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Reciprocal Regulation of the Mouse Protamine Genes by the Orphan Nuclear Receptor Germ Cell Nuclear Factor and CREMτ

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ABSTRACT Germ cell nuclear factor (GCNF) is a member of the nuclear receptor superfamily, which is expressed in the adult predominantly in the male and female germ cells. In the male, GCNF is expressed in spermatogenic cells. GCNF binds as a homodimer to direct repeat response elements of the consensus half-site sequence, AGGTCA, with 0 bp spacing (DR0). Using this information, a search of genomic databases was performed to identify candidate GCNF responsive, spermatogenic-specific, genes that contain DR0 sequences. The mouse protamine genes are the strongest candidates identified to date, as they are post-meiotically expressed in round spermatids and contain DR0 elements in their proximal promoters. Previous work has shown that both recombinant and endogenous GCNF bind to DR0 elements in the mouse protamine 1 and 2 (*Prm 1* and *Prm 2*) promoters with high affinity and specificity. The present work shows that in transient transfection assays in GC-1 and JEG-3 cells, co-transfection of a GCNF-VP16 expression plasmid with reporter plasmids containing either the wild type *Prm 1* or *Prm 2* promoter established that GCNF-VP16 is able to regulate transcription from both promoters in a DR0-dependent manner. Wild type GCNF, in contrast, acts as a repressor of basal transcription on both the *Prm 1* and *Prm 2* promoters in a DR0-dependent manner. Furthermore, CREMτ activation of these promoters is also repressed by wild-type GCNF, indicating that GCNF also acts as a repressor of activated transcription. GCNF therefore defines a novel nuclear receptor-signaling pathway that may regulate a subset of genes involved in the terminal differentiation process of spermatogenesis, exemplified by the protamines. *Mol. Reprod. Dev. 68: 394–407, 2004.* © 2004 Wiley-Liss, Inc.

Key Words: testis; gene regulation; spermatid; spermatogenesis; steroid hormone receptors

INTRODUCTION

Germ Cell Nuclear Factor, or GCNF (NR6A1) is an orphan member of the nuclear receptor superfamily of transcription factors (Chen et al., 1994; Committee, 1999). The original members of the superfamily were discovered as receptors for small, lipophilic ligands, such as the steroid hormones, the retinoids, vitamin D, and thyroid hormone (Tsai and O'Malley, 1994; Mangelsdorf et al., 1995). Over 48 members have been cloned, most, of which are termed orphans since they were cloned without knowledge of their ligands or functions. Extensive studies on the function of orphan receptors have been conducted in previous years in order to further our understanding of transcriptional regulation. Most nuclear receptors, including orphans and those with known ligands, are transcription factors, which act through specific DNA elements. GCNF binds to a direct repeat of the sequence AGGTCA spaced by zero nucleotides, known as a DR0 (Chen et al., 1994; Yan et al., 1997), as a homodimer (Cooney et al., 1998), and does not heterodimerize with RXR, as do most orphan receptors (Borgmeyer, 1997; Giguere, 1999). Based on sequence homology, GCNF is different enough from other members of the nuclear receptor superfamily to belong to its own subfamily (Cooney et al., 1999; Giguere, 1999). The function of GCNF is therefore not readily apparent by analysis of its primary sequence.

Using several approaches, our laboratory is attempting to determine what role GCNF plays in biology. Mice homozygous for a targeted mutation of GCNF die in

Abbreviations: GCNF, germ cell nuclear factor; NR6A1, nuclear receptor6a1; DR0, direct repeat 0 base pair spacing; CRE, cAMP response element; CREB, cAMP response element binding protein; CBP, CREB binding protein; tk, thymidine kinase; luc, luciferase; GC-1, germ cell-1; VP-16, viral protein 16; CaMKIV, calcium/calmodulin-dependent protein kinase IV; CREM, cAMP response element modulator; ACT, activator of CREM in testis; Prm, protamine; dpc, days post coitum; N-CoR, nuclear receptor co-repressor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptors; RT, reverse transcription; sFBS, charcoal-stripped fetal bovine serum; CMV, cyto megalo virus; TBE, tris-borate-EDTA buffer; TAE, tris-acetate-EDTA buffer.

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utero at approximately 10.5 days post-coitum (dpc) with multiple phenotypic defects (Chung et al., 2001). The function of GCNF in vivo appears to be that of transcriptional repressor, since in transient transfection of cells in culture, GCNF is able to repress transcription mediated by its binding to the DR0 sequence (Cooney et al., 1998). GCNF also functions as a repressor during embryogenesis, repressing expression of the transcription factor Oct4 through a DR0 element in its promoter (Fuhrmann et al., 2001). The repression function of GCNF appears to be mediated by interaction with the co-repressors N-CoR and SMRT (Yan and Jetten, 2000; Fuhrmann et al., 2001).

In adult mice, GCNF is most highly expressed in the germ cells of both males and females (Chen et al., 1994). Expression of mouse *GCNF* in the testis is limited to post-meiotic round spermatids, with the highest levels of message observed at stages VII–VIII (Katz et al., 1997). GCNF protein is also observed in round spermatids (Hummelke et al., 1998). A similar pattern is observed in rat testes (Katz et al., 1997; Zhang et al., 1998). However, GCNF expression has been detected in spermatocytes (Bauer et al., 1998). A similar expression pattern is observed in the human, where the highest levels of *GCNF* mRNA appear to be in the late-stage primary spermatocytes (Agoulnik et al., 1998). So far, little is known about the specific role of GCNF in these cells. However, our laboratory has shown that GCNF does specifically bind to DR0 elements in the promoters of the mouse protamine genes (Fig. 1) (Hummelke et al., 1998). GCNF is a candidate repressor of spermiogenic-specific genes, such as the *Protamine* genes.

Spermatogenesis in animals is a highly ordered, species-specific process that involves the appropriate temporal and spatial expression of many genes in several cell types. Achieving the appropriate expression requires many levels of regulation, from transcriptional to post-translational. The two protamine genes encode proteins that ultimately replace the histones in the sperm chromatin structure, and are necessary for proper germ cell differentiation in male mice (Cho et al., 2001). The protamines are also critical for proper spermatogenesis in human males (Belokopytova et al., 1993; de Yebra et al., 1993). The protamines are transcribed post-meiotically; first being observed in stage I round spermatids (Mali et al., 1989). The mRNAs are then stored for later translation, after transcription has generally been shut down in elongating spermatids (Braun et al., 1989). In addition to GCNF, it has been suggested that the cAMP-responsive transcription factor CREMτ, a member of the CREB family of transcription factors, is involved in the regulation of the protamine genes (Blendy et al., 1996; Nantel et al., 1996). CREMτ has been shown to bind to cAMP-responsive elements (CRE) present in both promoters (Johnson et al., 1991; Delmas et al., 1993). In order to activate transcription by CREMτ, it needs to be activated either by phosphorylation by PKA (Sassone-Corsi, 1995) or calcium/calmodulin-dependent kinase IV (Sun et al., 1995), with subsequent interaction with CREB binding protein (CBP) (Montminy, 1997). CREMτ can also be activated in a phosphorylation-independent manner by the protein activator of CREMτ in testis (ACT) (Fimia et al., 1999). Furthermore, mice lacking both copies of the CREM gene are infertile, showing a phenotype of spermatogenic-arrest at the earliest round spermatid stage (Blendy et al., 1996; Nantel et al., 1996). No protamine transcripts are observed in these mice,

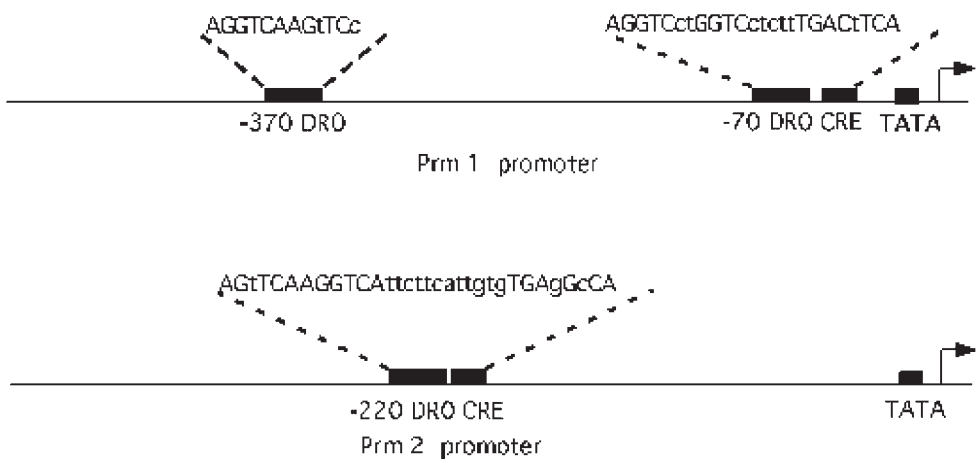

Fig. 1. DR0s and CREs in the mouse protamine promoters. The portions of the mouse protamine promoters that are necessary for appropriate temporal and spatial expression are represented schematically, with DR0, CRE, and TATA elements shown as boxes. The promoter for mouse protamine 1 (*Prm 1*) contains two DR0 sequences at approximately −370 and −70 relative to the transcriptional start site and a cAMP-Regulatory Element (CRE) at the −63 position. The mouse protamine 2 (*Prm 2*) promoter contains one DR0, at −220, and a CRE at −200. TATA boxes are also shown. Sequences for each of the naturally occurring elements, are shown above the diagrams. Differences from consensus for both DR0 and CRE sequences are in lower case. The arrow indicates the transcriptional start site.

along with other post-meiotically expressed genes (Blendy et al., 1996; Nantel et al., 1996). Whether this is directly due to the absence of CREMτ or the absence of the appropriate cell stages is not yet known. Further characterization of the effects of CREMτ on protamine gene expression will depend on a demonstration of direct activation.

