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# Functional cooperation between CREM and GCNF directs gene expression in haploid male germ cells

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## ABSTRACT

**Cellular differentiation and development of germ cells critically depend on a coordinated activation and repression of specific genes. The underlying regulation mechanisms, however, still lack a lot of understanding. Here, we describe that both the testis-specific transcriptional activator CREMτ (cAMP response element modulator tau) and the repressor GCNF (germ cell nuclear factor) have an overlapping binding site which alone is sufficient to direct cell type-specific expression *in vivo* in a heterologous promoter context. Expression of the transgene driven by the CREM/GCNF site is detectable in spermatids, but not in any somatic tissue or at any other stages during germ cell differentiation. CREMτ acts as an activator of gene transcription whereas GCNF suppresses this activity. Both factors compete for binding to the same DNA response element. Effective binding of CREM and GCNF highly depends on composition and epigenetic modification of the binding site. We also discovered that CREM and GCNF bind to each other via their DNA binding domains, indicating a complex interaction between the two factors. There are several testis-specific target genes that are regulated by CREM and GCNF in a reciprocal manner, showing a similar activation pattern as during spermatogenesis. Our data indicate that a single common binding site for CREM and GCNF is sufficient to specifically direct gene transcription in a tissue-, cell type- and differentiation-specific manner.**

## INTRODUCTION

Spermatogenesis is a complex process that involves the mitotic proliferation of spermatogonial stem cells, meiotic divisions of spermatocytes and morphological changes of haploid spermatids to highly specialized spermatozoa (1). To maintain a proper cellular differentiation and development, a defined set of genes needs to be expressed during a tightly defined time window. Deletion or mutation of just one of these critical genes within these developmental processes could be associated with male infertility or at least sub-fertility in animals and humans. Deletion of the protamine genes, e.g. leads to infertility, as these are essential for chromatin organisation in spermatozoa (2). Other examples involve genes that play an important role in energy metabolism or acrosome reaction of spermatozoa (3–5). Mechanisms must be in place to fulfil several functions at the same time: the activation of the gene in the correct cell type and the silencing of the same gene in all other tissues. The underlying regulation for these mechanisms is largely unknown so far.

The developmental program of spermatogenesis is regulated by several testis-specific transcription factors, including the cAMP response element modulator tau (CREMτ) and the germ cell nuclear factor (GCNF or NR6A1). CREMτ, the testis-specific transcriptional activator, is an alternative splice product of the CREM gene which belongs to the cAMP-regulated family of proteins and binds to the cAMP response element (CRE; consensus sequence 5′-TGACGTCA-3′) (6,7). CREMτ is highly expressed in spermatids during haploid germ cell development (8). Targeted gene disruption of the CREM gene leads to infertility in transgenic animals. Here, spermatogenesis comes to a halt at the round spermatid level (9,10). CRE or CRE-like sequences are frequently observed in haploid expressed genes (11)

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and have been shown to be critical for testis-specific expression in humans and mice (12–15).

The germ cell nuclear factor is a member of the nuclear receptor superfamily of ligand-activated transcription factors (16,17); however, a ligand for GCNF has still not been identified. Targeted gene disruption of GCNF leads to embryonic lethality (18). GCNF is suggested to be a transcriptional repressor. GCNF dimers bind to a direct repeat of the nuclear receptor half-site (5′-AGGT CA-3′) with zero base pairs spacing between the half-sites or to extended single half-sites (19–21). During embryonic stem cell differentiation, GCNF binds and represses pluripotency genes (22–24). Within an adult male organism GCNF is detectable in testis, where the protein is highly expressed in spermatids (25,26). GCNF binding sites have been described in a limited number of testis-specific gene promoters (19,27–29).

We previously described a dual DNA response element for the binding of CREMτ and GCNF within the testis-specific promoter of the mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) gene (30,31). mGPDH contributes to the aerobic metabolism and plays a role in capacitation of spermatozoa (32). Targeted gene disruption of mGPDH leads to male sub-fertility (33). Both transcription factors compete for binding to a composite CRE/nuclear receptor binding site (CRE/NR site). We were able to show in cell experiments that CREMτ activates gene transcription whereas GCNF suppresses this activation (31). Interestingly, a very similar regulation mechanism has been described for the protamine 1 and 2 genes (34). Since this reciprocal regulation pattern resembles the expression pattern of several testis-specific target genes in haploid male germ cells, we further characterized the CRE/NR site. In this report, we show that the CRE/NR site has an optimal structure for binding both factors. CREM and GCNF compete for binding to the DNA response element and also interact with each other via protein–protein interaction. A number of testis-specific target genes are regulated in a similar manner. Integration of the CRE/NR site upstream to a heterologous promoter is sufficient to specifically express a transgene in haploid germ cells. No detectable expression in any other somatic tissue or other stages of germ cell differentiation has been found. Our data indicate that a small DNA stretch of only 26 nucleotides is sufficient to direct testis-specific gene expression.

## MATERIAL AND METHODS

### Isolation and characterization of DNA sequences

Human mGPDH promoter sequence from −62 to −37 (5′-ggtatccttttgtgaggtcaacaatga-3′) was designed as double-stranded oligonucleotides and ligated upstream of the minimal rat prolactin core (PRL) promoter (from −38 to +36) into pGL3-basic (Promega, Mannheim, Germany), generating the construct CRE/NR-PRL-Luc. Mutant versions of this construct were similarly designed generating the constructs CRE/NR-4C-PRL-Luc and CRE/NR-5T-PRL-Luc, respectively (for oligonucleotide sequences see below). Expression plasmids for CREMτ (NM_183011) C-terminally fused with the FLAG epitope and GCNF (AF390896) N-terminally fused with the haemagglutinin (HA) epitope have been described (30,31). For bacterial expression full-length CREMτ (amino acids 1 to 283) was amplified by PCR and subcloned into pGEX-2T (Promega) generating a glutathione S-transferase (GST) fusion protein [GST–CREM (1–283)]. Various deletion fragments thereof were generated in a similar way resulting in fusion proteins GST–CREM (1–95), GST–CREM (90–220) and GST–CREM (215–283), respectively. Full-length GCNF was amplified by PCR and subcloned into pGEX-2T generating the construct GST-GCNF (1–495). Various deletion fragments thereof were generated in a similar way resulting in fusion proteins GST-GCNF (1–150), GST-GCNF (65–150) and GST-GCNF (140–495), respectively. A sequence portion of GCNF from amino acids 65 to 160 was additionally cloned in pcDNA3.1 (Invitrogen, Karlsruhe, Germany). Testis-specific promoter-reporter constructs of proacrosin (from −263 to +548), ELP (−652/+617) and SP-10 (−408/+28) in pGL3-basic were kind gifts of Sandra Danner (Fraunhofer Institute, Lübeck) (35). The testis-specific promoters of t-ACE (−114/+19) and Pdha2 (−236/+31) were amplified by PCR from mouse genomic DNA and subcloned into pGL3-basic (Supplementary Figure S1). For the generation of transgenic mice, the CRE/NR-PRL sequence portion were excised and introduced into the pWhere vector (InvivoGen, San Diego, USA) upstream of a LacZ gene. All constructs were confirmed by DNA sequencing. Methylated plasmids were obtained by incubation with SssI methylase (New England Biolabs, Frankfurt am Main, Germany) according to the manufacturer's recommendations and controlled by digestion with HpaII (methylation-sensitive) and the methylation-insensitive isoschizomer MspI. Quantitative real-time PCR was conducted as described (36). PCR primers were as follows: LacZ for: 5′-gacaccac agccacagacatc-3′; LacZ rev: 5′-cccagagagttgcccattgc-3′; β-actin for: 5′-tgatggtgggaatgggtcagaa-3′; β-actin rev: 5′-ccaagaaggaaggctggaaaag-3′.

