obtained by screening genomic or cDNA libraries and by RT-PCR from humans (Burkhardt et al. 1994) and a variety of other species (Bathgate et al. 1996, Spiess et al. 1999,

INSL3 plays important roles in male and female reproductive function. In males, INSL3 is involved in two very different, but critical, processes. During embryonic development, INSL3 was found to be a master regulator of gonadal descent, an essential step of the male sex differentiation process. Insl3-/- null mice present bilateral cryptorchid testes, located high in the abdominal cavity close to the kidneys (Nef & Parada 1999, Zimmermann et al. 1999). Furthermore, INSL3 expression in pancreatic β-cells of Insl3 null mice restored normal testis descent (Adham et al. 2002), while its expression in females led to descended ovaries (Adham et al. 2002, Koskimies et al. 2003). In adults, the role of INSL3 in males is not yet fully understood. INSL3 was found to be involved in bone metabolism (Ferlin et al. 2008) and proposed to act as a germ cell survival factor (Kawamura et al. 2004). In adult females, INSL3 plays a role in oocyte maturation and in follicle selection and survival (Spanel-Borowski et al. 2001, Irving-Rodgers et al. 2002, Kawamura et al. 2004).

Despite its crucial roles in reproductive development and function, surprisingly, very little is known about the mechanisms regulating Insl3 expression in Leydig cells. To date, only two transcription factors have been shown to regulate Insl3 promoter activity in Leydig cells: the nuclear receptors steriodogenic factor 1 (SF1, Ad4BP, NR5A1) and NUR77 (NGFI-B, NR4A1) (Zimmermann et al. 1998, Koskimies et al. 2002, Sadeghian et al. 2005, Tremblay & Robert 2005, Robert et al. 2006). As these nuclear receptors are co-expressed in other cell types that do not produce INSL3, other transcription factors must participate in Insl3 transcription in Leydig cells.

The orphan nuclear receptor, chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII, NR2F2), is strongly expressed in tissues originating from the mesenchyme and plays critical roles during mouse development (Tsai & Tsai 1997, Lin et al. 2011). Coup-tfii null mice die before E10.5 due to angiogenesis and cardiovascular defects (Pereira et al. 1999). Temporal and tissues-specific Coup-tfii ablation in the stomach, limbs, diaphragm, uterus, and endothelial cells have revealed important roles in cell growth and differentiation and in organogenesis (Lee et al. 2004, Takamoto et al. 2005, You et al. 2005, Kurihara et al. 2007, Petit et al. 2007). It was previously reported that COUP-TFII might be involved in Insl3 expression (Qin et al. 2008). Indeed, inactivation of Coup-tfii during pre-pubertal stages of male development in mice led to a blockade in Leydig cell differentiation at the progenitor stage. This resulted in infertility, hypogonadism and spermatogenetic arrest due to defective testosterone synthesis in Coup-tfii-/- male mice. A decrease in the expression of several genes (Hsd3b1, Cyp11a1 and Cyp17a1) required for testosterone biosynthesis was observed in these mice (Qin et al. 2008). A decrease in Insl3 mRNA levels was also reported (Qin et al. 2008). However, it remains unknown whether the decrease in gene expression, particularly Insl3, was due to a defect in the Leydig cell differentiation process and/or to a direct role of COUP-TFII in regulating their expression.

COUP-TFII regulates gene expression by binding to direct repeat (DR) motifs (AGGTCA) separated by variable spacing (from zero to six nucleotides) between the two half-sites (Pereira et al. 2000). Herein, we report that COUP-TFII directly and positively regulates Insl3 gene expression by binding to a previously uncharacterised DR0-like response element in the Insl3 promoter. In addition, we found that COUP-TFII cooperates with the nuclear receptor SF1 to further enhance Insl3 transcription.

Materials and methods

Double immunohistochemistry

Immunohistochemistry was done using an anti-COUP-TFII antiserum (1:100, R&D Systems, Minneapolis, MN, USA) and was carried out as described in Martin et al. (2008). For double immunodetection, the counterstaining with haematoxylin following COUP-TFII detection was omitted and replaced by the incubation with an anti-INSL3 antiserum (1:100 dilution, M-122, Santa Cruz Biotechnology) overnight at 25°C. The samples were washed twice with 1× PBS for 10 min each time and an alkaline phosphatase (ALKP)-conjugated secondary antibody (1:1000, Sigma–Aldrich Canada) was added and incubated for 1 h at 25°C. Final detection was done using

http://jme.endocrinology-journals.org
DOI: 10.1530/JME-13-0290
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Printed in Great Britain
Published by Bioscientifica Ltd.
an ALKP detection solution (Tris–HCl 0.1 M, pH 9.2, 1 mM levamisole, naphthol 0.04% and Fast Blue BB diazonium salt 0.2%). The negative control corresponds to the same procedures with the omission of anti-COUP-TFII antiserum (data not shown). All experiments were conducted according to the Canadian Council for Animal Care and have been approved by the Animal Care and Ethics Committee of Laval University (protocol #20090911). Images were obtained using an Axioskop2 Plus microscope (Carl Zeiss Canada, Toronto, ON, Canada) and the Image-Pro Plus Software (MediaCybernetics Rockville, MD, USA).

