The Nuclear Receptor COUP-TFII Regulates Amhr2 Gene Transcription via a GC-Rich Promoter Element in Mouse Leydig Cells

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The nuclear receptor chicken ovalbumin upstream promoter–transcription factor type II (COUP-TFII)/NR2F2 is expressed in adult Leydig cells, and conditional deletion of the Coup-tfii/Nr2f2 gene impedes their differentiation. Steroid production is also reduced in COUP-TFII–depleted Leydig cells, supporting an additional role in steroidogenesis for this transcription factor. COUP-TFII action in Leydig cells remains to be fully characterized. In the present work, we report that COUP-TFII is an essential regulator of the gene encoding the anti-Müllerian hormone receptor type 2 (Amhr2), which participates in Leydig cell differentiation and steroidogenesis. We found that Amhr2 mRNA levels are reduced in COUP-TFII–depleted MA-10 Leydig cells. Consistent with this, COUP-TFII directly activates a ∼1486 bp fragment of the mouse Amhr2 promoter in transient transfection assays. The COUP-TFII responsive region was localized between −67 and −34 bp. Chromatin immunoprecipitation assay confirmed COUP-TFII recruitment to the proximal Amhr2 promoter whereas DNA precipitation assay revealed that COUP-TFII associates with the −67/−34 bp region in vitro. Even though the −67/−34 bp region contains an imperfect nuclear receptor element, COUP-TFII–mediated activation of the Amhr2 promoter requires a GC-rich sequence at −39 bp known to bind the specificity protein (SP)1 transcription factor. COUP-TFII transcriptionally cooperates with SP1 on the Amhr2 promoter. Mutations that altered the GCGGGGCGG sequence at −39 bp abolished COUP-TFII–mediated activation, COUP-TFII/SP1 cooperation, and reduced COUP-TFII binding to the proximal Amhr2 promoter. Our data provide a better understanding of the mechanism of COUP-TFII action in Leydig cells through the identification and regulation of the Amhr2 promoter as a novel target.

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Anti-Müllerian hormone (AMH), produced by Sertoli cells in males and granulosa cells in females, is a glycoprotein that belongs to the TGFβ family of proteins (reviewed in [1]). In fetal males, AMH is best known for its essential function in promoting the regression of the Müllerian ducts that would otherwise develop into the fallopian tubes, uterus, and upper...
vagina (reviewed in [2]). AMH has also been described as an important regulator of Leydig cell differentiation and function. For instance, AMH inhibits steroidogenesis in fetal and adult primary Leydig cells [3–5] as well as in various Leydig cell lines [6, 7]. This inhibitory role of AMH on Leydig cell steroidogenesis was also reported in animal studies. Administration of AMH to adult rodents was found to inhibit testosterone biosynthesis [8, 9], and male transgenic mice overexpressing AMH exhibit feminized genitilia caused by reduced serum testosterone levels and Leydig cell numbers [10, 11]. The reduced number of Leydig cells in these mice was attributed to AMH-mediated inhibition of the differentiation of mesenchymal stem cells into Leydig cells [4]. Conversely, inactivation of the Amh gene in mice results in the retention of Mullerian duct derivatives as well as impairment in the differentiation of the adult Leydig cell population [12]. In Amh-deficient male mice, plasma testosterone concentrations are reduced in pubertal animals but are normal in adults [13]. This is thought to be due to Leydig cell hyperplasia where individual Leydig cells have a reduced capacity for testosterone biosynthesis leading to a deficit in androgen production, which is later compensated by an increase in the number of Leydig cells [13].

In Leydig cells, AMH acts via a heterodimeric receptor composed of the AMH type 2 receptor (AMHR2) [14, 15] and the activin receptor-like kinase 3 (ALK3). Amhr2 mRNA is present in Leydig cells as well as in several rodent Leydig cell lines [16, 17]. At the protein level, AMHR2 is found in both the fetal and adult Leydig cell populations in rodents [15, 18]. Deletion of the Amhr2 gene in male mice causes pseudohermaphroditism, infertility, seminiferous tubule atrophy, and Leydig cell hyperplasia [14], whereas Leydig cell–specific ablation of Alk3 causes impairments in Leydig cell differentiation and androgen metabolism [19]. In humans, mutations inactivating the AMH or AMHR2 gene lead to the development of persistent Mullerian duct syndrome in males characterized by infertility, inguinal hernias, and cryptorchidism [20], and in some cases Leydig cell hyperplasia, azoospermia, and low serum testosterone levels [21].

Despite the important role for the AMH/AMHR2 system in regulating the differentiation and function of both Leydig cell populations, much remains to be understood regarding the mechanisms governing Amhr2 gene expression in these cells. The Amhr2 promoter has been reported to be regulated by the nuclear receptor steroidogenic factor 1 (SF1/Ad4BP/NR5A1) acting via two conserved nuclear receptor binding motifs [17, 22, 23]. SF1 also cooperates with β-catenin to synergistically activate Amhr2 transcription [23]. The transcription factor GATA4 was also found to activate the Amhr2 promoter in Leydig cells [24]. Other regulators of Amhr2 promoter activity include Wilms tumor 1 [25] and early growth response 1 (EGR1) in murine LβT2 gonadotrope cells [26].

The chicken ovalbumin upstream promoter–transcription factor type II (COUP-TFI/ NR2F2) is a member of the steroid/thyroid nuclear receptor superfamily. COUP-TFI is expressed in numerous tissues (reviewed in [27–29]), including the testis, where it is found in the nucleus of interstitial/mesenchymal cells throughout development and into adulthood [30]. Homozygous Coup-tfi/Nr2f2 null mice die at embryonic day 10 due to the requirement of COUP-TFI for angiogenesis and heart development [31]. Tissue-specific ablation of COUP-TFI in the stomach, uterus, diaphragm, limbs, skeletal muscle, and endothelial cells also revealed essential roles in cell differentiation and organogenesis of these tissues [32–37]. In female mouse embryos, temporal ablation of COUP-TFI disrupts female sex differentiation by the abnormal retention of the male Wolffian ducts [38]. In males, timed inactivation of COUP-TFI during prepubertal stages of male sexual development results in infertility, hypogonadism, and a block in spermatogenesis due to a failure of progenitor Leydig cells to mature and, ultimately, to produce adequate testosterone levels [39]. These data indicate that COUP-TFI is essential for proper differentiation of adult Leydig cells. Moreover, diminished steroidogenesis in these mice was shown to be associated with decreased mRNA levels for several steroidogenic enzymes, including Hsd3b1, Cyp11a1, and Cyp17a1 [39]. This is consistent with the fact that COUP-TFI is expressed in steroidogenically active Leydig cells from the adult, but not the fetal population [30]. In Leydig cell lines, we also reported COUP-TFI to be a direct activator of the promoter for the steroidogenic acute
regulatory protein (Star) gene [30], which encodes a cholesterol transporter essential for testosterone synthesis.

