Cell-Specific and Hormonally-Regulated Expression of Gonadotropin-Regulated Testicular RNA Helicase Gene (GRTH/Ddx25) Resulting from Alternative Utilization of Translation Initiation Codons in the Rat Testis

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Running title: GRTH protein expression in the adult rat testis

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ABSTRACT

Gonadotropin-regulated testicular RNA Helicase (GRTH) is a novel DEAD-box protein with ATPase and RNA helicase activities. GRTH gene transcription is stimulated by gonadotropin (hCG) via cyclic AMP-induced androgen formation in testicular Leydig cells. In this study, immunocytochemical and Western analyses identified GRTH as a developmentally regulated protein in Leydig cells and in germ cells (pachytene spermatocytes and round spermatids) of the rat testis. Three ATGs with potential for generation of multiple protein species were identified. Germ cells primarily utilized the 1\textsuperscript{st} ATG codon (+1) and contained major proteins of 61/56 kDa, while Leydig cells utilized preferentially the 2\textsuperscript{nd} ATG codon (+343) with expression of 48/43 kDa species. A 3\textsuperscript{rd} ATG was weakly utilized and yield a 33 kDa only in germ cells. The increased in GRTH 43 kDa protein in Leydig cells caused by hCG treatment was prevented by the androgen receptor antagonist, Flutamide. In round spermatids, hCG caused a significant decrease of 61 kDa species and an induction 48/43 Kda species while no changes were observed in pachytene spermatocytes. Reversal of this hormone-induced switch of expression by Flutamide indicated a role of androgen in utilization of the 2\textsuperscript{nd} ATG. These studies have demonstrated a cell-specific and hormone-dependent alternative usage of ATG codons in the testis. They have also revealed that the androgen-dependent transcription of GRTH expression in Leydig cells is accompanied by a marked increase of 43 kDa species. The findings indicate that expression of GRTH proteins is regulated by gonadotropin/androgen at the translational level.
INTRODUCTION

RNA helicases are ribonucleic acid binding proteins which regulate RNA structure, possess ATPase activity, and unwind double stranded RNA in an ATP-dependent manner (1, 2). A large number of RNA helicases have been identified and these were classified into superfamilies based on specific amino acid sequences in the conserved motifs common to all family members. Among these proteins, DEAD-box proteins were grouped in a family of RNA helicases - the DEAD-box family (Asp-Glu-Ala-Asp), whose members exert regulatory roles in various aspects of RNA metabolism including translation of gene expression, nuclear transcription, pre-mRNA splicing, mRNA export and ribosome biogenesis (1, 2). In addition, some members of the DEAD-box family participate in regulatory events during organ maturation and cellular differentiation (1, 2).

The gonadotropin-regulated testicular RNA helicase (GRTH/DDX25), cloned from the rat Leydig cell, mouse and human testis cDNA libraries, is a novel member of the DEAD-box protein family of RNA helicases and is the first member found to be regulated by a hormone (3). Purified recombinant GST-GRTH displayed ATPase and ATP-dependent bi-directional RNA helicase activities. It also increased in vitro translation of luciferase RNA templates (3). Northern analysis indicated that this helicase is highly expressed in rat, mouse, and human testes, and is weakly expressed in the pituitary and hypothalamus. In vivo/in vitro studies demonstrated that GRTH is transcriptionally up-regulated by human chorionic gonadotropin at doses that cause down-regulation of LH/hCG receptors, steroidogenic enzymes and androgen formation (4). Furthermore, in vitro studies revealed that induction of GRTH mRNA by hCG is mediated via second messenger and androgen in Leydig cells. Inhibition of androgen
production by inhibitors of steroidogenic enzymes, or blockade of androgen action by a receptor antagonist, abrogated the stimulation of GRTH mRNA by gonadotropin or cAMP. *In situ* hybridization analysis demonstrated that GRTH is predominantly expressed in the testis in both somatic Leydig cells and meiotic spermatocytes and haploid germinal cells of the seminiferous epithelium, and is developmentally regulated. In the present study, we identified GRTH protein species, evaluated their protein levels, studied its cellular distribution within the testicular compartments, and its hormonal regulation in the adult rats testis. The regulation of this enzyme by gonadotropin and androgen, and its stage-specific localization in germ cells, indicate that GRTH could participate in the regulation of androgen-dependent steroidogenesis and spermatogenesis.
EXPERIMENTAL PROCEDURES

