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Gonadotropin-Regulated Testicular RNA Helicase (GRTH/DDX25) Gene: Cell-Specific Expression and Transcriptional Regulation by Androgen in Transgenic Mouse Testis

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ABSTRACT

Gonadotropin-regulated testicular RNA helicase is a multifunctional enzyme present in both Leydig and germ cells that is essential for the progress of spermatogenesis. GRTH gene expression is transcriptionally upregulated by human chorionic gonadotropin (hCG) via second messenger (cAMP) and androgen in Leydig cells. The regulatory region(s) in the GRTH gene that is/are required for its cell-specific expression in the testis and hCG/androgen dependent expression were investigated in transgenic mice carrying sequential deletions of 5′ flanking sequences of the GRTH gene. GFP-reporter gene expression directed by the GRTH 5′ flanking sequences extending to −3.6 kb was specifically located in Leydig cells and the 205 bp minimal promoter domain was sufficient for this cell-specific expression. The 1 kb (5′ to the ATG codon) transgene-directed expression was markedly increased by in vivo hCG treatment. Administration of the androgen receptor inhibitor Flutamide blocked the basal and hCG stimulated GFP expression in Leydig cells. We conclude that the expression of GRTH in testicular cells is differentially regulated by its 5′ flanking sequence and that the 1 kb fragment of GRTH gene contains sequences for androgen regulation of its expression in Leydig cells. J. Cell. Biochem. 109: 1142–1147, 2010.

KEY WORDS: DEAD-BOX RNA HELICASE; GRTH/DDX25; ANDROGEN REGULATION; LEYDIG CELLS; TRANSGENIC MICE; TESTIS

GRTH/Ddx25, a member of the DEAD-box protein family, is a testis-specific gonadotropin-regulated RNA helicase that is present in Leydig and germ cells (meiotic spermatocytes and spermatids). It is developmentally regulated and its expression is observed in the pubertal and adult testis [Tang et al., 1999; Sheng et al., 2003; Dufau and Tsai-Morris, 2007]. GRTH is an essential post-transcriptional regulator of spermatogenesis, present in the nucleus and cytoplasm of spermatocytes and round spermatids and confined to the cytoplasm of Leydig cells [Tang et al., 1999; Tsai-Morris et al., 2004b; Sheng et al., 2006]. In addition to its intrinsic helicase unwinding/ATPase activities and participation in translation [Tang et al., 1999], GRTH is a transport protein involved in gene-specific mRNA export and protein translation during spermatogenesis [Sheng et al., 2006; Dufau and Tsai-Morris, 2007]. It binds to specific set of mRNAs and as an integral component of RNP particles transports target mRNAs to cytoplasmic sites in a gene-specific manner (i.e., chromatoid bodies—storage/processing organelle homologue to somatic P-bodies; and polyribosomes—translation) during spermatogenesis [Sheng et al., 2006].

Male mice lacking GRTH are sterile due to lack of sperm resulting from failure of round spermatids to elongate [Tsai-Morris et al., 2004b]. The chromatoid body in spermatids is markedly reduced in size in GRTH null mice. This indicated that GRTH is necessary to maintain the structure and presumably the function of this organelle. In addition, severe apoptosis occurred in spermatocytes entering the metaphase of meiosis in the GRTH null mice. GRTH was found to negatively regulate the TNF-R1 and caspase pathways and

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to promote NF-κB function (nuclear translocation, changes of acetylation status) to prevent apoptosis in spermatocytes of adult mice [Gutti et al., 2008].

GRTH is the only member of the RNA helicase family of proteins that is known to be hormonally regulated [Tang et al., 1999; Sheng et al., 2003]. Physiological studies demonstrated that GRTH in Leydig cells is transcriptionally upregulated by human chorionic gonadotropin (hCG). Induction of GRTH gene expression by hCG is mediated via second messenger (cAMP) and androgen at the transcriptional level presumably by direct or indirect actions of androgen through androgen receptors (ARs) present in Leydig cells. Furthermore, because GRTH expression in germ cells (spermatocytes and round spermatids) is observed at puberty and adult life, and the type of germ cell arrest in GRTH null mice resembles that observed in a Sertoli cell-AR null model [Chang et al., 2004], a paracrine regulation of GRTH expression in germ cells, resulting from the actions androgen/AR in Sertoli cells is envisioned.