Despite the acknowledged role of COUP-TFII in the differentiation and function of the adult Leydig cell population, our understanding of its direct target genes, and therefore its mechanism of action in these cells, remains incomplete. In the current study, we report that the Amhr2 promoter is a novel target for COUP-TFII in MA-10 Leydig cells. COUP-TFII directly activates Amhr2 gene expression via a COUP-TFII–responsive, GC-rich sequence, present in the proximal Amhr2 promoter region, and that this transcriptional regulation involves a cooperation between COUP-TFII and specificity protein (SP)1. Importantly, these findings provide new insights not only into the mechanism of COUP-TFII action in adult Leydig cells but also help to better clarify Amhr2 transcription in these cells.

1. Materials and Methods

A. Plasmids

The −1486/+77, −758/+77, −486/+77, −257/+77, −151/+77, −122/+77, −67/+77, −51/+77, −45/+77, and −34/+77 bp mouse Amhr2 promoter constructs were generated by PCR using an Amhr2 reporter construct as template provided by Dr. Jose Teixeira (Michigan State University, East Lansing, MI) and sequence-specific forward primers containing a BamHI cloning site and a common reverse primer containing a XhoI cloning site. The primer sequences are listed in Table 1. PCR fragments were enzyme digested, gel purified, and cloned into the BamHI/SalI sites of a modified pXP1 luciferase reporter vector [40]. Mutations within the promoter region were generated using the PfuUltra High-Fidelity DNA Polymerase AD according to the manufacturer’s instructions (Agilent Technologies, Santa Clara, CA) with the primer sets listed in Table 1. A plasmid containing the mouse COUP-TFII cDNA [31] was obtained from Dr. Ming Tsai (Baylor College of Medicine, Houston, TX) and the COUP-TFII cDNA was subsequently subcloned into the pcDNA3.1 mammalian expression vector (Invitrogen Canada, Burlington, ON, Canada). All generated plasmids and PCR products were verified by an on-site sequencing service.

B. Cell Culture, Transfections, and Reporter Assays

Mouse MA-10 Leydig cells (American Type Culture Collection, Manassas, VA, catalog no. CRL-3050, RRID:CVCL_D789) [41], provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA), were grown in DMEM/F12 medium supplemented with 2.438 g/L sodium bicarbonate, 3.57 g/L HEPES, and 15% horse serum on plates coated with 0.1% gelatin. Penicillin and streptomycin sulfate were added to a final concentration of 50 mg/L, and cells were incubated at 37°C and 5% CO2 in a humidified incubator. MA-10 Leydig cells were isolated from mouse Leydig cell tumors (M5480P), and they represent an immature Leydig cell population [42] as characterized by the high expression of 5α-reductase [43]. Stimulation of MA-10 Leydig cells by LH/human chorionic gonadotropin, forskolin, and cAMP results in increased steroid production [42]. MA-10 cells were transiently transfected using polyethylenimine hydrochloride (Sigma-Aldrich Canada, Oakville, ON, Canada) as described in Longo et al. [44] with minor modifications. Briefly, the cells were plated in 24-well plates 24 hours prior to the transfection. To determine COUP-TFII responsiveness, the cells were cotransfected on the next day with 400 ng of reporter vector along with different amounts of expression vectors and polyethylenimine dissolved in Opti-MEM medium (GIBCO by Life Technologies, Burlington, ON, Canada). To determine relative promoter activity, the cells were cotransfected with 400 ng of reporter vector, 10 ng of phRL-TK Renilla luciferase expression vector, and 90 ng of an inert pSP64 plasmid (Promega, Madison, WI). Sixteen hours after transfection, the media were replaced and the cells were grown for an additional 32 hours. After the cells were lysed, the cell lysates were collected and analyzed using Tecan Spark 10M multimode plate reader (Tecan, Morrisville, NC) as previously described [45, 46].
C. Small Interfering RNA Transfection and Reverse Transcription- Quantitative PCR

MA-10 Leydig cells were transfected with 150 nM small interfering RNA (siRNA) directed against Coup-tfii transcripts (Nr2f2-MSS235957, Life Technologies, Carlsbad, CA) or with Stealth RNAi™ siRNA Negative Control, Med GC (Thermo Fisher Scientific, Mississauga, ON, Canada) using JetPRIME transfection reagent (PolyPlus-transfection, Illkirch, France) according to the manufacturer’s protocol. The cells were incubated for 48 hours and total RNA was extracted using TRIzol (Thermo Fisher Scientific) following the supplier’s instructions. Amhr2 mRNA levels were determined by reverse transcription- quantitative PCR (qPCR) as previously described [24, 45]. Relative expression of Amhr2 was normalized to the expression of Rpl19, used as internal control, and is plotted as a ratio of Amhr2 to Rpl19 levels; primer sets are listed in Table 1.

