A novel gonadotropin-regulated testicular RNA helicase

A new member of the DEAD-box family*

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A gonadotropin-regulated testicular RNA helicase (GRTH) was identified and characterized. GRTH cloned from rat Leydig cell, mouse testis, and human testis cDNA libraries is a novel member of the DEAD-box protein family. GRTH is transcriptionally up-regulated by chorionic gonadotropin via cyclic AMP-induced androgen formation in the Leydig cell. It has ATPase and RNA helicase activities and increases translation in vitro. This helicase is highly expressed in rat, mouse, and human testes and weakly expressed in the pituitary and hypothalamus. GRTH is produced in both somatic (Leydig cells) and germinal (meiotic spermatocytes and round haploid spermatids) cells and is developmentally regulated. GRTH predominantly localized in the cytoplasm may function as a translational activator. This novel helicase could be relevant to the control of steroidogenesis and the paracrine regulation of androgen-dependent spermatogenesis in the testis.

The closely related gonadotropins, luteinizing hormone (LH) and human chorionic gonadotropin (hCG), exert their functions through specific G protein-coupled receptors in gonadal cells. Physiological concentrations of gonadotropins maintain the steroidogenic function of the Leydig cells of the testis (see Ref. 1 for review). However, high concentrations of gonadotropins cause desensitization of steroidogenic enzymes of the androgen pathway, with consequent reduction of testosterone formation (2–5). The attenuation of steroidogenesis results from receptor activation by the gonadotropic hormone but is independent of the subsequent phase of receptor down-regulation. The control of the enzymes involved in this regulation including 3β-hydroxysteroid dehydogenase types I and II (3), 17α-hydroxylase/17, 20 lyase (5), and 17β-hydroxysteroid dehydrogenase type III (4) operates at the transcriptional level. Because this process affects several enzymes, the direct or indirect involvement of a master switch has been proposed (3).

Likely candidates for this regulation include the active steroid metabolites that are produced during gonadotropin stimulation, i.e. androgen (3) and estrogen (6–8). To identify potentially relevant proteins that participate in gonadotropin up- or down-regulation of steroidogenic enzymes at the transcriptional or post-transcriptional level, we utilized differential display analysis of mRNA from intact Leydig cells subjected to a single exposure of gonadotropin in vivo. We report the demonstration of a novel gonadotropin-regulated testicular RNA helicase (GRTH) that belongs to the family of DEAD-box proteins and is predominantly expressed in Leydig and germinal meiotic cells of the testis. This protein was found to be markedly up-regulated in the Leydig cell by hCG doses that cause steroidogenic desensitization. It is likely that GRTH serves in general to maintain receptors, enzymes, and factors that support testicular functions and spermatogenesis.

EXPERIMENTAL PROCEDURES

Animal Treatment and Cell Preparation—Gonadotropin-induced LH receptor and steroidogenic enzymes down-regulation of Leydig cells was produced as described previously (2) by administration of a single 2.5-μg subcutaneous injection of hCG (Pregnyl, Organon) to adult male rats (200–250 g) (Charles River Laboratories Inc., Wilmington, MA). Animals were sacrificed 24 h after hCG treatment, and testes were removed. Leydig cells were prepared by collagenase dispersion and purified by centrifugal elutriation as described previously (9). The cells were frozen at –70 °C until the further extraction of RNA. Following the dispersion to obtain Leydig cells, the seminiferous tubules were further treated with collagenase (0.05%) until no interstitial cells remained adhered to the tubules as assessed under inverted microscope. Tubules were minced into 1–2-mm segments, resuspended in Medium 199, 0.1% BSA, and shaken at 120 rpm/min for 20 min at 37 °C. The fragments were allowed to settle for 5 min, and germ cells were collected from supernatant by centrifugation.

In Vitro Study of the Hormonal Regulation of GRTH Leydig Cells in Primary Culture—Leydig cells were plated (1 × 10⁶ cells/ml) for 1 h and incubated with hCG or 8-bromo-cAMP or buffer (control) for 20 h. In studies conducted to examine the effects of endogenous or exogenous steroids, cells were preincubated with or without inhibitors of steroid biosynthesis (aminogluthethimide, 100 μg/mL; cyanokekten, 1 μM, and spironolactone, 10 μM (Sigma) for 20 min at 34 °C and kept throughout the culture. The cells were then incubated with hormones for 20 h. These compounds were previously found to inhibit effectively the enzymes of the steroidogenic pathway in the Leydig cells and consequently inhibited testosterone production (9–12). The enzymes blocked by the inhibitors included cholesterol side chain cleavage and aromatase (aminogluthetimide), 3β-hydroxysteroid oxidoreductase Δ5 isomerase (cyanokekten), and 17α-hydroxylase/17–20 desmolase (spironolactone). Total RNA was isolated from Leydig cells using TRIzol RNA reagent (Life Technologies, Inc.). Levels of GRTH mRNA were analyzed by Northern blot.