The basal transcriptional activity of the TATA-less GRTH gene is driven by GC-rich Sp1/Sp3 in the promoter region (−205/+63 bp) and is differentially regulated by upstream sequences in expressing (pituitary, αT3 and hypothalamic, GT1-7 cells) and non-expressing cells (mouse Leydig tumor cells [MLTC]) [Tsai-Morris et al., 2004a]. Sequences −852/−354 and −501/−354 caused 40–60% and >80% inhibition of transcription in expressing and non-expressing cells, respectively. Transcriptional activity was recovered only in expressing cells by the addition of upstream sequences (−1085/−852 bp). Thus, upstream sequences to the promoter domain of GRTH gene could contribute to the lack of expression in non-expressing MLTC cells.

To proceed with studies on basal and hormonally regulated transcription of the GRTH gene and to define regulatory regions required for cell-specific gene expression, we generated transgenic mice carrying deletions of the GRTH 5′ flanking sequence upstream to the promoter domain with GFP as the reporter gene. Our findings indicated that cell-specific expression of the GRTH gene is differentially directed by its 5′ flanking sequence and revealed that the 1 kb sequences adjacent to ATG codon contain the elements required for the transcriptional activation of GRTH gene by hCG/androgen in Leydig cells.

MATERIALS AND METHODS

PLASMID CONSTRUCTION

5′ flanking GRTH DNA fragment (−3600/+63, −1085/+63, and −205/+63 bp) were prepared by restriction enzyme digestion using previous established genomic clone in pZero vector and pGL plasmids for transcriptional analysis of mouse GRTH gene expression [Tsai-Morris et al., 2004a]. −1085/+63 and −205/+63 bp were isolated by restriction digestion of *Nhe*I and *Bgl*II from p-1085/+63GL and p-205/+63GL followed by filling the 3′ recessed termini with Klenow fragment and consequently subcloned into *Sma*I cloning site of the promoterless vector pEGFP-1 (#6086-1, Clontech, Mountain View, CA) upstream of the coding GFP sequence to produce transgenic plasmid p-1085/+63GFP and p-205/+63GFP, respectively. A 3.6 kb fragment was isolated by *Xho*I/*Apa*I cut of pZero plasmid containing a genomic fragment spanning from −4000 to first intron sequence of GRTH gene. It was then ligated to *APa*I/*Xho*I restriction fragment of p-1085/+63GFP containing the backbone of pEGFP with GRTH promoter sequence and resulted in the fusion construct of p-3600/+63GFP.

GENERATION OF GRTH TRANSGENIC MICE

DNA fragments were first isolated by restriction digestion of fusion construct by *Xho*1/*SSP*1 (p-3600/+63GFP) or *Bgl*II/*AF1*II (p-1085/+63GFP and p-205/+63GFP) to produce transgene fragment 5.2, 2.1, and 1.2 kb, respectively. Transgenic mice were generated by microinjection of respective purified fragment into fertilized eggs in Transgenic Core Facility of National Institute Mental Health, NIH. Mice carrying GRTH–EGFP fusion genes were identified by PCR-based screening to produce 420 bp fragment using primer sets located in −18 bp of 5′ flanking region of mouse GRTH gene (GAGCGGAGACCGCAGCTATGGCG) and +420 of pEGFP-1 (CTTGTAGTTGCCGTCGTCCTTGAAGA) using isolated mouse tail DNA. At least four separate mouse lines were created and out of these, three lines ($GRTH^{tg}$) with similar high levels of transgene expression were used as founders and maintained in C57/BL6 mouse strain. Animals were housed in pathogen-free, temperature- and light-controlled conditions (22°C), with an alternating light–dark cycle with 14 h of light and 10 h of darkness. All of the animal studies were approved by National Institute of Child Health and Human Development Animal Care and Use Committee.

