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Follicle-Stimulating Hormone Increases Testicular Anti-Müllerian Hormone (AMH) Production through Sertoli Cell Proliferation and a Nonclassical Cyclic Adenosine 5′-Monophosphate-Mediated Activation of the AMH Gene

CÉLINE LUKAS-CROISIER*, CELINA LASALA, JULIETTE NICAUD, PATRICIA BEDECARRÁS, T. RAJENDRA KUMAR, MARTIN DUTERTRE, MARTIN M. MATZUK, JEAN-YVES PICARD, NATHALIE JOSSO, AND RODOLFO REY

Centro de Investigaciones Endocrinológicas (Consejo Nacional de Investigaciones Científicas y Técnicas; C.L., P.B., R.R.), Hospital de Niños "R. Gutiérrez," C1425EFD Buenos Aires, Argentina; Unité de Recherches sur l'Endocrinologie du Développement (Intitut National de la Santé et de la Recherche Médicale; C.L.-C., J.N., M.D., J.-Y.P., N.J., R.R.), Département de Biologie, Ecole Normale Supérieure, 92120 Montrouge, France; and Departments of Pathology and Molecular and Cellular Biology (T.R.K., M.M.M.) and Department of Molecular and Human Genetics (M.M.M.), Baylor College of Medicine, Houston, Texas 77030

Anti-Müllerian hormone (AMH) production by testicular Sertoli cells is high before puberty and can be further induced by FSH. Our objective was to delineate the mechanisms by which FSH stimulates AMH production. Assay of serum AMH levels and histological morphometric analysis in prepubertal FSH-deficient transgenic mice showed that serum AMH and testicular mass were decreased owing to reduced Sertoli cell number. All parameters resumed normal values in mice treated with recombinant FSH. We also analyzed the ability of FSH and the factors involved in its signaling pathway to activate AMH transcription by transfecting AMH promoter-luc reporter constructs of different lengths in a prepubertal Sertoli cell line. Our results showed that FSH activates AMH transcription via adenylate cyclase, cAMP, and protein kinase A but involving a nonclassical cAMP-response pathway requiring nuclear factor-κB and activating protein 2 binding sites, which lie more than 1.9 kb upstream of the AMH transcription start site. This is the first report showing the importance of distant sequences in the regulation of AMH expression. We conclude that prepubertal testicular AMH production is increased by FSH stimulation through Sertoli cell proliferation and an enhancement of AMH gene transcription. (*Molecular Endocrinology* 17: 550–561, 2003)

ANTI-MÜLLERIAN HORMONE (AMH), also called Müllerian-inhibiting substance (MIS), is a member of the TGF-β superfamily of growth and differentiation factors, involved in the hormonal control of sex differentiation in the male fetus (reviewed in Refs. 1 and 2). In the testis, AMH is synthesized by Sertoli cells, the somatic component of the seminiferous tubules, from the very onset of fetal testicular differentiation (3). Upon binding to a specific membrane receptor in Müllerian ducts, AMH induces the regression of the anlagen of the uterus and Fallopian tubes in the male fetus (reviewed in Refs. 1 and 2). A growing amount of evidence indicates that AMH also acts as a negative modulator of Leydig cell differentiation and testosterone secretion in the male (reviewed in Refs. 2 and 4).

The ontogeny of AMH expression in the postnatal male is characterized by the existence of high levels until puberty; then, AMH expression is significantly down-regulated (5, 6), which results in a decrease of serum AMH to very low levels (reviewed in Refs. 2, 7, and 8). The regulatory mechanisms involved in the activation of AMH expression in the fetal Sertoli cell have been extensively studied (reviewed in Ref. 2), but less is known concerning the regulation of testicular AMH production after birth. Using clinical and experimental approaches, we have shown that pubertal elevation of the androgen concentration within the testis is responsible for the inhibition of AMH production (reviewed in Ref. 7). When the negative effect of androgens is absent owing either to impaired testosterone synthesis (9, 10) or to the lack of androgen receptor expression in Sertoli cells, *e.g.* in the normal fetus and neonate (6) and in the androgen insensitivity syndrome (6, 11), testicular AMH output can be stimulated

Abbreviations: AMH, Anti-Müllerian hormone; AP2, activating protein 2; dbcAMP, dibutyryl cAMP; CRE, cAMP-responsive element; FSH-R, FSH receptor; IκB, inhibitor of κB; luc, luciferase; MIS, Müllerian-inhibiting substance; NFκB, nuclear factor-κB; PI3-K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKB, protein kinase B; SF-1, steroidogenic factor 1; SOX9, SRY-related HMG-box gene 9; SRY, sex-determining region Y.

by FSH (6). The cellular and molecular mechanisms underlying FSH stimulation of AMH production are not known.

Sertoli cells express the FSH receptor, a seven-transmembrane-domain receptor belonging to the superfamily of G protein-coupled receptors. The best characterized pathway of FSH action in Sertoli cells is mediated through a G_s heterotrimeric protein, formed by α-, β-, and γ-subunits. The activated receptor catalyzes the exchange of GTP for GDP and the dissociation of the G protein heterotrimer. The GTP-bound $G_s\alpha$ protein activates adenylate cyclase resulting in increased intracellular cAMP levels, responsible for the stimulation of protein kinase A (PKA). In turn, PKA phosphorylates a class of transcription factors binding to cAMP-responsive elements (CREs) in FSH-target genes (reviewed in Ref. 12). More recently, alternative pathways have been described for FSH signaling, involving phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (PKB; Refs. 13 and 14). In the prepubertal Sertoli cell, FSH has also been shown to increase DNA and protein synthesis resulting in cell proliferation and protein secretion (reviewed in Ref. 15). The FSH-dependent elevation of serum AMH we have previously reported (6) could be explained by an increased AMH expression in individual Sertoli cells or by an increase in the number of Sertoli cells, or both. The purpose of this work was to delineate the mechanisms by which FSH stimulates testicular AMH output. Furthermore, because no consensus CRE sequences have been found in the 5′-flanking sequences of the AMH gene studied to present, we investigated other potentially involved molecular pathways.

RESULTS

Serum AMH Is a Marker of Sertoli Cell Number in the Prepubertal Male

To evaluate the effect of FSH on testicular AMH output and its correlation with Sertoli cell number before puberty, we used prepubertal FSH-deficient ($FSH^{-/-}$) transgenic male mice bearing a targeted deletion of exons 1, 2, and most of exon 3 of the gene encoding the FSH β-subunit (16). Results in Fig. 1A show that serum AMH was moderately but significantly lower in prepubertal $FSH^{-/-}$ mice than in normal littermates ($FSH^{+/-}$). Prepubertal $FSH^{-/-}$ mice also had lower testicular mass, owing to a lesser number of Sertoli cells (Fig. 1B). Both serum AMH (Fig. 1A) and Sertoli cell number (Fig. 1B) attained normal values in $FSH^{-/-}$ males treated with recombinant FSH for 6 d. These results lead us to the conclusion that, in prepubertal male mice, serum AMH levels are regulated by FSH and may reflect the total number of functional Sertoli cells, whose proliferation is FSH stimulated. Both the lack of FSH and its exogenous administration had a greater effect on testicular volume than on Sertoli cell number (Fig. 1B), which can be explained by the fact that FSH also regulates germ cell number (17).

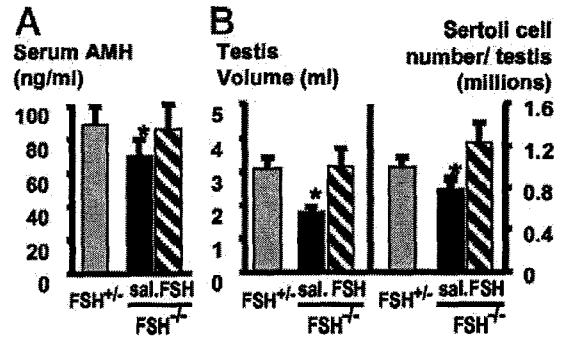

Fig. 1. Effect of FSH on Testicular AMH Production and Sertoli Cell Number

FSH-deficient ($FSH^{-/-}$) transgenic male mice and normal littermates ($FSH^{+/-}$) were killed at 7 d of age, serum AMH was assayed by ELISA (A), and morphometric studies of the testes were performed (B). FSH-deficient mice were divided into two groups: one of them received 0.5 U of recombinant FSH, and the other received the same volume of saline (sal.) every day from birth to d 6. Results were analyzed using a paired *t* test. *, $P < 0.05$ when compared with $FSH^{+/-}$.

