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Role of Winged Helix Transcription Factor (WIN) in the Regulation of Sertoli Cell Differentiated Functions: WIN Acts as an Early Event Gene for Follicle-Stimulating Hormone

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ABSTRACT

Members of the winged helix transcription factor family are known to regulate epithelial cell differentiation by regulating cell-specific gene expression. rWIN is a newly discovered member of the winged helix family shown to be present in the adult rat testis. In the testis the human homolog of rWIN, HFH-11, was localized to the germ cells (*i.e.* spermatocytes and spermatids) undergoing spermatogenesis. In the present study we show that rWIN is also expressed in testicular Sertoli cells. Sertoli cells are the epithelial component of the seminiferous tubule and provide both the cytoarchitectural support and the microenvironment for developing germ cells. The presence of rWIN in Sertoli cells was confirmed by Northern blot and RT-PCR analysis. The rWIN transcript size in the Sertoli cells was different from the germ cell transcript that is probably due to alternative splicing or modifications of the 3′-untranslated region. At least two spliced variants of rWIN were observed in the Sertoli cells corresponding to the deletion of an exon in the DNA-binding region. Long term stimulation of cultured Sertoli cells with the gonadotropin FSH down-regulated rWIN expression. In contrast, short-term stimulation (2 h) transiently up-regulated rWIN expression. The FSH-induced transient stimulation of rWIN precedes expression of the transferrin gene that is a marker of Sertoli cell differentiation. FSH-induced transferrin promoter activity was inhibited when cultured Sertoli cells were treated with an antisense oligonucleotide to rWIN. Interestingly, the constitutive overexpression of the DNA-binding domain of rWIN also down-regulated transferrin promoter activity. Analysis of the transferrin promoter with various deletion mutations suggested that rWIN acts at an upstream gene of the transferrin promoter. The results indicate that a transient up-regulation of rWIN in part mediates the ability of FSH to activate the transferrin promoter, which can be inhibited with a rWIN antisense oligonucleotide or constitutive expression of the rWIN DNA-binding domain. The current study demonstrates that rWIN acts as an early event gene for FSH actions on Sertoli cells and that rWIN appears to have a role in the regulation of Sertoli cell differentiated functions. (*Endocrinology* **141:** 2758–2766, 2000)

WINGED HELIX (WH) proteins are a large family of putative transcription factors that may regulate mesenchymal to epithelial transitions and maintain cellular differentiation (reviewed in Refs. 1 and 2). Members of the WH gene family are expressed in a wide range of tissues during various stages of embryonic development. Family members share a highly conserved 100-amino acid DNA-binding domain, which was first identified in HNF-3 proteins (2). Targeted disruptions of a number of WH genes have revealed essential functions of WH proteins in development for cell fate determination, cellular proliferation, and cellular differentiation (2–5). The role of WH proteins in mesenchymal to epithelial transitions during kidney and brain development was established by targeted disruption of BF-2 and BF-1, respectively (6, 7). An increasing number of WH proteins are also involved in the transcriptional regulation of cell-specific genes, which suggests a role in integrating transcriptional gene networks (8–10).

Recently, a novel member of the WH family, rWIN, was cloned from a rat pancreatic endocrine cell line (11). During embryonic development, rWIN and its human homolog, HFH-11 (12), are expressed in proliferating mesenchymal and epithelial cells of the lung, liver, intestine, renal cortex, and pancreas. In the adult tissues, rWIN expression is high in the testis and lung, whereas the expression of HFH-11 is restricted to adult thymus and testis. During embryogenesis, the expression of Trident, the mouse homolog of rWIN/HFH-11, is observed in all proliferative cells, but not in resting cells (13). The role of Trident in cellular proliferation is based on the observation that Trident is expressed in the cells entering the S phase and is then phosphorylated in the M phase of the cell cycle (13). The targeted disruption of the Trident gene in mice results in postnatal death due to polyploidy in the developing myocardium, leading to circulatory defects (14). Therefore, Trident appears to have a role in DNA replication during the G_2 and M phases of the cell cycle. Whether Trident is expressed in the adult mouse testis remains to be determined, but, similar to HFH-11, it is highly expressed in the thymus (13). The particular cell type(s) in the testis that expresses rWIN is not known, but its human homolog, HFH-11, is highly expressed in spermatocytes and spermatids (12). In contrast, spermatogonia undergoing active proliferation reportedly do not express HFH-11. Another cell type in the seminiferous tubule is the Sertoli cell. The Sertoli cells have epithelial characteristics and in postpubertal testis provide cytoarchitectural support to the

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developing germ cells (15). Most, if not all, Sertoli cell functions are regulated by the gonadotropin FSH.

The Sertoli cells continue to proliferate prepubertally until the onset of puberty and formation of the blood-testis barrier (16). After formation of the blood-testis barrier, the Sertoli cells become postmitotic and terminally differentiated. Sertoli cell proliferation and pre- and postpubertal gene expression are in part regulated by the gonadotropin FSH (17). The molecular mechanisms regulating the embryonic mesenchymal to epithelial transition of Sertoli cells and maintenance of the epithelial phenotype of Sertoli cells are largely unknown. Therefore, transcription factors that may be potentially involved in regulating Sertoli cell differentiation are being investigated. One approach has been to use the promoters of a number of Sertoli cell genes and analyze for conserved *cis* elements. A response element found to be highly conserved in a number of Sertoli cell genes was an E box response element. The E box element is a hexanucleotide consensus that binds transcription factors of the basic helix loop helix family (18). The two ubiquitously expressed bHLH proteins, REBα and the E2A gene product E47, were shown to be expressed in Sertoli cells (19, 20). To identify Sertoli cell-specific bHLH transcription factors, a Sertoli cell complementary DNA (cDNA) library was screened with the conserved bHLH domain of REBα under conditions of low stringency. One of the clones isolated with a 1023-bp insert when searched for homology with known sequences in the GenBank emerged as the rWIN sequence (11). The isolation of rWIN clone from the Sertoli cell cDNA library suggested that rWIN may be expressed in Sertoli cells. The current study was designed to investigate the expression and action of rWIN in Sertoli cells and its potential role in regulating Sertoli cell differentiated functions. Interestingly, rWIN was found to have a role in mediating the actions of FSH on Sertoli cells.