**siRNA transfection**

MA-10 Leydig cells were transfected with 150 nM siRNA (Life Technologies) directed against Coup-tfii transcripts (sequence: ACU GGC CAU AUA UGG CAA UUC AAU A) or with a control non-targeting siRNA for 48 h using the JetPRIME Transfection Reagent according to manufacturer’s protocol for siRNA transfection (Polyplus, Illkirch, France). Three different siRNAs directed against different regions of COUP-TFII were used and they all decreased COUP-TFII protein levels, albeit with different efficacy. The one that decreased COUP-TFII protein by about 80% was chosen. Less than 10% difference was observed between scrambled siRNAs and no siRNA (data not shown). siRNA transfection had no effect on cell viability (Tripan Blue exclusion) and cell number.

**RNA isolation and real-time PCR**

Following siRNA treatment, RNA isolation and cDNA synthesis were carried out as previously described (Martin et al. 2008). Quantitative RT-PCR was carried out using a LightCycler 1.5 instrument and the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) according to a LightCycler 1.5 instrument and the LightCycler FastStart a protocol for siRNA transfection (Polyplus, Illkirch, France). Three different siRNAs directed against different regions of COUP-TFII were used and they all decreased COUP-TFII protein levels, albeit with different efficacy. The one that decreased COUP-TFII protein by about 80% was chosen. Less than 10% difference was observed between scrambled siRNAs and no siRNA (data not shown). siRNA transfection had no effect on cell viability (Tripan Blue exclusion) and cell number.

**Protein purification and western blotting**

After siRNA transfections, MA-10 Leydig cells were rinsed twice with ice-cold PBS and nuclear proteins were prepared as described previously (Martin et al. 2008). Protein concentrations were determined using standard Bradford assays. Nuclear proteins (15 μg) were boiled 10 min in a denaturing loading buffer, fractionated by SDS–PAGE, and transferred onto nitrocellulose membrane (Bio-Rad). Immunodetection was carried out using a horseradish peroxidase coupled antibody approach according to the manufacturer’s instructions (Amersham ECL and ECL Prime Western Blotting Detection Reagents, GE Healthcare Life Sciences, Baie-D’Urfé, QC, Canada). COUP-TFII and LAMIN B proteins were detected using a monoclonal anti-COUP-TFII antibody (1:1000, R&D Systems) and an anti-LAMIN B antiserum (1:1000, C-20, Santa Cruz Biotechnology) respectively.

**Plasmids**

The −1087 to +5 bp murine Insl3 luciferase promoter construct was generated by PCR amplification from mouse genomic DNA using the following set of oligonucleotide primers: forward containing a BamHI cloning site (italicised), 5′-GCG GAT CCT GGT TCC TAT TAT GAT GCT G-3′ and reverse containing a KpnI cloning site (italicised), 5′-GGA GTC TGG CAG GAG GCA GTG GGC AG-3′. Deletions to −800, −600, −400, −333, −284, −234, −186 and −79 bp were obtained by PCR using the −1087 bp Insl3 promoter as template, along with a common reverse primer mentioned earlier and the following forward primers containing a BamHI (italicised) cloning site: −800 bp, 5′-GCG GAT CCC CCT TGC TCC CCT GAC TTG G-3′; −600 bp, 5′-GCG GAT CCC TGG AGT AGA GGT CTT G-3′; −400 bp, 5′-GCG GAT GCA ACA AGA AAG GAA CCA TTT A-3′; −333 bp, 5′-GCG GAT CCC TGG AGT AGA GGT CTT G-3′; −284 bp, 5′-GCG GAT CCC TGG AGT AGA GGT CTT G-3′; −234 bp, 5′-GCG GAT CCC TGG AGT AGA GGT CTT G-3′; −186 bp, 5′-GCG GAT CCC TGG AGT AGA GGT CTT G-3′; −79 bp, 5′-GCG GAT CCC TGG AGT AGA GGT CTT G-3′. All promoter fragments were cloned into a modified pXP1 luciferase reporter plasmid (Tremblay & Viger 1999). The −1087 bp Insl3 reporter constructs harbouring inactivating mutations in both half-site of the DR0-like or of the DR3 elements were
generated in a two-step process using the QuikChange XL Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with the following oligonucleotides (mutated nucleotides are in bold, underlined and lowercase): first half-site mutation in DR3 (sense, 5′-CTT CGG AGA GGA CTT CAA aac acc AAG CTG GAC ACA CAG C-3′; antisense, 5′-GCT GTG TG TGT CCA GCT TGG tgt ttG TAA GTC CTC TCC CAG G-3′), second half-site mutation in DR3 (both half-site mutated; sense, 5′-CCG CAC CTG GGA Gct tAC TCC AAA ACA CC-3′; antisense, 5′-GCT GTG TTG AAG TaA gCt CCC AGG TGC GG-3′), first half-site mutation in DR0-like (sense, 5′-CGC TGA CTC GAG CCT C-3′; antisense, 5′-GCA AGC ACC CAA AAT tgt GAG GCT CGA GTC ACG G-3′) and second half-site mutation in DR0-like (both half-sites mutated; sense, 5′-CCG TGA CTC GAG Cct aAT tgt TTT GGG TGC TGC TTG C-3′; antisense, 5′-GCA AGC AGC ACC CAA AAT tgt GAG GCT CGA GTC ACG G-3′). The −1087 bp Insl3 reporter construct harbouring mutations in both DR0-like and DR3 elements was generated via the same two-step process using the −1087 bp Insl3 DR3 mutated construct as template and the sense and antisense oligonucleotides containing the mutations for the both-half site of the DR0-like element described above. The human −1137 bp INSL3 and rat −508 bp Insl3 promoter constructs have been described previously (Robert et al. 2006, Laguée & Tremblay 2008). The mouse COUP-TFI expression vector (Pereira et al. 1999) was obtained from Dr Ming Tsai (Baylor College of Medicine, Houston, TX, USA). The mouse SF1 expression plasmid has been described previously (Tremblay & Viger 2001). All plasmids were verified by sequencing (Centre de Génomique de Québec, CHUL Research Centre, Quebec City, QC, Canada).