AMH Promoter Activity Is Enhanced after Activation of the FSH Signaling Pathway in SMAT1 Cells

To test whether FSH is also capable of activating AMH gene transcription in prepubertal Sertoli cells, we examined its ability to enhance the transcription of human AMH promoter-luciferase reporter constructs [5′human AMH (hAMH)-luc] transfected into SMAT1 cells, a clonal, immortalized Sertoli cell line, derived from a prepubertal mouse testis and expressing the transcription factors needed for basal AMH production (18). The longest 5′hAMH-luc construct contained 3068 bp of the hAMH gene 5′-flanking region. Because SMAT1 cells do not express the FSH receptor (FSH-R), to test FSH action on 5′hAMH-luc activity, we cotransfected an FSH-R expression vector into SMAT1 cells and incubated them with or without 2 U/ml of recombinant human FSH. We observed a modest but consistent enhancement of the activity of −3068-5′hAMH-luc after 36 h of incubation with FSH (Fig. 2A). At 2 h, the enhancement was not uniformly observed, which explains the higher variability and lack of statistically significant difference when compared with the basal condition. An activation of the 5′AMH-3068-luc construct was also obtained when SMAT1 cells devoid of FSH-R expression were cotransfected with a $G_s\alpha$-protein expression vector (Fig. 2B), when adenylate cyclase was directly stimulated with cholera toxin, or when cells were incubated with dibutyryl cAMP (dbcAMP; Fig. 2C). The effect induced by the addition of dbcAMP was significant at 2-h incubation and was maximal at 24 h. Altogether, these results indicate that the FSH-R-dependent G protein

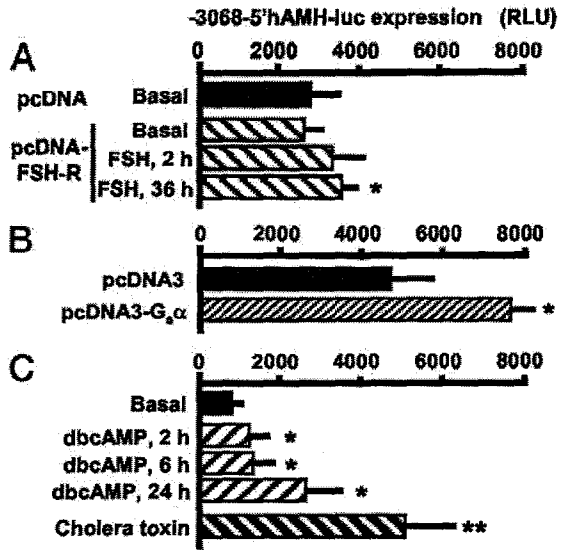

Fig. 2. AMH Promoter Activity Is Enhanced after Activation of the FSH Signaling Pathway at Various Levels

SMAT1 cells were transfected with 1 μg of the −3068-5′hAMH-luc construct and luc activity was assessed 48 h later. A, One microgram of an empty pcDNA or of a pcDNA-FSH receptor expression vector was cotransfected and cells were incubated, during the last 2 or 36 h, in medium with or without 2 U/ml of recombinant FSH. B, One microgram of an empty pcDNA or of a pcDNA-$G_s\alpha$ expression vector was cotransfected. C, Cells were incubated in basal medium or in medium containing 1 mM dbcAMP for 2, 6, or 24 h, or 1 μg/ml of cholera toxin for 24 h. In all cases, results (mean ± SEM of four experiments) were analyzed using a paired *t* test. *, $P < 0.05$ FSH *vs.* basal in pcDNA-FSH-R transfected cells, or pcDNA-$G_s\alpha$ *vs.* pcDNA, or dbcAMP or cholera toxin *vs.* basal.

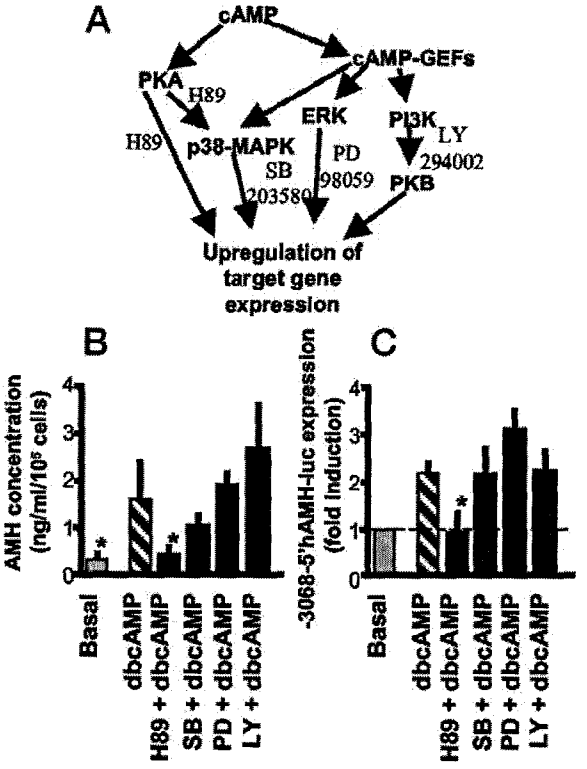

Fig. 3. cAMP-Dependent AMH Promoter Activation Is Blocked by an Inhibitor of PKA

A, FSH-mediated up-regulation of target genes via cAMP may involve PKA or more recently described signaling pathways involving cAMP-regulated guanine nucleotide exchange factors. Different inhibitors can be used to specifically block each of the various pathways (13, 14). B, SMAT1 cells were incubated for 24 h in basal medium or in medium containing 1 mM dbcAMP alone or with either 10 μM H89 (PKA inhibitor), 25 μM LY294002 (PI3-K inhibitor), 20 μM SB203580 (p38 kinase inhibitor), or 10 μM PD98059 (MAPK inhibitor), and murine AMH concentration was determined by ELISA in the culture medium. Cells were detached by mild trypsinization and counted. C, SMAT1 cells were transfected with −3068-5′hAMH-luc (1 μg), and luc activity was assessed 48 h later. In the last 24 h, cells were exposed to the same treatments as in B. Results are given as fold induction, considering as 1 the activity in the well with basal medium. In all cases, results (mean ± SEM of four experiments) were analyzed using Student's *t* test, in which *all columns* were compared with the *dbcAMP column*. *, $P < 0.05$.

adenylate cyclase pathway can be activated in SMAT1 cells and that it results in an increased transcriptional activity of a 3-kb hAMH promoter. For practical reasons, we performed most of the subsequent experiments using only dbcAMP for 24 h.

FSH Activates AMH Transcription through a Nonclassical PKA-Dependent Mechanism

Because the AMH promoter lacks a canonical CRE (2, 19), the classical pathway involving PKA-mediated phosphorylation of CRE-binding protein and subsequent binding to AMH 5′-flanking sequences seemed unlikely. Therefore, we searched for alternative molecular mechanisms that could explain the response to FSH. Signaling pathways recently described as intracellular targets of cAMP include PKB phosphorylation mediated by PI3-K, which can be blocked by LY294002, and activation of p38 MAPK and ERK cascades, which can be blocked by SB203580 and PD98059 respectively (Fig. 3A; Refs. 13 and 14). We incubated SMAT1 cells with dbcAMP in presence or absence of an inhibitor of PKA, H89, or of LY294002, SB203580, or PD98059, and measured AMH in the culture medium by ELISA (Fig. 3B). Only H89 was able to completely block cAMP-mediated AMH production. Similar results were observed in luc assays after transfection of −3068-5′hAMH-luc to SMAT1 cells (Fig. 3C). These results indicate that cAMP-mediated activation of the AMH promoter in the immature Sertoli cell line SMAT1 involves PKA and not PKB, ERK, or p38 MAPK.

Sequences Lying More Than 1.9 kb Upstream of the AMH Transcription Start Site Are Involved in the Response to cAMP

The proximal promoter of the murine AMH gene has been extensively studied, and response elements for

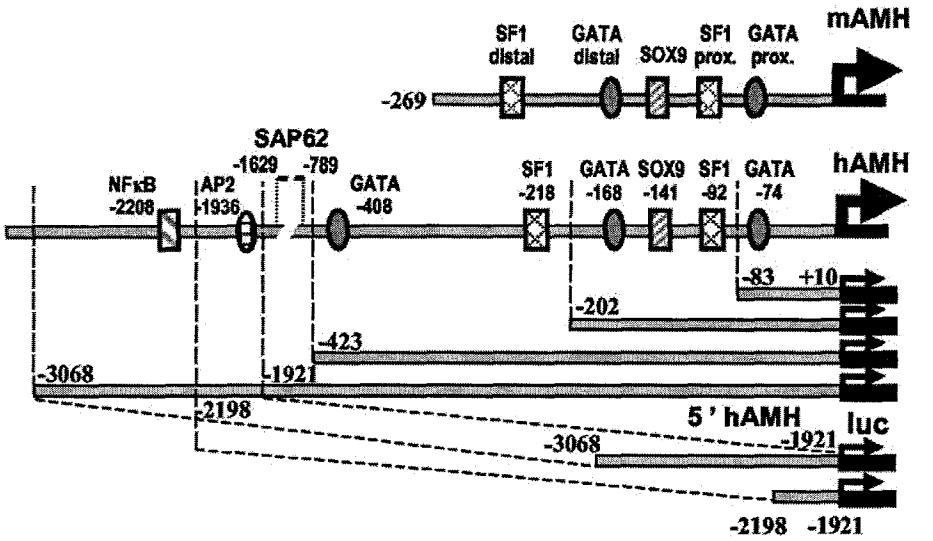

Fig. 4. Schematic Representation of the Mouse AMH (mAMH) and hAMH Gene 5′-Flanking Sequences and of 5′hAMH-luc Constructs Used in this Study

Localization of binding sites for SF-1, GATA-4, and SOX-9 in the mAMH promoter have been described (20, 22, 24, 25). Nucleotide positions of binding sites in the human promoter are according to the sequence published by Guerrier *et al.* (42). Localization of the human SAP62 gene is indicated according to Dresser *et al.* (44).

