Steroidogenic Factor-1 Regulates Transcription of the Human Anti-müllerian Hormone Receptor*

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Anti-müllerian hormone type II receptor (AMHRII) is a serine/threonine receptor and a member of type II receptors of the transforming growth factor β superfamily. AMHRII has been recently identified in humans, mice, rats, and rabbits. In the male embryo, the AMHRII gene has been shown to be expressed in Sertoli’s cells, in Leydig’s cells and in the mesenchymal cells surrounding the müllerian duct. To determine the functional region of the AMHRII promoter as well as the factors controlling AMHRII gene expression, we used a 1.1-kilobase DNA fragment from the 5′-flanking region of the human AMHRII gene to generate a series of deletion or mutation and analyzed the resulting transcriptional activities after transfection of the NT2/D1 teratocarcinoma cell line. Our results indicate that maximal expression of the AMHRII promoter in this particular cell line, a cell line positive for endogenous AMHRII expression, requires a conserved estrogen receptor half-site element (AGGTCA) identical to the binding element for steroidogenic factor-1 (SF-1). Studies of this SF-1 binding element using gel mobility shift, antibody supershift assays, and transient transfections of reporter constructs indicate that SF-1 can bind and transactivate the AMHRII promoter. Finally, SF-1 protein expression in human male embryos was shown to display a good coincidence with the previously reported AMHRII expression profile. We then propose that SF-1 may be a key transcriptional regulator of AMHRII gene expression during early human development.

Anti-müllerian hormone (AMH), also known as müllerian inhibiting substance, is a glycoprotein dimer that belongs to the growing family of the transforming growth factors β (1). AMH, the earliest product secreted by Sertoli’s cells, plays a critical role during early male fetal sex differentiation by inducing the regression of the müllerian ducts, the anlagen of the uterus, the Fallopian tubes, and the upper vagina. In the last 4 years, major progress has been realized in our understanding of AMH action by the isolation of its receptor (2–4). Members of the transforming growth factor β family signal through a heteromeric signaling complex composed of two related type I and II receptors, both transmembrane serine/threonine kinases (5). Type II receptors appear able to bind their ligands independently of type I components, but activation of the corresponding signal transduction pathway will require the presence of the two types of receptor (6). As a transforming growth factor β family member, AMH does not escape this rule and the receptor cloned in humans, mice, rats, and rabbits is an AMH type II receptor (AMHRII). Formal proof to equate this receptor with the AMH receptor came from the production of AMHRII mouse mutants using targeted mutagenesis (7). The mutant mice harbor the same resulting phenotype observed in AMH mutant as internal pseudohermaphrodites with uterine and oviductal tissues, a condition known in humans as persistent müllerian duct syndrome (8, 9). Simultaneously, analysis of developmental expression of AMHRII was reported by many groups in different models but always at the transcript level (2–4). All these data agree with an expression for AMHRII at the right time and right place in male embryos, i.e. in the window of sensitivity of the müllerian duct to AMH within the mesenchyme cells surrounding the male müllerian epithelium where AMH elicits its action (10). In these studies, other cellular sites of AMHRII expression are also depicted in Sertoli’s and granulosa cells of both fetal and adult gonads. More recently, expression of AMHRII on Leydig’s cells was also reported (11). A striking coincidence between AMH and AMHRII receptor expressions has led to diverse speculations on a putative autocrine feedback control of AMH (3, 12). These results also open the possibility that a similar combinatorial transcription factor complex is used to control both genes.

Previous studies reported that AMH expression is under the control of a 180-bp DNA fragment including a single estrogen receptor half-site (AGGTCA) recognized by a protein designated steroidogenic factor 1 (SF-1) (13). The functional importance of the AMH proximal promoter was supported by several observations including the coincident expression profile between SF-1 and AMH (13, 14). Beyond this particular function during the sex determining process in Sertoli’s cells, SF-1 was also shown to be important in the development of the hypothalamic-pituitary-gonadal axis or in the development of different endocrine tissues (15, 16).