**Cell culture, transfections and reporter assays**

Mouse MA-10 Leydig cells (Ascoli 1981), provided by Dr Mario Ascoli (University of Iowa, Iowa City, IA, USA) were grown in DMEM/F12 medium supplemented with 2.438 g/l sodium bicarbonate, 3.57 g/l HEPES, 15% horse serum and 50 mg/l of gentamicin and streptomycin sulfate. African Green monkey kidney fibroblast CV-1 cells were grown in DMEM medium supplemented with 3.7 g/l HEPES, 10% newborn calf serum and 50 mg/l of gentamicin and streptomycin sulfate. The MA-10 Leydig cells were originally derived from the mouse MS480P tumour (Ascoli 1981) and have since been well characterised. They produce steroids and respond to luteinizing hormone/human chorionic gonadotrophin (as well as Forskolin and CAMP) stimulation. The MA-10 Leydig cell line corresponds to the adult Leydig cells (reviewed in Rahman & Huhtaniemi (2004)). The expression of *Insl3* in MA-10 Leydig cells is similar to what has been reported in primary Leydig cell cultures and in rodents. For instance, *Insl3* expression is known to be repressed by estradiol *in vivo* in mice and rats (Nef et al. 2000, Cederroth et al. 2007, Strauss et al. 2009, Zhou et al. 2011) and this repression was also observed in MA-10 Leydig cells (Lagué & Tremblay 2009). *Insl3* expression is upregulated by androgens *in vivo* (Zhou et al. 2010) as well as in primary Leydig cells and in MA-10 Leydig cells (Lagué & Tremblay 2008, Tremblay et al. 2009). Therefore, the MA-10 Leydig cell line is an appropriate model to study *Insl3* gene regulation in the adult Leydig cell population. MA-10 and CV-1 cells were maintained at 37 °C in 5% CO₂. MA-10 and CV-1 cells were transfected and lysates were analysed as described previously (Martin et al. 2008, 2009). Data reported represent the average of at least three experiments, each performed in triplicate. The cell passage number was lower than 35 for all experiments.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assays were carried out essentially as described previously (Robert et al. 2006) with some modifications. Briefly, MA-10 Leydig cells were fixed with 1% formaldehyde at 20 °C for 10 min. After harvesting in PBS, pelleted cells were washed for 10 min at 4 °C each time with buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES and pH 6.5), buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES and pH 6.5) and protease inhibitors. The samples were then centrifuged at 5900 g for 5 min at 4 °C. The pellets were resuspended (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0, protease inhibitors) and incubated on ice for 10 min. The samples were next sonicated with the Misonix S-400 sonicator at 80% power and the lysates were centrifuged at 15 700 g for 10 min. The supernatants were diluted and incubated at 4 °C for 2 h with 30 μl of magnetic beads (Dynabeads, Life Technologies) which were previously blocked (with 0.2 μg/μl of sheared salmon sperm DNA and 0.5% BSA in PBS for 1 h at 4 °C) along with 5 μg of an anti-NUR77 (M210, Santa Cruz Biotecnologies) antiserum (used as an immunoprecipitation positive control), an anti-COUP-TFII antisemur or non-specific immunoglobulin Gs (IgGs) (Santa Cruz Biotechnologies). The precipitates were washed four times in a rotating wheel with ChIP wash buffer (100 mM Tris, pH 7.4, 500 mM LiCl, 1% Igepal and 1% sodium deoxycholate) for 5 min at 4 °C and once again with TE (10 mM Tris, pH 8 and 1 mM EDTA).
They were extracted with freshly prepared Elution buffer (1% SDS and 0.1 M NaHCO₃). The eluates were kept at 65 °C overnight with 0.2 M NaCl to reverse the crosslinks. DNA was purified by phenol/chloroform extraction and ethanol precipitation and resuspended in 10 mM Tris, pH 8. COUP-TFIImmunoprecipitated DNA fragments were analysed by PCR using primers specific for the proximal (−225 to −26 bp) (forward: 5'-CCC TTA CAA AGG GGC GCT TGG C-3' and reverse, 5'-GGA TGC TCC TTA TAA CTG CCT CG-3') or distal (−3133 to −2923 bp) (forward, 5'-GGT ACA AGA CCA AGA CTC CAG ACG TCA CG-3' and reverse, 5'-GGG GTA GTC CTG ACT AAG TTG AGA GGG G-3') region of the mouse Ins3 promoter. The PCRs were carried out on a T gradient thermocycler (Biometra, Montreal Biotech, Dorval, Québec, Canada) using Vent polymerase (New England Biolabs, Whitby, ON, Canada) and under the following conditions: 5 min at 95 °C followed by 40 cycles of denaturation (60 s at 95 °C), annealing (proximal region 30 s at 60 °C and distal region 60 s at 64 °C) and extension (30 s at 72 °C). The specificity of the PCR products was confirmed by agarose gel electrophoresis and sequencing. Input DNA represents 10% of total DNA used for a ChIP experiment. ChIP results were confirmed by three separate experiments.