### Electrophoretic mobility shift assay

*In vitro*-translated CREMτ-Flag and GCNF-HA were synthesized by a transcription/translation-coupled reticulocyte lysate system (TNT kit, Promega) according to the manufacturer's instructions. One microlitre of *in vitro*-translated CREMτ or GCNF was incubated with 10 fmol of radioactively labelled double-stranded wild-type oligonucleotide probe 5′-ggtatcctttgtgaggtcaaca atgacatta-3′ in the presence of 1 μg of poly(dA-dT)* poly(dA-dT) as described previously (30,31). For competition experiments, a 50-fold molar excess of double-stranded wild-type or mutated oligonucleotides were added to the binding reaction. Oligonucleotide sequences are as follows (italic letters indicate the central CRE/NR site, bold face letters indicate point mutations): wild-type, 5′-ggtatcctttg*tgaggtca*acaatgacatta-3′; mutation 1T → 1A, 5′-ggtatcctttg***a***gaggtcaacaatgacatta-3′; 4G → 4C, 5′-ggtatc ctttg*tgacgtca*acaatgacatta-3′; 5G → 5T, 5′-ggtatcctttg

*tgag**t**tca*acaatgacatta-3′; 7C → 7T, 5′-ggtatcctttg*tgaggt**t**a* acaatgacatta-3′; 8A → 8C, 5′-ggtatcctttg*tgaggtc**c***acaatga catta-3′. For supershift experiments, 1 µl of anti-HA (#2367, Cell Signalling Technology, Frankfurt am Main, Germany) or anti-Flag (M2) antibodies (#F1804, Sigma-Aldrich, Taufkirchen, Germany) were added to the binding reaction mixtures and pre-incubated for 60 min at room temperature. The shifted DNA bands were separated on 5% polyacrylamide gels and visualized by autoradiography on Kodak XAR films (Kodak, Rochester, USA).

### Transient transfection experiment

Human embryonic kidney HEK293 and human hepatocarcinoma HepG2 cells were cultured under standard conditions in Dulbecco's modified Eagle's medium plus Glutamax (Invitrogen) and 10% fetal calf serum as described. Transient transfection experiments were performed using a modified calcium phosphate technique (30,31,37). Typically, $\sim 7 \times 10^5$ cells per 9.6 $cm^2$ well were incubated with 2 µg promoter-containing pGL3 luciferase plasmid and 0.2 µg CREM or GCNF expression plasmids. Expression plasmids have been described above. For trichostatin A (TSA) inhibition experiments, TSA was added to a final concentration of 100 ng/ml 24 h before the cells were harvested. Luciferase assays were carried out in duplicates as described and normalized to the total protein concentration of the samples, which were determined by the Bradford method (Bio-Rad). Each construct was tested in at least three independent transfection experiments with two to three culture dishes per experiment ± SD.

### Chromatin immunoprecipitation assay

For chromatin immunoprecipitation (ChIP) analyses, 1 µg of CRE/NR-PRL-Luc reporter vectors, 0.1 µg CREMτ plus optionally 0.1 µg GCNF expression plasmids were transfected into HepG2 cells as described (38). Chromatin was immunoprecipitated by anti-acetylated histone H3 (#06-599, Upstate, Hamburg, Germany), anti-Flag (Sigma, #F1804), anti-HA (Santa Cruz, #Y-11), anti-GCNF (gift of Uwe Borgmeyer) or irrelevant antibodies (untreated non-specific rabbit antiserum). Immunoprecipitated or input DNA was purified using Qiaquick spin columns (Qiagen, Hilden, Germany) according to the instructions of the manufacturer amplified by quantitative PCR using pGL3forward (5′-ctagcaaaataggctgtccc-3′) and pGL3reverse primers (5′-ctttatgtttttggcgtcttc-3′) as described.

### Protein–protein interaction

A GST-pull down was used to access the physical interaction between CREM and GCNF. GST fusion proteins (see above) were bacterially expressed in BL21 *Escherichia coli* according to standard protocols and extracted in extraction buffer (150 mM NaCl, 10 mM $Na_2HPO_4$, 1 mM DTT, 0.5 mM AEBSF, pH 7.4). The expression of correctly sized proteins was monitored by SDS–PAGE. One hundred micrograms protein of total bacterial lysate was coupled to 150 µl glutathione sepharose 4B beads (GE Healthcare, München, Germany) for 1 h at 4°C with constant shaking. The GST fusion protein coupled beads were intensively washed with PD buffer (100 mM NaCl, 50 mM Tris–HCl, 0.1 mM EDTA, 0.1% BSA, 0.05% Triton X-100, 1 mM DTT, 0.2 mM AEBSF, pH 7.5). *In vitro*-translated L-[$^{35}$S] methionine-labelled protein (TNT kit, Promega) was added, incubated for 2 h at 4°C and afterwards washed three times with PD buffer. The coupled beads were boiled in loading buffer and separated by SDS–PAGE. The gel was dried and autoradiographed.

For co-immunoprecipitations, expression plasmids of Flag-tagged CREMτ and HA-tagged GCNF were transfected into HepG2 cells. Proteins were extracted in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 1 mM EDTA, 1% Triton X-100, 0.5 mM AEBSF, pH 7.4) and coupled to anti-Flag M2 affinity gel (Sigma) according to the instructions of the manufacturer in the presence of 160 U DNase I. The coupled beads were washed three times with TBS (150 mM NaCl, 50 mM Tris–HCl, pH 7.4) and applied to SDS–PAGE. Western blot analysis were performed using anti-Flag antibodies (Sigma, #F1804) and anti-HA antibodies (Santa Cruz, #Y-11).

### Transgenic mouse lines

A sequence portion of CRE/NR-PRL-LacZ was excised from the plasmid CRE/NR-PRL-pWhere by PacI digestion and gel eluted. The excised fragment contains the sequences from −62 to −37 of the human mGPDH gene upstream of the minimal PRL promoter (−38/+36) upstream of the LacZ gene. Pronucleus injection and generating of founder animals were conducted by the transgenic core facility at the ZMNH (University Hamburg, Germany) according to standard protocols. Animal experiments have been approved by local authorities. Three founder mice were proven to carry the transgene and two transgenic mouse lines were established (#14 and #58). Both lines show essentially identical results and data are shown from line #58. Transgenic mice were identified by PCR screening of genomic DNA isolated from tail biopsies.

### Histological staining of LacZ activity in transgenic mice

For LacZ staining, transgenic animals or wild-type littermates were killed by decapitation. Testis was prepared, incubated in 4% paraformaldehyde for 1 h at 4°C and washed in PBSmnn (150 mM NaCl, 10 mM $Na_2HPO_4$, 2 mM $MgCl_2$, 0.02% NP40, 0.01% Na-deoxycholate, pH 7.4). Tissue was incubated with 0.01 U Proteinase K in PBSmnn for 2 min at 37°C, washed again with PBSmnn and incubated over night at 37°C in X-Gal staining solution [PBSmnn + 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/ml X-Gal]. Testis tissue was again washed in PBSmnn and incubated overnight in 4% paraformaldehyde at room temperature. After dehydrating in ascending solutions of isopropanol the tissue was embedded in paraffin. Ten micrometre sections were dried and hydrated with decreasing concentrations of isopropanol, counterstained with nuclear fast

red (Roth, Karlsruhe, Germany) and analysed by microscopy (Zeiss, Oberkochen, Germany).

## RESULTS

### Reciprocal regulation of a testis-specific promoter by CREM and GCNF

We previously described the reciprocal regulation of the post-meiotically expressed target gene mGPDH by CREMτ and GCNF. In cell experiments, we demonstrated a CREM-mediated activation of an mGPDH promoter-driven reporter construct, whereas additional co-transfection of GCNF reversed this activity (31). These assays were limited by the low basal activities of used promoters and it was impossible to detect GCNF repression without preceding CREM activation. To overcome this problem, we introduced the dual CREM and GCNF binding site (CRE/NR site) upstream of the minimal prolactin (PRL) promoter, generating the construct CRE/NR-PRL-Luc. Due to the heterologous promoter portion, this construct shows significant activities above background levels in HEK293 cells. Co-transfection of CREMτ further activated the CRE/NR-PRL-Luc construct, whereas additional co-transfection of GCNF reversed the reporter activity (Figure 1A) as observed previously. Western blot analysis indicated no changes in CREMτ expression levels, thus GCNF-mediated down-regulation is not due to altered CREM concentrations (not shown). Transfection of GCNF alone dampened reporter activities suggesting a direct interaction of GCNF with the response element and application of trichostatin A (TSA), an inhibitor of histone deacetylases, partially relieves GCNF-mediated repression (Figure 1A). To get further insight into the repression mechanism of GCNF, we took advantage of a GCNF-VP16 fusion protein. In this construct, the full-length GCNF protein (including the repressor domain) has been fused to the viral activator domain VP16. As shown in Figure 1B, CRE/NR-PRL-Luc reporter activities were increased after cotransfection of GCNF-VP16 but not with VP16 alone. This indicates that GCNF directly binds to the reporter.