ANIMAL TREATMENT

Adult male transgenic mice were given subcutaneous injections of 0.5 μg of hCG (Pregnyl, Organon Pharmaceuticals, Roseland, NJ) in Dulbecco's phosphate-buffered saline or AR antagonist flutamide (2-methyl-*N*-[4-nitro-3-(trifluoromethyl)-phenyl]propanamide,) (Sigma–Aldrich, St. Louis, MO) (5 mg, two times a day for 3 days). In the group treated with both hCG and flutamide, animals were first treated by two s.c. injections of flutamide with a 12-h interval for 2 days followed by a single subcutaneous 0.5-μg dose of hCG 24 h with additional two injections of flutamide before sacrifice. Animals were killed by asphyxiation with CO_2 and decapitated 24 h after hCG or vehicle (controls) treatment. Testes were removed for histological and Western blot analyses.

IMMUNO-HISTOCHEMISTRY STUDY

Testes dissected from adult animals were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections were used for immuno-staining with a 1:2,000 dilution of rabbit anti-GFP antibody (Genetex, Inc., Irvine, CA) developed with a peroxidase-labeled avidin–biotin detection system. The antibody–antigen complexes were visualized with diaminobenzidine to produce brown deposit (VECTASTAIN, Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin. For immuno-gold electron microscopic (EM) analysis, ultrathin sections (80 nm) were incubated sequentially with rabbit anti-GFP antibody (1:50), anti-rabbit biotinylated Ig antibody (Vector Laboratories; 1:100), and streptavidin-gold (10 nm, 1:20; EM Sciences). The sections were stained with uranyl acetate and lead citrate and examined under transmission EM.

WESTERN BLOT ANALYSIS

Total protein lysates from mouse testis were prepared by homogenization in lysis buffer T-PER (Pierce Chemical, Rockford, IL) containing protease inhibitor cocktail (Roche Applied Sciences) followed by centrifugation at 1,300*g* for 10 min. The supernatant was collected and protein concentration determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Protein extracts (20 μg) separated by 4–20% SDS–PAGE gel, and transferred to nitrocellulose membrane were assessed using the specific rabbit anti-GFP antibody (Genetex, Inc.) and β-actin antibody (Sigma–Aldrich). Immunosignals were detected by a super-signal chemiluminescence system (Pierce Chemical).

STATISTICAL ANALYSIS

The significance of the differences between treatment groups was determined by Tukey's multiple-comparison test (one-way ANOVA analysis) using the Prism software program (GraphPad Software, Inc., San Diego, CA).

RESULTS

LEYDIG CELL-SPECIFIC EXPRESSION OF GRTH TRANSGENE

Immuno-histochemistry analyses of three independent transgenic mice lines carrying serial deletion of 5′ flanking sequences including 3.6 kb, 1 kb, or 205 bp promoter using GFP as the reporter gene are shown in Figure 1. In all cases, GFP expression was solely confined to the interstitial cells (Fig. 1, upper panel—3.6 kb GFP; middle panel—1 kb GFP; and lower panel—0.2 kb GFP). Immuno-EM studies of 1 kb GFP transgenic mice testis (Fig. 2) demonstrated that GFP signals were present in Leydig cells but absent in macrophages within the interstitial compartment. Also, GFP signals were not detected in Sertoli and germ cells within the seminiferous tubule. These results indicate that the 205 bp proximal promoter of the GRTH gene contains the necessary elements for Leydig cell-specific expression whereas sequence/s required to direct germ cell specific expression may reside upstream to 3.6 kb 5′ flanking region.