Animal Treatment

Adult and 21 day old male rats (Charles River Laboratories Inc., Wilmington, MA) were housed in pathogen-free, temperature- and light-controlled condition (20°C; alternating light-dark cycle with 14 h of light and 10 h of darkness). All animal studies were approved by the NICHHD animal and care and use committee (protocol # 00-044). Adult male rats were given subcutaneous injections of 2.5 µg Human Chorionic Gonadotropin (hCG) (Pregnyl, Organon pharmaceuticals, The Netherlands) in Dulbeccos’ phosphate buffered saline. For in vivo studies to explore a gonadotropin-mediated androgen effect on GRTH expression in Leydig cells, adult male rats were injected with the androgen receptor antagonist Flutamide (2-Methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl]propanamide, Sigma, St. Louis, Mo) (5 mg, two times a day for three days) followed by two injections of Flutamide with a 12 h interval and a single subcutaneous 2.5 µg dose of hCG 24 h before sacrifice. Animals were killed by asphyxiation with CO2 and decapitated 24 h after hCG or vehicle (controls) treatment unless otherwise indicated. Leydig cells [interstitium] and germinal cells [spermiferous tubule] were prepared from decapsulated testes and further purified for protein extraction to be analyzed by Western blots (see below).

Testicular Leydig and Germ Cells Preparation

Leydig cells were prepared by collagenase dispersion and purified by centrifugal elutriation (5). Following collagenase dispersion, spermiferous tubules were minced and incubated in Medium 199 containing 0.1% BSA, 0.1% trypsin (Sigma, St. Louis, Mo) and 17 µg/ml DNase (Sigma, St. Louis, Mo) for 30 min in a rotary water bath (80 rpm, 35C). After addition of
soybean 0.04 % trypsin inhibitor, the sample was filtered through a 300µm mesh, 90µm mesh, 40µm mesh screen and glass wool and cells were pelleted and resuspended in elutriation buffer containing 2 µg/ml DNase. The relevant types of germ cells were subsequently separated and purified by centrifugal elutriation using Beckman Avanti 21B centrifuge with elutriator rotor model J 5.0 as described previously (6). The first two fractions (1 & 2) were collected with flow rates of 31.5 and 41.4 ml/min at 3000 rpm and two additional fractions (3 & 4) were obtained with flow rates of 23.2 and 40 ml/min at 2000 rpm. Cells were identified on air-dried smears, fixed in Bouin’s and stained with hematoxylin and periodic acid-Schiff. Fractions 2 and 4 containing round spermatids and pachytene spermatocytes at a purity of 84 % and 86 % respectively were used for Western blot analyses.

**Western Blot and Immunohistochemistry Analysis**

A polyclonal antibody was raised in rabbits against a GRTH peptide (amino acids 465-477) and purified by protein A Sepharose (Amersham Pharmacia, Piscataway, NJ). Protein extracts from testicular Leydig, germ cells and various tissues including adrenal, ovary, pancreas, brain, pituitary were assessed by Western blot analysis using the purified GRTH antibody (1/400 dilution, 1 µg IgG/µl). The specificity of the antibody-GRTH interaction was evaluated in presence of 60 µM of the GRTH peptide 465-477. Immunosignals were detected by a super-resolution chemiluminescent system (Pierce, Rockford, IL). GRTH values were normalized by the corresponding β-actin signals. For immunochemistry analysis, testes from adult rats were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections were incubated with GRTH antiserum at 1/500 dilution (1µg IgG/µl) and developed with a peroxidase-labeled avidin biotin detection system. Sections of seminiferous tubules were staged according to the method of Leblond and Clermont (7). Quantitation of relative immunostaining intensity was
performed by three independent observers using score (0-5) assigned for the signal varying from absence, weak, medium, high to maximum by vision inspection. At least 50 pachytene spermatocytes and round spermatids per stage in 10 different seminiferous tubules were evaluated by two observers. Maximum intensity observed at the stage IX pachytene spermatocyte was defined as score 5.