### CREM and GCNF modify chromatin structure

To further investigate the molecular consequences of CREMτ activation and GCNF repression we performed ChIP analysis. After co-transfection of CREMτ and GCNF chromatin was fixed by formaldehyde and precipitated with an antibody against acetylated histone H3 (α-acetyl H3), a marker of transcriptional open chromatin. As expected, CREM-action leads to higher acetylated chromatin. In contrast GCNF-action leads to deacetylation or at least impeded acetylation of core histone molecules (Figure 2). This demonstrates a close correlation between chromatin accessibility and reporter gene activities and further supports earlier observations of correct chromatin organisation at transfected DNA templates (38,39).

Promoter occupancy increases after co-transfection of CREM as expected. Interestingly, the amount of promoter-bound CREM is not reduced after additional co-transfection of GCNF. This indicates that GCNF-mediated repression is not due to release of CREM from DNA. Similar results have been observed by immunoprecipitating GCNF-bound DNA (Figure 2).

### Composition of the critical DNA binding site

Next, we intended to find out which part of the dual CRE/NR binding site is critical for binding of CREM and GCNF, respectively. To achieve this aim, we incubated *in vitro*-translated CREM or GCNF with labelled wild-type CRE/NR site and competed with wild-type

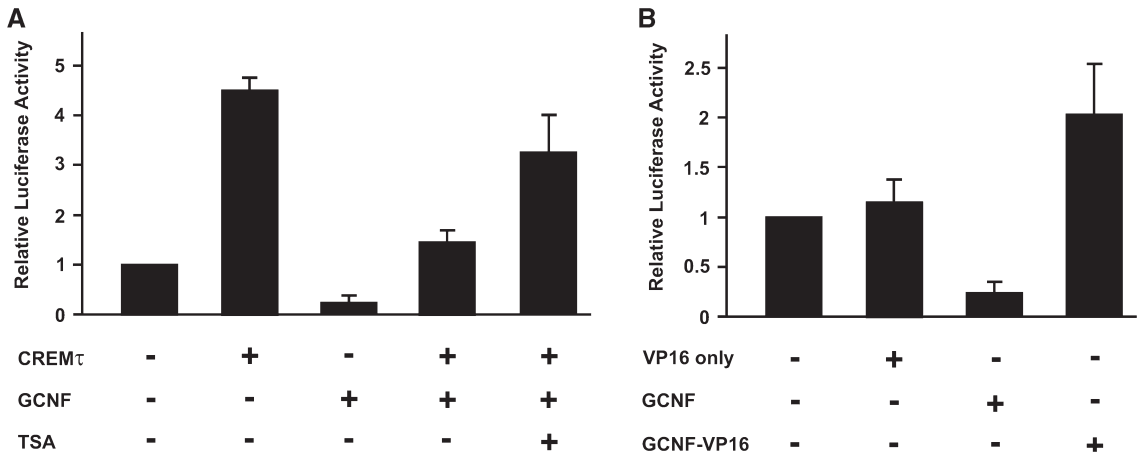


**Figure 1.** Transcriptional modulatory function of CREMτ and GCNF. Transient transfection experiments were performed in HEK293 cells. (**A**) Expression vectors for CREMτ and GCNF were co-transfected with a luciferase reporter vector carrying an mGPDH testis-specific promoter fragment from −62 to −37 upstream of the minimal prolactin promoter (CRE/NR-PRL-Luc). Optionally, cells were stimulated with TSA. (**B**) Expression vectors for the viral activator domain VP16 (VP16 only), full-length GCNF or a fusion construct of full-length GCNF and VP16 (GCNF-VP16) were co-transfected with the CRE/NR-PRL-Luc reporter. Promoter activities are presented relative to CRE/NR-PRL-Luc activities, normalized to the total protein concentration of the cell extract ± SD. Each construct was tested in five independent transfection experiments with three culture dishes per experiment.

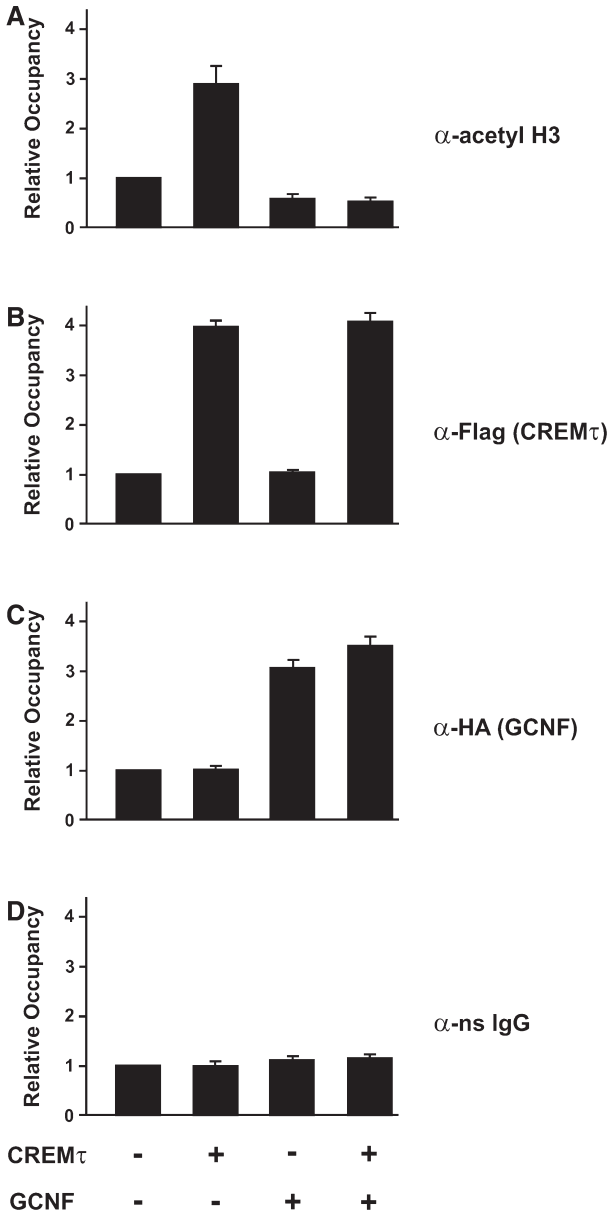


**Figure 2.** CREMτ and GCNF bind to the CRE/NR site. For ChIP analysis, expression plasmids for CREMτ, GCNF and the CRE/NR-PRL-Luc reporter were co-transfected in HepG2 cells. Chromatin was fixed by formaldehyde and preserved as input control or immunoprecipitated with antibodies against (**A**) acetylated histone H3 (α-acetyl H3), (**B**) Flag-CREMτ (α-Flag), (**C**) HA-GCNF (α-HA) or (**D**) non-specific antibody (α-ns IgG). Immunoprecipitated DNA was quantified by quantitative PCR and normalized to input controls of the same samples.

or various mutated versions of this site in an EMSA experiment. Under these experimental conditions, the binding to labelled wild-type sequence is not completely blocked by a molar excess of unlabelled sequence. Thus, we can distinguish between better and poorer binding sites in this competition assay. As shown in Figure 3A, mutation of the wild-type sequence 5′-*t*gaggtca-3′ to 5′-*a*gaggtca-3′ (mutation 1T → 1A, italic letters) and mutation 4G → 4C (mutation of 5′-tga*g*gtca-3′ to 5′-tga*c*gtca-3′) are not critical for binding to CREMτ. Both mutated versions remain suitable binding sites since they efficiently compete with the labelled wild-type fragment for CREM binding (Figure 3A, arrows). In contrast, mutations 5G → 5T, 7C → 7T and 8A → 8C seem to be critical for CREM binding. All three mutated versions could not prevent binding of CREM to the wild type sequence. To further specify the DNA–protein complexes, we incubated the reaction mixture with anti-Flag antibodies which super shifted the resulting Flag-CREM-DNA complexes to lower mobility (Figure 3A, arrowhead). Unspecific protein–DNA complexes are indicated by asterisks.