Because of the parallelism in the expression profile of both anti-müllerian hormone and AMHRII receptors, we now address the possibility that regulation of the two genes could use common factors. Thus, in the present study we first determined a minimal promoter activity within a 1.1-kb DNA fragment from the 5′-flanking of the human AMHRII gene. Then we identified the presence of a conserved SF-1 binding site in this sequence and demonstrated its functional requirement in the regulation of the AMHRII gene. Finally, using immunolocalization studies the developmental pattern of expression of the
SF-1 protein was shown to be coincident with AMHRII expression described in previous reports.

MATERIALS AND METHODS

Isolation of Human AMHRII Promoter and Plasmid Constructions—Human AMHRII DNA 5'-flanking sequences were generated by PCR using genomic DNA as a template and were next extended with a human PromoterFinder™ DNA Walking kit (CLONTECH). The sequences and positions of the two specific primers were as follows. The forward primer, 5'-CCACATAGCAAAAATTACCAATTTGC-3', is complementary to the human AMHRII genomic sequence deposited in GenBank™ under accession number AF074397, and corresponds to nucleotides -1244 to -722, whereas the reverse primer, 5'-CTTTGCGGACGGGCAGGAGGACAGTT-3', is complementary to that of nucleotides +61 to +110 (numbering from the transcription start site previously defined (21)). The amplification product was cloned into pUC18 (SureClone ligation kit, Amersham Pharmacia Biotech). DNA sequence was determined to verify identity with AMHRII sequence. The human PromoterFinder™ DNA Walking kit was used with the reverse GSP1 reverse primer 5'-GCCCTTCCCTGACCTTGGAAGAAGA-3', corresponding to nucleotides -253 to -277 in the first reaction and with the GSP2 reverse primer GAAAACAGTAACAATGGTAAATTT corresponding to nucleotides -511 to -534 (Fig. 1). The 1.1-kb AMHRII 5'-flanking region was finally reconstructed into the pUC18 plasmid with the help of an endogenous BglII restriction site located at position -375 bp and was fully sequenced.

The reporter gene construct was generated using the pGL family of luciferase reporter vectors (Stratagene). The 1.058-bp AMHRII promoter region was used as a template to amplify AMHRII promoter DNA from positions -1058 to +78 by PCR. The forward and reverse primers contain, respectively, the recognition sites for KpnI and XhoI. After KpnI-XhoI digestion, one copy of the 1136-bp PCR product was inserted in the KpnI-XhoI sites of pGL3-promoter vector. This construct will be referred to as -1058/178pGL3p. The same strategy was used to produce -546/178pGL3p and -257/178pGL3p deletion mutants. -1054/178 was created by BglII hydrolysis of the -1058/178pGL3p.
construct. Site-directed mutagenesis of the SF-1 binding site to produce 21058SF-1MUT-pGL3p was performed using the QuickChange™ site-directed mutagenesis kit (Stratagene) with the help of the RAMH-MUT oligonucleotide (see below). Full-length SF-1 cDNA was cloned as a BamHI and EcoRI fragment into the pcDNA3 vector, which directs transcription from the cytomegalovirus promoter as described previously.2

Preparation of Nuclear Extracts—Nuclear extracts from NT2/D1, HeLa, and transfected HeLa cells were prepared from freshly harvested cultured cells according to the method of Dignam et al. (17). For SF-1 transfected HeLa cells, 60–80% confluence cells were transfected with 2 μg of SF-1 expression plasmid and 7 μl of LipofectAMINE (Life Technologies) for 4 h. The nuclear extracts were then prepared after 48 h of culture.