GONADOTROPIN/ANDROGEN REGULATION IN 1 KB GRTH–GFP TRANSGENIC MICE

To assess the transcriptional regulation of GRTH by androgen in Leydig cells, transgenic mice carrying 1 kb sequence (promoter and upstream adjacent) of GRTH gene were treated in vivo with gonadotropin (hCG), AR antagonist Flutamide (Flut.), or in combination of hCG and Flutamide (Fig. 3). GFP protein expression was observed in three independent lines of transgenic mice in the absence of treatment, consistent with the GFP expression revealed by immuno-cytochemical studies (Fig. 1). hCG treatment of these animals caused a significant increase in GFP protein expression compared to untreated controls. Administration of the AR inhibitor Flutamide markedly decreased the basal level of GFP expression (Fig. 3, Flut vs. control) and the hCG stimulated GFP expression was inhibited by Flutamide to basal control levels (Fig. 3, hCG/Flut vs. control). The three independent lines of transgenic mice showed comparable responses to gonadotropin/androgen treatment. These results indicate that element/s required for androgen-induced AR action reside within the 1 kb 5′ flanking of GRTH gene for the transcriptional regulation by gonadotropin/androgen in Leydig cells.

DISCUSSION

Our transgenic mice studies have revealed the presence of a requisite sequence in the 5′ flanking region of the GRTH gene to direct its expression into the interstitial compartment of the testis. All transgene fragments (−3.6 kb /+63 bp, −1085/+63 bp, and −205/+63 bp) used in the study directed reporter gene EGFP expression specifically in Leydig cells of transgenic mice. When the 1 kb of GRTH 5′ flanking domain (−1085/+63 bp) was compared using the Needleman–Wunsch algorithm (GCG EMBOSS-lite-sequence comparison tool, helixweg.nih.gov) to the 2.2 kb murine LH receptor 5′ flanking region that is known to direct β-gal gene expression in adult Leydig cells in a transgenic mouse [Hamalainen et al., 1999], a high cluster identity of GRTH sequence to the LH receptor was found despite the presence of gaps between two sequences. Three alignments with minimum gap appeared downstream of the −200 bp of the GRTH gene. This is consistent with the finding that 205 bp promoter domain was found to be sufficient for Leydig cells specific expression (Fig. 1). The absence of expression in the tubular compartment observed in this study indicated that GRTH germ cell-targeted sequences (pachytene spermatocytes, spermatids) are localized upstream to 3.6 kb of the GRTH 5′ flanking region. The −6.4 kb region of GRTH is followed upstream by the coding sequences of the uridine synthetase gene which are unlikely to direct GRTH expression to germ cells. Transgenes containing either the −6.4 kb/+63 bp or with deletion of Leydig cell target sequence [−6.4/−3.6 kb] directly linked to the minimal promoter (−205/63 bp) could be useful to further elucidate germ cell specific targeting sequences in the GRTH. Specific *cis*-requirement(s) to direct GRTH gene expression in different testicular cells (interstitial—Leydig cell vs. tubules—germ cells) might be operative. An additional regulatory level through upstream interactions with cell-specific trans-factors at downstream sites (promoter (−205/+63) and/or domains within (−3.6 kb sequences)) could be required for GRTH expression in germ cells. The regulatory mechanism for cell-specific differential usage of 5′ flanking sequences in GRTH gene remains to be elucidated.

Transgenic mice bearing the 1 kb 5′ flanking fragment displayed significant increases of GFP protein expression in testis after in vivo gonadotropin (hCG) treatment. This is consistent with our previous observation of hormonally activated transcription of the GRTH gene in the adult male rat as revealed in nuclear run-off studies [Tang et al., 1999; Sheng et al., 2003]. In addition, our previous studies demonstrated that the actions of gonadotropin were mediated via cAMP through androgen/AR as the downstream effector. The stimulation of the expression of reporter GFP by gonadotropin within the 1 kb fragment was prevented by treatment with the AR inhibitor Flutamide in this study. Basal GFP levels were present in Leydig cells of transgenic mice and marked reductions were observed by treatment with Flutamide. This is reflective of the actions of endogenous androgens (testosterone) which are normally present in basal condition and maintained by the physiological