In a similar mutation analysis, we investigated the binding of GCNF to the CRE/NR site. As shown in Figure 3B (arrow), mutations 1T → 1A and 5G → 5T are still good binding sites for GCNF, since they efficiently block binding to wild-type CRE/NR. In contrast the mutations 4G → 4C and 8A → 8C are critical for appropriate binding of GCNF, in agreement with previous data (19). Again, the specificity has been proven by incubation with anti-HA antibodies which super shifted the HA-GCNF-DNA complex (Figure 3B, arrowhead).

These data indicate that the CRE/NR site appears to be a mixed optimum sequence for binding to both transcription factors. There are specific nucleotides within this motif which are essential for either CREM or GCNF binding. In particular, the mutation 4G → 4C remains an appropriate binding site for CREM but not for GCNF. In contrast, the mutation 5G → 5T preserved GCNF binding but lost the ability to bind CREM. Based on this observation, we introduced the indicated mutations into the reporter construct CRE/NR-PRL-Luc. We expected that the variant CRE/NR-4C-PRL-Luc remains activated by CREM but is not repressed by GCNF any more. In contrast, we expected that the variant CRE/NR-5T-PRL-Luc is not activated by CREM but remains a target of GCNF repression (compare to Figure 3A and B). However, this was not the case. Cell experiments demonstrated that both constructs are still activated by CREM and repressed by GCNF (Figure 3C and D). Although the induction rates are lower for both mutated constructs and the basal reporter activities are higher (4C) and lower (5T) compared to the wild-type construct.

## CREM and GCNF interact physically

To investigate whether mechanisms other than direct DNA binding to CRE/NR might be involved, we studied whether CREM and GCNF interact physically. We coupled bacterially expressed CREMτ to glutathione sepharose beads via a glutathione S-transferase (GST) fusion protein and incubated the coupled beads with radioactively labelled GCNF. As shown in Figure 4A, GCNF binds physically to CREM. To delineate the interacting domain of CREM, we divided the protein into three overlapping protein fragments. Pull-down experiments localized the interacting domain to the C-terminal portion of the protein from amino acids 215 to 283. This domain contains the DNA binding/leucine zipper domain (bZip) of CREM.

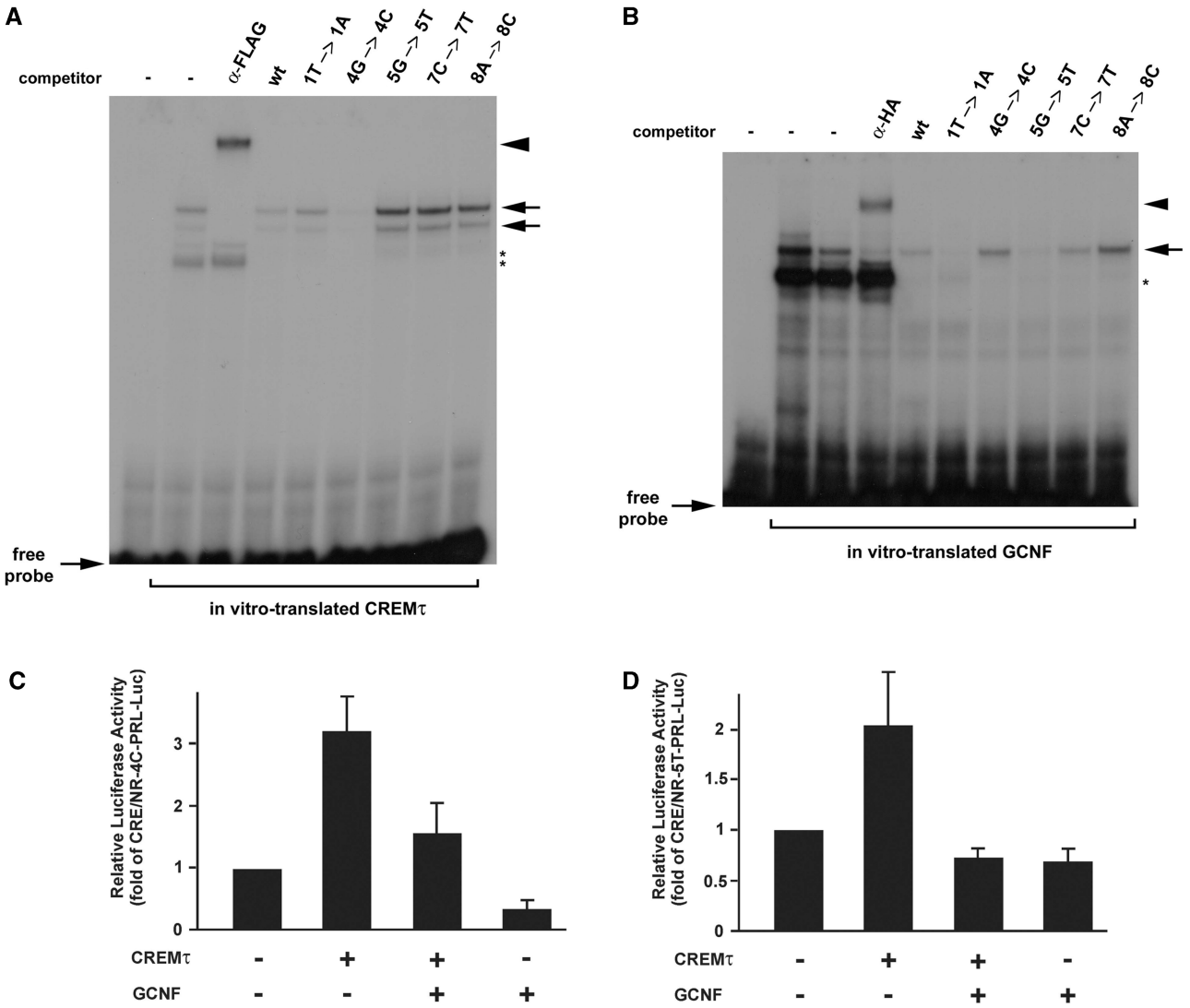


**Figure 3.** Point mutations within the CRE/NR site are critical for CREMτ and GCNF binding. *In vitro*-translated Flag-tagged CREMτ (**A**) or *in vitro*-translated HA-tagged GCNF (**B**) was incubated with the labelled CRE/NR site. For competition experiments, a 50-fold molar excess of wild-type or mutated unlabelled CRE/NR site was added (for sequences see 'Material and Methods' section). Antibodies used for complex interference experiments are indicated. Specific protein–DNA complexes are indicated by arrows, non-specific complexes are indicated by asterisks, and supershift complexes are indicated by arrowheads. (**C** and **D**) Expression plasmids for CREMτ, GCNF and reporter CRE/NR-4C-PRL-Luc (carrying the point mutation 4G → 4C) or reporter CRE/NR-5T-PRL-Luc (carrying the point mutation 5G → 5T) were transfected into HEK293 cells. Promoter activities were determined as described in Figure 1.

In a reciprocal approach, we coupled GCNF to sepharose beads via a GST fusion protein and incubated with radioactively labelled CREM. Again, we demonstrated a protein–protein interaction between CREM and GCNF (Figure 4B). Deletion mutation analysis suggested that a N-terminal portion of GCNF from amino acids 1 to 150, which contains the activator domain AF-1 and the DNA binding domain (DBD) of the protein preserved binding activity, whereas the DBD alone (amino acids 65–150) is not sufficient for CREM binding. However, this domain is sufficient to repress CREM-mediated activation in cell experiments suggesting that the repression activity of GCNF did not completely depend on protein binding to CREM (Figure 4D).

An interaction between CREM and GCNF has been reproduced using co-immunoprecipitation (Figure 4C) and mammalian two-hybrid assays (not shown) indicating that CREM and GCNF interact physically via protein–protein interactions. Since we pre-treated the co-IP samples with DNase, we have no indication that CREM-GCNF binding occurs via a contaminating piece of DNA.