**Over-expression of GRTH-GFP and GRTH-PBK protein in COS-1 cells**

The full length of the GRTH cDNA coding region including the 1st ATG codon [newly identified] or from 2nd ATG codon [1st ATG in our previous report, (3)] were subcloned into the pEGFP-N2 (GRTH-GFP) or pBK (GRTH-PBK) (2 µg) and transfected into COS-1 cells with Lipofectamine Plus (Invitrogen, Carlsbad, CA). After 36 h, cells were harvested for Western blot analyses by using a GFP monoclonal antibody (Clontech, Palo Alto, CA) or purified GRTH antibody.
RESULTS

Western analysis of GRTH gene expression

In the process of cloning and analyzing the promoter domain of GRTH gene, we encountered a single nucleotide (G) deletion at nt -272 bp in our previous reported cDNA sequence (3). This was verified at the cDNA level by sequencing clones isolated from a rat testis library. With this single base pair addition, the GRTH cDNA contains 357 additional base pair and an ATG codon (+1) 5' in frame to the original cDNA ATG codon (earlier defined as +1 bp, now revised as +343 bp). This sequence of 1630 bp contains an open reading frame encoding 483 amino acids with the additional of 114 amino acids resulting from 5' sequences to our earlier reported clone (Fig. 1B). Motif search by the GCG program of the 114 aa N-terminal sequence identified a consensus leucine zipper pattern (L6XL6XL6XL) with the last leucine missing (aa 18-32) in rat, mouse and human species. A data base search revealed high amino acid sequence homology of GRTH in rat, mouse and human species (93-98%). Four regions (aa. 65-71, 82-86, 91-97 and 99-105) within the N-terminal sequences are conserved between GRTH of rat, mouse and human, and DBP5 of mouse and human (8, 9). Furthermore, our recent studies demonstrated that the TATA-less GRTH gene contains multiple transcriptional start sites located within 80 bp 5' to the 1st ATG codon (+1) (C.H.Tsai-Morris et al, unpublished).

To further analyze the expression of this full-length cDNA we performed Western analysis using either specific GRTH antiserum or GFP monoclonal antibody for GRTH-GFP fusion protein over-expressed in COS-1 cells. Western blot analyses using a GFP monoclonal antibody showed expression of GRTH-GFP fusion proteins with construct A which contains both 1st and 2nd ATG (new clone) and construct B, our previous clone.

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with only the 2nd ATG (Fig. 1A, left panel, lanes A and B). Expression of construct A revealed a predominant 83 kDa band (56 kDa GRTH, 27 Kda GFP), and a weak 70 kDa protein band (43 kDa GRTH, 27 kDa GFP) and that of construct B a 70 kDa band. This indicated utilization of both ATG in the translation process. A 27 kDa GFP band was detected in cells transfected with the GFP construct without the GRTH insert (Fig. 1A, left panel, lane GFP). An additional 88 kDa species (61 kDa GRTH, 27 kDa GFP) was also observed. All protein species corresponding to the over-expressed GRTH-GFP fusion protein were also detected with a specific GRTH polyclonal antibody (Fig. 1A, middle panel, lanes A and B). In addition to the predominant band of 88/83 kDa, a second less prominent protein band of 70 kDa was detectable by the GRTH antibody from the expression of the full length GRTH-GFP fusion construct A (Fig. 1A, middle panel, lane A). This corresponded to the deduced protein size derived from the amino acid sequence reported in our early studies (3) (construct B, lane B), suggesting that the 2nd ATG in the coding region can also function in the translation process.