Materials and Methods

Isolation of Sertoli cells

Sertoli cells were isolated from the testis of 20-day-old rats by a modified procedure described previously (21, 22). The isolated Sertoli cells were more than 98% pure and were then plated under serum-free conditions in six-well tissue culture plates (Nunclon, Nunc, Naperville, IL) at a concentration 10^7 cells/plate. Cells were maintained in a 5% CO_2 atmosphere in Ham's F-12 medium (Life Technologies, Inc., Gaithersburg, MD) with 0.01% BSA at 32 C. Sertoli cells were treated with FSH (100 ng/ml; o-FSH-16, National Pituitary Agency), $(Bu)_2$cAMP (100 μM), or vehicle alone (Ham's F-12, control). These optimal concentrations of FSH and cAMP have previously been shown to dramatically stimulate cultured Sertoli cell differentiated functions (23, 24). The cells were cultured under serum-free conditions for a maximum of 5 days, with a medium change and treatment after 48 h of culture. Cell number, purity, and viability did not change during culture in the absence or presence of treatment.

Sertoli cell cDNA library

The rat Sertoli cell cDNA library was constructed in HybriZapII by Stratagene from polyadenylated RNA isolated from purified Sertoli cells of 20-day-old rats. The HybriZap library is primarily designed for use in the yeast two-hybrid screening, but also allows for nucleic acid screening. The standard procedures for nucleic acid screening were followed as mentioned previously (25). Individual plaques (1.5×10^6) were screened with the 170-bp REBα PCR fragment corresponding to the bHLH domain and end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The positive clones were excised from the Hybrizap II library to obtain pAD plasmid-containing clones. The pAD plasmids were analyzed by restriction mapping and sequencing. The sequences were subjected to a FASTA and BLASTn searches against known sequences in GenBank.

RNA preparation

Freshly isolated or cultured Sertoli cells were lysed directly using Tri-Reagent (T9424, Sigma, St. Louis, MO). The cell lysate was then passed several times through a Pasteur pipette to form homogenous lysate. The whole tissues (skeletal muscle, brain, and detunicated testis) were placed in Tri-Reagent (5%, wt/vol) and homogenized in a tissue homogenizer (Tissue Tearor, BioSpec Products, Inc., Bartlesville, OK). To avoid any possible contamination of extracellular material and high mol wt DNA in the final RNA samples, the homogenate was centrifuged at 12,000 $\times$ *g* for 10 min at 4 C. Total RNA was then isolated from the cell lysate and whole tissue homogenate following the manufacturer's protocol for RNA isolation using Tri-Reagent. The final RNA pellet was dissolved in distilled water at a concentration of 1 mg/ml.

PCR

Total RNA (2 μg) was reverse transcribed in a final volume of 20 μl containing 20 U RNasin (Promega Corp., Madison, WI); 200 μM each of deoxy (d)-ATP, dCTP, dTTP, and dGTP; 1 μg oligo(deoxythymidine) (Pharmacia, Piscataway, NJ), 10 μM dithiothreitol, and 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (BRL, Gaithersburg, MD) in the MMLV first strand synthesis buffer supplied by the manufacturer (BRL). The RNA and oligo(deoxythymidine) primer in the buffer were first denatured for 5 min at 65 C, then cooled on ice before addition of nucleotides and enzyme. The reverse transcriptase reaction was carried out at 37 C for 1 h. PCR was performed using the GeneAmp kit (Perkin-Elmer Corp./Cetus, Emeryville, CA) with 30 cycles as follows: 94 C for 1 min (denaturation), 58 C for 2 min (primer annealing), and 72 C for 1 min (primer extension). Each PCR reaction contained 250 pg reverse transcribed DNA, 1 μM of each 5′- and 3′-oligonucleotide primer, 2.5 U *Taq* polymerase (AmpliTaq, Perkin-Elmer Corp.), and 200 μM of each dATP, dCTP, dGTP, and dTTP. After amplification, the product of each reaction was subjected to electrophoresis through 1.5% agarose gel in buffer, and the products were visualized by ethidium bromide staining.

The primer pair sequences used were obtained from published sequences of rWIN (GenBank accession no. U83112) and synthesized from commercial sources. Primer pairs were designed to amplify the N-terminal region of the rWIN protein containing the winged helix domain (Fig. 1). The possible contamination of RNA with DNA was distin-

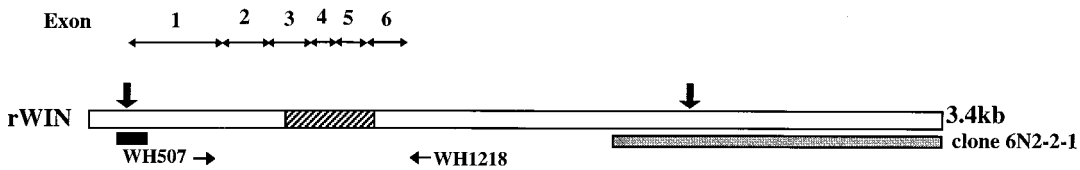

FIG. 1. Schematic showing the potential spliced regions in the rWIN sequence. Also shown is the location of the PCR primers (*forward* and *reverse arrows*), predicted protein coding region (*enclosed within inverted arrows*), the winged helix domain (*hatched box*), and the area of homology with the entire sequence of clone 6N2–2-1 (*shaded bar*). The domain of WH to which the antisense oligonucleotide was designed is shown by the *black bar*.

guished by performing the RT reaction without MMLV reverse transcriptase. The absence of any product in the amplification reaction using such a reverse transcribed preparation indicated the absence of any contaminating DNA in our RNA samples. The sequence and position of the primers were as follows: WIN 5′-primer (507), 5′-TTG GGA CCA AAG CCA GGG GCT AAG; and WIN 3′-primer (1218), 5′-CTT TCG CCG TGC GCC TAG TGG GAG TTC.