### A common regulation mechanism for several testis-specific gene promoters?

A reciprocal regulation of post-meiotically expressed target genes has been described for mGPDH and the protamine 1 and 2 genes (31,34). To test whether this is a unique or rather common regulation mechanism, we

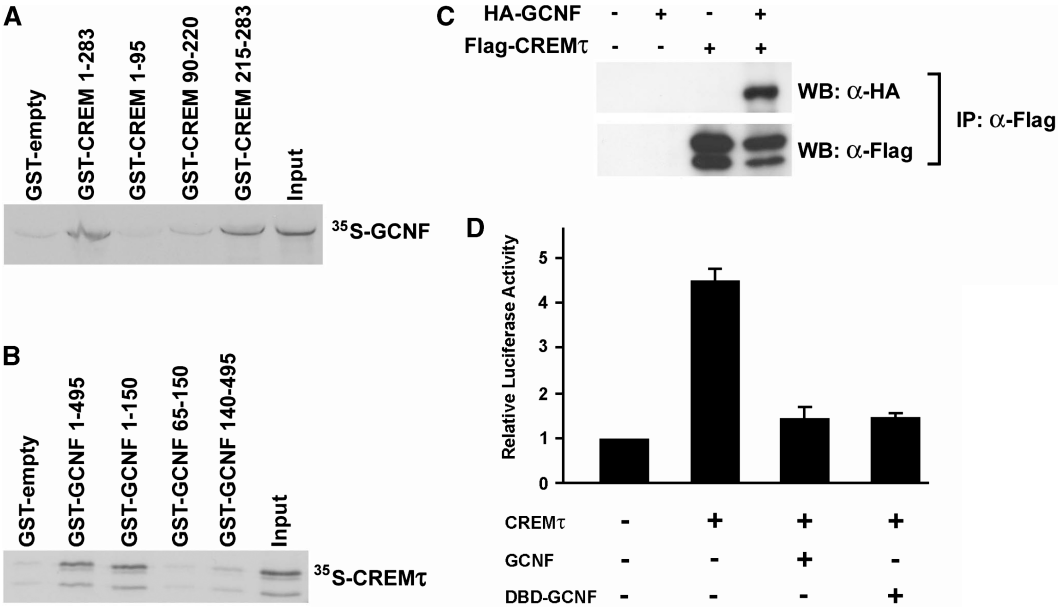


**Figure 4.** CREM and GCNF interact physically. (**A**) Fusion proteins of GST with full length and the indicated deletion mutants of CREMτ were bacterially expressed and coupled to glutathione sepharose beads. Bound beads were incubated with radioactively labelled GCNF to assay protein–protein interactions between CREM and GCNF. (**B**) A fusion protein of GST with the indicated GCNF fragments were coupled to sepharose beads and incubated with radioactively labelled CREMτ. (**C**) Expression plasmids of Flag-tagged CREM and HA-tagged GCNF were transfected in HepG2 cells and immunoprecipitated (IP) with anti-Flag antibodies. Immunopreciptiated fractions were analysed by western blotting (WB) using anti-HA antibodies to detected HA-tagged GCNF and anti-Flag antibodies to detected Flag-tagged CREMτ. (**D**) Cell experiments were performed as described in figure legend of Figure 1A. Additionally, a GCNF deletion fragment containing solely the DNA binding domain of GCNF (DBD-GCNF) was co-transfected demonstrating that this fragment is sufficient for repressor function.

investigated several post-meiotically expressed targets with respect to their regulation by CREM and GCNF. For some of these target genes a CREM-mediated activation has been described [e.g. endozepine-like peptide (ELP), proacrosin, pyruvate dehydrogenase (Pdha2), SP-10, testis-specific angiotensin converting enzyme (t-ACE)], whereas a binding site for GCNF has only been described within the ELP gene promoter (12,28,40–42). However, a reciprocal regulation of CREM and GCNF has not been described for any of these testis-specific promoters. As shown in Figure 5, all five promoter–reporter constructs are activated by CREMτ. With the exception of SP-10 CREM-mediated activation could be reversed after additional co-transfection of GCNF. Nevertheless, reporter activities of all five post-meiotically expressed genes could be blocked by GCNF, although this issue is difficult to address due to the low expression rates of testis-specific promoters in permanent cell lines. Of note, the promoters of proacrosin, t-ACE and mGPDH contain identical CRE/NR sites (Supplementary Figure S1). These data suggest that the reciprocal regulation by CREM and GCNF might be a general principle of gene regulation for at least some post-meiotically expressed target genes.

### Functionality of CRE/NR site *in vivo*

To finally test whether the CRE/NR site is functionally active *in vivo*, we generated a transgenic mouse line. The CRE/NR-PRL sequence portion has been integrated upstream of a LacZ reporter gene. This construct was excised from plasmid DNA, injected into the pronucleus of early mouse embryos and two transgenic mouse lines were established. Different tissues from transgenic animals and wild-type littermates were prepared and RNA was isolated, reverse transcribed and adjusted to quantitative real-time PCR. LacZ expression levels, which are exclusively driven by the CRE/NR-PRL promoter in transgenic animals, were normalized to the expression levels of the housekeeping gene β-actin. In a second normalization step, LacZ expression of transgenic animals were normalized to those of wild-type littermates. As shown in Figure 6A, LacZ specific expression was solely detectable from testis tissue in transgenic animals. Thus the CRE/NR site directs gene expression in a testis-specific manner.

We next wanted to know which particular cell type within the testis is stained by CRE/NR-driven LacZ expression. To this aim, we prepared testis from transgenic animals and wild-type littermates. The tissue was pre-fixed and stained for LacZ activity. After final fixation the tissue was embedded and analysed by microscopy. As shown in Figure 6B–D, we detected LacZ staining solely in different stages of spermatid development (arrows). LacZ staining was neither detectable in earlier developmental steps of germ cells (arrowheads) nor in any other somatic cell type in testis of transgenic animals (asterisks) nor in any cell type of wild-type littermates (Figure 6E).

## DISCUSSION

In this report, we describe a DNA response element which is sufficient to regulate a heterologous promoter activity,