D. Protein Purification and Western Blots

Nuclear proteins from MA-10 Leydig cells were extracted as previously described [47] and quantified using a Bradford protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada). In vitro translated COUP-TFII was generated using the Promega TnT quick coupled transcription/translation system (Thermo Fisher Scientific) according to the manufacturer’s protocol without additional purification or quantification. Denatured proteins were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore, Etobicoke, ON, Canada). Immunodetection was performed using horseradish peroxidase–conjugated antibodies according to the manufacturers’ protocols and the following reagents: ECL and ECL Prime Western blotting detection reagents (GE Healthcare Life Sciences, Mississauga, ON, Canada), Clarity Western ECL substrate (Bio-Rad Laboratories), Clarity Max Western ECL substrate (Bio-Rad Laboratories). Detection of COUP-TFII and lamin B proteins was performed using a mouse monoclonal anti–COUP-TFII antibody (dilution 1:1000; R&D Systems, Minneapolis, MN; catalog no. PP-H7147-00, RRID:AB_2155627) [48] and a goat polyclonal anti–lamin B antibody (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc-6216, RRID:AB_648156) [49], respectively.

E. Chromatin Immunoprecipitation–qPCR Assay

Three million MA-10 Leydig cells were plated on a 0.1% gelatin-coated 100-mm plate and incubated for 24 hours. Once the cells reached 80% confluency, the protein/DNA complexes were crosslinked by supplementing the media with 1% formaldehyde and incubating for 10 minutes at 37°C. The crosslinking reaction was stopped by the addition of glycine to a final concentration of 125 mM with shaking for 10 minutes at 25°C. Chromatin immunoprecipitation (ChIP) experiments were performed as described previously [30]. ChIP DNA fragments were analyzed by qPCR using specific primer sets for proximal and distal regions of the Amhr2 gene regulatory region (Table 1). The qPCR amplifications were done on a C1000 thermal cycler (Bio-Rad Laboratories) using PerfeCTa SYBR Green FastMix (Quantabio, Beverly, MA) and the following conditions: 3 minutes at 95°C, followed by 46 cycles of denaturation (10 seconds at 95°C), annealing (30 seconds at 59°C), and extension (20 seconds at 72°C). Specificity of the primer sets for the target regions were verified by agarose gel electrophoresis and sequencing. Results are represented as fold enrichments and were calculated from ChIP anti–COUP-TFII (R&D Systems, Minneapolis, MN; catalog no. PP-H7147-00, RRID:AB_2155627) [48] samples relative to the ChIP control IgG (Santa Cruz Biotechnology; catalog no. sc-2025, RRID:AB_737182) [50] after both samples were normalized to the input. Fold enrichment was calculated as follows: fold enrichment = \(2^{-\Delta\Delta Ct}\), where each target gene region was first normalized to the input following the formula “normalized to input” \(\Delta Ct = \Delta Ct\) (ChIP) − \(\Delta Ct\) (input − log dilution factor). Then, \(-\Delta\Delta Ct = normalized to input \Delta Ct (\text{NR2F2}) − normalized to input \Delta Ct (\text{IgG})\). The ChIP results were confirmed in three separate experiments.
| Purpose                   | Description                        | Template   | Sequence                                                                 |
|--------------------------|------------------------------------|------------|--------------------------------------------------------------------------|
| Promoter constructs      |                                    |            |                                                                          |
| -1486/+77 bp             | Amhr2 reporter construct [17]      | F:         | CGGGATCCAGGCCCTTTAACCTTTTG                                               |
| -758/+77 bp              |                                    | CG         | CGGGATCCAGAGGAGGTAGTCGAAATC                                              |
| -486/+77 bp              |                                    | CGGG       | CGGGATCCAGAGGAGGTAGTCGAAATC                                              |
| -257/+77 bp              |                                    | CGGG       | CGGGATCCAGAGGAGGTAGTCGAAATC                                              |
| -151/+77 bp              |                                    | CGGG       | CGGGATCCAGAGGAGGTAGTCGAAATC                                              |
| -122/+77 bp              |                                    | CGGG       | CGGGATCCAGAGGAGGTAGTCGAAATC                                              |
| -67/+77 bp               |                                    | CGGG       | CGGGATCCAGAGGAGGTAGTCGAAATC                                              |
| -51/+77 bp               |                                    | CGGG       | CGGGATCCAGAGGAGGTAGTCGAAATC                                              |
| -45/+77 bp               |                                    | CGGG       | CGGGATCCAGAGGAGGTAGTCGAAATC                                              |
| -34/+77 bp               |                                    | CGGG       | CGGGATCCAGAGGAGGTAGTCGAAATC                                              |
| -1486/+77 bp SF1         | distal mutant                      | -1486/+77 bp| AGAAGGTCGGCAGCCCTTTACCTCCATCAAAACAGTAGGGGATAGAGATTTC                     |
| -1486/+77 bp SF1         | proximal mutant                    | -1486/+77 bp| GTTCTCAAGTTGCAAGCCAANACCTTTACCTTCATCTCCATCCACCTTC                        |
| -1486/+77 bp SF1         | proximal mutant                    | -1486/+77 bp| GTTCTCAAGTTGCAAGCCAANACCTTTACCTTCATCTCCATCCACCTTC                        |
| -67/+77 bp M1            | proximal mutation                  | -67/+77 bp | AGAAGGTCGGCAGCCCTTTACCTCCATCAAAACAGTAGGGGATAGAGATTTC                     |
| -67/+77 bp M2            |                                    | -67/+77 bp | GTTCTCAAGTTGCAAGCCAANACCTTTACCTTCATCTCCATCCACCTTC                        |
| -67/+77 bp M3            |                                    | -67/+77 bp | GTTCTCAAGTTGCAAGCCAANACCTTTACCTTCATCTCCATCCACCTTC                        |
| -67/+77 bp M4            |                                    | -67/+77 bp | GTTCTCAAGTTGCAAGCCAANACCTTTACCTTCATCTCCATCCACCTTC                        |
| DNA Pull-down            |                                    |            |                                                                          |
| HA-DR1 WT                |                                    |            |                                                                          |
| HA-DR1 mutation          |                                    |            |                                                                          |
| -67/-34 bp WT            |                                    |            |                                                                          |