SOX-9 (20, 21), steroidogenic factor 1 (SF-1; Refs. 22–24), and GATA factors (24–26) have been shown to play important roles in the regulation of AMH expression. Sequence analysis of the 5′-flanking region of the hAMH gene revealed that GATA, SF-1, and SOX-9 binding sites are present within 220 bp of the transcriptional start site, in the same 5′-to-3′ order as in the mouse (Fig. 4). To map the sequences responsible for cAMP responsiveness in the hAMH 5′-flanking region, we generated several luc reporter constructs containing fragments of various lengths of the hAMH promoter (Fig. 4) that were used for transfections into SMAT1 cells. First, we observed that in basal conditions the 3068-bp promoter was the most active, with a clearly higher *trans*-activating capacity than the 423- and the 202-bp promoters (Fig. 5A). The 83-bp promoter had no *trans*-activating ability, as compared with the empty pGL2B luc plasmid. Second, cAMP enhanced the activity of the 3068 bp but not that of the shorter promoters. Furthermore, FSH increased the *trans*-activation of the 3068-bp promoter, but not that of the 423-bp promoter (Fig. 5B). These results strongly suggested that sequences of the AMH promoter involved in the response to FSH lie between positions −428 and −3068 relative to the transcription initiation site.

To test this hypothesis, we generated two short 5′AMH-luc constructs lacking the proximal promoter sequences: one spanning from −3068 to −1921 and a shorter one spanning from −2198 to −1921 (Fig. 4). As seen in Fig. 6, both the −3068–1921-5′hAMH-luc and the full-length −3068-5′hAMH-luc constructs, with intact nuclear factor (NF)-κB and activating protein 2 (AP2) sites, showed a similar response to cAMP. The response of the −2198–1921-5′hAMH-luc construct

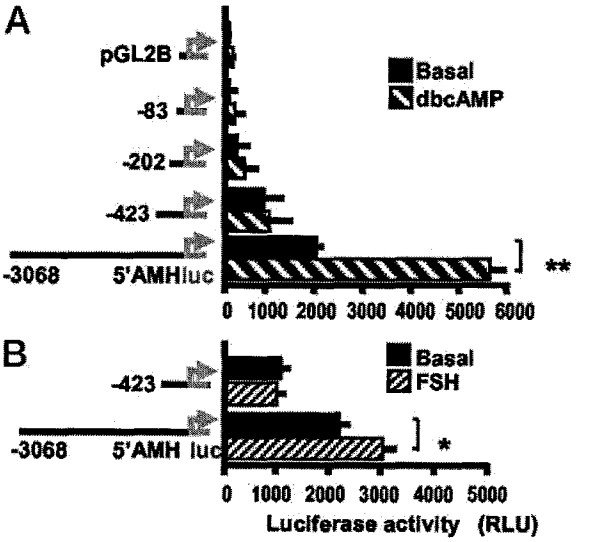

Fig. 5. FSH and cAMP Enhance the Activity of the 3068-bp AMH Promoter

SMAT1 cells were transfected with 1 μg of an empty pGL2B plasmid or of pGL2B containing different lengths of the hAMH 5′-flanking sequences, and luc activity was assessed 48 h later. Cells were incubated with or without 1 mM dbcAMP for 24 h (A) or 2 U/ml of recombinant FSH for 36 h (B). Results (mean ± SEM of seven experiments in A and four in B) were analyzed using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$, dbcAMP or FSH *vs.* basal.

was somewhat lower but still significant. Altogether, these data indicate that sequences lying upstream of position −1921 contribute to, and are sufficient for, cAMP enhancement of the AMH promoter activity. Furthermore, relevant sequences must exist between −2198 and −3068.

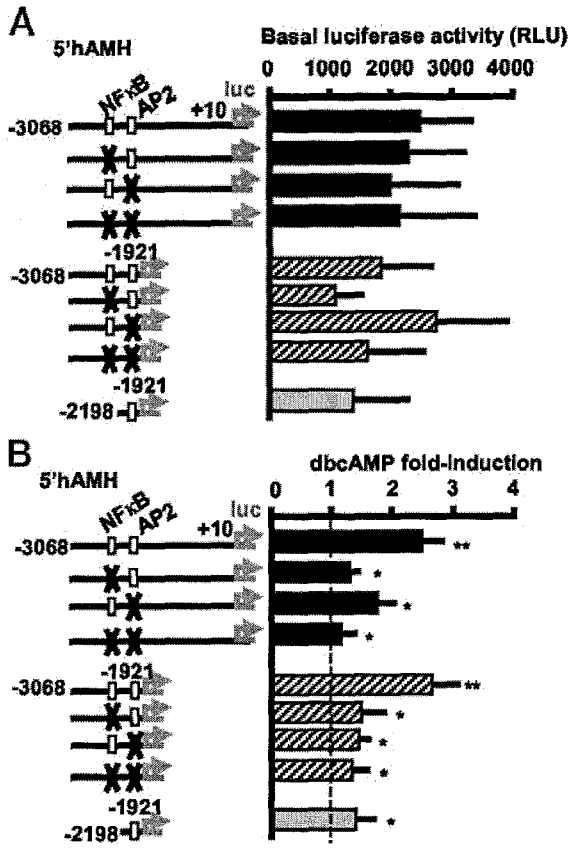

Fig. 6. cAMP Enhancement of the AMH Promoter Activity Involves NFκB and AP2 Binding Sites Lying More than 1921 bp Upstream of the Transcription Start Site

SMAT1 cells were transfected with 1 μg of the full-length −3068-5′hAMH-luc construct or of constructs consisting of sequences between −3068 and −1921 or between −2198 and −1921 with wild-type (*open boxes*) or mutated (*crossed boxes*) AP2 and/or NFκB sites, and luc activity was assessed 48 h later. A, Basal activity of the different constructs. B, Effect of the incubation with 1 mM dbcAMP during the last 24 h. Results are given as fold induction (mean ± SEM of four experiments), considering as 1 for each construct (*dotted line*) the luc activity without dbcAMP. Results were analyzed using a one-sample *t* test, in which the mean for each construct was compared with 1, which means no activation after dbcAMP addition. *, $P < 0.05$; **, $P < 0.01$.

An NFκB Binding Site at Position −2208 and an AP2 Binding Site at Position −1936 Are Required for the Enhancement of the AMH Promoter Activity by cAMP

A detailed analysis of the distal sequences between −1921 and −3068 upstream of the hAMH start initiation site revealed the existence of a consensus NFκB binding site at −2208 and a consensus AP2 site at −1936 (Fig. 4). We performed targeted mutagenesis of these sites in both the full-length 3068-5′hAMH-luc and the −3068–1921-5′hAMH-luc constructs. Mutations of NFκB and/or AP2 sites did not significantly affect the basal activity of the full-length promoter (Fig. 6A). Mutation of the NFκB site resulted in a dramatic decrease in, although not a complete abolition of, the response to cAMP (Fig. 6B). Note that deleting the −3068–2198 region, which encompasses the NFκB site, had the same effect. Mutation of the AP2 site also impaired the responsiveness to cAMP, although in the context of the full-length promoter this effect seemed less pronounced. A complete abrogation of cAMP responsiveness could not be obtained by mutating both NFκB and AP2 sites.

To further investigate the possible involvement of these two factors in AMH response to cAMP-mediated FSH stimulation in Sertoli cells, we investigated NFκB and AP2 expression in prepubertal mouse testes and in SMAT1 cells by immunocytochemistry (Fig. 7). In paraffin sections of 6-d-old mouse testes, NFκB reaction was intense in Sertoli, germ, peritubular, and interstitial cells, whereas AP2 reaction was faint in the basal cytoplasm of Sertoli cells and more intense in peritubular and interstitial cells. SMAT1 cells showed cytoplasmic expression of both factors in basal conditions. After incubation with dbcAMP, the immunoreactivity was concentrated in the perinuclear cytoplasm and extended into the nucleus.