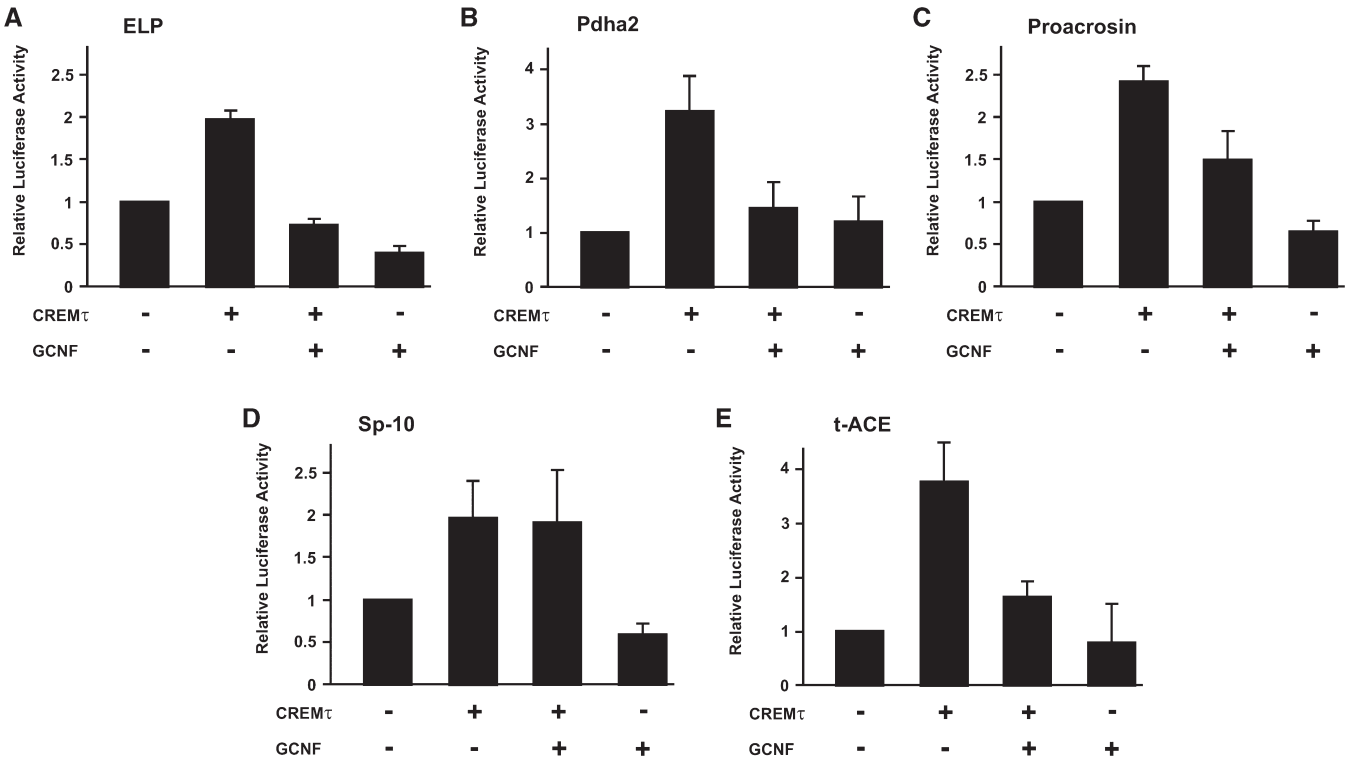


**Figure 5.** CREMτ and GCNF regulate post-meiotically expressed target genes. Expression plasmids for CREMτ, GCNF and promoter constructs for the post-meiotically expressed target genes endozepine-like peptide (ELP) (**A**), pyruvate dehydrogenase 2 (Pdha2) (**B**), proacrosin (**C**), SP-10 (**D**) or testis-specific angiotensin converting enzyme (t-ACE) (**E**) were co-transfected in HepG2 cells. Promoter activities are presented relative to unstimulated luciferase activities, normalized to the total protein concentration of the cell extract ± SD.

resulting in a defined expression in a tissue-, cell-type- and differentiation-specific manner.

Within the testis, several fertility-relevant target genes show a coordinated peak expression during spermatid development in germ cells. These testis-specific target genes contribute to a variety of physiological pathways, such as energy metabolism, chromatin organisation or acrosome reaction of spermatozoa. The coordinated expression pattern during spermiogenesis thus appears to be essential for male fertility. However, the underlying mechanisms for peak expression (in particular up-regulation in round spermatids and down-regulation during spermatid elongation) are unknown. Here, we report a short DNA response element of only 26 nucleotides (CRE/NR site) which serves as dual binding site for the transcriptional activator CREMτ and the transcriptional repressor GCNF. Both transcription factors are highly expressed during the developmental time window (8,25,26). CREMτ activates a CRE/NR-driven heterologous promoter construct in cell experiments, while additional co-transfection of GCNF suppresses this activation (Figure 1A). Both factors compete for binding to the same DNA response element, thus mimicking the expression pattern during spermatid development (Figure 3A and B) and modifying chromatin conformation (Figure 2). Although *in silico* analysis did not reveal transcription factor binding sites other than for CREM and GCNF, we cannot completely rule out this possibility. However, no other CRE-like binding factors (e.g. c-fos, c-jun) or other nuclear receptors (e.g. thyroid hormone receptor, estrogen receptor) have been described to contribute to the promoter activity of mGPDH, including the CRE/NR site (30,37,43).

The CRE/NR response element appears to present an optimal setting for binding both the CRE-binding factor CREM and the nuclear receptor GCNF. Accordingly, the consensus sequence of the CRE/NR site differs for both factors. Specific nucleotides are critical for binding CREM and GCNF, respectively. E.g. a point mutation in the CRE/NR site 5′-tga*g*gtca-3′ to 5′-tga*c*gtca-3′ (mutant 4G → 4C) converts it into the CRE consensus sequence. This CRE consensus site appears to be an even better binding site for CREM (Figure 3A and Supplementary Figure 2A), while GCNF could not bind to this sequence motif any more (Figure 3B). In a reciprocal manner, the mutant 5G → 5T remains a GCNF binding site but is defective to bind CREM (Figure 3A and B). Based on these *in vitro* binding studies it could be suggested that the 4C mutant remains a target for CREM activation but, as GCNF binding capacity is missing, is not being blocked by GCNF any more. On the other hand one could expect that the mutant 5T is not activated by CREM (due to the lack of CREM binding capacity) but remains repressed by GCNF in cell experiments. However, our cell experiments did not confirm this expectation (Figure 3C and D). Therefore, competition of two transcription factors for binding to the same DNA response element appears not to be the sole mechanism of action.

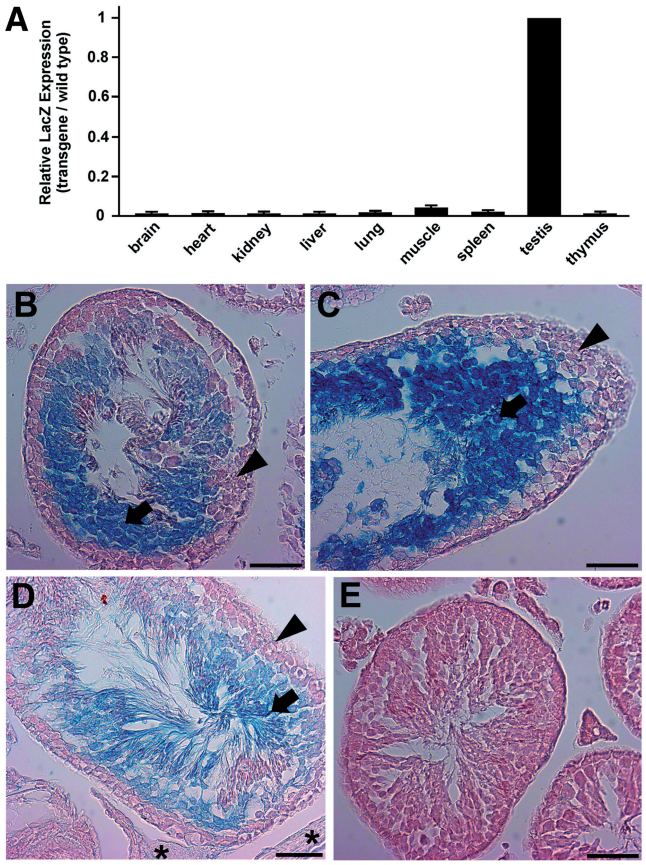


**Figure 6.** Testis-specific expression of a CRE/NR-driven transgene *in vivo*. A transgenic mouse line was constructed carrying the CRE/NR site upstream of a heterologous core promoter driving the expression of LacZ. (**A**) Several tissues from transgene-carrying and non-transgene-carrying mice were prepared and LacZ expression was studied by quantitative PCR. LacZ expression levels were normalized to β-actin expression levels. In a second normalization step, the normalized LacZ expression of transgenes were normalized to those of wild type littermates in a given tissue. Expression levels were expressed relative to testis expression rates. Data are shown from one representative pair of transgene-carrying versus non-transgene-carrying siblings and analyses are performed in triplicates ± SD. Testis of transgene-carrying mice (**B–D**) and wild-type littermate (**E**) were prepared and LacZ activity was monitored in histological sections. LacZ activity was detectable in spermatids (arrows) but neither in earlier steps of germ cell development (arrowheads) nor in other somatic tissues (asterisks). LacZ activity was also absent in non-transgene-carrying wild type littermates (**E**). Sections were counterstained with nuclear fast red and bars represent 50 μm.

Our next approach was to investigate whether CREM and GCNF show direct protein–protein interaction. We were indeed able to demonstrate an interaction between the two transcription factors, and interestingly enough, this interaction is mediated via the DBD of the proteins (Figure 4). Interaction between members of the CRE binding protein family and the nuclear receptor family has been described previously; e.g. the CRE binding protein (CREB) has been shown to interact with the thyroid hormone receptor, thus blocking the thyroid hormone pathway (44). On the other hand, shared DNA response elements of two different transcription factors have also been described (45). We therefore conclude that DNA binding competition represents one mode of regulatory action, while protein–protein interaction could be another one. Interestingly, the DBD of GCNF is sufficient to repress gene transcription in cell experiments (Figure 4D). This part of the protein has previously been shown to be the minimal binding domain for dimeric binding of GCNF to DNA (21,46). Although CREM is known to prefer dimeric binding to DNA as well, we did not observe a heterodimer consisting of the bZip-containing protein CREM and the nuclear receptor GCNF. ChIP and EMSA data indicate that CREM and GCNF are able to bind DNA in the presence of each other; however, binding appears to be mutually exclusive (Figure 2 and Supplementary Figure S3).