Also the expressed protein species resulting from transfecting the construct containing full length GRTH in PBK vector (construct C) were evaluated. We observed 61/56 kDa (predominant) and 43 kDa (minor) bands of size comparable to that calculated from the expressed GRTH-GFP construct (A), that corresponded to usage of the 1st and 2nd ATG codons, respectively (Fig. 1A, middle panel, lane C).

These findings demonstrate that newly identified ATG is the major translation initiation codon in over-expression studies in COS1 cells. However, the 2nd ATG codon appears to function in a cell specific manner in Leydig cells (Fig. 1A, right panel) and it may also play a significant role under hormonal stimulation (see Fig. 6). There are
specific endogenous 56-61 Kda protein complexes detected in the whole testis and purified adult rat testicular germ cells (round spermatids and pachytene spermatocytes) (Fig. 1A, right panel, lanes RS and P), while the 43 kDa is the major protein species in Leydig cells. Other minor protein bands of 48 and 56 kDa are also expressed in Leydig cells (Fig. 1A, right panel, lane LC). An additional minor protein band 33 kDa was also noted in germ cells (Fig. 1A, right panel, lanes RS and P). The later may result form utilization of ATG codons at +568 or +598. The specificity of the endogenous GRTH protein bands was confirmed by inhibition of the antibody-GRTH complex by the peptide used to prepare the antibody (Fig. 1A, right panel).

Developmental studies revealed that endogenous testicular GRTH protein was expressed predominantly as 61/56 kDa form observed in testis of pubertal and adult animals but was not present in immature animals (Fig. 2A) while a weak 43 kDa protein was expressed in the adult testis. This is reasonable since Leydig cells which are the major source of the 43 kDa species represent about 4 % of the total testis cell volume. (10). GRTH was not observed in other organs examined including adrenal, ovary, heart, hypothalamus or pituitary (Fig. 2B).

**Immunocytochemical analysis of GRTH in adult rat testis**

Immunohistochemistry studies showed that GRTH immuno-reactive protein was predominantly present in the interstitial cells of the adult rat testis and weakly expressed in the seminiferous tubules (Fig. 3.1, A and B). Subsequent studies revealed that the intensity of positively stained germ cells varied during the spermatogenic cycle (see Figs. 3.2 and 4).
To determine the cellular expression patterns of GRTH protein in the seminiferous tubules during germ cells development, we performed a detailed analysis of immunoreactive staining present in different types of germ cells during the spermatogenic cycle of the adult rat testis (Fig. 3.2). Both pachytene spermatocytes and round spermatids expressed GRTH protein and the intensity of staining in the individual cell types varied at different stages of the spermatogenic cycle. GRTH immunoreactive protein staining was significantly higher in round spermatids when compared to pachytene spermatocytes only at early stages (I-III). GRTH levels in round spermatids reached peak levels at stages VIII and IX and were diminished in late stages of elongating spermatids. In pachytene spermatocytes, signals were minimally detectable in stage I and gradually increasing at stage II through IX. The strongest immunoreactive signals were observed in stage VIII, and IX, where levels were significantly higher compared to round spermatids. GRTH immunoreactivity in pachytene spermatocytes was gradually decreased from stages X through stage XIII. It was noted that immunoreactive signals were prominent in stage XIV of pachytene spermatocytes when cells entered the metaphase of meiotic division. GRTH was not expressed in Sertoli cells or other types of germ cells.