Finally, we analyzed the effect of NFκB or AP2 overexpression on the activity of the 3-kb AMH promoter with wild-type or mutated NFκB or AP2 sites (Fig. 8). Overexpression of AP2 or NFκB resulted in an en-

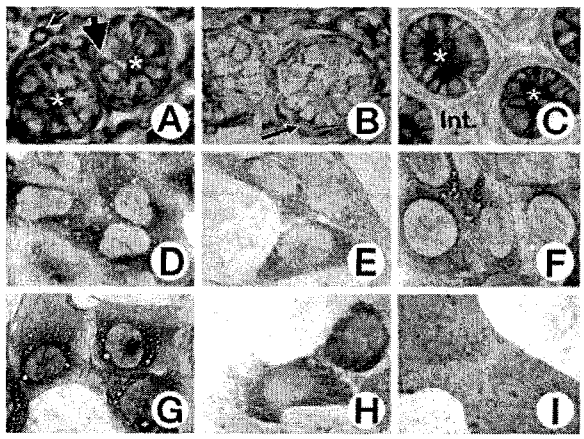

Fig. 7. AP2 and NFκB Are Expressed in Prepubertal Sertoli Cells and in the SMAT1 Cell Line

Paraffin-embedded testicular tissue obtained from 6-d-old mice (A–C), and SMAT1 cells (D–I) were submitted to immunohistochemical staining using antibodies for NFκB p65 (A, D, G) or AP2 (B, E, H) and AMH (C, F), used as a specific marker of Sertoli cells (3), or noninmune serum (I), as a negative control. NFκB (A) was positive in Sertoli cells (*asterisks*), germ cells (*large arrowhead*), and interstitial cells (*arrow*). AP2 (B) was slightly positive in the basal area of Sertoli cell cytoplasm (*arrow*), and more intensely positive in peritubular and interstitial cells. AMH (C) was positive in the cytoplasm of Sertoli cells (*asterisks*) but negative in the interstitial tissue (Int.). SMAT1 cells were cultured in basal medium, except for panels G and H, where 1 mM dbcAMP was added for 24 h.

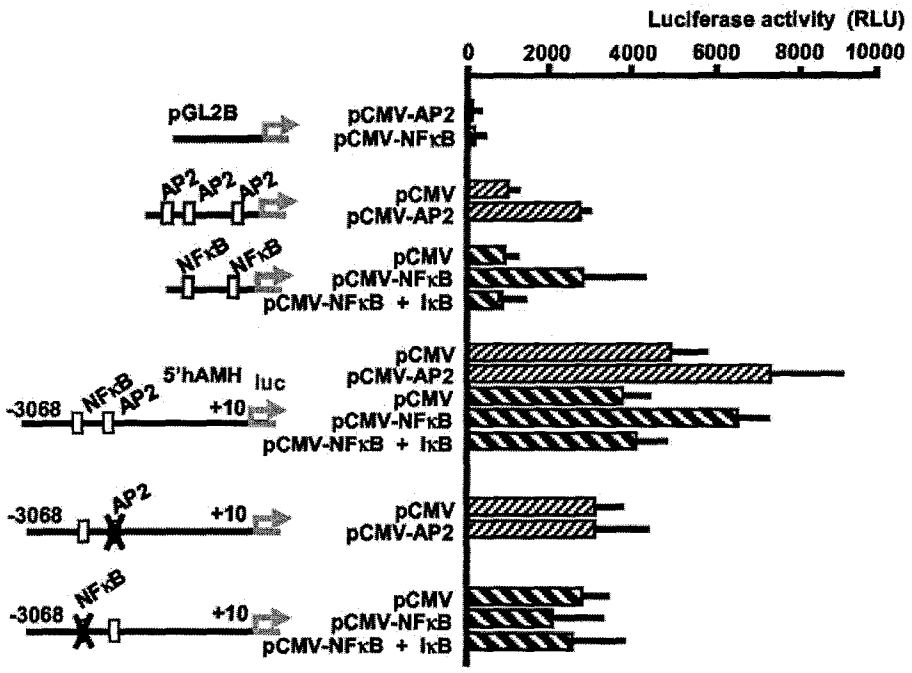

Fig. 8. AP2 and NFκB Factors Enhance the Activity of the 3068-bp AMH Promoter

SMAT1 cells were cotransfected with 0.5 μg of AP2 or NFκB expression plasmids, or their respective empty vectors, and 1 μg of a −3068-5′hAMH-luc construct carrying either wild-type (*open boxes*) or mutated (*crossed boxes*) AP2 or NFκB sites, and luc activity was assessed 48 h later. An IκB expression plasmid (2 μg) was cotransfected with NFκB (0.5 μg) where indicated. Total amount of transfected DNA per well was completed to 3.5 μg using pCMV vector in all cases. An empty pGL2B vector was used a negative control for −3068-5′hAMH-luc construct, and luc constructs with proven response elements for either AP2 or NFκB were used to monitor *trans*-activation efficacy.

hanced activity of the wild-type AMH promoter, whereas cotransfection of an excess of the inhibitor of κB (IκB) abrogated the effect induced by NFκB. Moreover, AP2 and NFκB were unable to increase the basal activity of the AMH promoter when their respective binding sites were mutated. Altogether, our results show that cAMP induction of the hAMH gene promoter activity in the prepubertal Sertoli cell line SMAT1 involves far upstream sequences (*i.e.* −3068/−1921) and requires NFκB and AP2 binding sites located in this region, although other sequences may also be involved (Fig. 9).

DISCUSSION

In fetal and postnatal life, testicular AMH production is hormonally regulated by androgens and FSH. Elevated intratesticular testosterone concentration has a potent inhibitory effect on AMH at puberty (6, 27), but in the absence of androgen action on Sertoli cells (9–11) FSH up-regulates testicular AMH production (6, 28). In this work, we provide new insight into the cellular and molecular mechanisms that explain the elevation of serum AMH after FSH stimulation. Based on results obtained from a clinical study on pediatric patients with various testicular disorders, we previously proposed that serum AMH levels are highly indicative of the number of functional Sertoli cells (9). Here, we provide further experimental evidence that serum AMH levels are correlated with the size of the Sertoli cell population; we also show that FSH is capable of enhancing AMH gene transcription. We can therefore conclude that the positive effect of FSH on testicular AMH production is due both to the proliferation of Sertoli cells and to the increase in AMH transcriptional activity per Sertoli cell. Furthermore, we show that sequences lying more than 1.9 kb upstream from the AMH transcription start site are involved in the activation of AMH gene expression in response to FSH.

Testicular function has classically been associated with androgen and sperm production. Because these two functions are quiescent before pubertal onset, the testis has been considered to be an inactive organ during childhood. Owing to the lack of an experimentally proven and clinically widely accepted marker of FSH action on prepubertal Sertoli cells, evaluation of the existence and functional potential of testicular tissue in boys has exclusively relied on Leydig cell testosterone secretion in response to human chorionic gonadotropin administration. However, Sertoli cells are very active before puberty: they proliferate and have an intense protein synthesis activity (reviewed in Refs. 29 and 30), including the secretion of high