Production and Purification of Bacterially Expressed pGEX-SF-1 Fusion Protein—The pGEX-4T3-SF-1 expression vector was used to produce the recombinant protein in the bacterial strain BL21 (DE3) after induction by 0.5 mM isopropyl-β-D-thiogalactopyranoside. After 2 h of induction at 30 °C, cells were collected by centrifugation, resuspended in lysis buffer (150 mM NaCl, 1 mM dithiothreitol, 5 mM EDTA, 25% sucrose, and 50 mM Tris, pH 7.5) supplemented with bovine deoxyribonuclease I (Amersham Pharmacia Biotech) and 0.5 mM Pefabloc-SC-AEBSF (Interchim) and sonicated for 5 min at 4 °C. Bacterial debris were removed by centrifugation at 25,000 rpm for 20 min. The lysate was loaded on glutathione-Sepharose beads and washed three times with buffer I (5 mM EDTA, 120 mM NaCl, Tris 50 mM, pH 7.6) and three times with buffer II (5 mM EDTA, 120 mM NaCl, Tris 50 mM, pH 7.6). Purified proteins were checked by SDS-polyacrylamide gel electrophoresis analysis and then used directly for in vitro binding assay studies after elution from the matrix with buffer II plus 10 mM reduced glutathione after 30 min of incubation at 4 °C.

DNA Binding Assays—Protein binding to AMHRII promoter DNA probes was assessed by the electrophoretic mobility shift assay (EMSA). For these experiments, duplex oligonucleotides were labeled by end-filling of protruding ends in the presence of [α-32P]dCTP and DNA polymerase I-Klenow fragment for 1 h at 37 °C. The labeled probe was purified on a 5% nondenaturant acrylamide gel and eluted overnight in 0.8 M NH4Ac, 5 mM EDTA, 0.1% SDS buffer at 50 °C. Binding reactions were performed under standard conditions (4°C, 10 min) in 10 mM Tris · HCl, 50 mM NaCl, 0.1% Nonidet P-40, 0.01% sodium dodecyl sulfate, 50% glycerol, and 50 μM of each oligonucleotide. DNA-protein complexes were resolved on a 4% nondenaturing acrylamide gel and visualized by phosphorimaging.

Luciferase activity (% of wild type)

|   | Luciferase activity |
|---|--------------------|
| 1 | 100%               |
| 2 | 85%                |
| 3 | 6%                 |
| 4 | 5%                 |
| 5 | 12%                |

Fig. 2. The SF-1 binding site is involved in the activity of reporter promoter in the NT2/D1 cell line. A, 250 ng of the −1054/+78-pGL3p reporter constructs containing the 1136-bp promoter were transfected in three different cell lines (COS-7, HeLa, and NT2/D1). Luciferase assays were performed a minimum of three times as described under “Materials and Methods.” Mean Luciferase (± S.D.) of each construct in all three cell lines is given relative to the promoter-less vector. White bars, empty pGL3p; black bars, −1058/+78-pGL3p; error bars, the standard errors. B, the different deletion mutation promoter constructs (−1054/+78-pGL3p, −546/+78-pGL3p, −257/+78-pGL3p, −1054−374-pGL3p, and −1054SF-1MUT-pGL3p) were transfected in the NT2/D1 cell line to identify the DNA sequence involved in the promoter activity. Relative luciferase activities were normalized to the −1054−78-pGL3p construct. Luciferase assays were performed after at least two independent transfections, each done in triplicate.

2 de Santa Barbara, P., Bonneau, N., Boizet, B., Desclouseaux, M., Moniot, B., Sudbeck, P., Scherer, G., Poulat, F., and Berta, P. (1998) Mol. Cell. Biol., in press.
were carried out at room temperature for 15 min in 20 μl comprising 5–10 μg of either nuclear extracts or recombinant proteins mixed to 32P-labeled probe (10,000 cpm), 2 μg of poly(dI-dC)poly(dI-dC) (Amerham Pharmacia Biotech), 20 mm Hapes, pH 7.9, 20% glycerol, 0.1 M nucleotide (molar excess of unlabeled SF-1-BS oligonucleotide). Incubated with the labeled RAMH-BS oligonucleotide, 20 m M Hepes, pH 7.9, 20% glycerol, 0.1 M nucleotide (molar excess of unlabeled double-stranded competitor oligonucleotides RAMH-BS comprising the SF-1 binding site present in the SF-1 promoter (SF-1-BS) or the AMHRII promoter (RAMH-BS oligonucleotide) was α-32P-labeled and incubated with 2 μg of NT2/D1 nuclear extract in the absence (lane 2) or in the presence of 50 molar excess of unlabeled SF-1-BS oligonucleotide (lane 5) both used as competitors. Formation of the specific complex was abolished in the presence of 1 μl of rabbit purified polyclonal SF-1 antibody (lanes 3 and 6) or a mutated version of this site (RAMH-MUT) (lanes 4 and 7). D, 2 μg of nuclear extract from the SF-1 transfected HeLa cells were pre-incubated with 2 μg of unlabeled SF-1-BS oligonucleotide or unrelated antibody (lane 4) and then incubated with the labeled RAMH-BS oligonucleotide.