(Continued)
Table 1. Oligonucleotides Used in This Study (Continued)

| Purpose | Description | Template | Sequence |
|----------|-------------|----------|----------|
|        | -67/−34 bp M1 | F: /5Biosg/CCACAGAGACCGGGATTAaagCAGAGCGGGGCGG | R: CCGCCCGCTCTGTGctTATCCCCGCTCTGTG |
|        | -67/−34 bp M4 | F: /5Biosg/CCACAGAGACCGGGATAGGACAGAGCttGGCtt | R: aaGCCaaGCTCTGTCTATCCCCGCTCTGTG |
| SF1 distal | | F: /5Biosg/TTCTTTCAAGGTCAGTAGGGGCTAGAGAATTT | R: AAATCTCTACCCCTACTGACCTTGGAAGAA |
| SF1 proximal | | F: /5Biosg/TTCTTCAGCTGGACAGCCAAGGTCCCTTCCTCC | R: GGAGGAAGGGACCTTGGCTGCAGCTGAGAA |
| ChIP | -140/+29 bp Amhr2 promoter | F: ATTGATTCTCTGCTCCTCCCTTTC | R: CTCAGCAGAAGGTCTCCTACTCAAAATA |
|        | -3395/−3274 bp Amhr2 promoter | F: TCAAAAGAATAATGACCGAGGC | R: CAAATGGCTTCCTTTGGTGCTGGAAT |
| qPCR | Amhr2 | F: CCTCTGCCCCTCTGGGCC TT | R: ACTGGCCATCTGCCAAGCG |
|        | Rpl19 | F: CTGAAGGTCAAAGGGAATGTG | R: GGACAGAGTCTTGTAGATTC |

Abbreviations: ChiP, chromatin immunoprecipitation; qPCR, quantitative PCR.
F. DNA Pulldown Assay

DNA pulldown assays were performed as previously described [30] with minor modifications. Biotinylated and nonbiotinylated oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Table 1. Ten micrograms of biotinylated oligonucleotides and 10 μg of complementary oligonucleotides were annealed in 100 μL of Taq reaction buffer (New England Biolabs, Whitby, ON, Canada) using a Biometra TGradient thermocycler (Montréal Biotech, Dorval, QC, Canada) and stored at −20°C until use. Streptavidin magnetic beads from Promega (Thermo Fisher Scientific) were washed four times with B&W buffer [5 mM Tris-Cl (pH 7.5), 0.5 mM EDTA, and 1 M NaCl]. DNA probes were diluted to 20 ng/μL in B&W buffer. One microgram of the diluted DNA probe was added to 50 μL of prewashed magnetic beads and incubated at 24°C for 20 minutes. The beads were washed once with 300 μL of cold B&W buffer, once with 300 μL of protein binding buffer [20 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 0.15% Triton X-100, 100 mM NaCl, 4 mM MgCl₂, 5% glycerol]. After DNA probes were bound to the beads, the beads were blocked with 0.67% BSA in 300 μL of protein binding buffer by rotation at 23°C for 20 minutes. Next, the beads were washed once with protein binding buffer. To the beads, 130 μg of nuclear extract was added in 500 μL of protein binding buffer and incubated for 90 minutes (60 minutes at 4°C and 30 minutes at 23°C) with constant rotation, followed by seven washes in 300 μL of cold protein binding buffer. Bound proteins were eluted in 20 μL of 1× Laemmli buffer by incubating at 95°C for 10 minutes and analyzed by Western blotting.

G. Statistical Analyses

All statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA; RRID:SCR_002798) [51] or Microsoft Excel (Microsoft Corporation, Redmond, WA; RRID:SCR_016137) [52]. Statistical analyses between two groups were performed using a Student t test or by one-way ANOVA followed by a Newman–Keuls post hoc test for multiple comparisons between the groups. P values from each comparison are indicated in the figure legends.

2. Results

A. COUP-TFII Regulates Amhr2 Gene Transcription in Leydig Cells

Transcriptomic microarray analysis of COUP-TFII–depleted MA-10 Leydig cells revealed several genes whose expression was affected by the lower level of COUP-TFII protein (data not shown). Owing to its well-established role in Leydig cell differentiation and steroidogenesis [6, 7, 11, 53], an interesting candidate was the Amhr2 gene. We therefore decided to further investigate whether COUP-TFII could be involved in the regulation of Amhr2 gene expression. MA-10 Leydig cells were transfected with either control siRNA or siRNA directed against COUP-TFII. Decreased COUP-TFII protein levels were confirmed by Western blot (Fig. 1A, top panel). In COUP-TFII–depleted MA-10 Leydig cells, Amhr2 mRNA levels were reduced by ~36% compared with control cells (Fig. 1A, bottom panel). To test whether the decrease in Amhr2 mRNA levels in COUP-TFII–depleted cells was due to a direct action of COUP-TFII on the Amhr2 promoter, MA-10 Leydig cells were cotransfected with two mouse Amhr2 reporter constructs, −1486/+77 bp and −34/+77 bp, in the absence or presence of a COUP-TFII expression vector. As shown in Fig. 1B, the −1486/+77 bp Amhr2 reporter was activated up to fourfold by COUP-TFII, whereas the −34/+77 bp Amhr2 reporter was only weakly activated by COUP-TFII.

B. COUP-TFII–Dependent Activation of the Amhr2 Promoter Does Not Require SF1 Regulatory Elements

Previous studies have demonstrated that COUP-TFII can act via SF1 regulatory elements on target genes [54–56]. The Amhr2 promoter contains two previously characterized SF1 elements at −250 bp (5′-AGGTCA-3′) and at −200 bp (5′-AGGTCC-3′) [17]. To test whether
COUP-TFII regulates Amhr2 promoter activity through the two SF1 elements, Ahmr2 promoter constructs harboring mutations in the distal, proximal, or both SF1 elements were generated and transfected in MA-10 Leydig cells. Surprisingly, these mutations had no impact on COUP-TFII responsiveness (Fig. 2), which suggests that one or more unknown regulatory elements are required.