Differential Display Analysis—Three sets of total RNA samples (300 ng for each) independently prepared from Leydig cells of rats treated with and without hCG were analyzed by differential display essentially as described previously (13). Briefly, samples were reversed-transcribed using six downstream primers followed by PCR with the same downstream primer and 12 different upstream primers (random 10-mers). PCR products with incorporated [32P]dATP (2000 Ci/mmol, NEN Life Science Products) were separated in non-denatured 8% sequencing gels.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF142629, AF142630, AF155140.
Gels were dried and evaluated by autoradiography. Regions containing differential bands on the gel were excised and eluted by electrophoresis. The products were reamplified and resolved by single strand conformation polymorphism gels to confirm that a single differential fragment was contained in each excised band. The differential bands were eluted, reamplified by PCR, and separated on 1% agarose gel, and the agarose gel was hybridized to electrophoresis. The differential fragments were cloned and sequenced. The nucleotide sequences were compared with sequences in the GenBank™/EMBL data bases using FASTA program. The individual bands were verified by RNA protection analysis.

**RNase Protection Assay**—RNA protection assays were performed by previously published methodology (14). The 32P-labeled RNA probe was synthesized by PCR followed by subcloning and in vitro transcription. Primers were used to amplify a fragment complementary to the coding region (256–611 nt position), see Fig. 1. Also, primers located at exons 4 and 5 of the rat β-actin gene were employed to amplify the β-actin fragment (270 bp) used as control. The constructs were linearized and used as template for the in vitro transcription with RNA polymerase T7 (Life Technologies, Inc.) and labeled with [32P]UTP (800 Ci/mmol, ICN Biomed).

**Promoter-Luciferase Constructs**—Luciferase RNA poly(A) template was generated by the in vitro transcription of the full-length luciferase cDNA cloned in the pSP64 poly(A) vector (Promega). In vitro transcription assays were carried out in a 50-μl reaction with additions of purified recombinant GRTH-GST fusion protein or GST (250 and 500 ng), 20 μCi of [35S]methionine (1000 Ci/mmol, 10 μCi/ml, Amersham Pharmacia Biotech), 20 μM amino acid mixture lacking methionine, 40 units of ribonuclease inhibitor, 1 μl of luciferase RNA, and 25 μl of reticulocyte lysate that had been incubated with micrococcal nuclease containing 2.5-μg mixture of tRNAs, 1 μl of hemin, 4 μm potassium acetate, and ATP-regenerating system from Promega (phosphocreatine/phosphokinase kinase). Samples were incubated for 90 min at 30 °C. The 61-kDa radiolabeled luciferase translated protein was resolved on 10% SDS-polyacrylamide gel. Autoradiograms were quantitated by Imaging Densitometer model GS-700 (Bio-Rad) and expressed as percent of GST control.

**Expression and Purification of Rat GRTH from Bacteria**—The GRTH cDNA was subcloned into pGEX-2T glutathione S-transferase (GST) gene vector (GRTH-GST). The construct was verified by sequence and transformed into E. coli BL21. The MicroSpin™ GST Purification Module System (Amersham Pharmacia Biotech) was used for the purification of GRTH-GST fusion protein. The purified protein recovered from the columns was aliquoted and analyzed by SDS-PAGE and Western blot analyses.

**Preparation of Helicase Substrates**—The long strand of RNA (5'-GGCGGAAGUGGGUCACUACCAUGAGCCACCCCGGUGAAAG-UGAUUGUAUUU-UUUCU-UUAAUUU-3') with 5'-hydroxyl terminus complementary to the middle region of the long strand of RNA was synthesized by Craychum Inc. (Dulles, VA). All the DNA oligomers were synthesized by DNA Technologies Inc., a sequence of the long DNA oligomer corresponds to the short strand of RNA. The three short DNA oligomers (5'-TCTTAGGCGGCGGCAGCCGC-3', 5'-TAAGGTTACCCAAATGGGCCC-3', and 5'-TCTAGGCAGGGGCGCCG-3') were synthesized by Clontech (PALG-5') correspond to the mid-region, 5'-end, and 3'-end of the long RNA strand, respectively. The short strands of RNA and DNA were labeled by 32P using T4 polynucleotide kinase (Life Technologies, Inc.) and [γ-32P]ATP. Labeled short strands (15 pmol) were annealed to long strands (10 pmol) by gradually decreasing the temperature from 90 to 4 °C within 3 h in the buffer of 10 mTris-HCl (pH 7.5), 50 mM NaCl, 2 mM MgCl2, 1 mM EDTA. The annealed substrates were resolved on 4% agarose gel, eluted by electrophoresis, and precipitated by alcohol. The purified substrates were dissolved in the helicase assay buffer.