The current work examines the effects of both GCNF and CREMτ on protamine gene expression, as well as how these factors affect each other. In transient transfection experiments, we demonstrate that GCNF represses basal activity of the protamine promoters dependent on DR0 sequences within these promoters. Furthermore, we demonstrate direct activation of the protamine promoters by CREMτ? CREMτ activation of the *Prm 1* promoter is dependent on a CRE at −63 relative to the transcriptional start site. The promoter element that mediates CREMτ activation of the *Prm 2* promoter has not been elucidated. GCNF represses CREMτ mediated activation of both protamine promoters, and does so in a DR0-dependent manner. GCNF therefore is a response element-dependent repressor of protamine gene transcription.

MATERIALS AND METHODS

Expression Vector and Reporter Plasmid Construction

Reporter plasmids were made by PCR sub-cloning of the *Prm 1*, *Prm 2* promoters, or corresponding mutant promoters into *Xho*I and *Nhe*I sites in the pGL3 basic luciferase vector (Promega, Madison, WI). PCR amplification of the promoters was performed using plasmids containing *Prm 1* (courtesy of Dr. Richard Palmiter) or *Prm 2* (courtesy of Dr. Norman Hecht) as templates. The PCR was performed as above, using the following primers:

- *Prm 1* 5′ primer: ATGGATCCGTCTAGTAATGTCCAAC
- *Prm 1* 3′ primer: GAGCTGGCTCAGAGCAG
- *Prm 2* 5′ primer: TAAGCTAGCACTACTAAC
- *Prm 2* 3′ primer: CATCTCGAGCCTGCCCACCTGCTCTTGGTG

Mutants were created by PCR linker mutation, with either an *Xba*I, a *Hind*III, or an *Mlu*I site replacing the *Prm 1* −370, *Prm 1* −70, or *Prm 2* −220 DR0, respectively. Two PCR products were generated for each mutation, the products were digested with the appropriate enzyme, along with the pGL3 basic plasmid, and the digested DNA ligated together. The primers used to generate each mutation are listed below.

- 5′ *Prm 1* −370 *Xba*I primer: TATCTAGAGTTCCCTCAGCAGCATTC
- 3′ *Prm 1* −70 *Xba*I primer: TATCTAGATTTTAAGAAATTATCCAACGCCC
- 5′ *Prm 1* −70 *Hind*III primer: TAAAGCTTGTCCTCTTTGACTTCAT
- 3′ *Prm 1* −70 *Hind*III primer: TAAAGCTTCTCACAGACCATGGCC
- 5′ *Prm 2* −220 *Mlu*I primer: AGAAGACGCGTGTCATTCTTCATTGTGTGAGGCCG
- 3′ *Prm 2* −220 *Mlu*I primer: GACACGCGTCTTCTAACCCTGGTCCCTTTAC

The *Prm 1* double DR0 mutant was generated in a similar manner, using primers containing mutation in both DR0 sequences in the PCR, and ligating all three products into the pGL3 basic plasmid.

Reporter plasmids containing two copies of the protamine DR0 sequences in front of the thymidine kinase minimal promoter were generated by removing hormone response element sequences from the plasmid HREtkluc (courtesy of Dr. Rainer Lanz), and ligating oligonucleotides containing two copies of each DR0 in place of the HRE. Single-stranded oligonucleotides were annealed in the following manner: Complementary single-stranded oligonucleotides were added to 0.25 M Tris-Cl (pH 7.5) in a 1:1 molar ratio and boiled for 5 min in 1 L of dH_2O. Boiling dH_2O was then removed from the heat source, and tubes containing the oligonucleotides were left in the dH_2O as it gradually cooled to RT. Oligonucleotides were then stored at −20°C. Sense strands of oligonucleotide sequences were:

- *Prm 1* −70: CTAGCTGTGAGGTCCTGGTCCATGCATCCAGGTCTTGGTCCTCTTAAC
- *Prm 1* −370: CTAGCTAAAAGGTCAAGTTCCATGCATGCAGGTCAAGTTCCCTCCAAAC
- *Prm 2* −220: CTAGCTAGAAGTTCAAGGTCAATGCATGCAGTTCAAGGTCATTCTAAC

Annealed oligonucleotides were then digested with *Xho*I and *Nhe*I, and ligated into the tkLuc plasmid digested with the same enzymes.

Cloning of the expression plasmids pCMVGCNF and pCMVGCNF-VP16 is described in Cooney et al. (1998). pCMVCREMτ was generated in a similar manner. CREMτ was liberated from the expression vector pSG5-CREMτ (courtesy of Dr. Anthony Means) by digestion with *Eco*RI, and was then ligated into *Eco*RI-digested pCMV4 (Andersson et al., 1989). Clones in the correct orientation were chosen after restriction digestion with *Sma*I, and then sequenced. PKA catalytic subunit expression plasmid was obtained from Dr. Wenlong Bai.

Transient Transfections

GC-1 cells (Hofmann et al., 1995) were plated at 4.5×10^4 cells/well, and JEG-3 cells were plated at 4.6×10^5 cells/ well, in 6-well plates in DMEM with 10% FBS, and allowed to attach overnight. Cells were then transfected the next day using Superfect transfection reagent according to manufacturers protocol (Qiagen, Valencia, CA). A total of 2.1 μg plasmid DNA was used for each well, with the empty pCMV4 expression plasmid used as balance DNA. Superfect/DNA/DMEM

mix (1 ml total volume) was left on the cells for 3 hr at 37°C in a tissue culture incubator, at which time it was aspirated and 3 ml of DMEM with either 10% FBS or 10% charcoal-stripped FBS (sFBS) were added to each well. Cells were incubated at 37°C for 48 hr, and then harvested using the Reporter Lysis Buffer from Promega. Protein extracts were then stored at −80°C overnight. Luciferase assays were then performed on thawed extracts, using the Dual Luciferase Reporter kit (Promega, Madison, WI). Results were plotted relative to reporter activity transfected with pCMV4 empty expression vector.

Gel mobility shift assays. To generate wild type, and −370DR0 and −70 DR0 mutant, *Prm 1* promoter probes Prm1Luc, Prm1m1Luc, and Prm1m2Luc reporter plasmids were linearized with Avr II. Each linearized plasmid was then labeled by a fill-in reaction with either Klenow or Sequenase (Amersham, Arlington Heights, IL) and radiolabeled [α-^{32}P]dCTP (ICN, Costa Mesa, CA). The various *Prm 1* probes were liberated from the Luciferase vectors by digestion with *Ban*II and gel purified as previously described (Cooney et al., 1991). The plasmid NSR13 containing the full mGCNF open reading frame was in vitro-translated using a TNT T3-coupled reticulocyte lysate system (Promega, Madison, WI), as previously described (Chen et al., 1994). An aliquot of in vitro translated protein (2 μl) was incubated with probe (6×10^4 cpm) in binding buffer (Chen et al., 1994) for 15 min at room temperature in the presence or absence of GCNF antibody. DNA–protein complexes were separated by electrophoresis in a 4.5% polyacrylamide gel in 0.5 × TBE at 10 V/cm, which was dried and exposed to X-ray film overnight at −70°C.

Reverse Transcription-Polymerase Chain Reaction

Analysis of genes expressed in GC-1 and JEG-3 cells was performed by RT-PCR. Total RNA of both cell lines was extracted using the Trizol method (Life Technologies, Inc., Rockville, MD). Ten 100 mm tissue culture dishes were each plated with 5×10^6 cells in DMEM (Life Technologies, Inc.) media, and incubated in a tissue culture (5% CO_2) for 48 hr at 37°C. At that time, the DMEM was aspirated, and the cells were washed three times with 3 ml of 1× PBS. Three milliliter of Trizol was then added, and the cells allowed to lyse for 15 min at RT. The cell-lysate containing-Trizol was then transferred to a 13 ml screw cap Sarstedt tube, and shaken vigorously for 2 min. The samples were prepared according to the Trizol protocol from the manufacturer. RNA was resuspended in RNAse-free dH_2O and stored at −20°C.

RNA samples from both GC-1 and JEG-3 cells were then treated with DNase 1. Reaction mixtures contained 500 μg of RNA, 10× DNase reaction buffer (Life Technologies, Inc.), 1 μg DNase/1 μg RNA, and RNAse-free dH_2O to bring the total volume to 1.0 ml. Reactions were then incubated at 37°C for 30 min. Reaction was stopped with 0.1 M EDTA (pH 8.0) and 1 mg/ml glycogen. DNase-treated RNA samples were then used in reverse transcriptase (RT) reactions. RT reactions consisted of 50 μg of DNase-treated RNA, 5× reaction buffer (Clontech, Palo Alto, CA) 1.0 μl 10 mM dNTP mix, 1 U RNAse inhibitor, 1.0 μl, 20 μM oligo-dT primers, and 200 U of MMLV RT in a total volume of 20 μl. Reactions were incubated in a 42°C water bath for 60 min, after which time they were stopped by heating to 94°C for 5 min. Reactions were spun down in a microcentrifuge, and diluted with RNAse-free dH_2O to 100 μl. PCR was then performed in a RoboCycler using specific primers (below). PCR conditions were as follows: 25 pmol of each primer, 1 μl of the appropriate RT-generated cDNA template, 5 μl 2 mM dNTP mix, 10 U of Advantage 2 PCR enzyme mix (Clontech), 5 μl 10× reaction buffer, and dH_2O to 50 μl. The thermal cycler was programmed for the following conditions: step 1, 94°C for 7 min; step 2, 94°C for 1 min; step 3, annealing temperature for 1 min; step 4, for 72°C 1 min; step 5, 72°C for 10 min. Steps 2–4 were repeated 30 times. PCR products were separated on a 1.5% agarose gel in 1× TAE (see above) at 2 V/cm for 75 min, and then visualized by ethidium bromide staining under UV light.