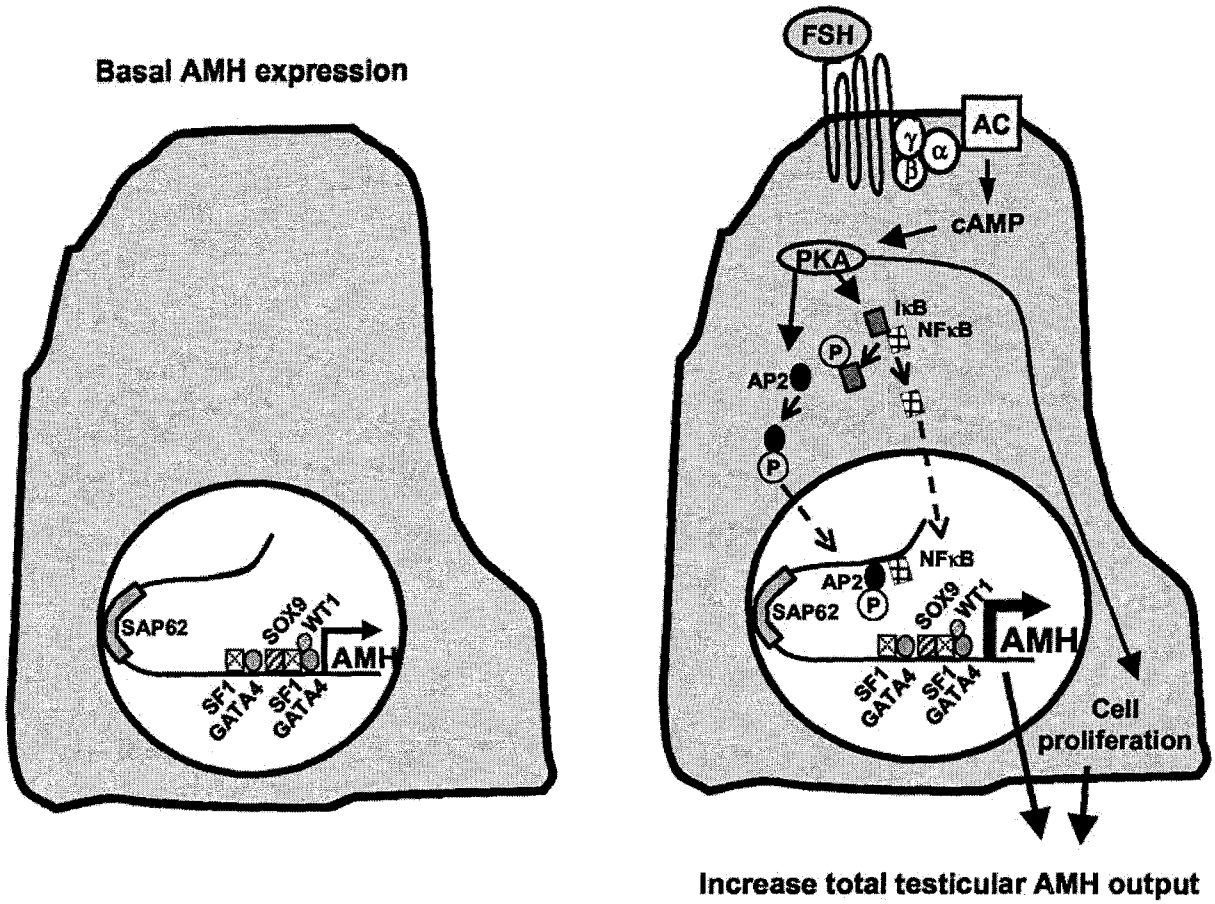

Fig. 9. Schematic Model to Explain the Increase of Testicular AMH Production after FSH Stimulation

In basal conditions, AMH expression depends on the *trans*-activating potential of several nuclear factors, SOX-9, GATA-4, SF-1, and WT-1 (20–25). FSH binds to a seven-transmembrane-domain G protein-coupled receptor; the α-subunit of the heterotrimeric G_s protein induces adenylate cyclase (AC) activity resulting in an increase of cAMP concentration and PKA activation. PKA is capable of phosphorylating AP2 (47, 48) and IκB (49, 50), which releases NFκB. Subsequently, the phosphorylated AP2 and NFκB translocate to the nucleus and enhance AMH transcription. FSH-dependent PKA activity also induces cell proliferation. Together, the increase in Sertoli cell number and the enhancement of AMH transcription in each Sertoli cell results in an increase of testicular AMH production and secretion to the circulation.

amounts of AMH. Serum AMH has been shown to be an extremely useful marker of prepubertal testicular Sertoli cell function, with no need for stimulation tests (31, 32). The fact that the number of spermatozoa produced by the adult testis is conditioned by the FSH-dependent, prepubertally attained size of the Sertoli cell population (33–36) reinforces the importance of Sertoli cell proliferation in fetal and early postnatal life in response to FSH. Previous work showed that testes of adult mice with a knockout of the FSHβ gene (16, 17) or of the FSH receptor gene (37, 38) were smaller in size owing to low germ cell number resulting from diminished Sertoli cell number and their capacity to nurture germ cells. Using the FSHβ knockout model, here we show that if no FSH is exogenously supplied the number of Sertoli cells per testis is already decreased at the onset of puberty, *i.e.* the age at which Sertoli cells stop dividing, providing further evidence of the importance of FSH in early life for the achievement of quantitatively normal sperm production and testicular size in adulthood. Furthermore, our data indicate that AMH is a marker of both Sertoli cell proliferation and protein synthesis activity in response to FSH before puberty and also support the hypothesis that serum AMH might be an useful marker of FSH action in the assessment of testicular function in prepubertal boys.

The activation and maintenance of basal levels of AMH expression in fetal life, experimentally studied by several groups (21–26, 39), are mostly independent of gonadotropins, as also shown by clinical observations in patients with hypogonadotropic hypogonadism (40) and by our present results in FSHβ gene knockout mice. Indeed, testicular AMH production is not abolished in the absence of FSH action. However, as we show here, it is moderately but significantly reduced, and resumes normal levels after exogenous FSH administration.

Owing to its temporal correlation with fetal testicular differentiation, AMH expression was first thought to be directly regulated by SRY (41). However, subsequent experiments have ruled out a direct *trans*-activating potential of SRY on AMH and shown that the combined action of multiple transcription factors is required to activate and maintain AMH expression in the fetal testis. The SRY-related protein SOX-9 (20, 21), SF-1 (22–24), and GATA factors (24–26) have been shown to bind to specific response elements present in the 5′-flanking region of the AMH gene and regulate its transcription. Although more than 3 kb of 5′-flanking sequences of the hAMH gene have been known for several years (42), up to now all functional studies have investigated less than 400 bp of the AMH proximal promoter region. A recent study shows that a 370-bp promoter is not sufficient to maintain AMH expression postnatally (43), clearly indicating that other factors binding to DNA elements lying far upstream are also involved in the regulation of AMH expression. In the present work, we have extended the investigation of the regulatory potential of 5′-flanking sequences of the AMH gene to 3 kb upstream of the translation start site. The fact that the 3′ end of the gene coding for SAP62/SF3A2, a ubiquitously expressed spliceosome protein, is just 789 bp upstream from the ATG codon in the hAMH gene and 433 bp in the mouse (44) has nourished the hypothesis that 5′ regulatory sequences of the AMH gene are very short. However, we show here that a 3-kb AMH promoter has a more potent activating capacity than smaller promoters, at least in the prepubertal Sertoli cell line SMAT1. We also show that the sequences between 1.9 and 3 kb upstream of the hAMH transcription site can up-regulate the activity of the AMH promoter. Owing to their position, these sequences could also be involved in the regulation of SAP62. Because it was beyond the scope of our study, we did not analyze SAP62 expression in SMAT1 cells. Nonetheless, the luc gene was placed at the AMH −not the SAP62−gene position and its activity is, thus, indicative of the AMH gene transcription levels that could be expected. Therefore, we can conclude that, in the present experimental conditions, AMH expression is regulated by distant sequences. Other genes, like IL-8, have already been shown to be transcriptionally activated through distant NFκB and AP2 binding sites (45).

In this study, we clearly demonstrate that the AMH promoter activity can be enhanced by a classical FSH-regulated signaling cascade involving a G protein, adenylate cyclase and PKA (Fig. 9). The best known FSH signal transduction pathway to the nucleus involves PKA phosphorylation of transcription factors binding to CREs in the promoter of target genes. The hAMH promoter lacks a canonical CRE (2, 19); we therefore proposed that FSH action was either indirect or involved a different pathway (19). Here we show that the effect of dbcAMP addition to SMAT1 cells results in an increase of the AMH promoter activity at 2 h, which may indicate that the pathway involved is quite direct. However, an even higher activity was observed at 24 h. Although this may result from the accumulation of luc protein—whose half-life is between 3 and 4 h (46)—in SMAT1 cells, an additive, indirect effect through regulation of an upstream gene cannot be ruled out. Our present results suggest that AP2 and NFκB binding sites at positions −1936 and −2208, respectively, are, at least in part, responsible for the enhancement of AMH expression induced by FSH via cAMP-PKA. PKA-dependent phosphorylation of AP2 in response to the elevation of intracellular cAMP concentration has been shown to mediate *trans*-activation of target genes (47, 48) and could explain in part FSH action on AMH (Fig. 8). AP2 binding to consensus elements in the human chorionic gonadotropin β promoter is increased by cAMP only after 24 h (48); a similar time dependency in our model could be an alternative explanation for the latency observed to reach the maximal response in FSH- or cAMP-stimulated conditions.

The mechanism involving NFκB in FSH-dependent AMH activation is less clear. The transcriptional activity of NFκB may be regulated at multiple steps in response to more than 150 different stimuli (49, 50). NFκB is normally retained in the cytoplasm owing to its association with the inhibitory molecule IκB. Phosphorylation and subsequent proteolysis of IκB allow NFκB translocation to the nucleus, where NFκB induces transcription once associated with the transcriptional cofactor CRE binding protein-binding protein (50). It has also been shown that the stimulation of Sertoli cells with activators of the PKA signaling pathway, such as forskolin or FSH, increases NFκB DNA binding activity to enhancer motifs within the promoter of cAMP-regulated genes (51, 52). Further studies will be necessary to completely understand the possible mechanisms by which NFκB may be involved in the regulation of AMH gene expression.