DNA precipitation assays

DNA precipitation assays were carried out with 30 μl of streptavidin magnetic beads (Promega), which were washed twice with 1× B&W buffer (5 mM Tris, pH 7.5, 0.5 mM EDTA and 1 M NaCl), and then 100 ng of WT and mutated double-stranded biotinylated oligonucleotides were bound to the beads in 1× B&W buffer for 1 h at room temperature (beads without oligonucleotides were used as a negative control). The beads were washed twice in 1× B&W buffer, once with 1× binding buffer (5% glycerol, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.15% Triton X-100, 100 mM NaCl and 4 mM MgCl₂) and blocked with 1% BSA and sperm salmon DNA (0.2 mg/ml) for 1 h at 4 °C. The bound biotinylated oligonucleotides were then mixed with 10 μg of poly (dl-dC) and 100 μg of nuclear extracts from MA-10 Leydig cells in a total volume of 500 μl of 1× binding buffer with protease inhibitors, and incubated for 2 h (1 h 30 min at 4 °C and 30 min at room temperature) with rotation, followed by five washes in 1× binding buffer. The bound proteins were then eluted in western blotting-loading buffer and analysed by SDS-PAGE. The following oligonucleotides were biotinylated, annealed with the corresponding non-biotinylated oligonucleotide and used as probes containing: i) the WT COUP-TFIIm high-affinity response element (underlined; Kliwer et al. 1992) 5'-AGG TTC TCA GAG GTC AGA GTG CT-3'; ii) the mutated (shown in lowercase); COUP-TFIIm high-affinity response element 5'-AGG TTC Aaa TCA GAa aTC AGA GAG CT-3'; iii) the WT DR0-like from the Ins3 promoter 5'-CCG TGA TGC CAG CCT CGA CTT TTG CGG TGC-3'; iv) the mutated (shown in lowercase) DR0-like from the Ins3 promoter 5'-CCG TGA TGC CAG CCT CGA CTT TTG GGG TGC-3'; v) the WT DR3 element from the Ins3 promoter 5'-ACC TGG GAG AGG ACT TCA AGG TCC CAA GCT GGA-3' and vi) the mutated (shown in lowercase) DR3 element from the Ins3 promoter 5'-ACC TGG GAG Agg Act TCA Aaa TCC CAA GCT GGA-3'.

Statistical analyses

For all single comparisons between two experimental groups, paired Student's t-tests were carried out. For all statistical analyses, P < 0.05 was considered significant. For multiple group comparisons, statistical analyses were done using one-way ANOVA followed by the Newman–Keuls post hoc test. All statistical analyses were done using the GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

COUP-TFIIm present in MA-10 Leydig cells regulates Ins3 gene expression

To determine whether COUP-TFIIm is present in INS3-expressing Leydig cells within the mouse testis, double immunohistochemistry was carried out on mice testis sections at E19.5 and post-natal day 32 (P32). As shown in Fig. 1A, at E19.5 COUP-TFIIm was detected in interstitial cells that were negative for INS3 (red–brown nuclear staining, open arrowheads). On the other hand, P32, COUP-TFIIm and INS3 (blue–purple cytoplasmic staining) were co-localised in interstitial Leydig cells (solid arrowheads). COUP-TFIIm was also detected in some peritubular cells at E19.5 and P32 that do not express INS3 (arrows in Fig. 1A and B). Thus, COUP-TFIIm and INS3 are co-expressed in adult Leydig cells but not in foetal Leydig cells of the mouse testis.