Primers:

- GCNF forward: 5′-TCAGGATGAATTGGCAGAGC-3′; annealing temp. = 62°C
- GCNF reverse: 5′-TATCTGGACTGGTCCAATGC-3′; annealing temp. = 62°C
- *Prm 1* forward: 5′-AAATTCCACCTGCTCACAGC-3′; annealing temp. = 62°C
- *Prm 1* reverse: 5′-GAGATGCTCTTGAAGTCTGG-3′; annealing temp. = 62°C
- *Prm 2* forward: 5′-ATGGTTCGCTACCGAATGAG3′; annealing temp. = 62°C
- *Prm 2* reverse: 5′-TGCTCAGTACTCAGATCTCG-8′; annealing temp. = 62°C
- CREMτ forward: 5′-GTTTCTGTAGCTGGATCAGG-3′; annealing temp. = 62°C
- CREMτ reverse: 5′-GTTCCACCTTGAGCTATAGC-3′; annealing temp. = 62°C

β-Actin primers were taken from Fuhrmann et al. (2001). PKA subunit primers were taken from Laxminarayana et al. (1999).

RESULTS

GCNF Interacts in a Functional Manner With DR0 Sequences in the Mouse Protamine Promoters

Sequence analysis of the *Prm* 1 and 2 promoters showed that they both contained DR0 GCNF response elements and CREs (Fig. 1). Having identified and established the *Prm 1* and *Prm 2* genes as candidate GCNF target genes by demonstrating that GCNF binds to DR0 sequences located in the promoters of these genes in vitro (Hummelke et al., 1998), the function of GCNF on these promoters was next investigated. The ability of GCNF to functionally interact with the DR0 sequences from the protamine promoters in a cellular context was examined by whether GCNF-VP16 could activate

reporter gene expression through these elements. We used a constitutively active chimera of GCNF and the VP-16 activation domain to circumvent the lack of a ligand for GCNF. Using GC-1 cells, a cell line derived from spermatogonial stem cells immortalized with the viral SV40 middle T antigen (Hofmann et al., 1995), co-transfection of the pCMVGCNF-VP16 expression vector with a separate reporter plasmid for each of the protamine DR0 sequences was performed. Each reporter contains two copies of one of the DR0 sequences (*Prm 1* −70, *Prm 1* −370, and *Prm 2* −220) inserted in front of the herpes simplex virus thymidine kinase (tk) minimal promoter driving a luciferase reporter gene. As expected from the in vitro binding data, GCNF-VP16 is able to activate the *Prm 2* −220 reporter construct (Fig. 2A; 2X220tkluc) and the *Prm 1* −370 construct (2X370tkluc), but not the *Prm 1* −70 construct (2X70tkluc).

To determine whether GCNF-VP16 could activate through the protamine promoters, reporter constructs using either the *Prm 1* or *Prm 2* promoters were linked to the luciferase reporter gene in pGL3 basic (called *Prm 1*luc and *Prm 2*luc, respectively). These reporter plasmids were co-transfected with pCMVGCNF-VP16 expression vector into GC-1 cells. Experiments with *Prm 1*luc resulted in 4.5-fold activation by GCNF-VP16

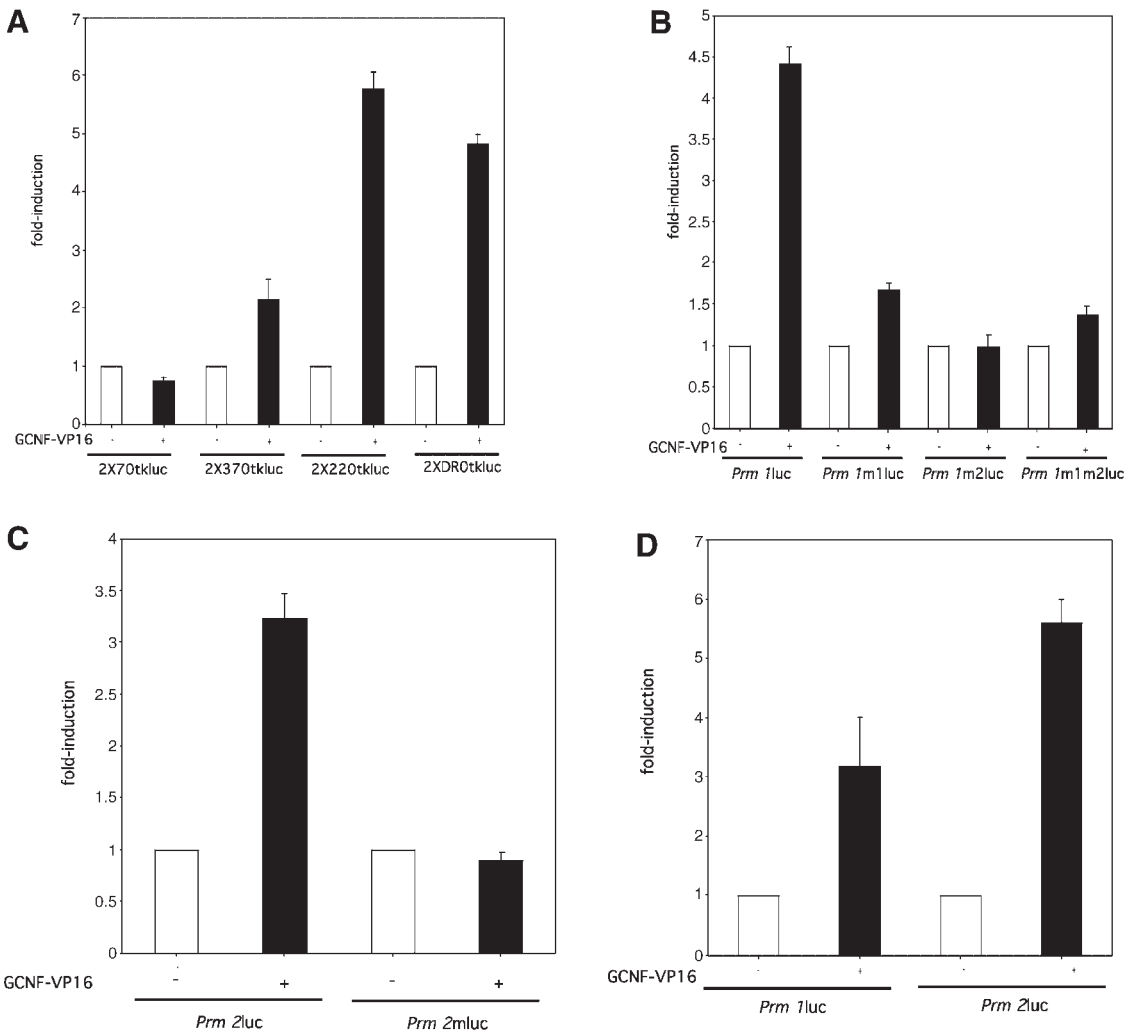

Fig. 2. GCNF-VP16 activates transcription through DR0s in the protamine 1 and 2 promoters. **A**: The expression plasmid pCMVGCNF-VP16 (250 ng) was co-transfected into GC-1 cells with 1.5 μg of each reporter plasmid individually. Each reporter plasmid contains two copies of the *Prm 1* −70 DR0 (2X70tkluc), *Prm 1* −370 DR0 (2X370tkluc), *Prm 2* −220 DR0 (2X220tkluc), or the consensus DR0 sequence (2XDR0tkluc), in front of the thymidine kinase (tk) minimal promoter. **B**: Similar to the experiment described in (B), GC-1 cells were co-transfected with 500 ng pCMVGCNF-VP16 expression vector and 500 ng *Prm 1*luc reporter plasmid, or each of the corresponding DR0 mutants (*Prm 1*m1luc, *Prm 1*m2luc, *Prm 1*m1m2luc). **C**: Co-transfection of GC-1 cells with 1.5 μg *Prm 2*luc or the −220 DR0 mutant construct, *Prm 2*mluc, −/+250 ng pCMVGCNF-VP16 expression vector. **D**: JEG-3 cells were co-transfected with 250 ng of pCMVGCNF-VP16 expression vector and 1.5 μg of either *Prm 1*luc or *Prm 2*luc reporter plasmids. In Panels (A–D), fold-induction was calculated for each reporter by dividing the activity observed for each set of conditions by the activity in the absence of pCMVGCNF-VP16. The results shown are representative of at least three experiments.

(Fig. 2B), while experiments with *Prm 2*luc resulted in threefold activation by GCNF-VP16 (Fig. 2C), indicating that GCNF-VP16 can bind to and activate both protamine promoters in a cellular context.

To demonstrate that activation of the protamine promoters by GCNF-VP16 in GC-1 cells is dependent on the presence of the DR0 sequences, these sequences were mutated by PCR-generated linker mutation. Mutation of either the *Prm 1* −370 or the *Prm 1* −70 DR0 sequences, alone or together, abrogates GCNF-VP16 activation of the *Prm 1* promoter (Fig. 2B). This was expected for the −370 DR0, but not for the −70 DR0, since GCNF neither binds this sequence in vitro (Hummelke et al., 1998), nor activates it in the heterologous promoter context (Fig. 2A). This result indicates that GCNF-VP16 needs both DR0 sequences for full activation of the *Prm 1* promoter, and that GCNF can bind the −70 DR0 in the full promoter context within cells. Similarly, activation of the *Prm 2* promoter by GCNF-VP16 is lost when the −220 DR0 is mutated (Fig. 2C). Both mutant and heterologous promoter experiments indicate that GCNF interacts with the *Prm 2* −220 DR0 sequence in cells as well as in vitro.

Another cell line that expresses endogenous GCNF, the choriocarcinoma cell line JEG-3, was also used to perform similar experiments. In these cells, it was observed that GCNF-VP16 also activates both protamine promoters, although in these cells GCNF-VP16 activates the *Prm 2* promoter to a slightly greater degree than the *Prm 1* promoter (Fig. 2D). From these results, it is clear that GCNF-VP16 activation of both protamine promoters is DR0-dependent, suggesting that GCNF regulates the expression of the protamine genes in vivo through these sequences.