**Figure 1.** COUP-TFII regulates mouse Amhr2 gene transcription in MA-10 Leydig cells. (A) Depletion of COUP-TFII in MA-10 Leydig cells reduces Amhr2 gene expression. MA-10 Leydig cells were transfected with control siRNA (open bar) or siRNA targeting COUP-TFII (filled bar). After 48 h, cells were collected and nuclear extracts as well as total RNA were prepared. The efficiency of COUP-TFII depletion was determined by Western blot using 5 μg of nuclear extracts from cells transfected with control siRNA (lane 1) and siRNA targeting COUP-TFII (lane 2). Lamin B was used as a loading control. Amhr2 mRNA levels were quantified by qPCR and normalized to Rpl19 mRNA. Data are represented as mean ± SEM. *P, 0.05. (B) COUP-TFII activates the mouse Amhr2 promoter in MA-10 Leydig cells. MA-10 Leydig cells were cotransfected with either 100 ng of an empty expression vector (control, open bars) or an expression vector for COUP-TFII (filled bars), along with 400 ng of Amhr2 promoter construct (−1486/+77 bp or −34/+77 bp, as indicated). Results are shown as fold activation over control ± SEM. *P < 0.05, vs its control (empty expression vector, value set at 1, filled bar over open bar); #P < 0.05, for activation by COUP-TFII between the −1486 and −34 bp reporters.
An Intact GC-Rich Sequence Is Essential for COUP-TFII–Dependent Activation of the Amhr2 Promoter

To locate the COUP-TFII response element, a series of 5’ progressive deletion constructs were generated and transfected in MA-10 Leydig cells. As shown in Fig. 3A, deletion from −1486 to −67 bp did not impair COUP-TFII–dependent activation of the Amhr2 promoter. However, a −34 bp deletion construct was only modestly activated by COUP-TFII (2-fold compared with empty vector; 20-fold with longer promoter constructs) (Fig. 3A). These results suggest that the critical COUP-TFII response element is located between −67 and −34 bp.

Analysis of the basal promoter activity of the same Amhr2 deletion constructs revealed a small but significant increase in activity with deletion from −1486 bp to −486 bp (Fig. 3B). Subsequent deletions to −122 bp and −67 bp caused a significant decrease in promoter activity (Fig. 3B), with the −67 bp reporter having only −30% activity of the −1486 bp reporter. Finally, a deletion to −34 bp abrogated Amhr2 promoter activity (Fig. 3B). These data indicate that the key regulatory elements required for Amhr2 promoter activity in MA-10 Leydig cells are located within −122 to −34 bp, the region that also contains the COUP-TFII response element.

We next determined whether COUP-TFII could directly bind to the −67/−34 bp region of the Amhr2 promoter. Using DNA pulldown assays and nuclear extracts from MA-10 Leydig cells, we found that COUP-TFII binds in vitro to oligonucleotides corresponding to the −67/−34 bp region (Fig. 4A, lane 4), as well as to a high-affinity direct repeat 1 (DR1) binding sequence used as positive control (Fig. 4A, lane 2). No significant binding was observed when only the magnetic beads were used as a negative control (Fig. 4A, lane 3). We subsequently performed ChIP-qPCR to determine whether COUP-TFII is recruited to the −67/−34 bp region of the Amhr2 promoter in a native chromatin environment in MA-10 Leydig cells. As shown in Fig. 4B, COUP-TFII was found to be associated with the proximal Amhr2 promoter.

Figure 2. COUP-TFII does not require the SF1 elements to activate the Amhr2 promoter. MA-10 Leydig cells were cotransfected with 100 ng of an empty expression vector (open bars) or 50 ng of an expression vector for COUP-TFII (filled bars) along with 400 ng of Amhr2 reporter constructs −1486/−77 bp, −34/−77 bp, or −1486/−77 bp harboring mutations (depicted by a large ×) within the SF1 elements (identified by open ovals) as indicated on the left side of the graph. Results are shown as fold activation over control ± SEM. For each reporter: *P < 0.05, vs its control (empty expression vector, value set at 1, filled bar over open bar); **P < 0.05, for activation by COUP-TFII between the −1486 bp reporters and the −34 bp reporter.
Figure 3. The COUP-TFII–responsive element is located within the proximal *Amhr2* promoter region. (A) MA-10 Leydig cells were cotransfected with 100 ng of an empty expression vector (−, open bars) or an expression vector for COUP-TFII (+, filled bars) along with a series of progressive 5’ deletion constructs of the mouse *Amhr2* promoter (400 ng) as indicated (left panel). The two previously reported SF1 elements are depicted by a white oval. Results are shown as fold activation over control ± SEM. For each reporter: *P < 0.05, vs its control (empty expression vector, value set at 1, filled bar over open bar); *P < 0.05, for activation by COUP-TFII of each reporter compared with that of the −34 bp reporter. (B) The regulatory elements required for maximal *Amhr2* promoter activity in MA-10 Leydig cells are located within the proximal −151 bp. MA-10 Leydig cells were transfected with a series of progressive 5’ deletion constructs of the mouse *Amhr2* promoter as indicated (left panel). Results are shown as relative activity compared with the activity of the −1486 bp reporter, which was arbitrarily set at 100%. A different letter indicates a statistically significant difference (P < 0.05).
between −140 and +29 bp, a region that contains the −67/−34 bp COUP-TFII–responsive sequence. In contrast, COUP-TFII was not significantly recruited to a distal region of the Amhr2 gene (−3395 to −3274 bp) that is devoid of AGGTCA-like motifs and was used as negative control.