**Helicase and ATPase Activity Assays**—The helicase assay was carried out in 15 μl of buffer (20 mM HEPES-KOH (pH 7.5), 50 mM KCl, 3 mM MgCl2) containing 0.1 mg/ml BSA, 1 unit/ml RNAse inhibitor (Promega), 0.4 μM dithiothreitol, 1 mM ATP, 0.2 μM double strand substrates, and different amounts (0.1 to 1.0 μg) of purified GRTH-GST or GST (control) proteins. The mixture was incubated for 30 min at 37 °C. The ATPase assay was performed by 32P-labeled substrates boiled for 3 min were used as positive controls. Aliquot (10 μl) of each reaction were separated by 8% native polyacrylamide gel and recorded by radiography. The ATP hydrolysis activity was analyzed as described previously (18). 1.5 μl of purified GRT-P-GST (control) proteins and 0.3 A260 units of different RNAs (mRNA and total RNA from rat Leydig cell, tRNA from yeast, polyA and polyU from Life Technologies, Inc.) were used for each reaction. All experiments were performed at least three times in triplicate. Statistical significance was evaluated by analysis of variance.
RESULTS

Identification, Isolation, and Cloning of a Novel Gonadotropin-regulated Testicular Gene GRTH—To identify genes that are regulated by gonadotropin, we compared differential display patterns of RNA obtained from Leydig cells of rats treated with a single desensitizing dose of hCG (2.5 μg) or vehicle alone 24 h prior to sacrifice. Differential display PCR using 6 downstream and 12 upstream primers (see “Experimental Procedures”) revealed 30 differential candidate bands that were observed in the three different RNA samples, of which 6 were up-regulated and 24 were down-regulated. After single-strand conformation polymorphism analysis, sequencing of the fragments, and RNA protection verification, one up-regulated fragment and six down-regulated fragments were obtained. PCR amplifications using primers 5'-dT11GA-3' and 5'-GATCATGGC-3' revealed a 84-bp fragment which appeared to be up-regulated by the hormone is shown in Fig. 1A, left. This 3' fragment displayed no similarity to any gene on the Data Bank. Subsequently, RNase protection assays using the 84-bp fragment and a 355-bp GRTH cDNA derived from the coding region (see below) confirmed that this gene was up-regulated by hCG (Fig. 1A, middle). The protected fragment of β-actin mRNA (290 bp) used as an internal control in this study showed no change.

To characterize the cDNA sequence of this hCG-regulated mRNA, the 32P-labeled 84-bp PCR fragment was used as a probe to screen a rat Leydig cell cDNA library. The full-length sequence (1629 bp) is contained an open reading frame encoding 369 amino acids (Fig. 1B) (GenBank accession number AF142629). The in vitro transcribed/translated rat GRTH cDNA yielded a protein of 43 kDa (Fig. 1A, right), in agreement with the expected size from the deduced amino acid sequence of 369 amino acids (Fig. 1B). Furthermore, mLTC cells transfected by rat GRTH cDNA with a V5 tag expressed a protein of molecular size comparable to the in vitro translated GRTH protein (Fig. 1A, right). Also, the mouse and human GRTH cDNA sequences were isolated from mouse and human testis cDNA libraries. The nucleotide and deduced amino acid sequences of the mouse (GenBank accession number AF142630) displayed 96 and 99% similarity to the rat sequence, respectively, and those of the human sequences (GenBank accession number AF155140) were 86 and 95% similar to the rat.

A database search using the FASTA program of GCG revealed similarity of the rat GRTH sequence to those members of the DEAD-box family of RNA helicases (19). A phylogram produced by the PILEUP program of the GCG package based on 116 members of the DEAD-box family revealed seven highly conserved signature regions of the family (Fig. 1A) including the (A/S)XGGXGKT and DEAD domains involved in ATP binding and/or ATP hydrolysis and SAT and HRGRXXR domains involved in RNA binding and unwinding (19). It was less related to initiator factors (eIF4As) of the translation complex in several species (41–42%) (22), the human oncogene RCK homolog DDX6 (36%) (22), Drosophila Me31B required for oogenesis (37%) (22), and yeast Ste13 required during mating (37%) (25). GRTH is more distantly related to mouse PL10, a protein found in cells of the germinal epithelium (35%) (26), and human p68, a protein related to cell growth and division (32%) (27).

Chromosomal Localization of GRTH—Chromosomal mapping using human, mouse, and rat lymphocytes revealed that this gene resides in chromosome 11q24 (human), 8q21 (rat), and mouse p12 (mouse), and human sequences of GRTH contain all conserved domains of DEAD-box family of RNA helicases (underlined).

![Fig. 1. A. up-regulation of GRTH by hCG identified by mRNA differential display analysis (left) and confirmed by RNase protection assay (middle) in rat Leydig cells and in vitro translation and mammalian expression (right). Differential display analysis of three sets of total RNA samples from Leydig cells of rats treated 24 h prior with a single dose of hCG (2.5 μg) (+) or vehicle alone (−). One PCR fragment (84 bp), indicated by arrows in the cDNA sequence below, was up-regulated by hCG treatment (left). Autoradiography of RNase protected fragments. Total RNA samples (10 μg) were hybridized to 32P-labeled cRNA GRTH probes: the 84-bp probe (differential display PCR product) and a 355-bp probe (+256 to +611 nt) from the GRTH coding region (shown by arrows with asterisks) simultaneously with a β-actin fragment (290 bp) probe as internal control (middle). In vitro transcribed/translated GRTH and GRTH cDNA (GRTH-V5) expressed in mLTC cells was detected by V5 antibody (right). B, rat, mouse, and human GRTH cDNA nucleotide and deduced amino acid sequences of the rat encoded protein. The complete nucleotide sequence of the rat GRTH (1629 bp) is derived from screening a rat Leydig cell cDNA library. An open reading frame of 369 amino acids is shown below the nucleotide sequence. Differences of the human (h) and mouse (m) GRTH from the rat sequence (r) are indicated in lowercase letters above the rat sequence. The deduced amino acid rat, mouse, and human sequences of GRTH contain all conserved domains of DEAD-box family of RNA helicases (underlined).
and 9A3-A5 (mouse) (Fig. 2). A search of mouse to human homology region indicated that genes mapped in the region of mouse 9A3-A5 mostly corresponded to the region of human 11q22–24, including the potassium inwardly rectifying channel subfamily J members 5 (GIRK4), Friend leukemia virus integration 1 (FLIL) genes, and DDX6 genes (23).