GCNF represses basal activity of the protamine promoters. In the absence of an exogenous ligand or other mode of activation, GCNF has been shown to repress basal transcription through a DR0 sequence (Cooney et al., 1998). Furthermore, GCNF-VP16 activates both protamine promoters through their DR0 sequences (Fig. 2). Thus, the ability of wild type GCNF to repress basal transcription of the protamine promoters through its interaction with the DR0 sequences was next studied. Co-transfecting GCNF expression vector with either *Prm 1*luc or *Prm 2*luc into GC-1 cells showed a 4–5-fold decrease in basal activity of both promoters compared to co-transfecting empty pCMV4 expression plasmid (Fig. 3A). For the *Prm 1* promoter in GC-1 cells, nearly full basal promoter activity is restored when either the −370 DR0 or the −70 DR0 elements are mutated (Fig. 3B). Thus, both *Prm 1* DR0 sequences are therefore necessary for GCNF to repress to its full ability, in agreement with the GCNF-VP16 data in Figure 1. Mutation of the *Prm 2* −220 DR0 also disrupts GCNF repression of basal activity of the *Prm 2* promoter in GC-1 cells (Fig. 3C). In addition, a decrease in the activity of both promoters in the presence of GCNF is also observed in JEG-3 cells (Fig. 3D), although to a lesser degree than in GC-1 cells, on the order of two-fold. Thus, the repression by GCNF is not cell-type dependent.

GCNF Binds to Both the *Prm 1* −70 and −370 DR0 Elements in the Context of the Full Promoter

The functional date shown in Figure 2A contradicts the data shown in Figures 2B and 3B, where the former suggests that GCNF cannot bind to the *Prm 1* −70 DR0, while the latter experiments clearly showed that in the context of the full promoter this element is functional. This suggested that GCNF could bind to the *Prm 1* −70 DR0 in the context of the full promoter. Thus, we set out to test this using gel mobility shift assays with radiolabeled probes to the full *Prm 1* promoter containing either a mutated −70 DR0, a mutated −370 DR0 or wild type DR0s. Incubating each probe with in vitro translated GCNF and in the presence of GCNF antibody we were able to show that GCNF could bind to the wild type *Prm 1* promoter, as previously shown (Fig. 4, lanes 1 and 2) (Hummelke et al., 1998). There is an endogenous DR0 binding activity in certain rabbit reticulocyte lysates that co-migrates with the GCNF complex, thus the antibody is used to clearly identify the presence of GCNF. As expected, when the *Prm 1* −70 DR0 was mutated binding was still observed to the *Prm 1* −370 DR0 (Fig. 4, lanes 3 and 4). However, in contrast to lack of binding to the *Prm 1* −70 DR0 oligonucleotides (Hummelke et al., 1998), GCNF could bind to the *Prm 1* −70 DR0 when the *Prm 1* −370 DR0 was mutated, albeit weakly, in the context of the full *Prm 1* promoter (Fig. 4, lanes 5 and 6). Thus, the binding data supports the functional assays in Figures 2C and 3D, confirming that the *Prm 1* −70 DR0 is indeed a functional GCNF response element in the context of the full promoter.

CREMτ Activates the Protamine Promoters

As a means of understanding the role of GCNF and its mechanism of action on the protamine promoters, its ability to function with CREMτ was examined. Since direct activation of the protamines by CREMτ has not been demonstrated, the ability of CREMτ to activate *Prm 1*luc and *Prm 2*luc in GC-1 cells was first examined. In order to activate transcription by CREMτ, we chose to activate it by phosphorylation with the PKA catalytic subunit. Each reporter plasmid was co-transfected with expression vectors for CREMτ, PKA catalytic subunit, or both together into GC-1 cells. When not bound to the PKA regulatory subunits, PKA catalytic subunit activates CREMτ, and therefore transfected PKA catalytic subunit should be able to activate CREMτ. The results of these experiments are seen in Figures 5A and B. For the *Prm 1* promoter, PKA alone does not significantly activate the *Prm 1* promoter, but unexpectedly CREMτ alone activates the promoter approximately 23-fold above basal (Fig. 5A). Transfection of CREMτ and PKA together activates *Prm 1* approximately 35-fold above basal (Fig. 5A). It was observed, rather unexpectedly, that PKA and CREMτ both individually activate the *Prm 2* reporter in GC-1 cells 4-fold and 10-fold, respectively (Fig. 5A). When PKA and CREMτ are transfected together, up to 70-fold activation is observed

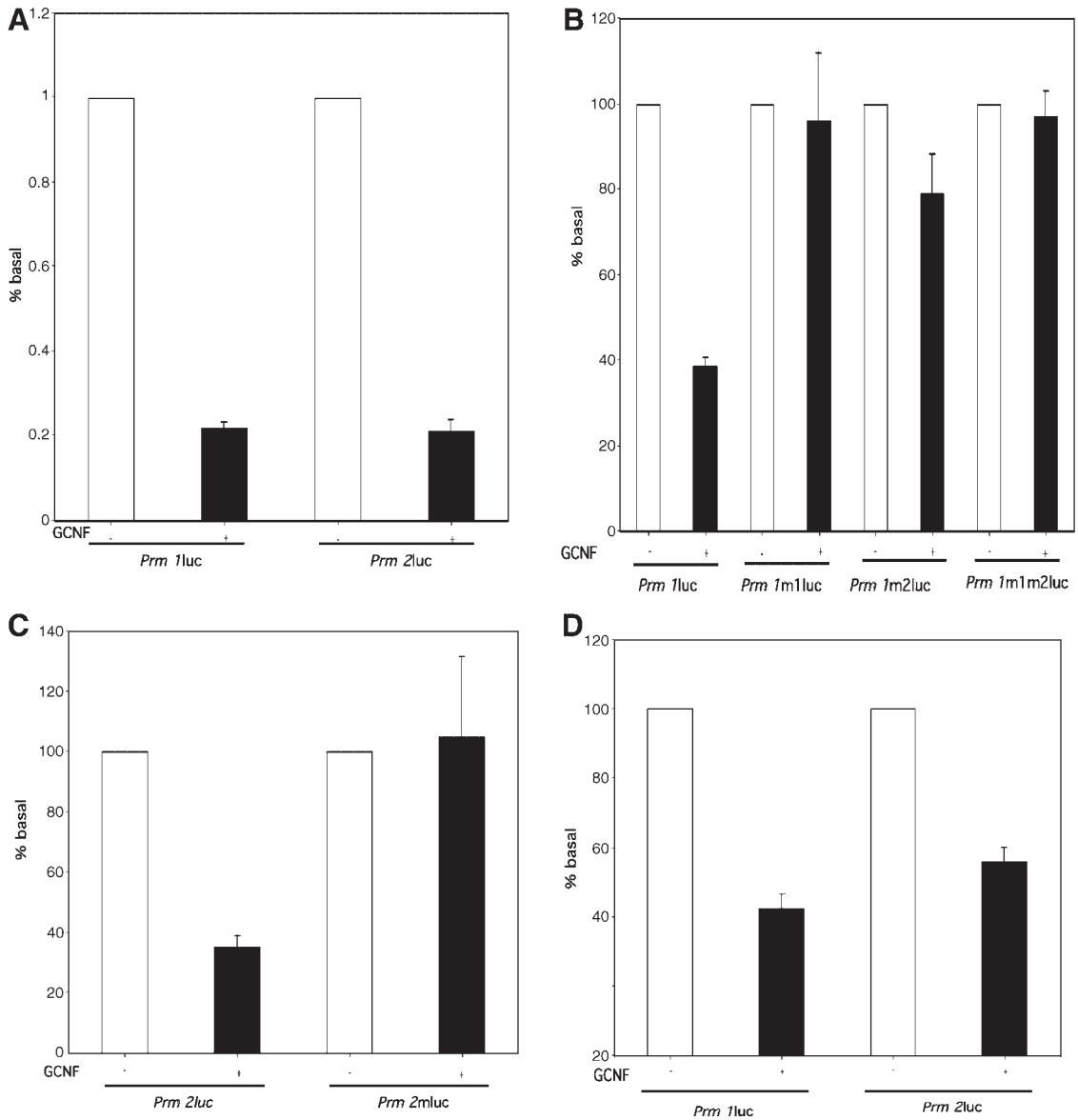

Fig. 3. GCNF represses basal activity of the protamine promoters in a DR0-dependent manner. **A**: The function of wild type GCNF on the protamine promoters was examined by co-transfecting GC-1 cells with 1.0 μg of either the *Prm 2*luc or *Prm 1*luc reporters and 50 ng of the pCMVGCNF expression vector. It was observed that GCNF can repress the basal activity of both promoters by as much as fivefold. **B**, **C**: To examine whether GCNF represses reporter activity by binding the DR0s in the protamine promoters, 1.0 μg of either *Prm 2*luc, *Prm 1*luc, or each of the DR0 mutant constructs (*Prm 2*mluc, *Prm 1*m1luc, *Prm 1*m2luc, *Prm 1*m1m2luc) reporter plasmids were individually co-transfected into GC-1 cells with 250 ng of pCMVGCNF expression vector. *Prm 1* reporters are shown in (B) and *Prm 2* reporters are shown in Panel (C). **D**: JEG-3 cells were co-transfected with 250 ng of pCMVGCNF expression vector and 1.0 μg of either *Prm 1*luc or *Prm 2*luc reporter plasmid. For Panel (A–D), % Basal was calculated by setting the activity of each reporter in the absence of pCMVGCNF to 100%. The results shown are representative of at least three experiments.

(Fig. 5B). This synergistic result indicates that CREMτ and PKA each may be involved in more pathways than the typical cAMP/PKA/CREMτ pathway in regulation of the protamine genes. These data demonstrate that CREMτ activates both protamine promoters in GC-1 cells, even in the absence of co-transfected PKA. Thus, GC-1 cells support CREMτ activity.