Next, to determine whether COUP-TFIIm is involved in Ins3 gene expression, MA-10 Leydig cells were transfected with siRNA directed against COUP-TFIIm or non-targeting siRNA, and Ins3 mRNA levels were determined by qPCR. COUP-TFIIm knockdown (to about 20%) was confirmed by...
COUP-TFII-depleted MA-10 Leydig cells, Insl3 mRNA levels were decreased by 40% when compared with cells transfected with scrambled siRNAs (Fig. 2). Thus COUP-TFII contributes to the regulation of Insl3 expression in MA-10 Leydig cells.

In order to determine whether COUP-TFII could activate an Insl3 promoter construct and to avoid non-specific activations, a dose-response assay was carried out. MA-10 Leydig cells were transfected with a −1087 bp mouse Insl3 reporter in the presence of increasing doses of a COUP-TFII expression vector. As shown in Fig. 3, the −1087 bp mouse Insl3 promoter was activated (3.5- to 4.3-fold) by COUP-TFII at all doses tested (25, 50, 125 and 250 ng). However, at 250 ng the minimal −79 bp Insl3 reporter was significantly activated by about sevenfold (Fig. 3), which indicates non-specific effects at a dose of 250 ng. A dose of 50 ng of COUP-TFII was therefore chosen as the optimal dose to significantly and specifically activate the −1087 bp Insl3 promoter.

COUP-TFII activates the Insl3 promoter via a novel DR0-like element at −97 bp

To locate the COUP-TFII-responsive element, a series of 5′ progressive deletions of the mouse Insl3 promoter were transfected in MA-10 Leydig cells. As shown in Fig. 4, deletion from −1087 to −186 bp did not significantly impair COUP-TFII responsiveness. However, further deletion to −79 bp resulted in a substantial decrease in COUP-TFII-dependent activation (Fig. 4). These results indicate that a COUP-TFII-responsive element is likely located between −186 and −79 bp. In silico analysis of this sequence revealed the presence of two previously uncharacterised DR elements, a DR0-like and a DR3, that could mediate the COUP-TFII-dependent activation of the
Insl3 promoter (Fig. 5A). Alignment of this sequence from different species (mice, rat, humans and bovine) revealed that the DR0-like element was well conserved in the Insl3 promoter of all species analysed, whereas the DR3 element was not (Fig. 5A). Consistent with this, COUP-TFII could equally activate the humans, rat and mice Insl3 promoter in transient transfections of MA-10 Leydig cells (Fig. 5B).

To determine the contribution of the DR0-like and DR3 elements to Insl3 promoter activity, MA-10 Leydig cells and CV-1 heterologous fibroblast cells were transfected with various −1087 bp Insl3 reporter constructs either WT harbouring mutations in the DR0-like and/or the DR3 elements. As shown in the left panel of Fig. 6A, mutation of the DR3 element led to a 75% decrease in Insl3 promoter activity in MA-10 Leydig cell. A mutation of the DR0-like element was more dramatic with a 93% reduction in Insl3 promoter activity, which is similar to what was observed with the minimal −79 bp Insl3 promoter. Mutation of both DR3 and DR0-like produced results identical to the DR0-like mutation (left panel of Fig. 6A). In CV-1 fibroblast cells, mutations of the DR3 and DR0-like elements also decreased Insl3 promoter activity, albeit to a much lesser extent than in MA-10 Leydig cells (right panel of Fig. 6A). These data indicate that the DR3 and DR0-like elements are both essential for maximal Insl3 promoter activity in MA-10 Leydig cells.

Next, we sought to determine whether the DR3 and/or the DR0-like elements were involved in the COUP-TFII-dependent activation of the Insl3 promoter. As shown in the left panel of Fig. 6B, mutation of the DR3 or the DR0-like individually or in combination (double DR3/DR1-like mutant) in the Insl3 promoter had no impact on COUP-TFII responsiveness in MA-10 Leydig cells. This could indicate that COUP-TFII activates the Insl3 promoter in association with other DNA-bound transcription factors in Leydig cells. In agreement with this, transfections in CV-1 fibroblast cells revealed that mutation of the DR0-like element at −103/−91 bp abrogated COUP-TFII responsiveness while mutation of the DR3 element had no impact (right panel in Fig. 6B). This indicates that the DR0-like element is necessary for the COUP-TFII-mediated activation of the −1087 bp Insl3 promoter and that in MA-10 Leydig cells, COUP-TFII may act independently of DNA binding.