Interestingly, not only FSH but testosterone and AMH itself can regulate NFκB signaling. AMH has been shown to regulate NFκB in the mammary gland (53) and the prostate (54). Because Sertoli cells express the specific AMH receptor type II (18, 55, 56) and the elements involved in its signaling pathway (57), it might be possible that AMH regulates its own expression through NFκB signaling. The interaction between testosterone and FSH in AMH regulation is intricate: whereas testosterone down-regulates AMH production, FSH induces AMH if the negative effect of androgens is absent (6). Mechanisms of physical interaction have been described between NFκB and nuclear steroid receptors, which explain their mutual antagonism (58, 59). Furthermore, the ligand-bound androgen receptor is capable of decreasing NFκB activity in the absence of classical androgen responsive elements in the promoters of target genes (60, 61). Because there is no direct binding site for the androgen receptor in the AMH promoter (19), the antagonism between FSH and testosterone action on AMH expression in puber-

tal Sertoli cells could be due to a cross-talk between the androgen receptor and NFκB.

MATERIALS AND METHODS

Animals

FSH-deficient ($FSH^{-/-}$) transgenic male mice bearing a homozygous ($fshb^{m1}/fshb^{m1}$) deletion of exons 1, 2, and most of exon 3 of the gene coding for the β-subunit of FSH, generated by homologous recombination (16), and heterozygous $FSH^{+/-}$ littermates ($fshb^{m1}/+$) were obtained by intercrossing homozygous males with heterozygous females. Genotyping was performed by PCR screening of genomic DNA using two primers (FSH 663S: 5′-GGACGTAGCTGTTTACTTCCC and FSH 848A: 5′-AGTGTAGCAGTAGCCCGCACA) allowing the amplification of the second exon of the mouse FSHβ gene (62). Agarose gel electrophoresis of the resulting PCR products were analyzed to distinguish between $FSH^{-/-}$ (homozygous for the deletion involving exon 2) and $FSH^{+/-}$ littermates (one deleted and one normal allele). In the same reaction, PCR amplification of 286 bp of the *Sry* gene (primers: MSRY1: 5′-TAGAGAGCATGGAGGGCCAT and MSRY2: 5′-TGCCACTCCTCTGTGACACT) was performed as control of all male DNAs. Heterozygous males have a normal gonadal function (16). All animal studies were conducted in compliance with European Community guidelines.

Treatment, Histological Analysis, and Serum AMH Determination

$FSH^{-/-}$ male mice were separated at birth in two groups: one group was injected sc with 0.5 U recombinant FSH (Gonal-F-75, Serono, Geneva, Switzerland) every day from postnatal d 1–6; the second group, serving as control, was injected with saline solution. All animals were killed on d 7; serum was extracted and stored at −20 C until assayed. Testes were weighed and either used for DNA extraction, or immersion-fixed in Bouin's fixative overnight, dehydrated in a graded series of ethanol, and finally embedded in paraffin wax.

Morphometric analysis was performed on one testis of seven $FSH^{-/-}$ mice (three treated with FSH and four non-treated) and of three $FSH^{+/-}$ littermates as previously described (63), with slight modifications. The number of Sertoli cells per testis was estimated by counting the number of nucleoli in a known area (10 fields representing 911,879 μm^2) using a 20× objective on a Reichert Diastar Microscope, and the images obtained with a Javelin Chromaspin-V CDD video camera (Javelin Electronics, Los Angeles, CA) were digitalized and analyzed using Optimas 4.0 for Windows (Bioscan, Inc., Edmonds, WA). As testicular specific gravity is approximately 1, testicular weight was used to estimate testicular volume. The total Sertoli cell number per testis was obtained by applying the Floderus equation as described (63). The person who performed Sertoli cell counts was blinded to the treatment status and genotype of the mice. Results were subjected to a paired *t* test.

AMH concentration was measured in the serum obtained from the same animals whose gonads were studied by histological morphometric analysis. Serum AMH was determined using an ELISA as previously described (6).

Plasmids

A 3078-bp fragment of the 5′-flanking region of the hAMH gene (position −3068/+10 relative to the major transcriptional initiation site; Ref. 42) was obtained from pGAMH1 construct (18) by restriction enzyme digestion using *Sac*I and *Avr*II. *Avr*II site was rendered blunt using the Klenow fragment of DNA polymerase I, and the resulting construct was subcloned between *Sac*I and *Bgl*II sites of luc vectors pGL2B and pGL2P (Promega Corp., Madison, WI) to obtain −3068-5′hAMH-luc plasmids. Four other deletion 5′hAMH-luc constructs, respectively, containing 1921, 423, 202, and 83 bp of the hAMH promoter, were also generated. A chimeric construct including a *Dra*III-*Sma*I fragment of pGL2B and a *Sma*I-*Nhe*I (−2198/−1921) fragment of 5′hAMH was subcloned between *Dra*III and *Nhe*I sites of −1921-5′hAMH-luc to obtain −2198-5′hAMH-luc. A similar construct was obtained using pGL2P. A *Nhe*I-*Avr*II (−1921/+10) fragment of pGAMH1 was subcloned in pGL2B linearized by *Nhe*I to obtain −1921-5′hAMH-luc. An *Eco*RV-*Avr*II (−423/+10) fragment of pGAMH1 was subcloned between *Sma*I and *Nhe*I sites of pGL2B to obtain −423-5′hAMH-luc. A 240-bp fragment of the proximal hAMH promoter was amplified by PCR using 5′-CAGCATGTTGACACATCAG and 5′-CTTTTTG-CAAAAGCCTAGGC primers. The amplified fragment was subsequently digested either by *Hin*cII and *Avr*II and subcloned between *Sma*I and *Nhe*I sites of pGL2B to obtain −202-5′hAMH-luc, or by *Bst*UI and *Avr*II and subcloned between *Sma*I and *Nhe*I sites of pGL2B to obtain −83-5′hAMH-luc. The −3068-5′hAMH-luc and the −2198-5′hAMH-luc constructs subcloned in pGL2P were digested by *Nhe*I and *Hin*dIII. The resulting longer fragments, respectively, containing −3068-1921-5′hAMH-luc and −2198-1921-5′hAMH-luc, were rendered blunt using the Klenow fragment of DNA polymerase I, and circularized by incubating with T4 DNA ligase.

The FSH receptor expression vector, pcDNA—FSH-R (64), and its control, pcDNA—CAT, were kindly provided by Drs. W. Tribley and M. Griswold (Pullman, WA). The Gsα protein expression vector, αs-WT (no. 63315; Ref. 65), was purchased from ATCC (Manassas, VA). The AP2 expression vector (66) and a proven AP2-responsive luc reporter, CGβ promoter-luc (67), were kindly provided by Drs. T. Williams (Denver, CO) and T. Kotlar and J. L. Jameson (Chicago, IL). Expression vectors for NFκB p65 and IκB (68) and a proven NFκB-responsive luc reporter plasmid (69) were kindly provided by Drs. A. Israël (Paris, France) and S. Maheswaran (Boston, MA).

Targeted Mutagenesis

Plasmids with mutations in binding sites for AP2 or NFκB of the hAMH promoter were generated using the pALTER1 site-directed mutagenesis system (Promega Corp.). One AP2 site was mutated (GGGAGGGG → TGGTACCG) at position −1936, and one NFκB site was mutated (GGGGTCCCC → ATGGTACCA) at position −2208.

Cell Culture, Transfections, Luc Assays, and AMH Determination in Culture Medium

SMAT1 cells, an immortalized immature Sertoli cell line (18), were cultured in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal calf serum, 1× amino acid mix (Eurobio, Les Ulis, France), 100 U/ml penicillin, and 100 μg/ml streptomycin (Eurobio). Cells were plated at 2×10^5 cells/well in six-well plates and transiently transfected with 1 μg of luc reporter plasmids, or at 0.5×10^5 cells/well in 24-well plates and transiently transfected with 0.5 μg of DNA using the LipofectAMINE PLUS Reagent package (Life Technologies, Inc.) according to the manufacturer's instructions. In brief, 1 d after initial plating, DMEM with fetal calf serum was changed for DMEM without serum. Twenty-four hours later, transfections were performed in DMEM without serum. dbcAMP (Sigma, St. Louis, MO) or cholera toxin (Sigma) was added at 1 mM or 1 μg/ml, respectively. Human recombinant FSH (Gonal-F-75, Serono) was used at 2 U/ml. Kinase inhibitors H89, LY294002, SB203580, or PD98059 (all from Calbiochem, San Diego, CA) were used at 10 μM, 25 μM, 20 μM, or 10 μM, respectively. Luc activity was determined with the

luc reporter gene assay kit (Roche Diagnostics, Indianapolis, IN) using a Lumat LB95507 luminometer (EG&G Berthold, Bad Wildbad, Germany) and normalized for protein concentration (all experiments) and for β-galactosidase activity where the activity of different reporter plasmids were compared (Fig. 5). Protein concentration was measured using the bicinchoninic acid kit for protein determination (Sigma), and β-galactosidase activity was estimated by spectrophotometry at 420 nm, using orthonitrophenyl-β-D-galactoside as substrate.