In competition assays, an unlabeled competitor oligonucleotide or an antibody solution was incubated for 15 min at room temperature before adding the probe. The DNA-protein complexes were resolved on a 5% polyacrylamide gel in Tris borate/EDTA buffer at 4 °C, fixed by 10% methanol/10% acetic acid, dried, and then autoradiographed.

The DNA probes used in these assays were complementary double-stranded DNA oligonucleotides including the SF-1 binding site from the AMHRII promoter (RAMH-BS) or the SF-1 binding site from the AMHRII promoter (SF-1-BS). The nucleotide sequences of the top strands of the double-stranded DNA oligonucleotides including the SF-1 binding site from the AMHRII promoter (RAMH-BS) or the SF-1 binding site from the AMH promoter (SF-1-BS). The nucleotide sequences of the top strands of the double-stranded DNA oligonucleotides are as follows: RAMH-BS, 5′-GCATCTTCTTCCAGAGTCAGGGAAG-3′; RAMH-MUT, 5′-GCATCTTCTTCCATTTTCAGGGAAG-3′; and SF-1-BS, 5′-GGCAGCCTTCCCCAAAGGTCGC-3′. The mutated nucleotides are in bold.

**Cell Culture and Transfection Assays**—The human NT2/D1 cell line (N-Tera 2, clone D1, a human pluripotent embryonic carcinoma cell line) was obtained from the American Type Culture Collection (number CRL 1973, ATCC, Biuvale, France). NT2/D1, HeLa, and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Imperial Laboratories, Flobio, France) containing 10% (v/v) fetal calf serum (Life Technologies Inc.), penicillin/streptomycin, and 2 mM glutamine with pCO2 of 5% at 37 °C in humidified air. Plasmids used for transfections were purified using the maxiprep reagent system (Qiagen). COS-7, HeLa, or NT2/D1 cells at 60–80% confluence were washed twice with serum-free medium and then cotransfected with 250 ng of reporter plasmid, 25 ng of Renilla luciferase control promoter vector (Promega) used as an internal control for transfection efficiency, and SF-1 expression plasmid with 3 μl of LipofectAMINE (Life Technologies Inc.) in 200 μl of serum-free medium. After 4 h of incubation, the medium was replaced with 500 μl of medium supplemented with 10% serum, and cells were harvested after 48 h of culture. Luciferase assays were checked with the Dual-Luciferase™ Reporter Assay (Promega). Promoter activities were expressed as relative luciferase activity units/Renilla unit, and each value represents the mean of six separate wells.

**Immunofluorescence Staining of Tissue Culture Cells**—Human embryonic gonadal tissues were obtained from therapeutic termination (week of gestation) as part of a program approved by both the ethics committee from CNRS and by the French National Ethics Committee. Embryos were staged according to the recognizes Carnegie stages (18). The youngest embryonic DNAs were extracted from limbs and demonstrated to be male by polymerase chain reaction as described (19). Sections were probed by an overnight incubation at room temperature with the appropriate antibody, anti-SF-1 (as previously described and demonstrated to be male by polymerase chain reaction as described (19).
fully characterized" or anti-AMH, respectively, with a 1:100 and a 1:400 dilution in phosphate-buffered saline-bovine serum albumin. After washing in phosphate-buffered saline, primary antibodies were visualized with Fluorolink Cy2-conjugated anti-rat labeled goat antibodies (dilution, 1:200) or with biotinylated anti-rabbit (dilution, 1:200) and Texas red-conjugated streptavidin-labeled sheep antibodies (dilution, 1:400). In each case incubations were performed for 90 min in the same conditions described for the primary antibodies. Sections were washed again and mounted in FluorSave reagent (Calbiochem). Images were collected and processed on a Bio-Rad confocal microscope or on a Zeiss Axiohot.