**COUP-TFII binds to the DR0-like element in the proximal Insl3 promoter**

Next, ChIP assays were carried out to determine whether COUP-TFII was recruited to the proximal Insl3 promoter. Proteins were cross-linked to the chromatin, which was then sheared and immunoprecipitated using an anti-COUP-TFII antiserum. By PCR, a band of ∼200 bp (−225 to −26 bp) was detected in the input sample (Fig. 7A, upper panel lane 4) as well as in the samples immunoprecipitated with the anti-COUP-TFII antiserum (Fig. 7A, upper panel lane 5) and with the anti-NUR77 antiserum (Fig. 7A, upper panel lane 7; used as a positive control). No bands were observed in the IgG control sample (Fig. 7A, upper panel lane 3). An upstream genomic region (−3133 to −2923 bp) that does not contain any COUP-TFII binding site was also used (Fig. 7A, lower panel). No specific recruitment of COUP-TFII was observed on this distal region located at ∼3000 bp upstream to the Insl3 transcriptional start site (Fig. 7A, lower panel lane 5) or when using an IgG as negative control (Fig. 7A, lower panel lane 3). COUP-TFII is thus recruited to the proximal Insl3 promoter in a native chromatin environment in MA-10 Leydig cells.

Although our ChIP data confirm the recruitment of COUP-TFII to the proximal Insl3 promoter, this approach cannot discriminate between direct DNA binding and indirect recruitment through protein–protein interactions.
interactions. Furthermore, our functional data (Fig. 6) suggest that COUP-TFII can act independently of DNA binding in MA-10 Leydig cells. Therefore, to determine whether COUP-TFII directly binds to the DR0-like element in vitro, we used a DNA precipitation approach. As shown in Fig. 7B, COUP-TFII was found to bind to oligonucleotides containing a high-affinity DR1 element (Fig. 7B, lane 3) as well as the DR0-like element from the Insl3 promoter (Fig. 7B, lane 5), but it did not bind the DR3 element from the Insl3 promoter (Fig. 7B, lane 7) or oligonucleotides harbouring mutations in these elements (Fig. 7B, lanes 4, 6 and 8). Taken together, these results indicate that COUP-TFII directly and specifically binds to the DR0-like element in the proximal Insl3 promoter.

COUP-TFII cooperates with SF1 on the Insl3 promoter

As our activation data (Fig. 6) indicate that COUP-TFII may act in association with other DNA-bound transcription factors and as the DR0-like element (−103/−91 bp) is in proximity to binding sites for the nuclear receptor SF1 (Zimmermann et al. 1998, Koskimies et al. 2002), one of which is located within the DR3 element (−151/−135 bp), we tested the possibility that these two nuclear receptors might cooperate to modulate Insl3 promoter activity. Co-transfections in MA-10 Leydig cells revealed that COUP-TFII and SF1 individually activate the −1087 bp Insl3 promoter about fivefold while both nuclear receptors in combination had additive effects (left panel in Fig. 8). This cooperation was more apparent in heterologous CV-1 cells, where co-transfection of both COUP-TFII and SF1 led to a synergistic activation (about 30-fold) of the Insl3 promoter (right panel in Fig. 8).

Discussion

INSL3 is produced exclusively by the male foetus primarily to control the first phase of testicular descent (Ivell & Anand-Ivell 2011). In adult males, INSL3 was identified as a major circulating testicular hormone that directly correlates with Leydig cell capacity and development (Ivell & Anand-Ivell 2011, Ivell et al. 2013a). Recent evidence has identified a novel role for INSL3 in bone metabolism (Ferlin et al. 2008) in addition to its proposed role in the prevention of male germ cells apoptosis (Kawamura et al. 2004, Del Borgo et al. 2006, Amory et al. 2007). In adult females, despite its low expression compared with males, INSL3 was found to be a paracrine factor within the ovary.
and may be involved in follicle selection and survival (Ivell & Anand-Ivell 2011). Despite these important physiological roles and the identification of INSL3 as a unique marker of Leydig cells, very little is known about the molecular mechanisms that regulate Insl3 expression in these cells.