Localization of GRTH by in Situ Hybridization—Cellular localization of GRTH mRNA within the rat testicular compartments revealed that GRTH transcripts were present in both interstitial and germ cells of the adult testis (Fig. 3). Some of the interstitial cells showed strong signals and others were negative. Variations were observed among the individual interstitial spaces (Fig. 3, C and D). In highly purified Leydig cell preparations (95% purity) isolated from the interstitial compartment of the adult rat testis, 60% of the cells (from 500 cells counted) were positive (Fig. 3A). Since these animals had not received exogenous hormone treatment, these observations likely reflect the stimulation/maintenance of expression by endogenous gonadotropin/androgen. Prominent and dense labeling within the seminiferous tubules of adult rats was highest in cells in meiotic phase, predominantly in pachytene spermatocytes (Fig. 3I). GRTH mRNA was also present in round spermatids of the adult rat testis (Fig. 3K). Cells within the basal compartment, including spermatogonia and preleptotene spermatocytes, were clearly negative as were the Sertoli cells (Fig. 3J). In the pubertal testis, only cells in meiotic phase, mainly pachytene spermatocytes, were positive. Again basally located cells, including Sertoli cells, were negative (Fig. 3H). Developmental studies demonstrated that GRTH was not present in the testis of immature animals (8 and 12 days old, data not shown), but positive signals were present in tubules and Leydig cells of pubertal animals (23 and 26 days) and in the adult testis. Under low magnification (×5), it was evident that at the pubertal stage some tubules were frankly positive, and others showed low positivity (indicated by the presence of a bright or pale ring, respectively, within the seminiferous tubule) (Fig. 3F). In contrast, in the adult testis all tubules were positive, with some gradations in grain density (not shown). These studies, and those presented in Fig. 4A, demonstrate that the ex-
expression of GRTH in the testis is developmentally regulated.

Tissue and Cell Distribution of GRTH Transcripts—The tissue distribution of the GRTH transcript studied by Northern blot analysis detected a single transcript of 1.6 kb. In Fig. 4A, it is shown that GRTH is predominantly expressed in the testis and less abundantly in the hypothalamus, pituitary, and brain but was not expressed in ovary or other tissues examined (Fig. 4A, left panel). GRTH mRNA was detected in Leydig cells (L.C.), and its abundance was comparable to that observed in germ cells (GC). In germinal cells, mRNA levels were very similar in both hCG-treated and untreated groups in contrast to the mRNA of Leydig cells from hCG-treated adult animals (+) which was significantly increased when compared with control. Northern blot analysis also detected a single transcript of 1.6 kb and rehybridized with cDNA β-actin probe. B, time course study of GRTH mRNA regulated by hCG. Leydig cells from male rats injected with 2.5 µg of hCG after 1–12 h treatment (short term) and 1–9 days (long term). RNase protection assays were performed for the short term study, and Northern blot analyses were performed for long term study. Data normalized by β-actin are presented as percent mean ± S.E. relative to saline control. C, stability of GRTH mRNA (left) and nuclear run-off assay (right). Gonadotropin-desensitized Leydig cells were obtained 12 h after treatment from rats with a single dose of hCG (2.5 µg). Cells were incubated with 10 µg/ml actinomycin D for 0–10 h. The mRNA samples (10 µg) were analyzed by Northern blot (left). Nuclei prepared from control and hCG-desensitized Leydig cells in the presence of [35S]UTP were analyzed by nuclear run-off assay (right). The newly transcribed RNAs were hybridized to a nitrocellulose membrane on which cDNAs of GRTH (20 µg), β-actin (20 µg), and vector (20 µg) have been immobilized. Results were recorded by autoradiography for visual display (upper) and quantified by PhosphorImager (lower). Data are percent of means ± S.E. relative to control.