The dependence of CREMτ activation on CRE sequences located in the protamine promoters was examined by co-transfection assays. Mutation of the −63 CRE in the *Prm 1* promoter (*Prm 1*CREmluc) resulted in a significant reduction of activation by CREMτ in GC-1 cells both alone and in the presence of the PKA catalytic subunit (Fig. 5A). This result indicates that the vast majority of CREMτ activity is mediated by the −63 CRE. A *Prm 2* reporter construct containing a mutation at the −200 CRE (*Prm 2*CREmluc) was co-transfected with CREMτ and PKA catalytic subunit expression

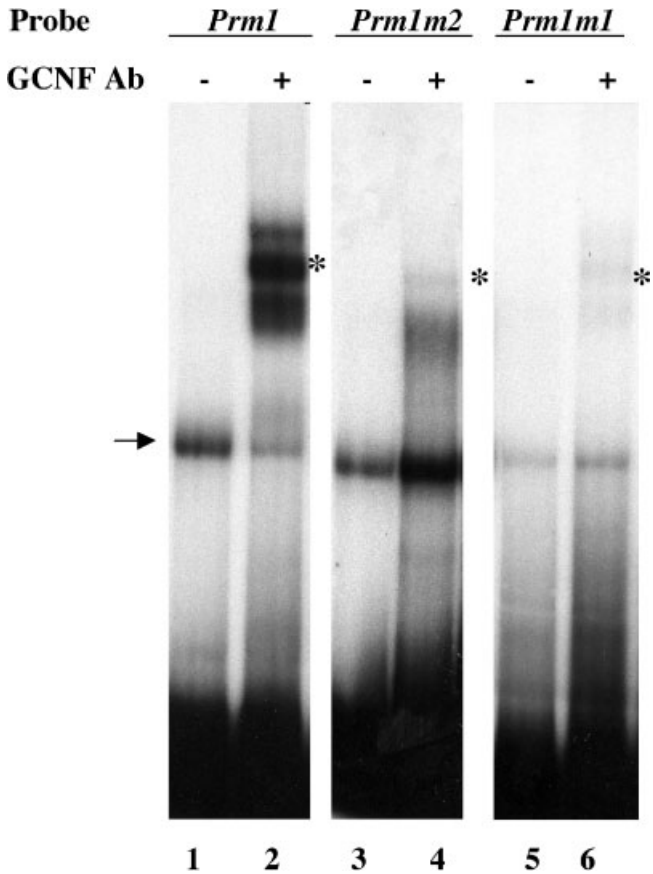

Fig. 4. Binding of GCNF to the −70 and −370 DR0 elements in the context of the full *Prm1* promoter. Gel mobility shift analysis of GCNF binding to the wild type or DR0 mutant Protamine 1 promoter. In vitro translated GCNF was incubated with labeled probes, as indicated, in the presence or absence of GCNF antibody (Ab). The arrow marks the migration of the GCNF complexes and the asterisks mark the migration of the GCNF:probe:antibody complex. *Prm1* = wild type *Prm 1* promoter, *Prm1m1* = −370 DR0 mutant *Prm 1* promoter and *Prm1m2* = the −70 DRO mutant *Prm 1* promoter.

vectors into GC-1 cells. CREMτ activation of the promoter was not significantly affected, either in the presence or absence of co-transfected PKA catalytic subunit (Fig. 5B). PKA alone was also able to activate the *Prm 2*CREmluc to the same extent as the wild type promoter in GC-1 cells, providing further evidence that PKA may act through an additional pathway.

The expression plasmids for CREMτ and the PKA catalytic subunit were also co-transfected into JEG-3 cells with each of the protamine reporters (Fig. 5C). CREMτ plus PKA activated the *Prm 1* promoter (Fig. 5C). Thus, CREMτ activation of *Prm 1* is cell-type dependent. However, in contrast to the GC-1 cells, the *Prm 2* promoter was not activated by CREMτ plus PKA in JEG-3 cells (Fig. 5C). Furthermore, neither CREMτ nor PKA individually activated either promoter in JEG-3 cells, as they had in GC-1 cells, indicating that there is most likely differences between these two cell lines in expression of the factors necessary for CREMτ activity. Additionally, in contrast to the GC-1 cells, the presence of PKA catalytic subunit is necessary for activation by CREMτ in JEG-3 cells. These results demonstrate that CREMτ regulates *Prm 1* through the −63 CRE sequence, but activates *Prm 2* through either a different element than the −200 CRE sequence in that promoter, or through a novel mechanism.

GCNF Represses CREMτ Activation of the Protamine Promoters in a DR0-Dependent Manner

The nature of the influence of GCNF on CREMτ activation of the protamines was studied next. In transient transfection assays, GC-1 cells were transfected with pCMV CREMτ expression vector and either the wild type or DR0-mutant protamine promoter reporters individually, with and without pCMVGCNF expression vector. The amount of activity of each reporter with CREMτ alone was compared with the activity of each reporter with both CREMτ and GCNF. For the *Prm 1* promoter GCNF is able to repress CREMτ activation of reporter activity greater than 60% (Fig. 6A). Repression of CREMτ activation by GCNF is lost when both DR0s are mutated (Fig. 6A). Similar results are observed with the *Prm 2* reporter, GCNF is able to repress CREMτ activation (Fig. 6B), and when the −220 DR0 is mutated, GCNF repression of CREMτ activation is lost (Fig. 6B).

Furthermore, when the PKA catalytic subunit expression vector is also transfected along with GCNF and CREMτ expression vectors, GCNF again is able to repress CREMτ activation of both protamine promoters, though to a lesser extent than in the absence of PKA (Fig. 6C). GCNF has a similar effect on CREMτ activity in JEG-3 cells, as it represses CREMτ activation of the *Prm 1* promoter (Fig. 6D). However, GCNF repression in these cells is less than in the GC-1 cells, as it is less than two-fold. These results show that GCNF is able to repress CREMτ-activated transcription of the protamine promoters through the DR0 sequences, in addition to repressing basal transcription. GCNF therefore plays a repressor function in regulating protamine expression.

Expression Profile of GC-1 and JEG-3 Cells

While GCNF acts as a repressor of transcription in both GC-1 and JEG-3 cells, CREMτ and PKA have differences in activity, both individually and together, when they are co-transfected with either *Prm 1*luc or *Prm 2*luc into each of these cell lines. Therefore, expression of the factors that have been studied in this work was characterized in both cell lines by RT-PCR (Materials and Methods). The results show several differences, and some similarities (Fig. 7). Surprisingly, GCNF is expressed in both the cell lines, CREMτ and *Prm 2* are both expressed only in GC-1 cells, and *Prm 1* is not expressed in either cell line (Fig. 7A). The absence of a *Prm 1* band in either cell line is not due to a failure of the PCR, as *Prm 1* is detected in a commercial made-for-PCR testis cDNA library (Fig. 7B). Expression of GCNF, CREMτ, and *Prm 2* in GC-1 cells is somewhat surprising, as these are all post-meiotically expressed genes,