As shown in Fig. 1, the rWIN-specific primers were designed to include all of the exons, as reported previously (11). Each RT reaction was performed using three different samples. The PCR-based amplification reactions were carried out in duplicate on each reverse transcribed RNA sample. Simultaneous PCR reactions were also carried out using primers designed to rat cyclophilin to monitor the efficiency of the PCR (26). Cyclophilin was faithfully amplified in all of the PCR reactions, indicating consistency in the quality of RT and PCR reactions. The data presented, therefore, are representative of three different RT-PCR reactions carried out in duplicate.

Subcloning and sequencing

The PCR fragments were run on 1.5% agarose gel, stained with ethidium bromide, and visualized. The bands were then dissected out, and the DNA was isolated from the gel using Glass MAX DNA isolation system (BRL). The purified DNA fragments were subcloned into pCR 2.1 (Invitrogen, San Diego, CA) plasmid. The cloned DNA fragments were sequenced using standard M13 forward and reverse primers in an automated fluorescence-based sequencer (PE Applied Biosystems, Foster City, CA). Internal sequences were obtained using nested primers that were designed based on previous sequences. All of the sequences reported are the consensus of two different experiments. The sequence alignments (GCG DNA analysis software, Genetics Computer Group, Madison, WI) were carried out using the available sequence of rWIN.

Northern blot analysis

Total RNA was extracted from cultured Sertoli cells [treated with FSH, cAMP, or vehicle alone (control)], freshly isolated Sertoli cells, germ cells, and various tissues as described above using Tri-Reagent (Sigma). For time-course experiments the RNA was extracted from cultured Sertoli cells treated with FSH or cAMP for various time intervals (see figure legends). Approximately 10 μg total RNA were fractionated on a 1% formaldehyde-agarose gel. After fractionation, the RNA in the gel was transferred onto a nylon membrane (Hybond$^+$ N, Amersham Pharmacia Biotech, Arlington Heights, IL) in 10 $\times$ SSC (standard saline citrate) buffer and UV-cross linked as described previously (25). The membranes were then prehybridized in Quick Hybridization buffer (Stratagene) for 30 min at 60 C. The hybridization was carried out at 60 C for 1 h with ^{32}P-labeled rWIN probe obtained by RT-PCR of Sertoli cell RNA. The membrane was subsequently stripped and rehybridized with rat cyclophilin. All of the probes were labeled using the Prime-It II kit from Stratagene.

Plasmids and antisense oligonucleotides

The chloramphenicol acetyltransferase (CAT) reporter plasmid (pUC8-CAT) containing −581 bp (−581 bp mTf-CAT) was provided by Dr. G. Stanley McKnight (University of Washington, Seattle, WA) (27). The 3-kb mouse transferrin promoter was ligated into PGL2 basic plasmid (Promega Corp.) as previously described (28). The mouse transferrin promoter used in the present study included the transcriptional initiation site of the transferrin gene, which is 54 bp upstream of the start site of translation (29).

The antisense oligonucleotide to rWIN was designed to incorporate 15 bases around and including the translational initiation site. The scrambled oligonucleotide was generated using the GCG software analysis package with the rWIN antisense oligonucleotide. Both oligonucleotides were synthesized from commercial sources using phosphothioate modification. The expression plasmid WH-pREP7 was made by cloning the 711-bp PCR product into the *Not*I site of the pREP7 plasmid (Invitrogen).

Transfection

Sertoli cells cultured in 24-well plates at a density of 10^6 cells for 48 h were transfected with a reporter gene construct by the calcium phosphate method coupled with hyperosmotic shock (10% glycerol) as previously described (27). Briefly, 1.5 μg reporter plasmid in 150 μl transfection buffer [250 mM $CaCl_2$ mixed 1:1 (vol/vol) with 2 $\times$ Hebes (28 mM NaCl, 50 mM HEPES, and 1.47 mM Na_2HPO_4, pH 7.05)] were added to each well of a 24-well plate containing 1×10^6 Sertoli cells in 1 ml Ham's F-12 with 0.01% BSA, and incubation was performed at 32 C for 4 h. After incubation, the cells were subjected to a hyperosmotic shock. The medium was aspirated, and 1 ml 10% glycerol in HBSS (Life Technologies, Inc.) was added. The cells were incubated for 3 min, and the wells were washed twice before fresh Ham's F-12 was added. Various treatments were subsequently added, and cells were incubated for 48 h before harvesting for CAT assays. For antisense experiments the transfected Sertoli cells were treated with the antisense, sense, or scrambled oligonucleotide immediately after transfection and retreated with the oligonucleotide every 12 h for a total of 72 h. In each experiment the transfection efficiency was monitored by transfecting the Sertoli cells with a plasmid containing the β-galactosidase gene driven by a cytomegalovirus promoter. Subsequent staining and counting the cells expressing β-galactosidase (blue color) resulted in approximately 25% transfection efficiency.

CAT assay

Assay of CAT activity was performed as follows. Medium was removed from the wells, and the cells were washed once with PBS. One hundred microliters of the cell lysis buffer (Promega Corp.) were added to each well, and incubation was carried out for 15 min at room temperature. The wells were then scraped, and buffer was collected in 1.5-ml microfuge tubes. Tubes were heated to 65 C for 10 min to inactivate endogenous acetylases and then centrifuged at 12,000 $\times$ *g* for 10 min at 4 C to remove cell debris. An aliquot of cell extract (54 μl) was mixed with 65 μl 0.25 M Tris (pH 8.0), 25 μg *n*-butyryl coenzyme A (5 mg/ml; Sigma), and 0.1 μCi (1 μl) [^{14}C]chloramphenicol (ICN Biomedicals, Inc., Costa Mesa, CA) and incubated overnight at 37 C. The mixture was extracted once with 300 μl mixed xylenes and back-extracted with 100 μl 0.25 M Tris (pH 8.0). A 200-μl aliquot of the organic phase was counted in a scintillation counter to determine the relative amount of CAT activity. The average conversion of CAT substrate for treated cells ranged from 20–30%. This assay was linear with the protein concentration used.