Up-regulation of GRTH mRNA by hCG at Transcriptional Level—To explore whether increases in GRTH mRNA induced by hCG were related to changes in mRNA stability, we assessed GRTH mRNA degradation rates. The mRNA levels at different times after treatment with actinomycin D were generally higher in the samples from hCG-treated animals than in controls (Fig. 4C). Only 20% of GRTH mRNA was degraded after 10 h of actinomycin D treatment in samples from both treated and non-treated groups which indicated that the half-life of GRTH mRNA in Leydig cells was very long. The degradation rates between the two groups were very similar. This indicated that up-regulation of GRTH mRNA in the Leydig cells was not due to stabilization of the mRNA by hCG but rather to a change in transcriptional activity. Nuclear run-off assays demonstrated that newly synthesized GRTH 32P-labeled Leydig cell mRNA from hCG-treated animals increased when compared with non-treated controls, whereas β-actin (control) remained unchanged (Fig. 4C).

In Vitro Study of the Hormonal Regulation of GRTH in the Primary Cultures of Leydig Cells—To elucidate further the mechanism of hCG-induced GRTH mRNA increase in Leydig cells, in vitro studies were performed in primary Leydig cell.
Leydig cells treated with hCG (10 ng/ml) or 8-bromo-cAMP (1 mM) were quantified by PhosphorImager (counts were normalized by the regulation of transcription. This was supported by the observation that a construct bearing a promoter of a gene not expressed in the Leydig cell (SV-40) was also stimulated by GRTH. In contrast, simultaneous quantitation of luciferase activity was evaluated as indicative of luciferase protein expression. hLHR174 and r-LHR174, human and rat luteinizing hormone receptor promoters (36, 37); PRLR-P1, rat prolactin receptor promoter-1 (38); P450–17a, rat 17α-hydroxylase/17,20 lyase promoter (39); SV40, eukaryotic simian virus-40 promoter. Right, quantitation of luciferase mRNA by RNA protection assay. A portion of mLTC cells cotransfected with pBK-GRTH or pBK with r-LHR174/luciferase was used for luciferase mRNA quantitation. Data are presented as percent of means ± S.E. relative to pBK control. B, GRTH effect on in vitro translation. Data are expressed as percent increases of luciferase RNA translation induced by GRTH-GST over the GST control (250 and 500 ng of GRTH-GST or GST). C, subcellular localization of GRTH in mLTC by confocal microscope. Expression of fusion protein GRTH-EGFP or EGFP alone (control) in the living cells was examined by confocal microscope under a 40 × oil objective lens. Green fluorescence signals from GRTH-EGFP with lower amplification (a, zoom = 2) and higher amplification (b, zoom = 5) and from EGFP (control) with higher amplification (c, zoom = 5) were recorded (bars = 10 μm). D, Western blot analysis. GRTH-GST and GST were revealed by a GFP antibody. Molecular mass values are indicated on the left.

Exploring the Function of GRTH—To examine whether GRTH was involved in the regulation of Leydig cell steroidogenic enzymes and receptors, and to gain further insights into the cellular function of GRTH, we evaluated the changes induced by cotransfected GRTH (GRTH-pBK) on luciferase activity generated from constructs containing the luciferase reporter gene driven by promoters of relevant Leydig cell genes in mLTC cells (Fig. 6A, left). An increase of 50–80% in luciferase activity over controls (pBK) was observed with the four constructs bearing relevant promoters. This finding demonstrated that the hCG-induced down-regulation of the expression of these genes observed in our previous studies (3–5) was not related to the up-regulation of GRTH. The similar increase in activity with the four gene promoters indicated that the GRTH action was related to a general mechanism not concerned with the regulation of transcription. This was supported by the observation that a construct bearing a promoter of a gene not expressed in the Leydig cell (SV-40) was also stimulated by GRTH. In contrast, simultaneous quantitation of luciferase activity with the four gene promoters indicated that the GRTH activity was mediated through the action of androgens in Leydig cells. GRTH levels in controls were due to the presence of endogenous androgens, estrogen, and their precursors (10). Dihydrotestosterone increased GRTH mRNA level by 40% in primary cultures of Leydig cells, in which the production of endogenous steroids had been blocked by the addition of inhibitors (Fig. 5B). In contrast, estradiol had no effect on GRTH expression. Thus, the fact that hCG was unable to stimulate GRTH expression when endogenous production of androgen action was blocked by the inhibitors of steroidogenic enzymes, coupled to the finding that dihydrotestosterone was able to elicit similar increases as hCG in these cells, strongly indicated that hCG-induced GRTH expression was mediated through the action of androgens in Leydig cells. GRTH levels in controls were due to the presence of endogenous androgens in the cells, and these were not changed in cultures where steroidogenesis was inhibited. This is expected since the incubation time of the primary cultures was relatively short and the half-life of GRTH is long to reveal the impact of the reduced androgen levels.

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mRNA by RNase protection assay in mLTC cells cotransfected with GRTH cDNA (pBK-GRTH) revealed no changes when compared with cells cotransfected with pBK empty vector, lacking GRTH (Fig. 6A, right). This finding supports the notion that the increase of luciferase activity induced by GRTH was not due to transcriptional stimulation of the gene promoter but likely to changes at the translational level. In vitro translation studies demonstrated that recombinant GRTH-GST (250 and 500 ng) increased translation of luciferase RNA template over GST control by 100 and 200%, respectively (Fig. 6B). This further indicated the potential role of GRTH in translation.