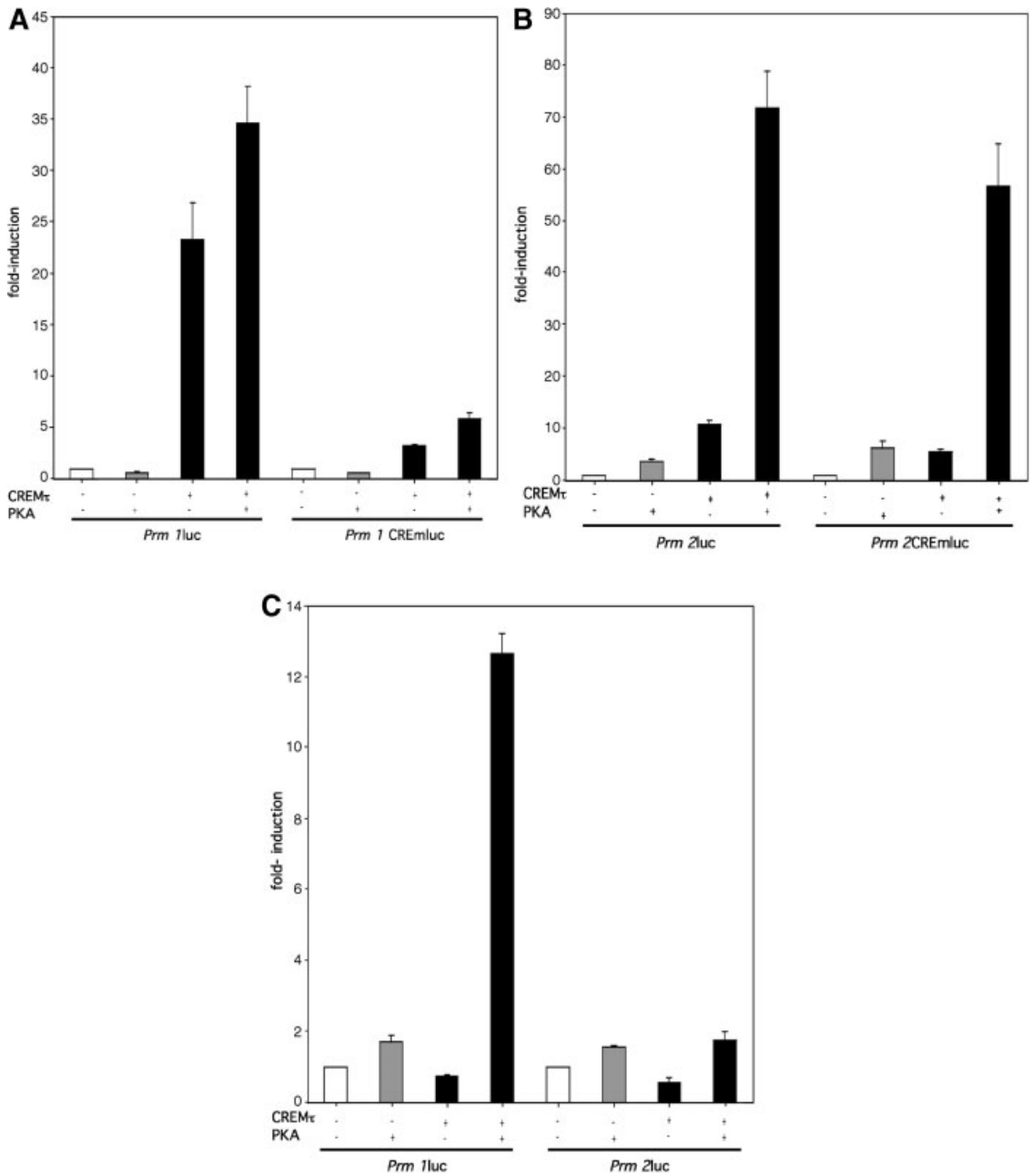

Fig. 5. Differential activation of prm 1 and 2 promoters by. **A**: The ability of CREMτ to activate the *Prm 1* promoter was examined by transient transfection of 500 ng of *Prm1* luc reporter plasmid ±50 ng of the pCMV CREMτ expression vector, ±250 ng of a PKA catalytic subunit expression vector in GC-1 cells. Either *Prm 1*luc or *Prm 1*CREmluc reporter plasmid were co-transfected into GC-1 cells. *Prm 1*CREmluc contains a linker mutation in place of the −63 CRE. **B**: The ability of CREMτ to activate the *Prm 2* promoter was examined by transient transfection of 500 ng of *Prm 2*luc reporter plasmid ±50 ng of the pCMV CREMτ expression vector, ±250 ng of a PKA catalytic subunit expression vector in GC-1 cells. The CRE-dependence of CREMτ activation was examined by co-transfection of 500 ng of a reporter plasmid containing a mutated −200 CRE (*Prm 2*CREmluc) ±50 ng of pCMV CREMτ expression vector ±250 ng of the PKA catalytic subunit expression vector. **C**: JEG-3 cells were co-transfected with 1.5 μg of either the *Prm 1*luc or the *Prm 2*luc reporters ±250 ng of the pCMV CREMτ expression vector, ±250 ng PKA catalytic subunit expression vector. For Panels (A–C), fold-induction is calculated relative to the basal activity of each reporter in the presence of only the empty expression vector pCMV4. The results shown are representative of at least three experiments.

and the GC-1 cells are derived from mitotic, diploid spermatogonial stem cells (Hofmann et al., 1995). These cells may therefore have a somewhat differentiated phenotype. The difference in CREMτ expression may explain why PKA catalytic subunit is constitutively active in GC-1 cells. However, the presence of CREMτ in GC-1 cells does not rule out PKA activating another factor, since mutation of CRE sequence in the *Prm 2* promoter does not diminish the PKA-alone activity (Fig. 5A).

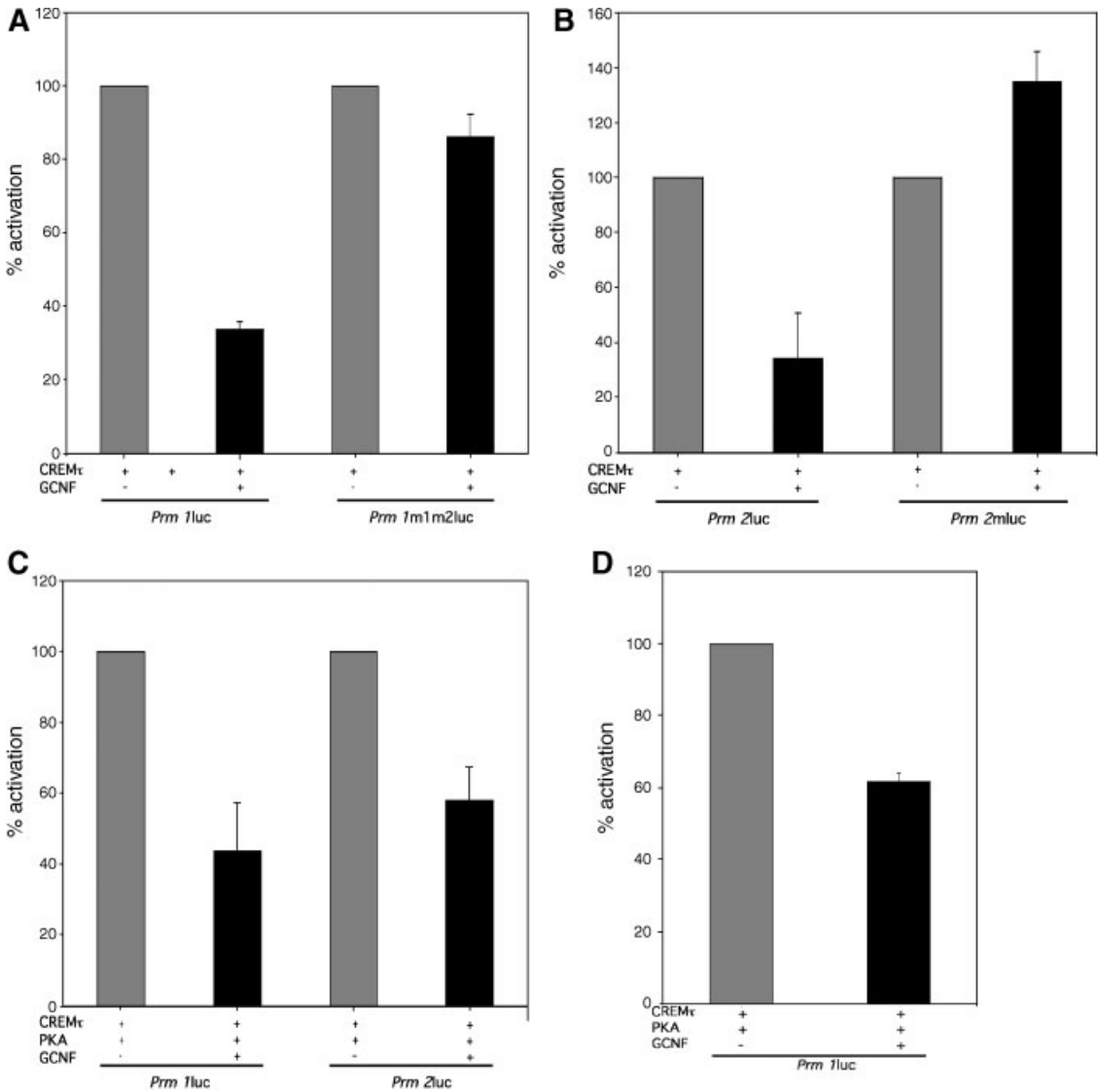

Fig. 6. GCNF represses CREMτ activation of the protamine promoters in a DR0-dependent manner. **A**: To examine the ability of GCNF to repress CREMτ activation of the *Prm 1* promoter, co-transfection of 50 ng of pCMV CREMτ expression vector with 500 ng of either the *Prm 1*luc or *Prm 1*m1m2luc, ±500 ng of pCMVGCNF expression vector, was performed in GC-1 cells. For Panels (**A**) and (**B**), % activation is calculated by setting activity of each promoter in the presence of pCMV CREMτ to 100%. **B**: To examine the ability of GCNF to repress CREMτ activation of the *Prm 2* promoter, co-transfection of 50 ng of the pCMV CREMτ expression vector with 250 ng of either the *Prm 2*luc or *Prm 2*mluc reporters, ±500 ng of the pCMVGCNF expression vector, was performed in GC-1 cells. **C**: The ability of GCNF to repress PKA-induced CREMτ activation of the protamine promoters was examined by co-transfecting 50 ng pCMV CREMτ and 250 ng pCMVPKA expression vectors with either *Prm 1*luc or *Prm 2*luc reporters, ±500 ng pCMVGCNF, into GC-1 cells. **D**: The ability of GCNF to repress CREMτ activation of the *Prm 1* promoter in JEG-3 cells was tested. JEG-3 cells were co-transfected with 250 ng pCMV CREMτ and 250 ng pCMVPKA expression vectors ±500 ng of pCMVGCNF. For Panels (C) and (D), % activation is calculated by setting the activity of each promoter in the presence of both pCMV CREMτ and pCMVPKA to 100%. The results shown are representative of at least three experiments.

The expression profile of the seven major PKA subunits in both GC-1 and JEG-3 cells is shown in Figure 7C. Two striking differences are immediately obvious. First, all seven subunits are expressed in JEG-3 cells. Second, of the regulatory subunits, GC-1 cells express only RIα but not RIβ, RIIα, or RIIβ. GC-1 cells do express two of the catalytic subunits, Cα and Cγ (Fig. 7C). It would appear then that the CREMτ constitutive activation of the protamine promoters observed in the GC-1 cells might be due to insufficient regulatory subunits of PKA for the amount of catalytic subunits present. Furthermore, the presence of all the regulatory subunits in the JEG-3 cells would then account for the inactivity of CREMτ on the protamine promoters without benefit of transfected PKA catalytic subunit. The differences in gene expression observed thus may be responsible for the differences in transcriptional activity of the protamine promoters observed