Statistical analysis

All transfection data were obtained from a minimum of three different experiments unless otherwise stated. Each data point (from treatments) was converted to a relative CAT activity (control CAT activity = 1), with the mean and SEM from multiple experiments determined as indicated in the figure legends. Data were analyzed by an ANOVA and/or Student's *t* test with the SAS statistical package (SAS Institute, Inc., Cary, NC) as indicated in the figure legends. The CAT reporter plasmids without mTf promoter were used as negative controls. In response to FSH and $(Bu)_2$cAMP, the relative CAT activity of the negative control plasmid ranged from 1.5–2.

Results

A member of the WH transcription factor family, rWIN, was unexpectedly identified from the 20-day-old cultured rat Sertoli cell cDNA library while screening with the probe corresponding to the basic helix loop helix domain of REBα (30) under conditions of low stringency. The rWIN was identified due to nonspecific interactions in the screen. The rWIN clone, initially termed 6N2–2-1 in the plasmid pAD-GAL4, had an insert of 1023 bp. Sequence analysis and translation in all reading frames indicated that a consensus bHLH domain was absent from the clone 6N2–2-1. Comparison of the 6N2–2-1 sequence against known sequences in GenBank and EST databases revealed that 6N2–2-1 was identical to the

recently reported gene rWIN (GenBank accession no. U83112) isolated from the rat insulinoma (pancreatic endocrine) cell line. The reported full length of rWIN is approximately 3439 bp and codes for a 771-amino acid protein with potential start and stop nucleotide sites positioned at 87 and 2400 bp, respectively (11). The clone 6N2–2-1 therefore consisted entirely of the 3′-untranslated region of rWIN (Fig. 1).

Two different approaches, RT-PCR and Northern blot analysis, were used to confirm that rWIN is expressed in Sertoli cells. Oligonucleotide primers were designed toward the 5′-end of the rWIN sequence to amplify a 711-bp region by RT-PCR. The 711-bp PCR product corresponds to the nucleotides between 507 and 1218 and spans the DNA-binding domain of rWIN, including the potential exons 4, 5, and 6 as reported previously (11) (Fig. 1).

As shown in Fig. 2, two PCR products, designated A and B, of approximately 700 bp were detected in the reverse transcribed RNA obtained from the whole testis, cultured Sertoli cells, and germ cells. To determine their identities, PCR products A and B were isolated, subcloned, and sequenced. Sequence analysis revealed that both PCR products were nearly identical, except that the smaller PCR product B lacked the 45-bp exon 6 (Fig. 3). Interestingly, exon 4 was absent in both PCR products A and B (Fig. 3). rWIN expression was not observed in other tissues examined, such as brain, liver, kidney, lungs, and muscle (Fig. 2). The PCR data shown in Fig. 2 suggest that rWIN is also expressed in Sertoli cells. The expression of rWIN, as determined by PCR, was also observed in the testis of prenatal day 18 embryos and postnatal day 0 and 10 rats (Fig. 2). The rWIN transcript was previously shown to be expressed in the adult rat testis (11), and its human homolog HFH-11 was localized to the spermatocytes and spermatids within the seminiferous epithelium (12). The embryonic testis primarily consists of mitotically active and differentiating Sertoli cells, interstitial cells, and spermatogonia in a stage of mitotic arrest. Previous studies have shown that HFH-11 in the testis is expressed primarily in differentiating germ cells, but not spermatogonia, which suggests that the cell type(s) expressing rWIN in the embryonic testis is potentially the Sertoli cells and/or interstitial cells (12).

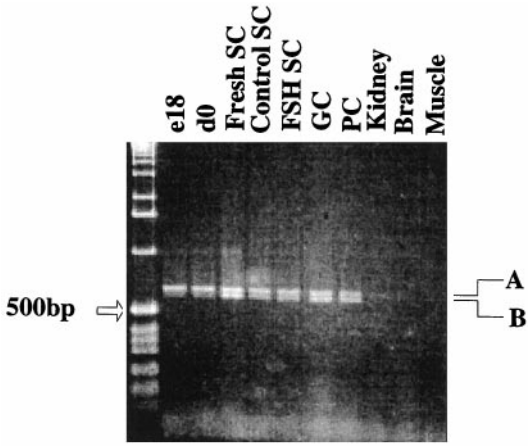

FIG. 2. RT-PCR using WH-specific primers (5′-WH507 and 3′-WH1218) on the RNA obtained from embryonic day 18 testis (e18), testis from the newly born rat (d0), freshly isolated Sertoli cells (Fresh SC), cultured Sertoli cells treated with vehicle alone (Control SC), cultured Sertoli cells treated with FSH (FSH SC), freshly isolated germ cells (GC), peritubular cells (PC), kidney, brain, and muscle. Two PCR products were obtained, which are marked A and B. The data are representative of three separate PCRs carried out on at least three different RT messenger RNA samples.

Northern blot analysis was performed to confirm that Sertoli cells express rWIN. As shown in Fig. 4A, rWIN expression was observed in the cultured Sertoli cells (control) or in cultured Sertoli cells treated with the gonadotropin FSH. Interestingly, the rWIN transcript was also observed in the RNA obtained from germ cells. The fact that only a single transcript was seen in Sertoli cells with the Northern blot as opposed to two transcripts seen in the PCR reaction suggests that the size fractionation of RNA was not sufficient to resolve the two transcripts with a 45-bp size difference due to the presence of exon 6 (Fig. 4A).

The gonadotropin FSH is known to stimulate a number of Sertoli cell genes, including c-*fos* and transferrin. The Northern blots at different exposures and from different experiments were subjected to a densitometric scan to determine whether FSH had an effect on the levels of rWIN expression. Surprisingly, stimulation of Sertoli cells with FSH for 72 h down-regulated rWIN expression ($P < 0.001$) compared with that in untreated controls (Fig. 4B).