INSL3 is constitutively expressed in Leydig cells and, unlike testicular steroids, it is regulated independently of the hypothalamo–pituitary–gonadal axis (Anand-Ivell et al. 2006, Atlantis et al. 2009, Ivell & Anand-Ivell 2009). This absence of direct hormonal regulation was also reported in studies of the rat and mice Insl3 gene promoter (Zimmermann et al. 1998, Koskimies et al. 2002). From these studies, a relatively short promoter region of ~200 bp was sufficient to drive Insl3 gene transcription in various Leydig cell lines. Within this proximal region, three binding sites for the transcription factor SF1 were

Figure 5
Species conservation of COUP-TFII responsiveness. (A) Sequence alignment of the two direct-repeat (DR) elements (grey shaded boxes) present in the Insl3 promoter from the mice, rat, human and bovine. The DR3 element is located at −143 bp and the DR0-like element is located at −97 bp. Conserved residues are indicated by capital letter while one mismatch is represented by a lowercase letter. Non-conserved residues are indicated by asterisks. The two potential DR elements are represented by the white (DR3) and grey (DR0-like) diamonds. (B) MA-10 Leydig cells were co-transfected with either an empty expression vector (−) or a COUP-TFII expression vector (+), along the mice (black bars), human (open bars) or rat (grey bars) Insl3 promoter as indicated. The number of experiments, each performed in triplicate, is indicated. Results are shown as fold activation over control (±S.E.M.). An asterisk (*P<0.05) indicates a statistically significant difference from control.

Figure 6
COUP-TFII activates the Insl3 promoter via the DR0-like element at −97 bp. (A) The DR0-like and DR3 elements are critical for Insl3 promoter activity. MA-10 Leydig (left panel) and CV-1 fibroblast (right panel) cells were co-transfected with either a WT −1087 bp Insl3 reporter, reporters harbouring mutations (depicted by a large X) in the DR0-like and DR3 elements as indicated, or a minimal −79 bp reporter. The two DR elements are represented by the white (DR3) and grey (DR0-like) diamonds. The number of experiments, each performed in triplicate, is indicated. Results are shown as relative activity (±S.E.M.). A different letter indicates a statistically significant difference (P<0.001). (B) MA-10 Leydig (left panel) and CV-1 (right panel) cells were transfected with the same reporters described in A along with either an empty expression vector (−) or an expression vector for COUP-TFII (+). The number of experiments, each performed in triplicate, is indicated. Results are shown as fold activation over control (±S.E.M.). A different letter indicates a statistically significant difference (P<0.001).
Figure 7
COUP-TFII is recruited to the proximal Insl3 promoter and binds specifically to the DR0-like element. (A) COUP-TFII recruitment to the proximal Insl3 promoter in MA-10 cells was determined by ChIP. An aliquot of chromatin preparation before immunoprecipitation (input) was used as positive control. A 200-bp DNA fragment containing the DR0-like element present in the mouse Insl3 promoter was amplified by PCR in the input and in the sample immunoprecipitated with the anti-COUP-TFII antiserum but not in the IgG control sample. Recruitment of NUR77 in the same region was used as positive control. The recruitment of COUP-TFII and NUR77 was also assessed on the distal Insl3 promoter region (−136 to −3000 bp) and the signals were similar to those of the IgG-negative control sample. Water was used as negative control for the PCR (Ctrl (−) PCR). The PCR is representative of three independent experiments. (B) DNA precipitation assays were carried out using oligonucleotides containing a high affinity DR1 element (HA DR1, AGGTCAnAGGTCA, positive control), the DR0-like element and the DR3 element from the Insl3 promoter either WT or with a mutation that destroys the binding site (Mut) along with nuclear extracts from MA-10 Leydig cells. Western blottings were used to detect COUP-TFII. Input corresponds to 15 µg of the nuclear extracts used in the DNA precipitation assay. The western blotting shown is the representative of three independent experiments.

dependently identified (Zimmermann et al. 1998, Koskimies et al. 2002). Mutagenesis studies showed that all three SF1 elements were needed for maximal SF1-dependent activation of the Insl3 promoter. However, SF1 displayed differential binding affinities for the three SF1 elements and was found to have the highest affinity for the most distal element at −144 to −136 bp (which represents a half-site of the DR3 we have identified in our study; Koskimies et al. 2002). Yet it was the element at −115 to −107 bp that showed the strongest response to SF1 in terms of transcriptional activation (Koskimies et al. 2002). In addition to the mice and rat Insl3 promoter, SF1 was also identified as a potent activator of the canine INSL3 promoter (Truong et al. 2003). Binding of SF1 to each site may very well differ in intensity between species as shown by Sadeghian et al. (2005), but the motif at −115 to −107 bp was always found to be critical for Insl3 gene expression in all species analysed (Zimmermann et al. 1998, Koskimies et al. 2002, Truong et al. 2003, Sadeghian et al. 2005, Robert et al. 2006). In addition to SF1, our laboratory has identified the orphan nuclear receptor NUR77 as a positive regulator of human and mice INSL3 transcription in Leydig cells. NUR77 was found to bind to an element located at −100 bp within the proximal INSL3 promoter, an element essential and sufficient to confer NUR77 responsiveness (Robert et al. 2006).