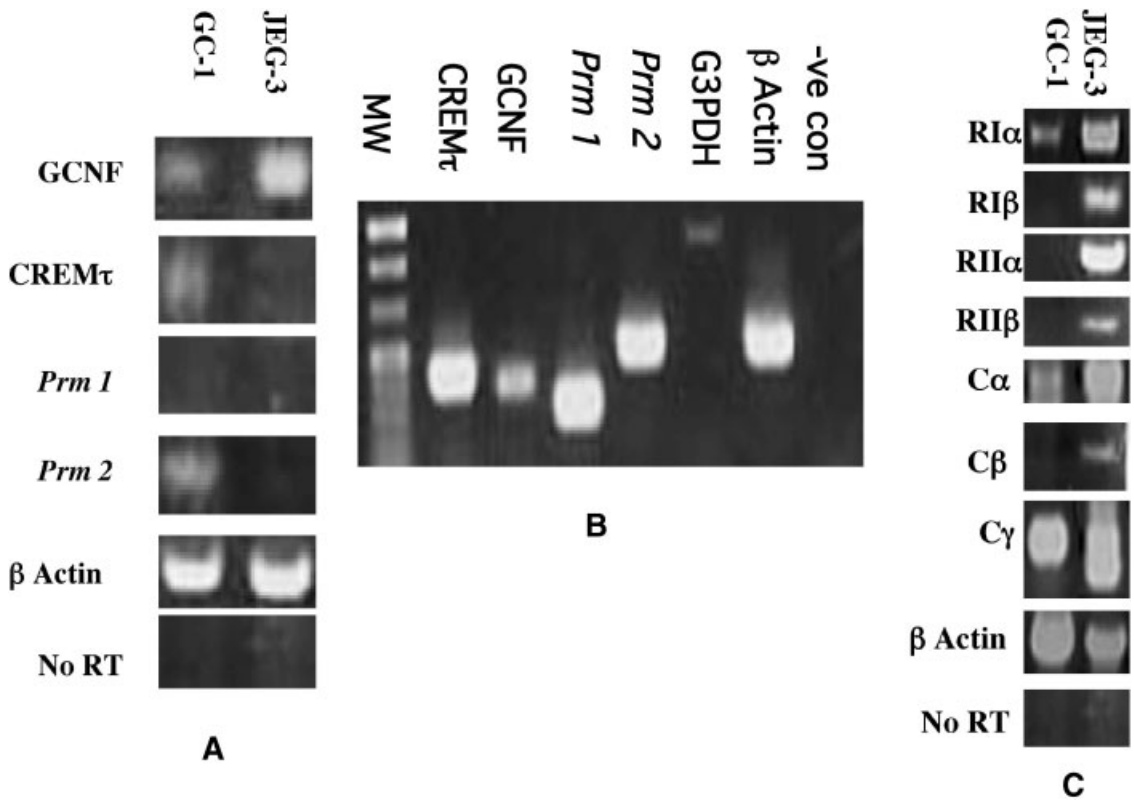

Fig. 7. Expression of GCNF, CREMτ, Protamines, and PKA regulatory and catalytic subunits in GC-1 and JEG-3 Cells. **A**: Agarose gel electrophoresis of RT-PCR performed on RNA from either GC-1 (**left lanes**) or JEG-3 cells (**right lanes**) using primers specific for GCNF, CREMτ, *Prm 1*, and *Prm 2*. β-Actin was used as a loading control. **Bottom panel** shows PCR of samples minus reverse transcriptase in the RT reactions. **B**: Agarose gel electrophoresis of PCR performed on cDNA from Marathon-ready testis cDNA library (Clontech). Primers specific for GCNF, CREMτ, *Prm 1*, *Prm 2* were used. G3PDH and β-actin primers were used as positive controls. −ve con = no template reaction. MW = Lifetech molecular weight marker VIII. **C**: Agarose gel electrophoresis of RT-PCR of RNA from GC-1 or JEG-3 cells using primers specific for PKA regulatory subunits RIα, RIβ, RIIα, RIIβ, and catalytic subunits Cα, Cβ, and Cγ. β-Actin was used as a loading control. No RT indicates samples of RNA were not given reverse transcriptase during RT reactions.

between GC-1 and JEG-3 cells. Differential gene expression in GC-1 and JEG-3 cells provides one tool by which the repressor function of GCNF can be studied.

DISCUSSION

The process of spermatogenesis in male animals is a highly organized, multi-step terminal differentiation program that ultimately produces viable, haploid germ cells capable of fertilization from diploid, undifferentiated cells. For spermatogenesis to occur appropriately, the proteins and genes involved have to be exquisitely regulated. Achieving the appropriate expression requires many levels of regulation, from transcriptional to post-translational. Transcriptional regulation plays an important role, especially in the post-meiotic process of spermiogenesis (Sassone-Corsi, 1997). Here, we have demonstrated that the transcription factors GCNF and CREMτ play reciprocal roles in regulating expression of the *Protamine* genes.

Expression of both GCNF and CREMτ in spermatids correlates with their having a role in regulating spermiogenesis (Delmas et al., 1993; Katz et al., 1997). In this study, several insights into the regulation of the post meiotically expressed *Protamine* genes by GCNF and CREMτ have been elucidated. GCNF represses basal transcription of the mouse *Prm 1* and *Prm 2* genes, dependent on the presence of the naturally occurring DR0 sequences found in the promoters. We had previously demonstrated that GCNF can bind in vitro to the *Prm 1* −370 and the *Prm 2* −220 DR0 sequences, but not to the *Prm 1* −70 DR0 sequence (Hummelke et al., 1998). The relative activation of each of the DR0-tk reporters agrees both with the in vitro binding data (Hummelke et al., 1998), and with the degree of difference from the consensus DR0 sequence (2XDR0tkluc; Fig. 1A). This data suggested that the former two, but not the latter, DR0 sequences would be responsible for GCNF action on these promoters. However, the results shown here indicate that all three DR0 sequences in the protamine promoters are capable of supporting GCNF action. Mutation of either of the *Prm 1* DR0s precludes repression by wild type GCNF, as well as activation by the chimeric GCNF-VP16 (Figs. 2, 3, and 6).

This finding indicates that in the context of the full promoter, GCNF is able to bind to the *Prm 1* −70 DR0 sequence, which was demonstrated using gel mobility shift assays with recombinant GCNF (Fig. 4). The function of GCNF therefore requires binding to both

DR0 sequences. Loss of one of the GCNF binding sites may disrupt formation of the appropriate complex on the *Prm 1* promoter. Repression of the *Prm 2* promoter by GCNF appears to be a little more straightforward than that of *Prm 1*. Having only one DR0 sequence, at −220, repression of the *Prm 2* promoter by GCNF is dependent on the presence of this sequence (Fig. 3). Additionally, GCNF-VP16 activates the 2X220tkluc reporter construct (Fig. 2), indicating that the −220 DR0 sequence is sufficient for GCNF action. Thus, GCNF has been shown to be a repressor of transcription through naturally occurring DR0 sequences in their native promoter contexts.

CREMτ Regulation of *Prm 1* and *Prm 2* Promoters

We also demonstrate that CREMτ does directly activate the mouse protamine promoters (Fig. 5). This result confirms CREMτ binding studies of the protamine promoters (Johnson et al., 1991; Delmas et al., 1993), and mutation of the CREM gene in mouse models (Blendy et al., 1996; Nantel et al., 1996). The fact that there may be a difference in the mechanism by which CREMτ regulates these two promoters is interesting in and of itself. However, even though there may be differential regulation of the protamine promoters individually, their location in a gene cluster on chromosome 16 in mice (Hecht et al., 1986) suggests that their promoters may both contribute to the coordinate expression of these genes.

Two conclusions can be drawn from the results observed in Figure 5. First, CREMτ is constitutively active in GC-1 cells, probably due to the presence of an activating factor that is not present in the JEG-3 cells. Second, PKA is activating a factor that is able to activate both promoters. Constitutive activation of the protamines by CREMτ in GC-1 cells is surprising. One reason for this occurrence may be the presence of endogenous catalytic subunits of PKA in the GC-1 cells, which are basically unopposed due to the virtual absence of regulatory subunits (Fig. 7B). GC-1 cells express two PKA catalytic subunits, Cα and Cγ, but only one regulatory subunit, RIα. Therefore, endogenous RIα in GC-1 cells may be saturated in its ability to inhibit the endogenous catalytic subunits, leaving an excess of Cα and Cγ to partially activate transfected CREMτ. Furthermore, transfected PKA catalytic subunit is therefore able to further activate CREMτ in GC-1 cells. In JEG-3 cells, CREMτ is not constitutively active, because these cells have the full complement of regulatory subunits (Fig. 7B). Transfected PKA catalytic subunit is still able to activate CREMτ in the JEG-3 cells, as expected, due to overexpression of the catalytic subunit from the CMV promoter in the expression plasmid (Materials and Methods). It is therefore possible that the constitutive activation of the protamines by CREMτ in GC-1 cells is due to unopposed endogenous PKA catalytic subunit.

Another explanation for the constitutive activation of the protamine promoters by CREMτ in GC-1 cells is the presence of the calcium/calmodulin-dependent protein kinase IV (CaMKIV) in these cells (data not shown). CaMKIV has been shown to activate transcription by phosphorylating CREMτ in vitro (Sun et al., 1995). RT-PCR analysis of the RNA from the GC-1 and JEG-3 cells indicates that CaMKIV is expressed in the GC-1 cells, but not the JEG-3 cells (data not shown). Activation by transfected CREMτ alone in GC-1 cells therefore may be due to the presence of endogenous CaMKIV in these cells. Endogenous CaMKIV and unopposed PKA catalytic subunits may both contribute to active CREMτ in the GC-1 cells.

It is also observed here that the PKA catalytic subunit alone activates the *Prm 2* promoter in GC-1 cells (Fig. 5A). This is not observed in JEG-3 cells (Fig. 5C). While there is endogenous CREMτ in the GC-1 cells that could activate the promoter in response to PKA (Fig. 7), it would be expected that PKA would have the same affect on the *Prm 1* promoter. The fact that PKA alone does not activate both promoters in GC-1 cells suggests that it is acting through another factor endogenous to these cells, which is specific for the *Prm 2* promoter. The nature of this factor is currently unknown.

GCNF May be Involved in General Transcriptional Shut-Down in Spermiogenesis

The role of GCNF in regulation of both protamine promoters is that of repressor of both basal (Fig. 3), and CREMτ-activated transcription (Fig. 6) in both GC-1 and JEG-3 cells. If CREMτ truly is activating transcription of *Prm 1* and *Prm 2* by different mechanisms, then the ability of GCNF to repress activated transcription of both promoters suggests that GCNF is likely acting directly on the promoters rather than disrupting CREMτ access to the promoter.