RESULTS AND DISCUSSION

The human AMH receptor type II gene has been previously cloned (2–4). As a first step to understand its transcriptional control, we synthesized the recently published 5'-flanking sequence (21) by polymerase chain reaction, and this sequence was subsequently extended by a walking procedure. As shown in Fig. 1, sequence analysis of the resulting 1.1 kb did not reveal any canonical TATA or CCAAT box. A computer search (20) for regulatory elements identified two Sp1 sites (at −42 and −634), one SOX site (at −525), and two GATA motifs lying very closely to the transcription initiation site recently defined (21). Through this analysis and previous analysis (21), a perfect consensus SF-1 response element (CAGAAGGTTA) conserved in the mouse was detected at positions −270 to −262. An identical DNA element located in the AMH minimal promoter has led recently to the implication of SF-1 in the regulation of AMH expression (13). To assess its transcription activity, this 1.1 kb DNA fragment was fused to the luciferase reporter gene and then transiently transfected in NT2/D1, HeLa or COS-7 cell lines. As shown in Fig. 2, the 1.1 kb DNA sequence increased luciferase expression in NT2/D1 cells about 4.5-fold. In contrast, this fragment did not enhance pGL3p expression in HeLa or COS cells. These results document that a promoter activity is indeed present in the 1.1 kb of upstream sequence but also the cell selectivity of this activity. The NT2/D1 cell line has been shown to express most (if not all) the genes described so far as involved in the sex determination cascade (Refs. 22 and 23 and data not shown). Moreover, an endogenous expression of the AMHRII gene in the NT2/D1 cells was detected by reverse transcription-PCR assay confirming that this cell line is a convenient model to explore AMHRII transcriptional regulation (data not shown). A series of reporter constructs was designed to sequentially delete putative transcription factor binding sites within the promoter. The results of this analysis are summarized in Fig. 2B. Deletion or site-directed mutagenesis of the SF-1 binding site resulted in a significant reduction in the AMHRII promoter activity relative to the 1.1-kb intact promoter. All the activities were estimated using transient transfection assays in the NT2/D1 cell line.

To assess whether the SF-1 motif delineated in the above study might effectively bind the corresponding protein, we examined the binding of SF-1 to this sequence derived from the human AMHRII promoter. Therefore, EMSAs were carried out using GST-SF-1 fusion protein and a synthetic oligonucleotide probe corresponding to the AMHRII promoter SF-1 motif (Fig. 3A). A band containing the SF-1/DNA complex was clearly observed, competed specifically by an unlabeled probe, and ablated by including anti-SF-1 polyclonal antibody. Similar results were obtained when EMSAs were carried out using nuclear extracts prepared from NT2/D1 cell line, a cell line expressing endogenous SF-1. The gel shift band was again competed by unlabeled probe with a sequence derived from either the AMHRII receptor or the AMH promoter and was abolished by including the SF-1 antibody (Fig. 3B). Finally, as a control, EMSAs were also performed using nuclear extracts prepared from HeLa cells or from HeLa cells after transfection with a SF-1 expression plasmid pCDNA3-SF-1 (expressing SF-1 under the control of the cytomegalovirus minimal promoter; see "Materials and Methods") (Fig. 3, C and D). Together, these results demonstrated that SF-1 transcription factor can bind in vitro to the AMHRII gene promoter.