In our present work, we found that the orphan nuclear receptor COUP-TFII was essential for maximal Insl3 expression in MA-10 Leydig cells. Our results complement the in vivo findings by Qin et al. (2008) which showed that Insl3 mRNA levels were decreased in Coup-tfii−/− mice at the pre-pubertal stage. However, as Leydig cell differentiation was impaired in these animals, it remained unknown whether the decrease in Insl3 was due to improper Leydig cell differentiation and/or to a direct action of COUP-TFII in Insl3 transcription. The fact that depletion of COUP-TFII in MA-10 Leydig cells using siRNA led to a significant decrease in Insl3 mRNA levels indicates that COUP-TFII could directly regulate Insl3 gene expression in these cells. This is further supported by the
fact that COUP-TFII and INSL3 co-localise in Leydig cells of the adult mouse testis. In foetal testis, however, COUP-TFII is not present in INSL3 positive cells, indicating that this nuclear receptor does not directly regulate Insl3 expression in the foetal testis.

A previously uncharacterised DR0-like sequence (GAGCCTGACCT) was identified for COUP-TFII binding and promoter activation in the proximal Insl3 promoter, the region known to be sufficient for the Insl3 promoter activity (Zimmermann et al. 1998, Koskimies et al. 2002, Sadeghian et al. 2005). This DR0-like sequence at −97 bp was also present in the Insl3 promoter from various species including mice, rat, human and bovine, and thus has been evolutionarily conserved supporting a crucial function for the associated DNA-binding protein. Consistent with this, the human and rat Insl3 promoter were also significantly activated by COUP-TFII.

COUP-TFII is known to bind to a variety of DR elements with variable spacing. Although it shows the highest affinity for a DR1 element, COUP-TFII can also bind to DR0 and DR2 elements with the second highest affinity (Cooney et al. 1992, Kadokawa et al. 1992, Klewer et al. 1992). Mutation of the DR0-like element severely decreased Insl3 promoter activity in MA-10 Leydig cells thus identifying this element as critical for Insl3 transcription. Furthermore, DNA–protein interaction experiments revealed that COUP-TFII binds to the DR0-like element. Yet, mutation in the DR0-like element that prevents COUP-TFII binding had no effect on COUP-TFII-dependent activation of the Insl3 promoter in MA-10 Leydig cells. However, the same mutation abrogated COUP-TFII responsiveness of the Insl3 promoter in heterologous CV-1 fibroblast cells. These results indicated that although COUP-TFII can bind to and activate the Insl3 promoter via the DR0-like element, it may also do so in a DNA binding-independent manner most likely via interactions with other Leydig cell-enriched, DNA-bound transcription factors. The proximity of the SF1 binding site within the DR3 element, which is also important for Insl3 promoter activity in Leydig cells (this study and Koskimies et al. (2002)), prompted us to test whether COUP-TFII and SF1 function together on the Insl3 promoter. We found that COUP-TFII and SF1 had additive effects on Insl3 promoter activity when transfected in MA-10 Leydig cells whereas in CV-1 heterologous cells, the nuclear receptors had strong synergistic effects. This is the first report of a transcriptional cooperation between COUP-TFII and SF1. Additional work is needed to fully decipher the mechanisms of COUP-TFII and SF1 cooperation in Insl3 gene expression.

In conclusion, we reported that Insl3 mRNA levels were decreased in COUP-TFII-depleted MA-10 Leydig cells and found that the Insl3 promoter is directly and positively regulated by COUP-TFII. Furthermore, we identified a transcriptional cooperation between the nuclear receptors COUP-TFII and SF1 to further enhance Insl3 promoter activity. It is noteworthy that the concentration of INSL3 circulating in the bloodstream parallels the mRNA levels found within the testes (Irving-Rodgers et al. 2002, Ivell et al. 2013b) further supporting that this hormone is constitutively secreted as soon as it is synthesised. For this reason, quantification of Insl3 mRNA levels in the testis, or of INSL3 as the secreted peptide circulating in the bloodstream, provides an excellent assessment of Leydig cell differentiation and function (Ivell et al. 2013a). Our findings contribute to a better understanding of the regulation of INSL3 expression, a hormone now considered as an informative clinical parameter of gonadal function (Ivell et al. 2013a).

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
R E M-V is the recipient of a doctoral studentship from the Réseau Québécois en Reproduction. J J T holds a Chercheur-Boursier Scholarship from the Fonds de Recherche du Québec – Santé. This work was funded by grants from the Canadian Institutes of Health Research (funding reference number MOP-81387) and from the Natural Sciences and Engineering Research Council of Canada (funding reference number 262224) to J J T.

Acknowledgements
The authors thank Drs Ming Tsai and Mario Ascoli for generously providing the COUP-TFII expression plasmid and the MA-10 cell line used in this study. They also thank Eric Boucher for his technical assistance with animal tissues and Caroline Daems for an Insl3 promoter construct.

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Received in final form 29 March 2014

Accepted 25 April 2014

Accepted Preprint published online 29 April 2014