A time-course experiment was performed to determine the dynamics of the down-regulation of rWIN expression by FSH. Cultured Sertoli cells were treated with FSH for different periods of time, and the total RNA was used to measure the expression of rWIN and transferrin genes (Fig. 5A). Transferrin was used as a marker for Sertoli cell differentiation and as an example of a gene that is up-regulated by FSH and cAMP. The quantitation of Northern blot data by densitometric analysis indicated that FSH maximally stimulated rWIN expression within 30 min to 1 h ($P < 0.001$ compared with WIN expression at time zero), which returned to basal levels by 4 h. In contrast, an increase in transferrin expression was observed only after 8 h of FSH treatment (Fig. 5B). Similar results were observed with $(Bu)_2$cAMP stimulation of Sertoli cells (Fig. 5B). Therefore, the expression of rWIN precedes the expression of transferrin and suggests a role for rWIN as a potential immediate early gene involved in the cellular/nuclear events leading to activation of the transferrin gene. The role of rWIN as an immediate early gene was supported by the observation that treatment of Sertoli cells with cycloheximide (5 μg/ml) for 2 h superinduced rWIN expression (data not shown).

An antisense approach was used to test the hypothesis that rWIN may function as an immediate early gene with a potential role in the regulation of transferrin gene expression. Cultured Sertoli cells were transiently transfected with the CAT reporter construct under the control of the proximal −581 bp of the mouse transferrin promoter. After transfection, the Sertoli cells were treated with 15 bp rWIN antisense phosphothioate-modified oligonucleotide and FSH (Fig. 1). The cells were treated with the antisense oligonucleotide (4 μM) every 12 h for a total of 72 h before harvesting cells for the CAT assay. As a control the cells were either not treated with an antisense oligonucleotide or were treated with scrambled rWIN or a sense oligonucleotide. FSH and cAMP significantly stimulated CAT activity above basal levels in

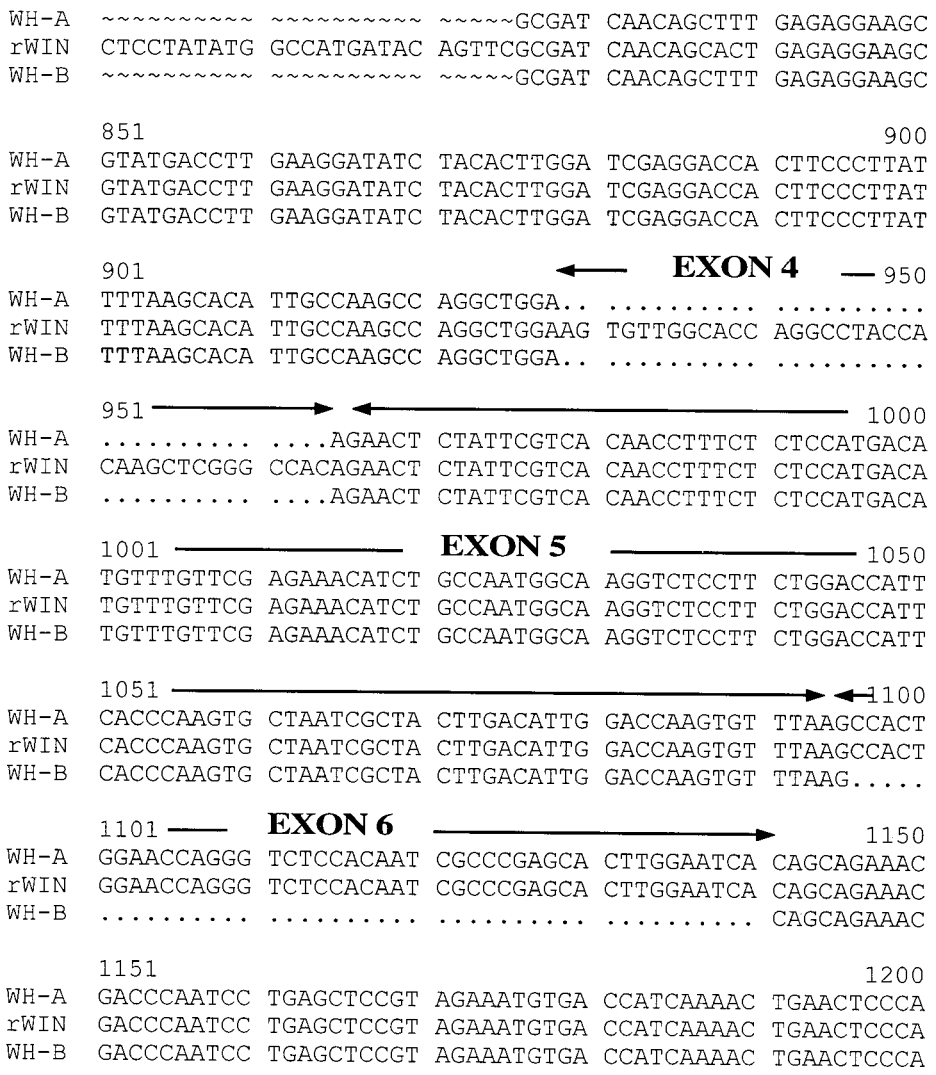

FIG. 3. Alignment of the partial nucleotide sequence of PCR products A (WH-A) and B (WH-B) obtained by PCR using primers 5′-WH507 and 3′-WH1218 to the published sequence of rWIN (accession no. U83112). PCR product A lacked exon 4, whereas PCR product B lacked both exons 4 and 6.

the absence of any oligonucleotide (Fig. 6). The presence of scrambled or sense oligonucleotide did not significantly change FSH- or cAMP-stimulated transferrin promoter activity compared with the control value (Fig. 6). However, in the presence of an antisense oligonucleotide a significant repression of FSH-stimulated transferrin promoter activity was observed (Fig. 6). The observations suggest that rWIN expression is sensitive to antisense inhibition and appears to be involved in FSH-stimulated transferrin promoter activation.