Analysis of the intensity of GRTH immunoreactive signals at different stages of round spermatids and pachytene spermatocytes during the spermatogenic cycle and the corresponding pattern of individual cell types are shown in Figure 4A and B. The specific cell- and stages- of GRTH protein expression during germ cells development suggests that GRTH may play an important role in spermatogenesis.
Gonadotropin up-regulation of GRTH expression in adult rat testes

The GRTH protein levels were significantly increased in adult rat Leydig cells (43 kDa) at 12 h after gonadotropin treatment of adult rats, further increased at 24 h and returned to near control level at 96 h (Fig. 5A). The weaker 48 and 56 kDa species were unchanged at 12 h but were not detectable at 24 h and were again observed at 96 h after treatment (Fig. 5 B). The increase of the 43 kDa GRTH species in Leydig cells caused by gonadotropin was prevented by Flutamide treatment (Fig. 5 C). Flutamide treatment per se also reduced GRTH protein to 56% of controls (p < 0.01). These findings indicated that GRTH protein expression is up-regulated by the action of androgen induced by gonadotropin stimulation in the Leydig cells.

In germ cells the GRTH protein (61/56 kDa) were markedly affected by gonadotropin stimulation in a cell-specific manner (Fig. 6). While there were no changes observed in pachytene spermatocytes, gonadotropin treatment significantly altered the pattern of protein expression in round spermatids. In these cells the 61 kDa species was significantly decreased to barely detectable levels, while only minor reduction in the 56 kDa species were observed. Most notably was the marked induction of the 48/43 kDa species that were undetectable in round spermatids of control animals. Moreover treatment with Flutamide prevented the induction of these species by hCG in the round spermatids. This indicated alternative utilization of GRTH translation initiation codon governed by direct or indirect action of androgen in these cells.
DISCUSSION

This study has identified GRTH cDNA clones that contain an in frame ATG codon 5' upstream to the translation initiation codon present in the cDNA previously cloned in our laboratory. This additional codon extends the open reading frame of GRTH by 114 amino acids. Both ATG codons are actively translated in the adult rat testis, and the GRTH protein is expressed in both Leydig cells and germinal cells. Also, there is a cell-specific and hormone-dependent usage of ATG codons in the testis. Our findings have revealed up-regulation of GRTH in the Leydig cells and a switch of GRTH protein species expressed in round spermatids induced by gonadotropin treatment, and a direct/or indirect involvement of androgen in this regulation. These results support the notion that androgen exerts regulatory function in GRTH gene expression.

The primary antibody used in this study is a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the unique C-terminal amino acid sequence (aa. 465-477) with no similarity to any other member of RNA helicase gene family or known proteins. Western blots analyses of transiently transfected COS-1 cells with various constructs (Fig. 1) confirmed the antibody specificity since both GRTH and GFP antibodies revealed major 88/83 kDa species and a minor 70 kDa protein band from expression of GRTH-GFP fusion construct A. Moreover, the 70 kDa species was detected from expression of the 5' truncated GRTH-GFP, construct B. In cells transfected with construct C, both 61/56 kDa and 43 kDa species were present. The observed sizes of the endogenous proteins (61/56 kDa and 43 kDa species) were consistent with those derived from the deduced amino acid sequences and the expressed GRTH-GFP fusion proteins corrected for the GFP contribution. The ~5 kDa difference
observed in the 61 vs 56 kDa and 48 vs 43 kDa species may result from post-translational modification (i.e. N-glycosylation at aa. 426 or 472 site). The difference of the 61/56 kDa and 48/43 kDa species can be accounted for by codon usage at ATG +1 and +343 nt, respectively. The intensity of the 61 kDa species is stronger than the 56 kDa species in the testis and round spermatids while the pachytene spermatocytes do not contain 56 kDa species. In Leydig cells, the 43 kDa protein is the dominant species. The mechanism involved in the formation of these various GRTH protein species and the significance of their respective function in specific cell types remain to be resolved.

GRTH was present in testis and not detected in other tissues examined including ovary, hypothalamus and pituitary. The lack of detectable protein expression in these tissues is of interest since our previous study demonstrated mRNA expression at these sites (3) and indicates lack of translatability or very low levels of the protein, undetectable by the method employed.