To better locate the COUP-TFII binding region, we performed an *in silico* analysis of the −67/−34 bp region of the Amhr2 promoter across different species. This analysis revealed the presence of a potential nuclear receptor element (NRE) 5′-AGGACA-3′ (Fig. 5A) at −48 bp that could potentially be recognized by COUP-TFII. To test the functional importance of this
Figure 5. The AGGACA sequence within the −67/−34 bp region is not required for COUP-TFII binding and activation of the Amhr2 promoter. (A) Representation of the −1486/+77 bp Amhr2 promoter showing the two previously characterized SF1 elements (open oval) and the COUP-TFII–responsive region (−67/−34 bp, filled rectangle). Sequence alignment of the −67/−34 bp region from various species (mouse, rat, human, orangutan, chimp) revealed a potential binding site (box) for nuclear receptors (NREs; AGGACA or AGGATG). An asterisk corresponds to a species-conserved nucleotide compared with the mouse sequence. (B) MA-10 Leydig cells were cotransfected with 50 ng of an empty expression vector (−, open bars) or an expression vector for COUP-TFII (+, filled bars) along with 400 ng of various Amhr2 reporter constructs: −67/+77 bp WT, −67/+77 bp harboring a mutation (AGGACA → AaagCA) in the potential NRE (M1), and −34/+77 bp. The NRE is represented by a black diamond (WT), and a large × represents the mutation. Results are shown as fold activation over control (±SEM). For each reporter, *P < 0.05, vs its control (empty expression vector, value set at 1, filled bar over open bar); +P < 0.05, for the activation by COUP-TFII of each reporter compared with that of the −34 bp reporter. (C) DNA pulldown assays were performed using oligonucleotides containing a high-affinity COUP-TFII direct repeat 1 sequence (HA-DR1) or oligonucleotides that contain the −67/−34 bp sequence of the Amhr2 promoter either WT or harboring a trinucleotide mutation in the potential NRE (AGGACA → AaagCA, M1) along with nuclear extracts from MA-10 Leydig cells. Western blots were used to detect COUP-TFII. Input corresponds to 25 μg of the nuclear extracts used in the DNA pulldown assay (n = 3).
predicted nuclear receptor motif in COUP-TFII responsiveness, we used site-directed mutagenesis to mutate the NRE sequence (AGGACA to AaagCA, M1) in the context of the −67/+77 bp Amhr2 promoter. Surprisingly, mutation of the NRE did not significantly impair the ability of COUP-TFII to activate the Amhr2 reporter (Fig. 5B), suggesting that the 5′-AGGACA-3′ motif is not responsible for the COUP-TFII responsiveness. In agreement with this observation, we found that COUP-TFII present in nuclear extracts could still bind to the mutated −67/−34 bp sequence in DNA pulldown assays (Fig. 5C, lane 4). To ultimately pinpoint the COUP-TFII response element within the −67/−34 bp region of the Amhr2 promoter, we generated two additional 5′ deletion constructs of the Amhr2 promoter: −51/+77 bp (retains the NRE) and −45/+77 bp (removes the NRE). As shown in Fig. 6, both −51 bp and −45 bp reporters were still activated by COUP-TFII, although the −34 bp was no longer responsive. This indicated that the COUP-TFII response element most likely resides between −45 and −34 bp. Sequence analysis of the −45 to −34 bp region identified a GC-rich sequence (5′-GCGGGGCGG-3′) at −43/−34 bp that is adjacent to the NRE and well conserved across species (Fig. 7A). To validate the requirement of this GC-rich sequence, different mutations were introduced within the −67/+77 bp construct: GCGGGGCGG to GCttGGCGG (M2), GCGGGGCGG to GCGGGGCtt (M3), and GCGGGGCGG to GCGtGGCtt (M4). Cotransfections in MA-10 Leydig cells showed that all three mutations reduced COUP-TFII-mediated activation to background levels, as observed with the minimal −34 bp reporter (Fig. 7B). An intact GC-rich sequence at −39 bp is therefore essential for COUP-TFII–dependent activation of the Amhr2 promoter.

D. COUP-TFII Binds to the GC-Rich Sequence

To confirm whether COUP-TFII directly binds to the GC-rich sequence in vitro, we performed DNA pulldown assays using either nuclear extracts from MA-10 Leydig cells that endogenously express COUP-TFII (Fig. 8A) or recombinant COUP-TFII in vitro transcribed and translated (Fig. 8B). As expected, endogenously expressed COUP-TFII was found to bind to oligonucleotides containing −67/−34 bp wild-type (WT) and −67/−34 bp M1 (mutated NRE) (Fig. 8A, lanes 5 and 7, respectively). COUP-TFII from MA-10 nuclear extracts could still bind to oligonucleotides −67/−34 bp M4 containing a mutated GC-rich

Figure 6. The COUP-TFII response element is located within −45 bp to −34 bp of the Amhr2 promoter. MA-10 Leydig cells were cotransfected with 50 ng of an empty expression vector (−, open bars) or an expression vector for COUP-TFII (+, filled bars) along with 400 ng of different 5′ progressive Amhr2 deletion constructs as indicated. Results are shown as fold activation over control ± SEM. For each reporter: #P < 0.05, vs its control (empty expression vector, value set at 1, filled bar over open bar); *P < 0.05, for difference in the activation by COUP-TFII of each reporter compared with that of the −34 bp reporter.
sequence (above background observed with the beads), albeit to a lesser extent than the WT or M1 (mutated NRE) sequences (compare lanes 5, 6, and 7 in Fig. 8A). The residual binding observed with M4 could be due to low-affinity binding of COUP-TFII to the NRE. We also found that COUP-TFII from MA-10 Leydig cell nuclear extracts can bind to oligonucleotides containing the SF1 distal (5′-AGGTCA-3′) and SF1 proximal (5′-AGGTCC-3′) sequences (Fig. 8A, lanes 8 and 9, respectively) of the Amhr2 promoter, with COUP-TFII having a higher affinity for the distal site. A high-affinity DR1 (HA-DR1) WT binding site was used as positive control (Fig. 8A, lane 2), whereas magnetic beads (Fig. 8A, lane 4) and a mutated HA-DR1 sequence (Fig. 8A, lane 3) were used as negative controls. Because COUP-TFII can bind to DNA in association with other transcription factors present in nuclear extracts, we used in vitro transcribed/translated COUP-TFII in DNA pulldown assays to study the binding properties of the COUP-TFII protein by itself. As expected, in vitro–produced COUP-TFII could bind to the HA-DR1 sequence (Fig. 8B, top panel, lane 2). COUP-TFII binding was also observed on the SF1 distal sequence (Fig. 8B, top panel, lane 7). To detect

**Figure 7.** An intact GC-rich sequence is required for the COUP-TFII–mediated activation of the −67 bp Amhr2 promoter. (A) *In silico* sequence analysis of the −52/−34 bp Amhr2 revealed the presence of a highly conserved GC-rich sequence located from −43 to −34 bp (gray box) adjacent to the potential NRE (bolded nucleotides). An asterisk corresponds to a species-conserved nucleotide compared with the mouse sequence. (B) MA-10 Leydig cells were cotransfected with an empty expression vector (−, open bars) or an expression vector for COUP-TFII (+, filled bars) along with various Amhr2 reporter constructs: −67/+77 bp WT, −67/+77 bp harboring different mutations within the GC-rich sequence (M2, M3, M4), and −34/+77 bp. The potential NRE (AGGACA) is represented by a filled diamond, and the GC-rich sequence is depicted by the gray rectangle. The mutated nucleotides are in lowercase and underlined. Results are shown as fold activation over control ± SEM. For each reporter: *P < 0.05, vs its control (empty expression vector, value set at 1, filled bar over open bar); †P < 0.05, for the activation by COUP-TFII between the −67 bp reporters and the −34 bp reporter.