To determine the effect of the various kinase inhibitors on AMH secretion to the culture medium, SMAT1 cells were plated and treated as described and AMH was assayed using the ELISA described above. Cells were detached from the plate by gentle trypsinization and counted in a Malassez chamber.

Immunocytochemistry

Paraffin-embedded sections of testicular tissue fixed in paraformaldehyde (4% in PBS) from 6-d-old mice were used for immunocytochemistry using the antigen retrieval technique as previously described (6), with slight modifications. SMAT1 cells, cultured on four-chamber Lab-Tek Permanox slides (Nunc, Naperville, IL) at 2–5 × 10^4 cells per chamber, were fixed in 4% paraformaldehyde and submitted to immunocytochemistry without antigen retrieval. Primary antibodies were: rabbit polyclonal antirecombinant hAMH L40 (1 μg/ml), rabbit polyclonal anti-NFκB p65 sc-109 (2 μg/ml, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit polyclonal anti-AP2 sc-184 (2 μg/ml, Santa Cruz Biotechnology, Inc.). A negative control reaction was performed by replacing the primary antibody with nonimmune rabbit serum. The reaction was revealed using peroxidase Vectastain Elite ABC kit PK-6101 (Vector Laboratories, Inc., Burlingame, CA) and DAB Plus Reagent K-3467 (DAKO Corp., Copenhagen, Denmark).

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Address all correspondence and requests for reprints to: Dr. Rodolfo Rey, Centro de Investigaciones Endocrinológicas, Hospital de Niños, Gallo 1330, C1425EFD Buenos Aires, Argentina. E-mail: rodolforey@cedie.org.ar.

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* Present address: Service d'Endocrinologie et Maladies Métaboliques, Centre Hospitalier Universitaire, Reims, France.

REFERENCES

1. Josso N, Cate RL, Picard JY, Vigier B, di Clemente N, Wilson C, Imbeaud S, Pepinsky RB, Guerrier D, Boussin L 1993 Anti-Müllerian hormone: the Jost factor. Recent Prog Horm Res 48:1–59
2. Teixeira J, Maheswaran S, Donahoe PK 2001 Mullerian inhibiting substance: an instructive developmental hormone with diagnostic and possible therapeutic applications. Endocr Rev 22:657–674
3. Tran D, Meusy-Dessolle N, Josso N 1977 Anti-Müllerian hormone is a functional marker of foetal Sertoli cells. Nature 269:411–412
4. Josso N, di Clemente N, Gouédard L 2001 Anti-Müllerian hormone and its receptors. Mol Cell Endocrinol 179: 25–32
5. Münsterberg A, Lovell-Badge R 1991 Expression of the mouse anti-Müllerian hormone gene suggests a role in both male and female sexual differentiation. Development 113:613–624
6. Al-Attar L, Noël K, Dutertre M, Belville C, Forest MG, Burgoyne PS, Josso N, Rey R 1997 Hormonal and cellular regulation of Sertoli cell anti-Müllerian hormone production in the postnatal mouse. J Clin Invest 100: 1335–1343
7. Rey R 1998 Endocrine, paracrine and cellular regulation of postnatal anti-Müllerian hormone secretion by Sertoli cells. Trends Endocrinol Metab 9:271–276
8. Lane AH, Lee MM 1999 Clinical applications of Mullerian inhibiting substance in patients with gonadal disorders. Endocrinologist 9:208–215
9. Rey RA, Belville C, Nihoul-Fékété C, Michel-Calemard L, Forest MG, Lahlou N, Jaubert F, Mowszowicz I, David M, Saka N, Bouvattier C, Bertrand AM, Lecointre C, Soskin S, Cabrol S, Crosnier H, Leger J, Lortat-Jacob S, Nicolino M, Rabl W, Toledo SP, Bas F, Gompel A, Czernichow P, Josso N 1999 Evaluation of gonadal function in 107 intersex patients by means of serum antimüllerian hormone measurement. J Clin Endocrinol Metab 84: 627–631
10. Stuchi-Perez EG, Lukas-Croisier C, De Castro M, Baptista MT, Ribeiro Scolfaro M, Marques-De-Faria AP, Hackel C, Maciel-Guerra AT, Guerra Junior G 2000 Evaluation of the tubular and interstitial functions of the testis in 46,XY patients with ambiguous genitalia. J Pediatr Endocrinol Metab 13:605–612
11. Rey R, Mebarki F, Forest MG, Mowszowicz I, Cate RL, Morel Y, Chaussain JL, Josso N 1994 Anti-Müllerian hormone in children with androgen insensitivity. J Clin Endocrinol Metab 79:960–964
12. Moyle WR 2001 Gonadotropins. In: de Groot LJ, Jameson JL, eds. Endocrinology. Philadelphia: W. B. Saunders; 1895–1904
13. González-Robayna IJ, Falender AE, Ochsner S, Firestone GL, Richards JS 2000 Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-Induced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. Mol Endocrinol 14: 1283–1300
14. Richards JS 2001 New signaling pathways for hormones and cyclic adenosine 3′,5′-monophosphate action in endocrine cells. Mol Endocrinol 15:209–218
15. Griswold MD 1993 Actions of FSH on mammalian Sertoli cells. In: Russell LD, Griswold M, eds. The Sertoli cell. Clearwater, FL: Cache River Press; 493–508
16. Kumar TR, Wang Y, Lu N, Matzuk MM 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15:201–204
17. Wreford NG, Kumar TR, Matzuk MM, de Kretser DM 2001 Analysis of the testicular phenotype of the follicle-stimulating hormone β-subunit knockout and the activin