Production of multiple protein isoforms could result from transcriptional and/or translational mechanism. Different promoter usage, alternate mRNA splicing, utilization of alternative translation initiation codon and proteolytic cleavage could generate protein diversity and ultimately fulfill unique biological functions. Since GRTH mRNA is a single transcript of 1.6 kb, one of the mechanisms for the differential expression of GRTH protein species in the testis is the alternative initiation of translation from different ATG codons. Initiation of translation at alternative ATG codons in a single transcript has been documented for a number of genes including intronless members of C/EBP family, C/EBPβ (11) and C/EBP α (12), and intron containing Egr3 gene and eukaryotic translation initiation factor 4GI (13, 14). In the case of the GRTH gene, the
usage of alternative ATG codons is supported by findings from overexpression studies of engineered constructs containing different GRTH translational initiation codons. It is also indicated by the size of the specific GRTH protein species endogenously expressed in the testis, which corresponded to the proposed ATG initiation codons (Fig. 1). The overexpression studies indicated that both ATG codons at +1 and +343 nt of GRTH actively initiated the synthesis of different protein species. Testicular germ cells preferentially utilize the +1 ATG codon while Leydig cells use the 2nd ATG codon at nt +343 in the translation process. In addition, there is a 3rd functional ATG codon at nt 568 or 598 which is utilized in germ cells exclusively yielding a 33 kDa species. All the sequences surrounding these different initiation codons contain consensus Kozak sequences (GCC(A/G)CCATGG) with 2-4 nt mismatches 5' to ATG (Fig. 1, right).

Although the 1st ATG codon at +1 nt position appears to better match the consensus Kozak sequence, our study indicated that all the proposed ATG codons are functional depending on the cell type.

Although proteolytic cleavage could also be an alternative mechanism for generation of different protein isoforms, we believe this is unlikely in the case of GRTH gene products. The rationale is based on the fact that ATG codons are not only utilized in a cell specific manner but also appear to be hormonally sensitive in a specific type of germ cells (round spermatids). The switch between 1st and 2nd ATG usage in round spermatids is dependent on the gonadotropin stimulation and androgen production. In presence of hormone, 48/43 kDa protein species were generated presumably using the translation initiation codon located at nucleotide 343. The possibility of generating identical 48/43 kDa protein species as the Leydig cells through a proteolytic process induced by
gonadotropin would be very low. It is important to note that the Leydig cell is tonically regulated by gonadotropin, a hormone and that promotes androgen (testosterone) formation in these cells. Autocrine actions of testosterone in Leydig cells and paracrine actions at tubule sites (round spermatids) exert increases in GRTH and promotes the utilization of the 2nd ATG codon in both cell types. The usage of alternative ATG codon by a stimulant was also reported for isoform formation of the Murine IL 12 gene during lipopolysacharide stimulation (15).

The action of androgen on GRTH protein expression in germ cells appears to be cell specific, although germinal cells do not exhibit either gonadotropin or androgen binding activity (16, 17). In contrast to the up-regulation of GRTH gene expression caused by hCG induced androgen action in the Leydig cells, GRTH protein levels in purified pachytene spermatocytes were not affected by hormonal stimulation. However, the 48/43 kDa protein species were induced by gonadotropin treatment in round spermatids while the 61 kDa protein species was markedly diminished in this cell type. We propose that a specific androgen response factor(s) that is/are present in both Leydig cell and round spermatids but not in pachytene spermatocytes promotes utilization of the 2nd ATG codon of GRTH mRNA for the synthesis of 48/43 kDa GRTH protein.

The up-regulation of GRTH expression by gonadotropin/androgen Leydig results from transcriptional regulation (3) and this also applicable to the GRTH induction observed during development in the various cell types. In preliminary studies we have determined that the GRTH gene, that lacks consensus androgen/glucocorticoid responsive element, is not modulated by androgen in COS-1 cells co-transfected with androgen receptor and GRTH promoter/5' flanking construct (unpublished observations). This indicated indirect
cell-specific actions of androgen on GRTH transcription in the testis. On the other hand, the prevalence of the 48/43 kDa species in Leydig cells and the hormonal switch of expressed species in round spermatids is reflective of alternative promoter usage. Thus, this indicates that the 48/43 kDa protein is an androgen responsive protein that may participate in both metabolic function of the Leydig cells and spermatogenesis.