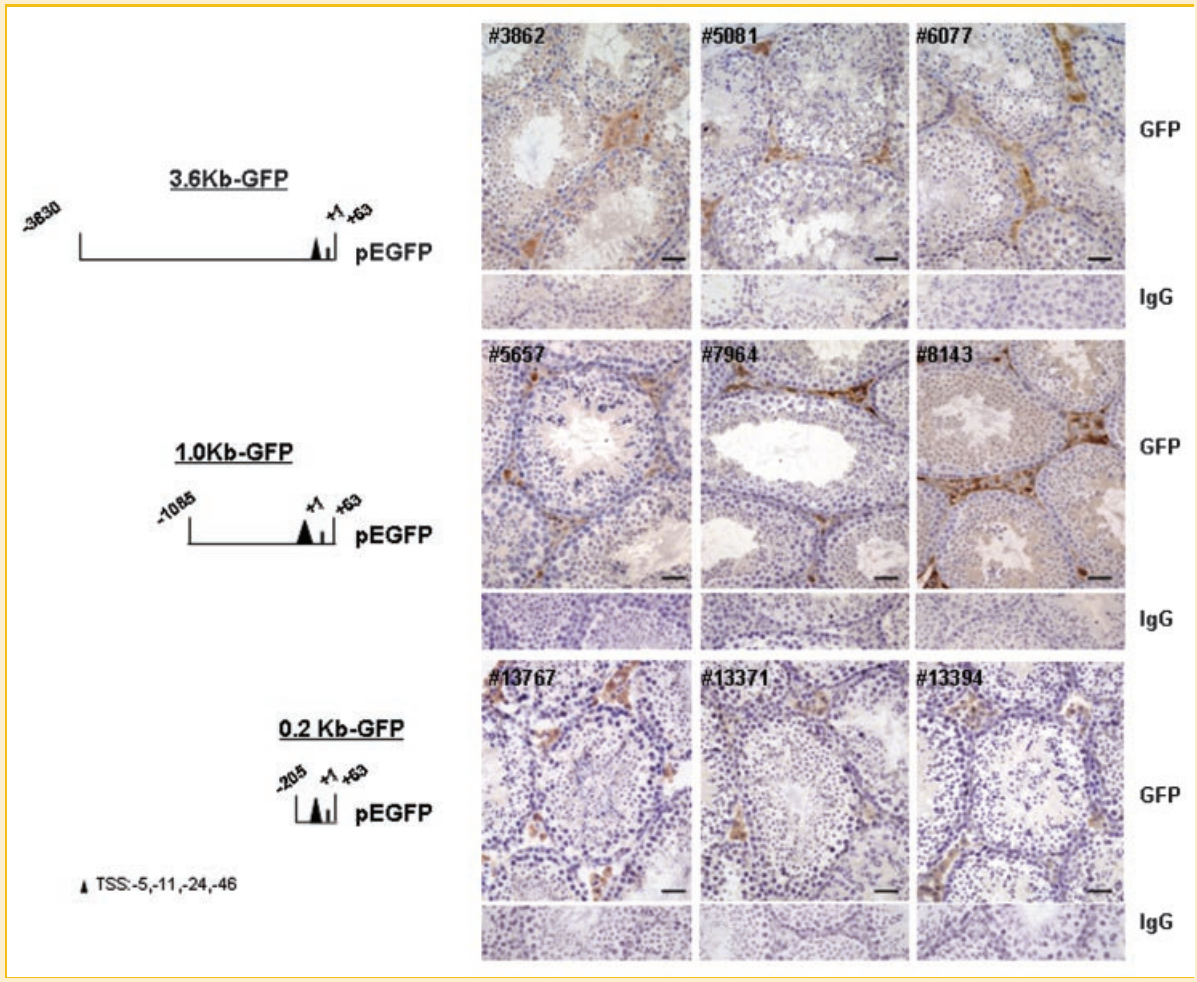

Fig. 1. 5′ Flanking sequences of GRTH gene required for Leydig cell specific expression. Immuno-histochemistry analysis of the GFP expression directed by 3.6 kb (top panel), 1.0 kb (middle panel) of 5′ flanking sequences or 205 bp promoter (lower panel) of mouse GRTH gene were performed in the testis isolated from transgenic mice. Representative micrographs of three independent lines generated from each transgene showed Leydig cell specific staining visualized by the brown staining. IgG was used as the negative control. Bar = 40 μm. Left schematic representation of GRTH 5′ flanking gene constructs of used. TSS, transcriptional start sites.