Subcellular localization of transfected GRTH tagged with the enhanced green fluorescence protein (GRTH-EGFP) in mLTC cells by confocal microscopy illustrated that GRTH-EGFP was present predominantly in the cytoplasm (Fig. 6C, a and b), whereas EGFP (control) was present throughout the nucleus, membrane, and cytoplasm (Fig. 6C, c). It could not be excluded completely the presence of GRTH-EGFP in the nucleus since vestigial fluorescence was observed in some transfected cells. The specificity of GRTH-EGFP expression was assessed by Western blot using an anti-GFP monoclonal antibody (Fig. 6D). A single band of 59 kDa which corresponded to the GRTH-EGFP fusion protein (42 kDa, GRTH, 27 kDa, EGFP) was observed in cells transfected with GRTH-EGFP, whereas a band of 27 kDa was observed in EGFP-transfected cells. No bands were detected in the negative control cells.

**Purified Recombinant GRTH Displayed ATPase and RNA Helicase Activity**—The purified recombinant GRTH-GST fusion protein (71 kDa) and recombinant GST (29 kDa) resolved as single bands on stained SDS-PAGE (Fig. 7A, left) were utilized to assess the function of GRTH. Western blots displayed single immunostained bands that migrated at positions that corresponded to the stained bands (Fig. 7A, right). Whereas the purified GST used as control showed no ATP hydrolyzing activity (Fig. 7B), the GRTH-GST displayed significant ATPase activity, which was further stimulated 3-fold by the addition of mRNA and poly(A) nucleotides. Addition of poly(U), total RNA, and tRNA only increased the activity by 20–40%, whereas DNA caused a 2.5-fold increase in activity. Omission of MgCl2 or addition of EDTA in the reaction completely abolished the activity (data not shown). These results demonstrated that GRTH possessed intrinsic ATPase activity with an absolute requirement for Mg2+ and that RNA and DNA increase the ATP hydrolyzing activity of GRTH.

To evaluate the RNA helicase activity of GRTH, we synthesized different duplexes of RNA/RNA, RNA/DNA, and DNA/ DNA as substrates (Fig. 7C, upper panels) and incubated with purified GRTH-GST or GST (control). A single stretch of at least 30 bp at both 5' and 3'-ends of RNA or DNA was provided for the binding of GRTH-GST protein. The double strands of RNA/RNA and DNA/DNA were unwound by GRTH-GST in a dose-dependent manner (0.1–10 μg), whereas the GST (negative control) did not show activity (Fig. 7C). Omission of ATP from the reaction abolished GRTH unwinding activity. In contrast, double-stranded DNA could not be unwound by GRTH-GST. Duplex substrates with a 5'- or 3'-single-stranded RNA tail (provided as an unwinding initiation site) were utilized to determine the directionality of unwinding induced by GRTH. GRTH-GST catalytically unwind both 3'- and 5'-duplexes in a dose-dependent manner with the requirement of ATP, whereas GST controls were inactive. These results demonstrate that GRTH is an ATP-dependent bidirectional RNA helicase.

**DISCUSSION**

In this study, we have cloned and characterized a novel gonadotropin-regulated and developmentally expressed testicular RNA helicase (GRTH). This protein, which is expressed in the Leydig and germ cells of the testis, belongs to the DEAD (Asp-Glu-Ala-Asp)-box family within the superfamily of RNA helicases (28) and contains all the conserved domains of the family. GRTH differs from other members of this family in having high intrinsic ATPase activity in the absence of RNA but resembles several members (19, 21, 27), in that its ATPase activity was remarkably enhanced in presence of mRNA, synthetic poly(A), and DNA. Thus, GRTH not only catalyzed ATP hydrolysis to supply energy for RNA processes but perhaps also for some other biological processes. The DNA-enhanced ATP hydrolysis is not utilized for the process of unwinding DNA duplexes. Bidirectional unwinding of RNA duplexes suggested that GRTH is not only a less restricting ATPase but also a less restricting RNA helicase. These functional features indicate that GRTH could be involved in a variety of biological processes in the target tissues. It is very likely that GRTH participates in poly(A)+-related mRNA processes including melting the secondary structure of mRNA and initiation of translation.

GRTH is the first protein of the DEAD-box family reported to be regulated by a hormone. The initial goal of the study was to identify factor(s) responsible for gonadotropin down-regulation of gonadal receptors and steroidogenic enzymes of testicular...
Leydig cells that would consequently reflect the production of androgen. On the other hand, for maintenance of the expression of relevant and/or general cellular genes and the restoration of the cellular down-regulated functions, some activators may be induced or enhanced during the initial hormonal stimulus or early in the desensitization process. GRTH was the only displayed fragment that was verified as an hCG up-regulated gene. However, the regulation of GRTH increase by hCG did not preclude the reduction of these enzymes (2–5). This suggested that GRTH does not play a role in the down-regulation phase of the desensitizing process but perhaps in other gonadotropin-regulated cellular functions, including maintenance of steroidogenic enzymes and receptors and their recovery from desensitization. In this regard, the return of GRTH expression to control correlated with the temporal recovery of enzymes and receptors (3–5).