Yet another remarkable feature of the CRE/NR site (in contrast to the CRE consensus site alone) is lacking of a central CpG nucleotide. CpGs are almost absent within the testis-specific promoter of mGPDH. CpG dinucleotides are putative targets of DNA methylation which is an epigenetic marker frequently associated with gene silencing. Numerous testis-specific promoters contain a low number of CpGs, whereas others promoters contain clusters of CpGs (know as CpG islands). The CpG-containing promoters are hypo-methylated in testis, in contrast to their hyper-methylated status in somatic tissues (12,47,48). Random methylation of a CpG within a CRE site of a testis-specific promoter would lead to a defective activation by CREM, since CREM cannot bind and activate gene transcription from methylated templates (Supplementary Figure S2). Furthermore, GCNF initiates a cascade of events during embryonic development which leads to DNA methylation and consequently to gene silencing of target genes (24,49). Therefore, it might be an advantage to have a CpG-free CRE site in testis-specific promoters. This argumentation is supported by Yasunaga and co-workers, who found that specific 8-mers are enriched in testis-specific promoters but not in somatic promoters. They discovered that CpG-free CRE-like sites are over-represented in testis-specific promoters, among them the sequences of the CRE/NR site 5′-tgaggtca-3′ and its 5′-extended version 5′-tgtgaggt-3′. In contrast, the CRE consensus and other CpG-containing CRE-like sites are enriched in somatic promoters but under-represented in testis-specific promoters (11). Similar results have been observed in a recent study (50).

It is remarkable that a small DNA fragment of only 26 nucleotides is sufficient to specifically regulate a heterologous promoter, resulting in gene expression in a given developmental stage of germ cell differentiation. First, this construct is sufficient to maintain appropriate expression in spermiogenesis; and second, it blocks expression in any other somatic cell type (Figure 6). The CRE/NR site appears to activate heterologous promoters independent of their sequence composition. For instance, the testis-specific promoter of mGPDH (the CRE/NR site was originally excided from here) does not contain a TATA box but a CAAT box, whereas the minimal prolactin promoter (which is used for the transgenic construct) does contain a TATA box but is deficient in a CAAT box. Several post-meiotically expressed target genes have

been described to contain CRE-like sequences and a number of these gene promoters are regulated by CREM and GCNF in a reciprocal manner (Figure 5). It has been long known that surprisingly small promoter fragments of ~100 nucleotides are sufficient to regulate a transgene, resulting in haploid gene expression in transgenic animals. For protamine 1, a sequence portion from −150 to −37 is sufficient for appropriate localisation. This sequence portion contains both a CRE site and a GCNF binding site (34,51) (Supplementary Figure S1). A second example is the testis-specific promoter of t-ACE which is also reciprocally regulated by CREM and GCNF (Figure 5E). Here, a sequence portion from −91 to +17 is sufficient for testis-specific expression. Interestingly, the sequence portion from −55 to −48 in the same promoter is 100% identical to the CRE/NR site described in this report (40,52) (Supplementary figure 1). Other examples are the promoters of SP-10, pyruvate dehydrogenase 2, lactate dehydrogenase c and β1,4-galactosyltransferase-I (12,15,41,48,53). These transgenic mouse models do show specific expression in testis but are devoid of reporter gene expression in any other somatic tissue. Maybe the transgene serves as an insulator in somatic tissues by tethering the transgene to the nuclear matrix (54). However, to our knowledge it has not been reported that both the testis-specific expression and the lack of expression in somatic tissues was mediated by only one response element in a heterologous context.

Sequence analyses suggested that the position of the CRE/NR site relative to the transcriptional start site (TSS) might be critical for appropriate function. All the CRE-like sequences mentioned above are located at −50 ± 20 nucleotides relative to TSS. This observation is supported by recent findings by Nozaki and co-workers, who examined EGFP-tagged promoter constructs in testis, using an *in vivo* electroporation method for transient transfection. Nozaki and co-workers (14) described that not only the position relative to the TSS but also the orientation of the CRE-like sequence appears to be important for CREM-mediated activation. The positional effect is further supported by the above mentioned *in silico* data. However, effects mediated by orientation appear to be unlikely since the reverse CRE/NR sequence 5′-tgacctca-3′ is also enriched in testis-specific promoters (11).

Conclusively, we suggest that the CRE/NR site represents a key compound for testis-specific expression. The site is a target for a coordinated action of CREMτ and GCNF. The activity of these two factors leads to a tissue-, cell-type- and differentiation-specific expression.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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## REFERENCES