Studies on the distribution of the GRTH immuno-reactive protein in the seminiferous epithelium of the adult rat testis have clearly demonstrated that GRTH expression is cell- and stage-specific during germ cell development. Immunocytochemical analysis showed the presence of high levels of GRTH in pachytene spermatocytes and haploid spermatids at stages VIII to IX. High level of GRTH immuno-staining was also observed in the metaphase of primary and secondary spermatocytes at stage XIV when chromosomes are condensed. The cell- and stage-specific GRTH expression in the germ cells maturation process suggests that GRTH may act either directly or indirectly on germ cells to regulate spermatogenesis.

Several candidate genes for the regulation of spermatogenesis have been reported. Within the DEAD-box protein helicase family, Mvh (18), a mouse homolog of the Drosophila maternal gene Vasa that is required for the completion of oogenesis (19), is expressed in male premeiotic germ cells and appears to be associated with the meiotic process. Moreover, homozygous knockout Mvh mice produced no sperm in the testis due to the failure of premeiotic cells to complete meiosis (18). Two other evolutionary DEAD-box RNA helicase genes distantly related to GRTH, mouse PL10 (20) and P68 (21), displayed testis-selective mRNA expression restricted to late pachytene spermatocytes and round spermatids in mouse. In other studies, male germ cells were
reported to contain protein(s) that are able to specifically bind to 3' UTR of mouse P68 helicase (22). Based on its structural characteristics and in vitro function (3) we proposed that GRTH might function as a translational activator to promote protein expression of crucial gene(s) at selective stages of the spermatogenic cycle. It is not known whether the above three distantly related RNA helicases have synergistic, independent or substitutive roles in the regulation on spermatogenesis. The similarity of these protein sequences with GRTH is only limited to the conserved motifs of the RNA helicase gene family and displayed very low overall similarities (32-35 %). The physiological significance and role of GRTH during the spermatogenic cycle remains to be determined.

The distribution and regulatory pattern of GRTH gene expression at the protein level is similar to its mRNA expression in tissues and cells examined (3). The testicular cell specific and hormonal dependent usage of translation codons for GRTH protein synthesis add complexity and diversity to regulatory mechanism(s) that may participate during testicular development. The up-regulation of GRTH protein by gonadotropin in the Leydig cells follows closely the down-regulation of receptors, steroidogenic enzymes and acyl CoA synthetase (GR-LACS) (4, 23), and its return to controls levels are concomitant to their recovery from down-regulation. These changes could be reflective of the existence of a regulated mechanism responsible for recovery of down regulated transcriptional events through increases in translatability of the reduced relevant messages. The findings presented in this study demonstrate a cell-, stage- and compartment- specific expression of GRTH protein in testicular cells and provides further evidence for potential roles of GRTH in the control of steroidogenesis and
spermatogenesis in the testis. Development of GRTH null mice will establish the physiological function of the protein.
FIGURE LEGENDS

Fig. 1A. Western analysis of over-expressed GRTH protein in COS-1 cells and endogenous level of GRTH protein in the adult rat testis. Western analysis of GRTH-GFP fusion protein (construct A & B), GFP control or GRTH-PBK (construct C) over-expressed in COS-1 cells. Diagram of the constructs used in the over-expressed study indicated in the bottom of the panel. Arrows indicate the position of initiation ATG nucleotides (nt) and amino acid (aa). Protein bands from extracts of transfected cells were detected using a GFP monoclonal antibody (Left panel) or GRTH specific polyclonal antiserum (Middle panel). Endogenous GRTH protein bands from whole testis, purified Leydig cells (LC) and germ cells (RS: round spermatids, P: pachytene spermatocytes) detected with GRTH polyclonal antibody. Complete inhibition of the antibody-GRTH interaction was observed in presence of the GRTH peptide (Right panel).