We also demonstrated that the up-regulation of GRTH gene expression results from direct and/or indirect actions at the transcriptional level of signaling molecules or metabolic products induced by hCG. The regulation of GRTH could share aspects of the control mechanism(s) responsible for hCG-induced down-regulation of steroidogenic enzymes and receptors. Our cotransfection studies excluded the involvement of GRTH as a transcriptional inhibitor responsible for hCG-induced desensitization of receptors and steroidogenic enzymes (Fig. 6A). The similar GRTH-induced increases of reporter gene activities driven by different promoters in the absence of mRNA changes implied that the stimulating action of GRTH was through a common mechanism at the post-transcriptional level. The increased in vitro translational activity induced by GRTH and the predominant localization of GRTH-GFP in the cytoplasm of transfected cells (Fig. 6, B and C) support its involvement in the translational process. However, its participation in other biological processes cannot be excluded, since weak nuclear localization was observed, and both RNA and DNA can stimulate ATPase activity of GRTH. Thus, it is conceivable that the up-regulation of the GRTH gene product induced by the hormonal stimuli may contribute to the translatability of hormone receptors and steroidogenic enzymes and other proteins.

GRTH is specifically expressed in male but not in female gonadal tissue. In addition to its expression in the Leydig cells of the interstitial compartment of the testis, GRTH is also highly expressed in meiotic and round haploid germinal cells (Fig. 3). Differentiation in germ cells requires enhanced activation of transcription and translation of specific genes in which GRTH may play important roles. Interestingly, some members of the RNA helicase family were expressed differentially during development or restrictively distributed in germ cells (24–26, 29), and it has been suggested that these proteins may actively participate in sexual development (25) and in the process of oogenesis (24, 29) and spermatogenesis (26). The structure and function of GRTH links this new helicase with other members of the RNA helicase family, including eIF4A (22, 30), Ded1p (31), and PL10 proteins (26).

The male gonad-specific expression of GRTH and the striking increase of its mRNA in pubertal and post-pubertal rodents suggest that GRTH is functionally related to gonadal maturational and spermatogenesis. The developmental increase in GRTH in the pubertal rodent testis occurs at the same time as gonadotropin-induced androgen responses appear in the Leydig cell (32), and when the differentiation of germ cells that ultimately leads to the development of haploid spermatids begins to occur (33). It is reasonable to propose that the steroid hormone produced in the gonadotropin-stimulated testis could not only exert intracrine or autocrine actions on GRTH gene expression in the Leydig cell but may also influence GRTH gene transcription within the seminiferous tubule. The notably high expression of the helicase in pachytene spermatocytes indicates that GRTH may be one of the genes involved in the meiotic process that has a crucial role in spermatogenesis (34).

The specific expression of GRTH in the testis, but not in ovary, implies that its gene is either suppressed by female-specific or induced by male-specific gonadal factor(s). Since estrogen is produced in both male and female gonads, it is unlikely to be involved in the induction of GRTH gene expression. Consistent with this, estradiol did not affect GRTH mRNA in cultured rat Leydig cells. In contrast, the non-metabolizable androgen, dihydrotestosterone, increased the expression of GRTH mRNA in Leydig cells. The stimulation effects of hCG in vivo on the GRTH gene expression were also observed in vitro, and cAMP, the second messenger of hCG action, had comparable effects. However, no stimulation was observed after inhibition of steroid biosynthetic enzymes, which abolished hCG- and cAMP-stimulated increases of testosterone in Leydig cells. The absence of a rapid change in basal GRTH mRNA levels during androgen suppression in vitro is not unexpected, since incubations could only be conducted with short term primary cultures and the GRTH mRNA is long-lived (Fig. 4C). Androgen induced by gonadotropin could act through its cognate receptors in the Leydig cell to increase GRTH gene expression. It could also exert actions in the germinal cells of the seminiferous epithelium through androgen receptors present in Sertoli cells, since germinal cells do not possess androgen receptors (35). Although androgens are not present in the pituitary, hypothalamus, and brain, where low GRTH mRNA levels are found, androgen receptors are present in these tissues. It is conceivable that androgen from the circulation may induce GRTH expression at these sites. Major developmental changes in GRTH mRNA were observed during puberty when predominant expression of GRTH mRNA was observed in the Leydig cell and germinal epithelium. Our findings suggest that this novel testicular helicase could serve to maintain testicular functions related to steroidogenesis and spermatogenesis.