The rWIN DNA-binding domain was constitutively expressed (WH-pREP7) in cultured Sertoli cells along with the 600-bp transferrin promoter-CAT reporter construct to further examine the role of rWIN in the regulation of transferrin promoter activation. This rWIN DNA-binding domain construct does not appear to contain the potential *trans*-activation domain, so it can be used as a dominant negative construct. The transiently transfected Sertoli cells were either left untreated or were treated with FSH or cAMP for 72 h before harvesting for the CAT assay. As shown in Fig. 7, overexpression of the rWIN DNA-binding domain significantly down-regulated the −581 kb proximal transferrin promoter activity in response to FSH or cAMP. Similar results were obtained when the 3-kb proximal transferrin promoter was cotransfected with WH-pREP7 overexpression plasmid (data not shown). Transfection of various amounts (200 ng to 2 μg) of WH-pREP7 plasmid showed a concentration-dependent decrease in transferrin promoter activity (data not shown). The optimum concentration of 1 μg rWIN expression plasmid was then used for all of the overexpression experiments. The activity of the mouse transferrin promoter in response to FSH did not change significantly in the presence or absence of pREP7 plasmid alone. To determine whether the down-regulation was due to the direct binding of rWIN to a corresponding *cis* element(s) in the transferrin promoter, the promoter was analyzed for the rWIN DNA-binding domain AGATTGAGTA (11). The absence of a consensus rWIN DNA-binding domain in the transferrin promoter suggests that the down-regulation may not be due to the direct binding of rWIN to its corresponding *cis* element on the transferrin promoter.

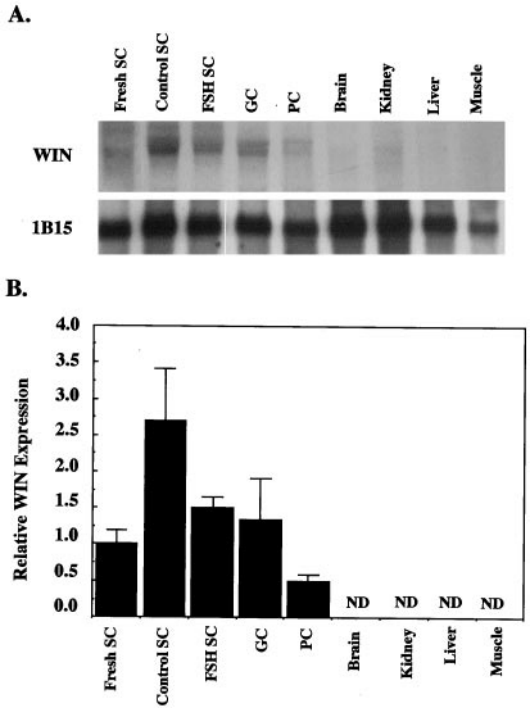

FIG. 4. A, Northern blot analysis of rWIN. The predicted transcript size of approximately 3.4 kb was detected with the 711-bp PCR probe (WH507-WH1218). Also at the *bottom* is the blot for the constitutively expressed cyclophilin gene (1B15). The data are representative of three different Northern blots performed on separate RNA samples collected at different times. B, Scanning densitometry of the blots was used to quantitate the relative expression of rWIN. Data were normalized for cyclophilin (1B15) expression. The data are presented as relative expression (mean ± SEM) in relation to the expression of WIN from freshly isolated Sertoli cells (which was set at 1). SC, Freshly isolated Sertoli cells or cultured Sertoli cells treated with FSH or vehicle alone (control); GC, germ cells; PC, peritubular cells.

Discussion

The current study identifies and examines the potential functional significance of rWIN, a newly discovered member of the WH transcription factor family, in testicular Sertoli cells. The WH proteins participate in the differentiation of diverse cellular lineages and function in pattern formation during embryogenesis (1, 2). The rat rWIN (11) and its human and mouse homologs, HFH-11 (12) and Trident (13), respectively, were recently identified and shown to have distinct patterns of expression during embryonic development and in the adult. The only tissue with overlapping patterns of expression for HFH-11 and rWIN is the adult testis. The specific cell types in the testis expressing rWIN are not known, but HFH-11 has previously been localized to the spermatocytes and spermatids, but not spermatogonia (12). The presence of contaminating Sertoli cells in freshly isolated germ cells may account for the rWIN PCR product in reverse transcribed germ cell RNA. The current study reports the presence of rWIN transcript in Sertoli cells, as determined by RT-PCR and Northern blot experiments. This was further confirmed by the observation that WIN expression is regulated by the gonadotropin FSH.

Long term stimulation (72 h) of cultured Sertoli cells with FSH decreased rWIN expression. The dynamics of the regulation of rWIN expression suggest that rWIN is transiently up-regulated within 30 min of FSH stimulation. Such a transient increase in rWIN expression is analogous to the induction of the immediate early gene c-*fos* (31). The expression of the transferrin gene correlates with Sertoli cell differentiation and is up-regulated only after rWIN expression approaches basal levels, which is approximately 4 h after FSH stimulation. The transient increase in rWIN expression precedes the increase in transferrin gene expression. Observations suggest a role for rWIN as a potential immediate early gene that may be required to switch on the network of regulatory genes involved in Sertoli cell differentiation.