type II receptor knockout mice by stereological analysis. Endocrinology 142:2916–2920
18. Dutertre M, Rey R, Porteu A, Josso N, Picard JY 1997 A mouse Sertoli cell line expressing anti-Müllerian hormone and its type II receptor. Mol Cell Endocrinol 136:57–65
19. Rey R, Josso N 1996 Regulation of testicular anti-Müllerian hormone secretion. Eur J Endocrinol 135: 144–152
20. de Santa Barbara P, Bonneaud N, Boizet B, Desclozeaux M, Moniot B, Sudbeck P, Scherer G, Poulat F, Berta P 1998 Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Müllerian hormone gene. Mol Cell Biol 18: 6653–6665
21. Arango NA, Lovell-Badge R, Behringer RR 1999 Targeted mutagenesis of the endogenous mouse Mis gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. Cell 99:409–419
22. Shen WH, Moore CC, Ikeda Y, Parker KL, Ingraham HA 1994 Nuclear receptor steroidogenic factor 1 regulates the Müllerian inhibiting substance gene: a link to the sex determination cascade. Cell 77:651–661
23. Giuili G, Shen WH, Ingraham HA 1997 The nuclear receptor SF-1 mediates sexually dimorphic expression of Mullerian inhibiting substance, in vivo. Development 124: 1799–1807
24. Watanabe K, Clarke TR, Lane AH, Wang X, Donahoe PK 2000 Endogenous expression of Müllerian inhibiting substance in early postnatal rat Sertoli cells requires multiple steroidogenic factor-1 and GATA-4-binding sites. Proc Natl Acad Sci USA 97:1624–1629
25. Tremblay JJ, Viger RS 1999 Transcription factor GATA-4 enhances Mullerian inhibiting substance gene transcription through a direct interaction with the nuclear receptor SF-1. Mol Endocrinol 13:1388–1401
26. Beau C, Rauch M, Joulin V, Jégou B, Guerrier D 2000 GATA-1 is a potential repressor of anti-Müllerian hormone expression during the establishment of puberty in the mouse. Mol Reprod Dev 56:124–138
27. Rey R, Lordereau-Richard I, Carel JC, Barbet P, Cate RL, Roger M, Chaussain JL, Josso N 1993 Anti-Müllerian hormone and testosterone serum levels are inversely related during normal and precocious pubertal development. J Clin Endocrinol Metab 77:1220–1226
28. Voutilainen R, Miller WL 1987 Human Müllerian inhibitory factor messenger ribonucleic acid is hormonally regulated in the fetal testis and in adult granulosa cells. Mol Endocrinol 1:604–608
29. Rey R 1999 The prepubertal testis: a quiescent or a silently active organ? Histol Histopathol 14:991–1000
30. Chemes HE 2001 Infancy is not a quiescent period of testicular development. Int J Androl 24:2–7
31. Lee MM, Donahoe PK, Silverman BL, Hasegawa T, Hasegawa Y, Gustafson ML, Chang YC, MacLaughlin DT 1997 Measurements of serum Müllerian inhibiting substance in the evaluation of children with nonpalpable gonads. N Engl J Med 336:1480–1486
32. Rey R 2000 Assessment of seminiferous tubule function (anti-Müllerian hormone). Baillieres Best Pract Res Clin Endocrinol Metab 14:399–408
33. Orth JM, Gunsalus GL, Lamperti AA 1988 Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. Endocrinology 122: 787–794
34. Orth JM 1984 The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. Endocrinology 115:1248–1255
35. Nistal M, Abaurrea MA, Paniagua R 1982 Morphological and histometric study on the human Sertoli cell from birth to the onset of puberty. J Anat 134(Part 2):351–363
36. Kluin PM, Kramer MF, de Rooij DG 1984 Proliferation of spermatogonia and Sertoli cells in maturing mice. Anat Embryol (Berl) 169:73–78
37. Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P 1998 Impairing follicle-stimulating hormone (FSH) signaling *in vivo*: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci USA 95:13612–13617
38. Krishnamurthy H, Babu PS, Morales CR, Sairam MR 2001 Delay in sexual maturity of the follicle-stimulating hormone receptor knockout male mouse. Biol Reprod 65:522–531
39. Morikawa N, Clarke TR, Novina CD, Watanabe K, Haqq C, Weiss M, Roy AL, Donahoe PK 2000 Human Müllerian inhibiting substance promoter contains a functional TFII-I-binding initiator. Biol Reprod 63:1075–1083
40. Young J, Rey R, Couzinet B, Chanson P, Josso N, Schaison G 1999 Anti-Müllerian hormone in patients with hypogonadotropic hypogonadism. J Clin Endocrinol Metab 84:2696–2699
41. Haqq CM, King CY, Ukiyama E, Falsafi S, Haqq TN, Donahoe PK, Weiss MA 1994 Molecular basis of mammalian sexual determination: activation of Müllerian inhibiting substance gene expression by SRY. Science 266:1494–1500
42. Guerrier D, Boussin L, Mader S, Josso N, Kahn A, Picard JY 1990 Expression of the gene for anti-Müllerian hormone. J Reprod Fertil 88:695–706
43. Beau C, Vivian N, Münsterberg A, Dresser DW, Lovell-Badge R, Guerrier D 2001 In vivo analysis of the regulation of the anti-Müllerian hormone, as a marker of Sertoli cell differentiation during testicular development, reveals a multi-step process. Mol Reprod Dev 59:256–264
44. Dresser DW, Hacker A, Lovell-Badge R, Guerrier D 1995 The genes for a spliceosome protein (SAP62) and the anti-Müllerian hormone (AMH) are contiguous. Hum Mol Genet 4:1613–1618
45. Smith RS, Fedyk ER, Springer TA, Mukaida N, Iglewski BH, Phipps RP 2001 IL-8 production in human lung fibroblasts and epithelial cells activated by the Pseudomonas autoinducer N-3-oxododecanoyl homoserine lactone is transcriptionally regulated by NF-κB and activator protein-2. J Immunol 167:366–374
46. Leclerc GM, Boockfor FR, Faught WJ, Frawley LS 2000 Development of a destabilized firefly luciferase enzyme for measurement of gene expression. Biotechniques 29: 590–591
47. Imagawa M, Chiu R, Karin M 1987 Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell 51: 251–260
48. Johnson W, Jameson JL 1999 AP-2 (activating protein 2) and Sp1 (selective promoter factor 1) regulatory elements play distinct roles in the control of basal activity and cyclic adenosine 3′,5′-monophosphate responsiveness of the human chorionic gonadotropin-β promoter. Mol Endocrinol 13:1963–1975
49. Pahl HL 1999 Activators and target genes of Rel/NF-κB transcription factors. Oncogene 18:6853–6866
50. Abraham E 2000 NF-κB activation. Crit Care Med 28: N100–N104
51. Delfino F, Walker WH 1998 Stage-specific nuclear expression of NF-κB in mammalian testis. Mol Endocrinol 12:1696–1707
52. Delfino FJ, Walker WH 1999 NF-κB induces cAMP-response element-binding protein gene transcription in Sertoli cells. J Biol Chem 274:35607–35613
53. Segev DL, Hoshiya Y, Stephen AE, Hoshiya M, Tran TT, MacLaughlin DT, Donahoe PK, Maheswaran S 2001 Mullerian inhibiting substance regulates NFκB signaling and growth of mammary epithelial cells *in vivo*. J Biol Chem 276:26799–26806

54. Segev DL, Hoshiya Y, Hoshiya M, Tran TT, Carey JL, Stephen AE, MacLaughlin DT, Donahoe PK, Maheswaran S 2002 Mullerian-inhibiting substance regulates NF-κB signaling in the prostate *in vitro* and *in vivo*. Proc Natl Acad Sci USA 99:239–244
55. di Clemente N, Wilson C, Faure E, Boussin L, Carmillo P, Tizard R, Picard JY, Vigier B, Josso N, Cate R 1994 Cloning, expression, and alternative splicing of the receptor for anti-Müllerian hormone. Mol Endocrinol 8:1006–1020
56. Baarends WM, Hoogerbrugge JW, Post M, Visser JA, De Rooij DG, Parvinen M, Themmen AP, Grootegoed JA 1995 Anti-Mullerian hormone and anti-Mullerian hormone type II receptor messenger ribonucleic acid expression during postnatal testis development and in the adult testis of the rat. Endocrinology 136:5614–5622
57. Gouédard L, Chen YG, Thevenet L, Racine C, Borie S, Lamarre I, Josso N, Massague J, di Clemente N 2000 Engagement of bone morphogenetic protein type IB receptor and Smad1 signaling by anti-Müllerian hormone and its type II receptor. J Biol Chem 275:27973–27978
58. McKay LI, Cidlowski JA 1999 Molecular control of immune/inflammatory responses: interactions between nuclear factor-κB and steroid receptor-signaling pathways. Endocr Rev 20:435–459
59. McKay LI, Cidlowski JA 1998 Cross-talk between nuclear factor-kappa B and the steroid hormone receptors: mechanisms of mutual antagonism. Mol Endocrinol 12: 45–56
60. Palvimo JJ, Reinikainen P, Ikonen T, Kallio PJ, Moilanen A, Jänne OA 1996 Mutual transcriptional interference between RelA and androgen receptor. J Biol Chem 271: 24151–24156
61. Keller ET, Chang C, Ershler WB 1996 Inhibition of NFκB activity through maintenance of IκBα levels contributes to dihydrotestosterone-mediated repression of the interleukin-6 promoter. J Biol Chem 271:26267–26275
62. Kumar TR, Kelly M, Mortrud M, Low MJ, Matzuk MM 1995 Cloning of the mouse gonadotropin β-subunit-encoding genes, I. Structure of the follicle-stimulating hormone β-subunit-encoding gene. Gene 166:333–334
63. Rey RA, Campo SM, Bedecarrás P, Nagle CA, Chemes HE 1993 Is infancy a quiescent period of testicular development? Histological, morphometric, and functional study of the seminiferous tubules of the cebus monkey from birth to the end of puberty. J Clin Endocrinol Metab 76:1325–1331
64. Maguire SM, Tribley WA, Griswold MD 1997 Follicle-stimulating hormone (FSH) regulates the expression of FSH receptor messenger ribonucleic acid in cultured Sertoli cells and in hypophysectomized rat testis. Biol Reprod 56:1106–1111
65. Iiri T, Herzmark P, Nakamoto JM, van Dop C, Bourne HR 1994 Rapid GDP release from Gs α in patients with gain and loss of endocrine function. Nature 371:164–168
66. Williams T, Tjian R 1991 Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. Genes Dev 5:670–682
67. Albanese C, Kay TW, Troccoli NM, Jameson JL 1991 Novel cyclic adenosine 3′,5′-monophosphate response element in the human chorionic gonadotropin β-subunit gene. Mol Endocrinol 5:693–702
68. Whiteside ST, Epinat JC, Rice NR, Israël A 1997 IκB epsilon, a novel member of the IκB family, controls RelA and cRel NF-κB activity. EMBO J 16:1413–1426
69. Ten RM, Paya CV, Israël N, Le Bail O, Mattei MG, Virelizier JL, Kourilsky P, Israel A 1992 The characterization of the promoter of the gene encoding the p50 subunit of NF-κB indicates that it participates in its own regulation. EMBO J 11:195–203