Fig. 1B. Sequences of extended 5’ open reading frame nucleotides (342 bp) of rat GRTH cDNA and deduced amino acids (114 aa). The complete open reading frame of GRTH contains 483 amino acids. Potential translation initiation codons indicated in nucleotides (atg) or methionine (M).

Fig. 2. Developmental analysis and tissue distribution of GRTH protein in the rat. Western analysis of GRTH expression using purified GRTH polyclonal antisera in the testes of different ages (1 week to 8 weeks old) (A) and various tissues from adult male rat (B).
Fig. 3.1. Immunohistochemical localization of GRTH in interstitial Leydig cells of adult testes. Immunostaining signals (A) are significantly stronger in interstitial Leydig cells (LC) compared to seminiferous tubules (ST). Purified rabbit IgG was used as the negative control (B).

Fig. 3.2. Immunohistochemical localization of GRTH in the seminiferous tubule of adult testes. Immunoreactive GRTH signals at the different identified stages of the spermatogenic cycle (I-XIV) determined using previously established criteria (7). Pachytene spermatocyte (P), Round spermatids (RS), Elongating/elongated spermatids (ES), Diplotene (Di), Leptotene (L) and Zygotene (Z) spermatocytes and Sertoli cells (S), Residual body (RB). Negative control using rabbit IgG (VIIIc, IXc and XIVc). Sm: Pachytene spermatocytes in the metaphase of meiosis.

Fig. 4A. Changes of the GRTH immunostaining signals in pachytene spermatocytes and round spermatids during the spermatogenic cycle of the adult rat testes. Results assessed at different stages of seminiferous tubules by scoring as described in Materials & Methods, are presented as arbitrary levels. Highest signals were observed at stages VIII-IX of pachytene spermatocytes and stage IX of elongating spermatids.

Fig. 4B. Schematic representation of the spermatogenic cycle of the adult rat. The box area indicates type of germ cells at the different stages to be correlated with GRTH protein expression revealed by immunostaining. The intensity of GRTH expression was arbitrarily scored. Maximal intensity observed at stage IX of pachytene spermatocyte was defined as 5.
Fig. 5A. Time course studies of GRTH protein level regulated by hCG in rat Leydig cells. Western blot analysis of GRTH protein from Leydig cells of adult male rats injected with 2.5 µg of hCG or vehicle (control) 24 h prior to sacrifice. The major 43 kDa band revealed by autoradiography (upper) was quantified by densitometry (lower). Counts were normalized by β-actin and presented as percent mean ± SE relative to controls.

Fig. 5B. Western blot showing changes induced by hCG in all protein species in Leydig cells. In addition of major increases of the predominant 43 kDa band induced by hormone treatment note the disappearance of the minor bands of 48 and 56 kDa at 24 h and return at 96 h.

Fig. 5C. In vivo study of hormone effects on GRTH protein level in Leydig cells. Adult rats were treated with Flutamide (Flut) or/ and hCG as indicated in (Materials and Methods). Protein samples from Leydig cells of adult rats were analyzed by Western blot. Results were recorded by autoradiography (upper) and quantified by densitometry (lower). Counts were normalized by β-actin and presented as percent mean ± SE relative to controls.

Fig. 6. Western analysis of GRTH expression in germ cells of adult rats. GRTH protein level (61/56KDa) found in the enriched pachytene spermatocytes (P) preparation from the adult rat testes and round spermatids (RS). Adult rats were treated with Flutamide (Flut) or/ and hCG as indicated in (Materials and Methods). Protein samples from purified germ cells of adult rats were analyzed by Western blot.
REFERENCES


Figure 1
Figure 2
Figure 4
Figure 5

A

B

C

Time after hCG treatment

GRTH/β-actin

kDa

43–

β-actin

Control

Flutamide

hCG

hCG + Flutamide

Control

Flutamide

hCG

hCG + Flutamide

GRTH/β-actin

kDa

43–

β-actin

Figure 5
Figure 6

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