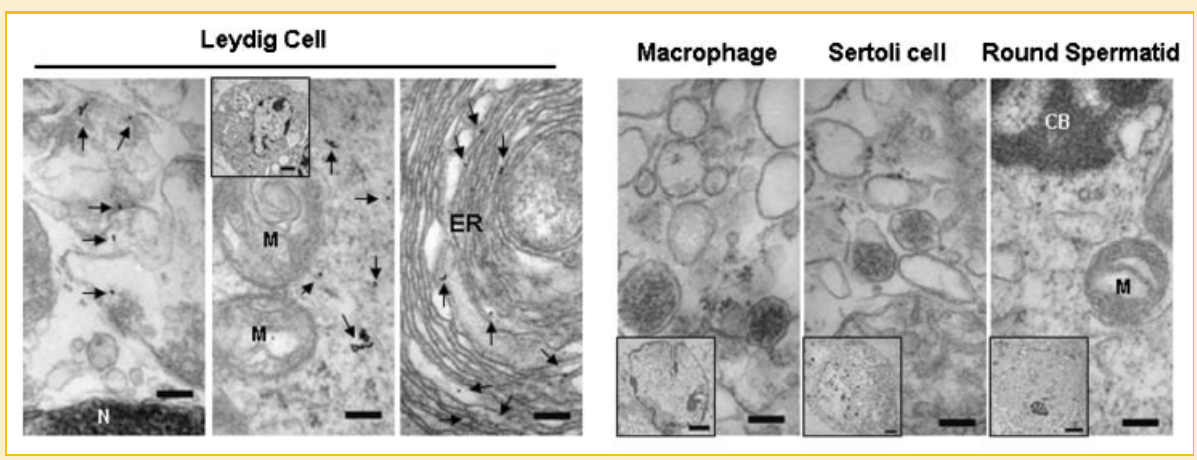

Fig. 2. Immuno-gold labeling analysis. Electron microscopic immuno-gold labeling analysis shows GFP signals are present in the cytoplasm of Leydig cells and absent in a representative macrophage, Sertoli cell, and round spermatids of 1.0 kb GFRTH-GFP transgenic mice. Gold particles are revealed as small intense dark dots (indicated by arrow). Bar = 15 μm in regular panel. Inset: lower magnification of cells. bar = 1.5 μm. N, nucleus; M, mitochondria; ER, endoplasmic reticulum; CB, chromatoid body.