We next examined the functional importance of this interaction in co-transfection assays. For this purpose, reporter plasmids containing the 1.1-kb AMHRII promoter region fused to the firefly luciferase (−1058/+78-pGL3p) were transfected into HeLa cells along with pCDNA3-SF-1. As shown in Fig. 4, if the 5'-flanking region of the AMHRII gene has failed again by itself to activate luciferase activity in HeLa cells, a cell line that lacks endogenous SF-1 protein (13), addition of increasing amounts of SF-1 expression plasmid has led to a 5-fold higher reporter activity. This activation was completely abolished after mutation of the SF-1 binding site (−1058SF-1MUT-pGL3p construct) to prevent SF-1 protein binding (Fig. 4). Even if the level of activation remains rather low with a large amount of exogenous SF-1, these results provide strong evidence on the ability of SF-1 to activate the AMHRII promoter and the requirement of an intact SF-1 binding site to maintain this transactivation capacity. They also suggest that SF-1 do not require a ligand or a cofactor to activate AMHRII expression. This contrasts with the fact that SF-1 by itself was unable to activate the AMH promoter in HeLa cells (13). However, contradictory data have been recently outlined on this requirement for SF-1 (24). In the case of AMHRII, our results do not exclude the involvement of other synergistic factors that could enhance but also direct AMHRII expression to specific sites, even if the AMHRII expression profile remains wider than the one described for AMH in urogenital ridges (25).

Further evidence that SF-1 controls AMHRII gene in vivo came from analysis of the expression pattern of SF-1 by immunohistochemistry to establish a correlation between SF-1 and AMHRII expression. Recently, expression of AMHRII has been extensively analyzed (2–4) and reviewed (26). In embryos, this expression appears localized in the urogenital ridges coincidently with Mullerian duct regression, in fetal Sertoli’s and granulosa cells. This expression was recently extended to the Ley-
dig’s cell compartment (11). Other sites of expression during the posnatal or adult life are also documented (26). The description of a specific anti-SF-1 polyclonal antibody provides us with an appropriate tool to describe SF-1 expression during human embryonic life at the level of the protein product. Using a 6.5–7-week-old human embryo we showed a nuclear expression for SF-1 in aligned Sertoli’s cells also characterized by their AMH expression (Fig. 5, panels 1 and 2). Similarly, a nuclear expression was also detected in cells outside the seminiferous tubes, presumably in the future Leydig’s cells (Fig. 5, panels 1 and 2). At this developmental stage, SF-1 expression was also detected in the mesenchymal cells surrounding the Müllerian duct, a location where the AMHRII is also known to be expressed (Fig. 5, panel 3). This is in agreement with previous reports analyzing AMHRII mRNA expression during Müllerian duct regression in the rat (2) or in the rabbit (3).

In conclusion, the present work suggests that proper AMHRII gene activation may require a perfectly consensual DNA binding element for the SF-1, a member of the orphan nuclear receptor family located in the AMH type II receptor proximal promoter. The present data with previous reports analyzing AMHRII gene expression (2–4, 26) provide the first evidence that SF-1 could have an essential role in the transcriptional control of the AMHRII promoter. Transgenic experiments using the AMHRII promoter described herein may now be a useful tool to confirm the role of the SF-1 binding site in this regulation, a strategy recently used for AMH (14). The nature of the conserved DNA binding sites present in their respective promoters suggest other potential similarities in their mode of control. In this respect both SOX9 (2) and GATA-4 described recently to contribute to AMH regulation (27) will provide some attractive candidates to contribute to AMHRII control. This intriguing possibility is now under investigation.

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Fig. 5. Immunolocalization of SF-1 protein in 6.5–7- and 8.5-week-old male urogenital ridges. Panels 1 and 2, immunolocalization of SF-1 in green and (panel 2) of AMH in red in a 6.5–7-week-old fetal human genital ridge (original magnification, ×100). Note that the seminiferous cords have developed and are separated by thick mesenchyme at this stage. Long arrow, Sertoli’s cells; short arrow, Leydig’s cells; ts, seminiferous tubules. Expression of SF-1 protein in the cells surrounding the fetal Müllerian duct (M) in a 6.5–7-week-old (3) and a 8.5-week-old (4) fetal human reproductive tract. Note the regression of the Müllerian duct in the later stage (panel 4 versus panel 3). Scale bars, 50 μm.
SF-1 and AMHRII in Testis Differentiation

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