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REFERENCES
1. Dufau, M. L. (1988) Annu. Rev. Physiol. 50, 483–508
2. Dufau, M. L., Cigorraga, S. B., Baikul, A. J., Bator, J. M., Sorrell, S. H., Neuhauer, J. F., and Catt, K. J. (1979) J. Steroid Biochem. 11, 193–199
3. Tang, P. Z., Tsai-Morris, C. H., and Dufau, M. L. (1998) Endocrinology 138, 4596–4605
4. Tsai-Morris, C. H., Khanam, A., Tang, P.-Z., and Dufau, M. L. (1999) Endocrinology 140 3534–3542
5. Nishihara, M., Wintem, C. A., Buko, E., Waterman, M., and Dufau, M. L. (1988) Biochem. Biophys. Res. Commun. 154, 151–158
6. Dufau, M. L., and Catt, K. J. (1978) Vitam. Horm. 36, 461–592
7. Nozu, K., Dehejia, A., Zawistowski, L., Catt, K. J., and Dufau, M. L. (1981) J. Biol. Chem. 256, 12875–12882
8. Nozu, K., Matsuura, S., Catt, K. J., and Dufau, M. L. (1981) J. Biol. Chem. 256, 10012–10017
9. Aguilano, D. R., and Dufau, M. L. (1984) Endocrinology 114, 499–510
10. Cigorraga, S. B., Dufau, M. L., and Catt, K. J. (1978) J. Biol. Chem. 253, 4297–4304
11. Aguiaino, D. R., Tsai-Morris, C. H., Hattori, M. A., and Dufau, M. L. (1985) Endocrinology 116, 1745–1754
12. Khanum, A., Buko, E., and Dufau, M. L. (1997) Endocrinology 138, 1612–1620
13. Liang, P., and Pardee, A. B. (1992) Science 257, 967–971
14. Gilman, G. (1987) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 4.10.1–4.10.9, Greene Publishing Associates and Wiley-Interscience, New York
15. Heng, H. H., Squire, J., and Tsai, L. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9509–9513
16. Greenberg, M. E., and Bender, T. P. (1990) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 4.10.1–4.10.9, Greene Publishing Associates and Wiley-Interscience, New York
17. Fox, C. H., and Cotter-Fox, M. (1993) in Current Protocols in Immunology (Coligan, J., and Kruisbeek, A., eds) pp. 2.8.1–2.8.21 John Wiley & Sons, Inc., New York
18. Merrick, W. C., and Sonenberg, N. (1997). Methods 11, 333–342.

19. Pause, A., and Sonenberg, N. (1992). EMBO J. 11, 2643–2654.

20. Gee, S. L., and Conboy, J. G. (1994). Gene (Amst.) 140, 171–177.

21. Tseng, S. S., Weaver, P. L., Liu, Y., Hitomi, M., Tartakoff, A. M., and Chang, T. H. (1998). EMBO J. 17, 2651–2662.

22. Jaramillo, M., Dever, T. E., Merrick, W. C., and Sonenberg, N. (1991). Mol. Cell. Biol. 11, 5992–5997.

23. Lu, D., and Yunis, J. J. (1992). Nucleic Acids Res. 20, 1967–1972.

24. de Valoir, T., Tucker, M. A., Belikoff, E. J., Camp, L. A., Bolduc, C., and Beckingham, K. (1991). Proc. Natl. Acad. Sci. U. S. A. 88, 2113–2117.

25. Maekawa, H., Nakagawa, T., Uno, Y., Kitamura, K., and Shimoda, C. (1994). Mol. Gen. Genet. 244, 456–464.

26. Leroy, P., Alzari, P., Sassoon, D., Wolgemuth, D., and Fellous, M. (1989). Cell 57, 549–559.

27. Hirling, H., Scheffner, M., Restle, T., and Stahl, H. (1989). Nature 339, 562–564.

28. Schmidt, S. R., and Linder, P. (1992). Mol. Microbiol. 6, 283–291.

29. Gururajan, R., Perry, O. K. H., Melton, D. A., and Weeks, D. L. (1991). Nature 349, 717–719.

30. Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W. C., and Sonenberg, N. (1990). Mol. Cell. Biol. 10, 1134–1144.

31. Chuang, R. Y., Weaver, P. L., Liu, Z., and Chang, T. H. (1997). Science 273, 1468–1471.

32. Huhtaniemi, I. T., Nozu, K., Warren, D. W., Dufau, M. L., and Catt, K. J. (1982). Endocrinology 111, 1711–1720.

33. Clermont, Y., and Perez, B. (1957). Am. J. Anat. 100, 241–268.

34. Edelmann, W., Cohen, P. E., Kane, M., Lau, K., Morrow, B., Bennett, S., Umar, A., Kunkel, T., Cattoretti, G., Chaganti, R., Pollard, J. W., Kolodner, R. D., and Kucherlapati, R. (1996). Cell 85, 1125–1134.

35. Brenner, W. J., Millar, M. R., Sharpe, R. M., and Saunders, P. T. (1994). Endocrinology 135, 1227–1234.

36. Tsai-Morris, C. H., Geng, Y., Xie, X. Z., Buczko, E., and Dufau, M. L. (1994). J. Biol. Chem. 269, 15868–15875.

37. Tsai-Morris, C. H., Geng, Y., Buczko, E., and Dufau, M. L. (1998). J. Clin. Endocrinol. & Metab. 83, 288–291.

38. Hu, Z., Zhuang, L., Guan, X., Meng, J., and Dufau, M. L. (1997). J. Biol. Chem. 272, 14263–14271.

39. Dufau, M. L., Miyagawa, Y., Takada, S., Khanum, A., Miyagawa, H., and Buczko, E. (1997). Steroids 62, 128–132.