![Figure 7](image-url)
any low-affinity binding of COUP-TFII, the same blot was exposed for a longer period (Fig. 8B, lower panel). This longer exposure revealed that COUP-TFII can bind weakly to the SF1 proximal sequence (Fig. 8B, lower panel, lane 8). Very weak binding of in vitro produced COUP-TFII to the WT −67/−34 bp sequence was also detected (Fig. 8B, lower panel, lane 5). Mutation of the GC-rich sequence (M4) within the −67/−34 bp sequence abolished the binding, as it was indistinguishable from the magnetic beads used as negative control (Fig. 8B, lower panel, lanes 4 and 6). The weak binding of in vitro–generated COUP-TFII by itself to the −67/−34 bp sequence of the Amhr2 promoter indicates that it likely requires the presence of another transcription factor present in MA-10 Leydig cell extracts to properly bind to this sequence.

E. COUP-TFII Cooperates With SP1 on the Amhr2 Promoter

GC-rich sequences are known to be bound by members of the SP family of transcription factors, such as SP1 and SP3 (reviewed in [57]). SP1 and SP3 are ubiquitously expressed, including in various Leydig cell lines such as MA-10 Leydig cells [58, 59]. Furthermore, SP1 and COUP-TF factors have been reported to cooperate to regulate gene expression (reviewed in [60]). We therefore examined the possibility that COUP-TFII and SP1 might cooperate on the Amhr2 promoter. As shown in Fig. 9, COUP-TFII can activate the Amhr2 promoter by ∼10-fold whereas SP1 is a weaker activator (∼1.6-fold). Combination of both transcription factors led to synergistic activation of 20-fold (Fig. 9). This COUP-TFII/SP1 cooperation was
lost when the GC-rich sequence was mutated (M4 in Fig. 9) and no cooperation was observed with the minimal 234/177 bp reporter (Fig. 9).

3. Discussion

AMH is abundantly produced by Sertoli cells during fetal life to ensure regression of the Müllerian ducts in developing male embryos. AMH is also present in the adult testis, albeit at lower level, where it is known to regulate Leydig cell steroidogenesis [4, 6–9] as well as postnatal Leydig cell differentiation [4, 8–11, 61]. The effects of AMH on Leydig cells are mediated via the AMH receptors AMHR2 [4, 15] and ALK3 [19]. Leydig cell lines, including rat R2C and mouse MA-10, are known to express Amhr2, and treatment of these cells or mice with AMH represses steroidogenesis [6–9].

A. Identification of COUP-TFII as a New Regulator of Amhr2 Expression in Leydig Cells

Despite the importance of AMH/AMHR2 in regulating Leydig cell differentiation and function, very little is known about the regulation of Amhr2 gene expression in these cells. Studies on the human and rat AMHR2 promoter have revealed that the nuclear receptor SF1 (NR5A1/Ad4BP) is important for AMHR2 promoter activity [17, 22].

The nuclear receptor COUP-TFII (NR2F2) is also expressed in Leydig cells from the adult population and is known to bind to DNA sequence similar to SF1 elements [54–56, 62]. As SF1, COUP-TFII regulates the expression of the Star [30] and Insl3 [63] genes in Leydig cells. Our analysis of COUP-TFII–depleted MA-10 Leydig cells revealed that Amhr2 mRNA levels were significantly reduced in the absence of COUP-TFII, indicating that COUP-TFII is also required for expression of the endogenous Amhr2 gene in these cells. Consistent with a direct action on Amhr2 transcription, we found that COUP-TFII could activate a −1486 bp mouse Amhr2 promoter construct in MA-10 Leydig cells. This promoter fragment contains the two

Figure 9. COUP-TFII and SP1 cooperate to activate the Amhr2 promoter. MA-10 Leydig cells were cotransfected with an empty expression vector (white bars) or expression vectors for COUP-TFII (black bars) and SP1 (hatched bars) alone or in combination (gray bars), along with various Amhr2 reporter constructs: −67/+77 bp WT, −67/+77 bp harboring a mutation within the GC-rich sequence (M4), and −34/+77 bp. The potential NRE (AGGACA) is represented by a black diamond, and the GC-rich sequence is depicted by the gray rectangle. Mutation of the GC-rich sequence is depicted by a large ×. Results are shown as fold activation over control ± SEM. For each reporter: #P < 0.05, vs its control (empty expression vector, value set at 1). For the −67/+77 bp WT reporter: *P < 0.05, for the activation between COUP-TFII alone and the combination of COUP-TFII and SP1.

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previously characterized SF1 elements at -250 bp (5'-AGGTCA-3') and at -200 bp (5'-AGGTCC-3') to which COUP-TFII can bind in vitro. However, mutation/deletion of either or both SF1 elements had no effect on the transactivation of the Amhr2 promoter by COUP-TFII, indicating that they are not required for COUP-TFII–mediated activation. Although another region of the Amhr2 promoter is required for COUP-TFII responsiveness, it remains possible that COUP-TFII might still contribute to Amhr2 transcription via the SF1 elements in a different context.

Results from functional studies in R2C Leydig cells revealed that a sequence located between -99 and -91 bp of the Amhr2 promoter and containing an AGG core nuclear receptor binding motif (5'-AGAGGTGGGT-3') is required for maximal activation by SF1 [17]. We hypothesized that the same region might be involved in COUP-TFII–dependent transactivation of the Amhr2 promoter. However, we found that deletion of that region did not affect Amhr2 promoter activation by COUP-TFII in MA-10 Leydig cells. Instead, we found that a region between -67 and -34 bp, to which COUP-TFII can bind, is essential for COUP-TFII–dependent activation of mouse Amhr2 promoter in MA-10 Leydig cells. This is also consistent with the fact that the proximal Amhr2 promoter (-151 to -34 bp) contains all of the regulatory elements for maximal activity in MA-10 Leydig cells.