The treatment of Sertoli cells with an antisense oligonucleotide down-regulates FSH-induced transferrin promoter activity and provides direct evidence for the role of rWIN in regulating Sertoli cell gene expression. Constitutive expression of the rWIN DNA-binding domain also down-regulated FSH-induced transferrin promoter activity. The data obtained from antisense and overexpression experiments support the time course of rWIN and transferrin gene expression in response to FSH. The transient up-regulation of rWIN within 30 min of FSH stimulation can be blocked by antisense oligonucleotides to rWIN, which may be required to switch on the subsequent regulatory network of genes involved in or leading to transferrin gene expression. The repression of transferrin promoter activity by the constitutively expressed rWIN DNA-binding domain represents a condition in which rWIN expression is neither transient nor under any hormonal control. The down-regulation of rWIN that precedes the transferrin expression may be a critical step, as has been shown with c-*fos* expression. Such a down-regulation of rWIN may be required for the initiation of Sertoli cell differentiated functions. The ability of rWIN to act as a transcriptional repressor is consistent with the proposed function of HFH-11. Overexpression of HFH-11B, a spliced variant that lacks exon B, is a transcriptional repressor, whereas the HFH-11A isoform, incorporating both exons A and B, has no transcriptional activity (12). The rWIN sequence used in the present study lacked both exon 4 and exon 6, which may have resulted in the transcriptional repression observed in the present study.

The regulatory network of genes controlling transferrin gene expression appear to involve intermediate transcription factors regulated by rWIN, because a consensus rWIN-binding domain (AGATTGAGTA, SAAB5–2) (11) was absent from the mouse transferrin promoter used in the present study. It is speculated that rWIN activity may be dependent on FSH-mediated phosphorylation events. Observations suggest that rWIN may function as an immediate early gene whose transient expression is important for FSH actions on Sertoli cell function. Both the time-course, antisense inhibition, and cycloheximide experiments support this conclusion.

The expression of HFH-11 in the cortical epithelium and mesenchyme of the embryonic kidney is transient and is absent in the adult (12). Such a transient expression of HFH-11 is also observed in the proliferating cells of the embryonic intestine, lung, and liver. The persistent expression of rWIN in both the embryonic and adult testes is a novel observation and suggests a role for rWIN in regulating the differentiation of a particular cell type(s) in the testis. The role

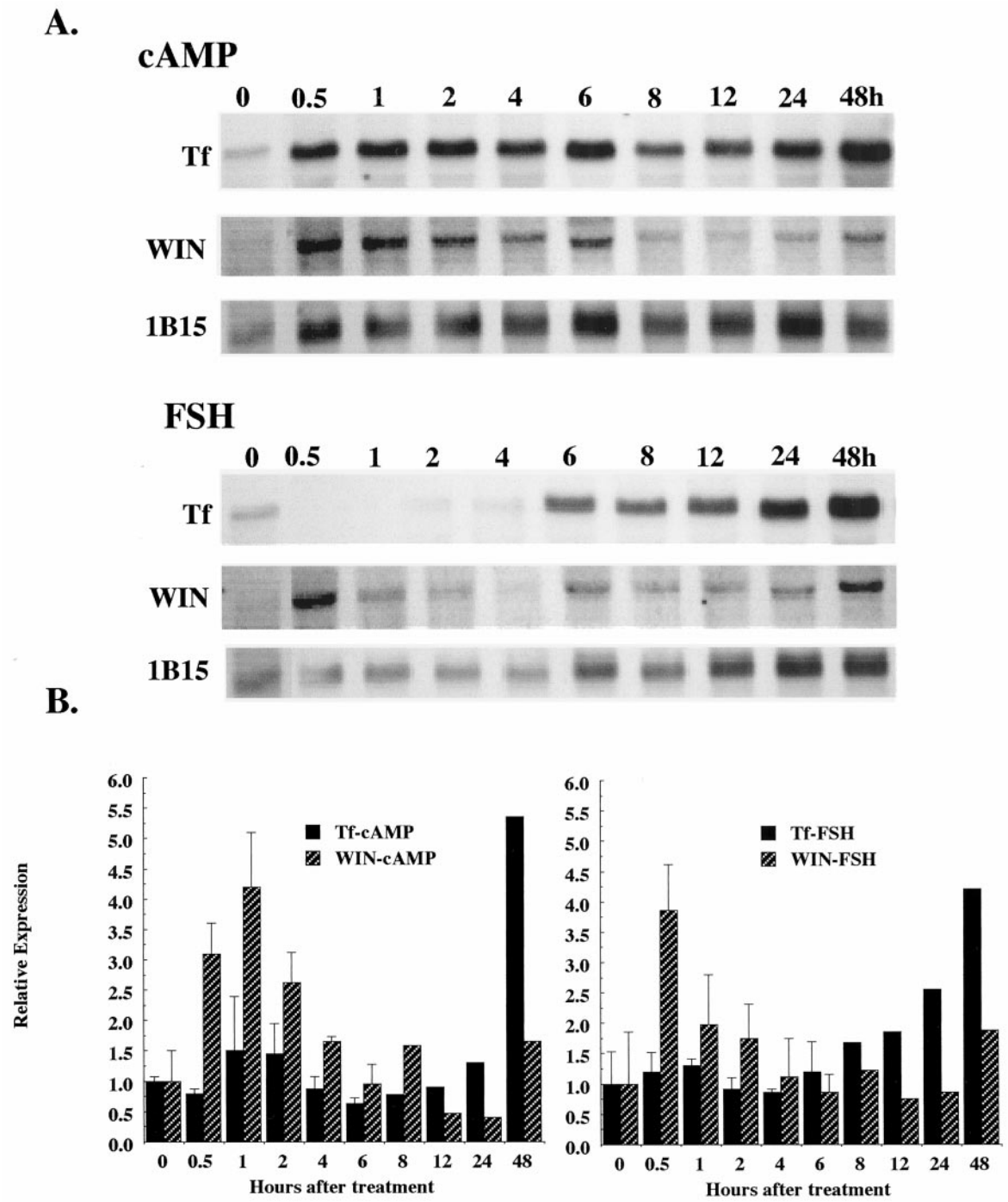

FIG. 5. A, Northern blot analysis for the expression of rWIN and transferrin messenger RNA by Sertoli cells in response to FSH and cAMP at various time intervals. The expression of the constitutively expressed gene cyclophilin (1B15), shown in the *bottom panel* of each treatment, was used to monitor the integrity and the amount of messenger RNA loaded for each treatment. B, The scanning densitometric analysis to quantitate the change in the expression of rWIN and transferrin in response to cAMP and FSH over the period of the treatment. The data are normalized to the cyclophilin (1B15) gene and are combined from three different experiments. Data are presented as relative expression (mean ± SEM) in relation to WIN and Tf gene expression (which was set at 1) at time zero.