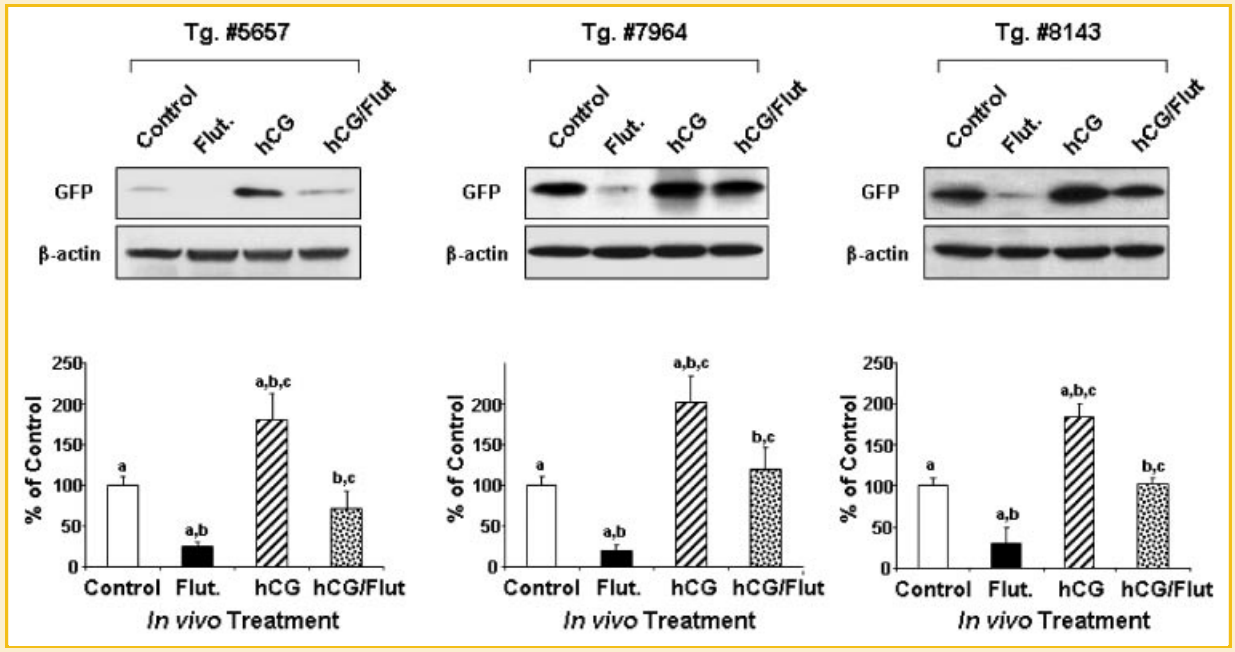

Fig. 3. Gonadotropin/androgen mediated action in 1.0 kb GFP transgenic mice. Top panel: GFP protein expression in three transgenic mice lines (Tg 5657, 7964, and 8143). Western blot analysis using anti-GFP antibody to assess GFP expression in the testis after in vivo hormone treatment. Flut.: flutamide. Three different lines of 5′ flanking 1 kb (−1085/+63) transgenic mice showed a similar response to gonadotropin/androgen treatment. Lower panel: summary of Western blot analyses. Signals from three independent experiments with four animals in each group were quantified and normalized by β-actin. Results are presented as percent of individual treatment relative to control sample. Mean ± SE of three independent experiments in triplicate. Identical superscripts indicate statistical significance between experimental groups ($P < 0.05$).

release of pituitary luteinizing hormone. These findings demonstrate that GRTH transcription can be upregulated by basal androgen and hCG-stimulated androgen-induced AR action. Also, these results suggest that DNA sequence(s) involved in androgen stimulation of transcription is/are located within the −1085/+63 bp of the GRTH gene. Several cAMP-like response elements (CRE: KWCGTCA), hormone response like element half-sites (HRE: A/G/TGWASA/GnnnKGTYCT) are found in this 1 kb sequence upstream to 205 bp promoter domain [Tsai-Morris et al., 2004a]. Although no androgen response elements are found, one potential candidate (ARE:TGTCCC) which is identical to that observed in the human secretory component (sc) gene is present at −827 bp within the −1085/−205 bp sequence [Haelens et al., 1999]. It is reasonable to assume that androgen action could occur through association of the activated AR with one of these elements or a not yet identified *cis*-element present in the GRTH gene. Alternatively, the activated AR could impact on the expression of a transcription factor or mediator which in turn could increase transcription of GRTH. Also, activation of the GRTH gene promoter by androgen through AR could be caused by a non-classical AR DNA dependent mechanism through association of activated AR with transfactor(s) bound to their DNA cognate sequence. Any of these modalities are expected to be enhanced by recruitment of co-regulators [Culig et al., 2004; Siriett et al., 2006; Chmelar et al., 2007; Peng et al., 2008]. In summary, the present study demonstrated cell-specific directed gene expression in the testis that is governed by 5′ flanking sequences of the gene. Functional studies with 1 kb 5′ flanking GRTH/DDX25 sequences in transgenic animals have provided relevant insights for elucidation of the regulation of the GRTH/DDX25 gene by gonadotropin-stimulated androgen action in the testis.

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