B. Mechanisms of COUP-TFII Action on the Amhr2 Promoter

Sequence analysis of the -67 to -34 bp region revealed the presence of a potential NRE that contains an AGG core motif for the binding of nuclear receptors (5'-AGGACA-3'). We initially hypothesized that COUP-TFII might regulate Amhr2 promoter activity via this NRE. Surprisingly, deletion or mutation (5'-AGGACA-3' to AaagCA-3') of the NRE did not reduce COUP-TFII–dependent activation. Similarly, mutation of the NRE did not drastically affect the binding of COUP-TFII present in nuclear extracts from MA-10 Leydig cells. These data indicate that COUP-TFII either directly binds to another sequence within the -67 to -34 bp region or is recruited to the proximal promoter via interaction with another DNA-bound transcription factor. The reduced binding of in vitro–produced recombinant COUP-TFII compared with COUP-TFII present in nuclear extracts from MA-10 Leydig cells supports the hypothesis of an interaction with, or stabilization by, another transcription factor. This is also consistent with the fact that COUP-TFII is known to regulate gene expression as homodimer or heterodimer with other transcription factors (reviewed in [29, 64, 65]).

Fine mapping of the Amhr2 promoter revealed that the COUP-TFII–responsive sequence is located between -45 and -34 bp. This sequence contains a species-conserved GC-rich sequence, and mutations within the GC-rich sequence abolished COUP-TFII–dependent activation of the Amhr2 promoter and reduced binding of COUP-TFII present in MA-10 Leydig cell extracts as well as in vitro–produced recombinant COUP-TFII, which indicates that COUP-TFII can bind directly to the GC-rich sequence, albeit with low affinity. Interestingly, footprinting analyses of the Amhr2 promoter incubated with nuclear proteins from R2C Leydig cells revealed that the GC-rich sequence is protected from DNase I digestion, which indicates the binding of a transcription factor thought to be SP1 [17]. SP1 belongs to a family of ubiquitous transcription factors that can drive gene expression by binding to GC-rich sequences located in promoter region of target genes (reviewed in [57]). SP1 is expressed in Leydig cells where it regulates expression of several genes, including the genes encoding the LH receptor [66], the nuclear receptor SF1 [67], and the PDGF-Ra [58].

Although the role of SP1 in Amhr2 expression in Leydig cells remained unexplored, it was proposed that it might participate in the initiation of transcription of this TATA-less promoter and/or interact with other transcription factors to contribute to the expression of the gene [17]. In agreement with this hypothesis, we found that COUP-TFII and SP1 functionally cooperate to further activate the Amhr2 promoter and that this activation required an intact GC-rich sequence. These data support a model (Fig. 10) whereby COUP-TFII by itself can bind with low affinity to the GC-rich sequence within the proximal Amhr2 promoter (Fig. 10A)
and is stabilized by an interaction with SP1 and/or another transcription factor (Fig. 10B), ultimately leading to increased Amhr2 gene transcription. In addition to the GC-rich sequence, we cannot exclude the possibility that the NRE is also involved in COUP-TFII binding to the Amhr2 promoter once SP1 has stabilized the complex. However, in our experiments, we consistently observed a significant decrease in COUP-TFII–dependent activation when the GC-rich sequence is mutated, indicating that the NRE sequence cannot compensate for the absence of an intact adjacent GC-rich sequence. A similar mechanism of COUP-TFII recruitment via DNA-bound SP1 was originally proposed by Pipaón et al. [68] to describe COUP-TFII action on the nerve growth factor–induced protein A (Ngfi-A/Egr-1) promoter. In their model, they proposed that SP1 serves as a docking protein for COUP-TF, as they found that COUP-TF cannot bind its responsive element alone. Since then, this mechanism of SP1/COUP-TF cooperation has been reported for the expression of several other genes in various tissues, including the human immunodeficiency virus type 1 long terminal repeat (HIV-1 LTR) [69], neuropilin 2 (Nrp2) [70], orthodenticle homebox 2 (Otx2) [71], EYA transcriptional coactivator and phosphatase 1 (Eya1), Wilms’ tumor 1 (Wt1) [72], E2F transcription factor 1 (E2f1) [73], angioptietin 1 (Ang1) [74], Glut4 [75], and IGF-1 (Igf1) [76]. These gene promoters, although activated by COUP-TFII, do not contain a classical COUP-TFII binding site. COUP-TFII responsiveness was rather found to require an intact SP1 element in the proximal promoter to which COUP-TFII is recruited. However, recruitment of COUP-TFII to those GC-rich sequences had so far only been demonstrated by ChIP, which does not discriminate between direct binding to DNA and indirect recruitment via interaction with another DNA-bound transcription factor. Our current work shows that COUP-TFII can directly bind to a GC-rich sequence. In addition to a functional cooperation, COUP-TFII and SP1 were found to physically interact [68], consistent with an SP1-mediated recruitment and/or stabilization of COUP-TFII to the promoter of target genes.

In conclusion, our present work establishes a role for the nuclear receptor COUP-TFII in the expression of the Amhr2 gene in Leydig cells. We found that COUP-TFII action does not require direct binding to DNA but rather involves a cooperation with the SP1 transcription factor bound to a GC-rich sequence within the proximal Amhr2 promoter.

**Figure 10.** Proposed model for the COUP-TFII–dependent activation of the Amhr2 promoter in MA-10 Leydig cells. (A) COUP-TFII is recruited to the proximal promoter region where it binds mainly to the GC-rich sequence and only with low affinity to the NRE. (B) Binding of SP1 to the GC-rich sequence and interaction with COUP-TFII stabilizes COUP-TFII, resulting in higher transcription. The DNA sequence for the NRE is shown in bold, and the GC-rich sequence is boxed.
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