The repression of basal transcription of both promoters supports the idea that GCNF may be repressing by inhibiting access to the promoters. Presumably, multiple factors endogenous to the transfected cells are responsible for the basal activity observed. GCNF is able to reduce this activity significantly (Fig. 3). GCNF repression of both basal and activated transcription may be mediated by interactions with co-repressors such as SMRT and N-CoR (Chen and Evans, 1995; Horlein et al., 1995; Yan and Jetten, 2000; Fuhrmann et al., 2001). SMRT and N-CoR not only play a role in repression of activated transcription through interactions with the general transcriptional machinery, but also are known to recruit histone deacetylase activity to promoters, thereby inducing chromatin silencing of transcription by deacetylation of the nucleosome (McKenna et al., 1999). Based on this information, GCNF may be involved in the shut down of transcription at the end of spermiogenesis by a methodical silencing of active genes, and an induction of a closed chromatin conformation.

While cross-talk between the signaling pathways of GCNF and other transcription factors in germ cells is still theoretical, the present study demonstrates that GCNF can influence one factor that is important to spermiogenic-gene expression, including the prota-

mines. The results of the co-transfection studies shown in Figure 5 show that CREMτ can activate both protamine promoters directly in both GC-1 and JEG-3 cells. Activation by CREMτ is repressed by GCNF (Fig. 6). GCNF therefore negatively influences activation of the protamine promoters by CREMτ, and may well do so with other factors. Whether GCNF is also able to positively influence CREMτ activation in response to an unknown signal is not yet known. Elucidation of interactions between GCNF and factors such as CREMτ, on the *Prm1* and *Prm 2* promoters, will give further insight into the role that GCNF plays on the protamine promoters in particular, and in the regulation of spermiogenic-specific genes in general. The ability of GCNF to regulate protamine gene expression would make it an excellent target for contraceptive intervention based on GCNF agonists and antagonists, as disruption of protamine expression leads to infertility, confirmation of which awaits the results of the Cre/Lox knockout of GCNF in the testes.

CONCLUSION

CREMτ is a positive regulator of protamine gene expression, turning it on at the beginning of the round spermatid phase. GCNF is a response element-dependent repressor of transcription, and plays a role in repressing both basal and CREMτ-activated transcription of these genes during spermatogenesis. Thus, GCNF may turn off protamine gene expression at the end of the round spermatid phase.

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REFERENCES

Agoulnik IY, Cho Y, Niederberger C, Kieback DG, Cooney AJ. 1998. Cloning, expression analysis and chromosomal localization of the human nuclear receptor gene GCNF. FEBS Lett 424:73–78.

Andersson S, Davis DL, Dahlback H, Jornvall H, Russell DW. 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J Biol Chem 264:8222–8229.

Bauer UM, Schneider-Hirsch S, Reinhardt S, Benavente R, Maelicke A. 1998. The murine nuclear orphan receptor GCNF is expressed in the XY body of primary spermatocytes. FEBS Lett 439:208–214.

Belokopytova IA, Kostyleva EI, Tomilin AN, Vorob'ev VI. 1993. Human male infertility may be due to a decrease of the protamine P2 content in sperm chromatin. Mol Reprod Dev 34:53–57.

Blendy JA, Kaestner KH, Weinbauer GF, Nieschlag E, Schutz G. 1996. Severe impairment of spermatogenesis in mice lacking the CREM gene. Nature 380:162–165.

Borgmeyer U. 1997. Dimeric binding of the mouse germ cell nuclear factor. Eur J Biochem 244:120–127.

Braun RE, Peschon JJ, Behringer RR, Brinster RL, Palmiter RD. 1989. Protamine 3′-untranslated sequences regulate temporal translational control and subcellular localization of growth hormone in spermatids of transgenic mice. Genes Dev 3:793–802.

Chen JD, Evans RM. 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457.

Chen F, Cooney AJ, Wang Y, Law SW, O'Malley BW. 1994. Cloning of a novel orphan receptor (GCNF) expressed during germ cell development. Mol Endocrinol 8:1434–1444.

Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB, Eddy EM. 2001. Haploinsufficiency of protamine-1 or -2 causes infertility in mice. Nat Genet 28:82–86.

Chung AC, Katz D, Pereira FA, Jackson KJ, DeMayo FJ, Cooney AJ, O'Malley BW. 2001. Loss of orphan receptor germ cell nuclear factor function results in ectopic development of the tail bud and a novel posterior truncation. Mol Cell Biol 21:663–677.

Committee NRN. 1999. A unified nomenclature system for the nuclear receptor superfamily. Cell 97:161–163.

Cooney AJ, Tsai SY, O'Malley BW, Tsai MJ. 1991. Chicken ovalbumin upstream promoter transcription factor binds to a negative regulatory region in the human immunodeficiency virus type 1 long terminal repeat. J Virol 65:2853–2860.

Cooney AJ, Hummelke GC, Herman T, Chen F, Jackson KJ. 1998. Germ cell nuclear factor is a response element-specific repressor of transcription. Biochem Biophys Res Commun 245:94–100.

Cooney AJ, Katz D, Hummelke GC, Jackson KJ. 1999. Germ cell nuclear factor: An orphan receptor in search of a function. Amer Zool 39:796–806.

de Yebra L, Ballesca JL, Vanrell JA, Bassas L, Oliva R. 1993. Complete selective absence of protamine P2 in humans. J Biol Chem 268: 10553–10557.

Delmas V, van der Hoorn F, Mellstrom B, Jegou B, Sassone-Corsi P. 1993. Induction of CREM activator proteins in spermatids: Downstream targets and implications for haploid germ cell differentiation. Mol Endocrinol 7:1502–1514.

Fimia GM, de Cesare D, Sassone-Corsi P. 1999. CBP-independent activation of CREM and CREB by the LIM-only protein ACT. Nature 398:165–169.

Fuhrmann G, Chung AC, Jackson KJ, Hummelke GC, Baniahmad A, Sutter J, Sylvester I, Scholer HR, Cooney AJ. 2001. Mouse germline restriction of Oct 4 expression by germ cell nuclear factor. Dev Cell 1: 377–387.

Giguere V. 1999. Orphan nuclear receptors: From gene to function. Endocr Rev 20:689–725.

Hecht NB, Kleene KC, Yelick PC, Johnson PA, Pravtcheva DD, Ruddle FH. 1986. Mapping of haploid expressed genes: Genes for both mouse protamines are located on chromosome 16. Somat Cell Mol Genet 12:203–208.

Hofmann MC, Abramian D, Millan JL. 1995. A haploid and a diploid cell coexist in an in vitro immortalized spermatogenic cell line. Dev Genet 16:119–127.

Horlein AJ, Naar AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Soderstrom M, Glass CK, et al. 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377:397–404.

Hummelke GC, Meistrich ML, Cooney AJ. 1998. Mouse protamine genes are candidate targets for the novel orphan nuclear receptor, germ cell nuclear factor. Mol Reprod Dev 50:396–405.

Johnson PA, Bunick D, Hecht NB. 1991. Protein binding regions in the mouse and rat protamine-2 genes. Biol Reprod 44:127–134.

Katz D, Niederberger C, Slaughter GR, Cooney AJ. 1997. Characterization of germ cell-specific expression of the orphan nuclear receptor, germ cell nuclear factor. Endocrinology 138:4364–4372.

Laxminarayana D, Khan IU, Mishra N, Olorenshaw I, Tasken K, Kammer GM. 1999. Diminished levels of protein kinase A RIa and RIb transcripts and proteins in systemic lupus erythematosus T lymphocytes. J Immunol 162:5639–5648.

Mali P, Kaipia A, Kangasniemi M, Toppari J, Sandberg M, Hecht NB, Parvinen M. 1989. Stage-specific expression of nucleoprotein mRNAs during rat and mouse spermiogenesis. Reprod Fertil Dev 1:369–382.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, et al. 1995. The nuclear receptor superfamily: The second decade. Cell 83:835–839.

McKenna NJ, Lanz RB, O'Malley BW. 1999. Nuclear receptor coregulators: Cellular and molecular biology. Endocr Rev 20:321–344.

Montminy M. 1997. Transcriptional regulation by cyclic AMP. Annu Rev Biochem 66:807–822.

Nantel F, Monaco L, Foulkes NS, Masquilier D, LeMeur M, Henriksen K, Dierich A, Parvinen M, Sassone-Corsi P. 1996. Spermiogenesis

deficiency and germ-cell apoptosis in CREM-mutant mice. Nature 380:159–162.

Sassone-Corsi P. 1995. Transcription factors responsive to cAMP. Annu Rev Cell Dev Biol 11:355–377.

Sassone-Corsi P. 1997. Transcriptional checkpoints determining the fate of male germ cells. Cell 88:163–166.

Sun Z, Sassone-Corsi P, Means AR. 1995. Calspermin gene transcription is regulated by two cyclic AMP response elements contained in an alternative promoter in the calmodulin kinase IV gene. Mol Cell Biol 15:561–571.

Tsai MJ, O'Malley BW. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451–486.

Yan Z, Jetten AM. 2000. Characterization of the repressor function of the nuclear orphan receptor retinoid receptor-related testis-associated receptor/germ cell nuclear factor. J Biol Chem 275:35077–35085.

Yan ZH, Medvedev A, Hirose T, Gotoh H, Jetten AM. 1997. Characterization of the response element and DNA binding properties of the nuclear orphan receptor germ cell nuclear factor/retinoid receptor-related testis-associated receptor. J Biol Chem 272:10565–10572.

Zhang YL, Akmal KM, Tsuruta JK, Shang Q, Hirose T, Jetten AM, Kim KH, O'Brien DA. 1998. Expression of germ cell nuclear factor (GCNF/RTR) during spermatogenesis. Mol Reprod Dev 50: 93–102.