of HFH-11 in cellular differentiation is suggested by the studies involving human colon carcinoma cell line Coco-2. HFH-11 is expressed in Coco-2 cells that are beginning to differentiate, implying that HFH-11 may regulate genes mediating the transition between proliferating intestinal epithelial cells and enterocyte differentiation that occurs after stimulation of the cell cycle (12). A similar role for rWIN in Sertoli cells may be predicted. In Sertoli cells, rWIN may be involved in initiating and subsequently maintaining Sertoli cell differentiation. Support for this hypothesis is provided

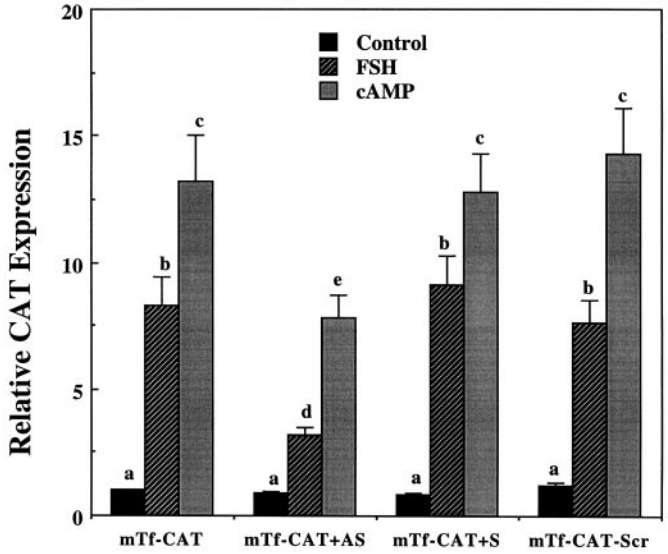

FIG. 6. Effect of antisense oligonucleotide to rWIN on the activity of the transferrin promoter-CAT construct. The cultured Sertoli cells were transfected with the proximal 600-bp mouse transferrin promoter-CAT construct (mTf-CAT). Immediately after transfection, rWIN antisense (AS), sense (S), or scrambled (Scr) phosphothiote-modified oligonucleotides (4 μM) were added. The cells were challenged with FSH or $(Bu)_2$cAMP 2 h after the addition of oligonucleotides. The ologonucleotides were subsequently added every 12 h until the cells were harvested for CAT assay (72 h). The data are presented as the relative CAT activity of the mTf-CAT control (without any treatment and in the absence of oligonucleotide; set at 1) and are the mean ± SEM of triplicate samples in three separate experiments. *Different superscript letters above error bars* represent a statistically significant difference ($P < 0.001$).

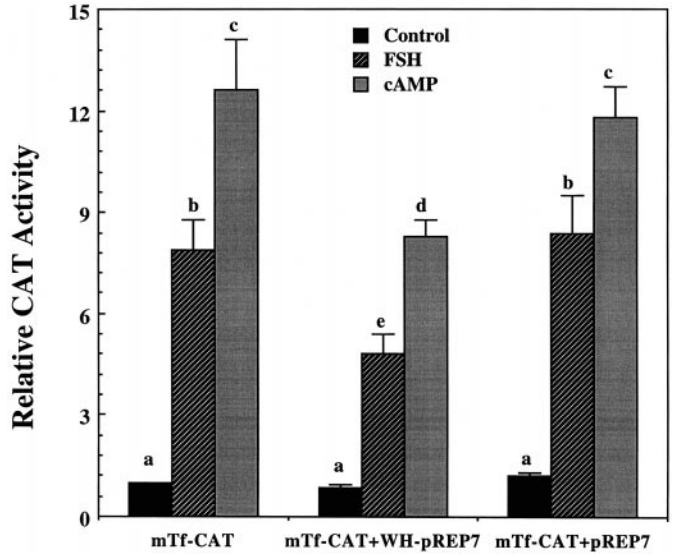

FIG. 7. Effect of rWIN overexpression on the activity of the proximal 600-bp mouse transferrin promoter-CAT. The cultured Sertoli cells were transfected with transferrin promoter-CAT construct or co-transfected along with pREP7 or the overexpression plasmid WH-pREP7. After transfection the cells were either untreated (control) or were treated with FSH or $(Bu)_2$cAMP (cAMP) as indicated. The data are presented as the relative CAT activity of the mTf-CAT control (without any treatment and in the absence of expression plasmid; set at 1) and are the mean ± SEM of triplicate samples in three separate experiments. *Different superscript letters above error bars* represent a statistically significant difference ($P < 0.001$).

by the observations that rWIN is involved in regulating the transferrin gene, which in the current study is used as a marker of Sertoli cell differentiation. Interestingly, the expression of rWIN is also regulated by FSH.

Apart from maintaining Sertoli cell differentiation in the postpubertal testis, FSH also regulates Sertoli cell division during embryonic and prepubertal stages of development (16). The molecular mechanisms involved in such a biphasic action of FSH from proliferation followed by differentiation are largely unknown. The expression of rWIN in postpubertal and possibly embryonic and postnatal Sertoli cells provides important insights into the molecular mechanisms involved in the control of Sertoli cell function. The low levels of rWIN and a transient increase in its expression may be critical events for the Sertoli cells to maintain their differentiated stage.

In conclusion, the observations presented demonstrate that the expression of rWIN in Sertoli cells is regulated by FSH. rWIN may regulate Sertoli cell gene expression and, in particular, the expression of transferrin. Future studies will be focused on understanding the possible role of rWIN during mesenchymal to epithelial transition of Sertoli cells, identification of intermediate gene targets of rWIN, the possible hormonal regulation of differential splicing, and its significance during various stages of Sertoli cell development. Analysis of testicular morphology in Trident$^{-/-}$ knockout mice will also be performed (14).

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