1. Russell,L.D., Ettlin,R.A., Hikim,A.P.S. and Clegg,E.D. (1990) *Histological and Histopathological Evaluation of the Testis*. Cache River Press, Clearwater, FL, USA, pp. 1–40.
2. Cho,C., Willis,W.D., Goulding,E.H., Jung-Ha,H., Choi,Y.C., Hecht,N.B. and Eddy,E.M. (2001) Haploinsufficiency of protamine-1 or -2 causes infertility in mice. *Nat. Genet.*, **28**, 82–86.
3. Krege,J.H., John,S.W., Langenbach,L.L., Hodgin,J.B., Hagaman,J.R., Bachman,E.S., Jennette,J.C., O'Brien,D.A. and Smithies,O. (1995) Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature*, **375**, 146–148.
4. Miki,K., Qu,W., Goulding,E.H., Willis,W.D., Bunch,D.O., Strader,L.F., Perreault,S.D., Eddy,E.M. and O'Brien,D.A. (2004) Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proc. Natl Acad. Sci. USA*, **101**, 16501–16506.
5. Nishimune,Y. and Tanaka,H. (2006) Infertility caused by polymorphisms or mutations in spermatogenesis-specific genes. *J. Androl*, **27**, 326–334.
6. Don,J. and Stelzer,G. (2002) The expanding family of CREB/CREM transcription factors that are involved with spermatogenesis. *Mol. Cell Endocrinol.*, **187**, 115–124.
7. Krausz,C. and Sassone-Corsi,P. (2005) Genetic control of spermiogenesis: insights from the CREM gene and implications for human infertility. *Reprod. Biomed. Online*, **10**, 64–71.
8. Delmas,V., van der,H.F., Mellstrom,B., Jegou,B. and Sassone-Corsi,P. (1993) Induction of CREM activator proteins in spermatids: down-stream targets and implications for haploid germ cell differentiation. *Mol. Endocrinol.*, **7**, 1502–1514.
9. Nantel,F., Monaco,L., Foulkes,N.S., Masquilier,D., LeMeur,M., Henriksen,K., Dierich,A., Parvinen,M. and Sassone-Corsi,P. (1996) Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. *Nature*, **380**, 159–162.
10. Blendy,J.A., Kaestner,K.H., Weinbauer,G.F., Nieschlag,E. and Schutz,G. (1996) Severe impairment of spermatogenesis in mice lacking the CREM gene. *Nature*, **380**, 162–165.
11. Yamashita,A., Goto,N., Nishiguchi,S., Shimada,K., Yamanishi,H. and Yasunaga,T. (2008) Computational search for over-represented 8-mers within the 5′-regulatory regions of 634 mouse testis-specific genes. *Gene*, **427**, 93–98.
12. Iannello,R.C., Gould,J.A., Young,J.C., Giudice,A., Medcalf,R. and Kola,I. (2000) Methylation-dependent silencing of the testis-specific Pdha-2 basal promoter occurs through selective targeting of an activating transcription factor/cAMP-responsive element-binding site. *J. Biol. Chem.*, **275**, 19603–19608.
13. Miyagawa,Y., Nishimura,H., Tsujimura,A., Matsuoka,Y., Matsumiya,K., Okuyama,A., Nishimune,Y. and Tanaka,H. (2005) Single-nucleotide polymorphisms and mutation analyses of the TNP1 and TNP2 genes of fertile and infertile human male populations. *J. Androl.*, **26**, 779–786.
14. Somboonthum,P., Ohta,H., Yamada,S., Onishi,M., Ike,A., Nishimune,Y. and Nozaki,M. (2005) cAMP-responsive element in TATA-less core promoter is essential for haploid-specific gene expression in mouse testis. *Nucleic Acids Res.*, **33**, 3401–3411.
15. Tang,H., Kung,A. and Goldberg,E. (2008) Regulation of murine lactate dehydrogenase C (Ldhc) gene expression. *Biol. Reprod.*, **78**, 455–461.
16. Zechel,C. (2005) The germ cell nuclear factor (GCNF). *Mol. Reprod. Dev.*, **72**, 550–556.
17. Zhao,H., Li,Z., Cooney,A.J. and Lan,Z.J. (2007) Orphan nuclear receptor function in the ovary. *Front Biosci.*, **12**, 3398–3405.
18. Chung,A.C., Katz,D., Pereira,F.A., Jackson,K.J., DeMayo,F.J., Cooney,A.J. and O'Malley,B.W. (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.
19. Yan,Z.H., Medvedev,A., Hirose,T., Gotoh,H. and Jetten,A.M. (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.
20. Susens,U., Aguiluz,J.B., Evans,R.M. and Borgmeyer,U. (1997) The germ cell nuclear factor mGCNF is expressed in the developing nervous system. *Dev. Neurosci.*, **19**, 410–420.
21. Borgmeyer,U. (1997) Dimeric binding of the mouse germ cell nuclear factor. *Eur. J. Biochem.*, **244**, 120–127.
22. Gu,P., LeMenuet,D., Chung,A.C., Mancini,M., Wheeler,D.A. and Cooney,A.J. (2005) Orphan nuclear receptor GCNF is required for the repression of pluripotency genes during retinoic acid-induced embryonic stem cell differentiation. *Mol. Cell Biol.*, **25**, 8507–8519.
23. Hentschke,M., Kurth,I., Borgmeyer,U. and Hubner,C.A. (2006) Germ cell nuclear factor is a repressor of CRIPTO-1 and CRIPTO-3. *J. Biol. Chem.*, **281**, 33497–33504.
24. Sato,N., Kondo,M. and Arai,K. (2006) The orphan nuclear receptor GCNF recruits DNA methyltransferase for Oct-3/4 silencing. *Biochem. Biophys. Res. Commun.*, **344**, 845–851.
25. Lan,Z.J., Gu,P., Xu,X. and Cooney,A.J. (2003) Expression of the orphan nuclear receptor, germ cell nuclear factor, in mouse gonads and preimplantation embryos. *Biol. Reprod.*, **68**, 282–289.
26. Yang,G., Zhang,Y.L., Buchold,G.M., Jetten,A.M. and O'Brien,D.A. (2003) Analysis of germ cell nuclear factor transcripts and protein expression during spermatogenesis. *Biol. Reprod.*, **68**, 1620–1630.
27. Hummelke,G.C., Meistrich,M.L. and Cooney,A.J. (1998) Mouse protamine genes are candidate targets for the novel orphan nuclear receptor, germ cell nuclear factor. *Mol. Reprod. Dev.*, **50**, 396–405.
28. Valentin,M., Balvers,M., Pusch,W., Weinbauer,G.F., Knudsen,J. and Ivell,R. (2000) Structure and expression of the mouse gene encoding the endozepine-like peptide from haploid male germ cells. *Eur J Biochem*, **267**, 5438–5449.
29. He,C., Ding,N. and Kang,J. (2008) Germ cell nuclear factor directly represses the transcription of peroxisome proliferator-activated receptor delta gene. *Acta Biochim. Biophys. Sin.*, **40**, 253–260.
30. Weitzel,J.M., Shiryaeva,N.B., Middendorff,R., Balvers,M., Radtke,C., Ivell,R. and Seitz,H.J. (2003) Testis-specific expression of rat mitochondrial glycerol-3-phosphate dehydrogenase in haploid male germ cells. *Biol. Reprod.*, **68**, 699–707.
31. Rajkovic,M., Middendorff,R., Wetzel,M.G., Frkovic,D., Damerow,S., Seitz,H.J. and Weitzel,J.M. (2004) Germ cell nuclear factor relieves cAMP-response element modulator tau-mediated activation of the testis-specific promoter of human mitochondrial glycerol-3-phosphate dehydrogenase. *J. Biol. Chem.*, **279**, 52493–52499.
32. Kota,V., Dhople,V.M. and Shivaji,S. (2009) Tyrosine phosphoproteome of hamster spermatozoa: role of glycerol-3-phosphate dehydrogenase 2 in sperm capacitation. *Proteomics*, **9**, 1809–1826.
33. Brown,L.J., Koza,R.A., Everett,C., Reitman,M.L., Marshall,L., Fahien,L.A., Kozak,L.P. and MacDonald,M.J. (2002) Normal thyroid thermogenesis but reduced viability and adiposity in mice lacking the mitochondrial glycerol phosphate dehydrogenase. *J. Biol. Chem.*, **277**, 32892–32898.
34. Hummelke,G.C. and Cooney,A.J. (2004) Reciprocal regulation of the mouse protamine genes by the orphan nuclear receptor germ cell nuclear factor and CREMtau. *Mol. Reprod. Dev.*, **68**, 394–407.
35. Danner,S., Kirchhoff,C. and Ivell,R. (2009) Seminiferous tubule transfection in vitro to define post-meiotic gene regulation. *Reprod. Biol. Endocrinol.*, **7**, 67.
36. Wulf,A., Harneit,A. and Weitzel,J.M. (2007) T3-mediated gene expression is independent of PGC-1alpha. *Mol. Cell Endocrinol.*, **270**, 57–63.
37. Weitzel,J.M., Grott,S., Radtke,C., Kutz,S. and Seitz,H.J. (2000) Multiple promoters direct the tissue-specific expression of rat mitochondrial glycerol-3-phosphate dehydrogenase. *Biol. Chem.*, **381**, 611–614.
38. Wulf,A., Harneit,A., Kroger,M., Kebenko,M., Wetzel,M.G. and Weitzel,J.M. (2008) T3-mediated expression of PGC-1alpha via a far upstream located thyroid hormone response element. *Mol. Cell Endocrinol.*, **287**, 90–95.
39. Buschhausen,G., Wittig,B., Graessmann,M. and Graessmann,A. (1987) Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene. *Proc. Natl Acad. Sci. USA*, **84**, 1177–1181.
40. Zhou,Y., Sun,Z., Means,A.R., Sassone-Corsi,P. and Bernstein,K.E. (1996) cAMP-response element modulator tau is a positive regulator of testis angiotensin converting enzyme transcription. *Proc. Natl Acad. Sci. USA*, **93**, 12262–12266.
41. Reddi,P.P., Flickinger,C.J. and Herr,J.C. (1999) Round spermatid-specific transcription of the mouse SP-10 gene is mediated by a 294-base pair proximal promoter. *Biol. Reprod.*, **61**, 1256–1266.
42. Schulten,H.J., Nayernia,K., Reim,K., Engel,W. and Burfeind,P. (2001) Assessment of promoter elements of the germ cell-specific proacrosin gene. *J. Cell Biochem.*, **83**, 155–162.
43. Weitzel,J.M., Kutz,S., Radtke,C., Grott,S. and Seitz,H.J. (2001) Hormonal regulation of multiple promoters of the rat mitochondrial glycerol-3-phosphate dehydrogenase gene: identification of a complex hormone-response element in the ubiquitous promoter B. *Eur. J. Biochem.*, **268**, 4095–4103.
44. Mendez-Pertuz,M., Sanchez-Pacheco,A. and Aranda,A. (2003) The thyroid hormone receptor antagonizes CREB-mediated transcription. *EMBO J.*, **22**, 3102–312.
45. Wehrenberg,U., Ivell,R., Jansen,M., von Goedecke,S. and Walther,N. (1994) Two orphan receptors binding to a common site are involved in the regulation of the oxytocin gene in the bovine ovary. *Proc. Natl Acad. Sci. USA*, **91**, 1440–1444.
46. Greschik,H., Wurtz,J.M., Hublitz,P., Kohler,F., Moras,D. and Schule,R. (1999) Characterization of the DNA-binding and dimerization properties of the nuclear orphan receptor germ cell nuclear factor. *Mol. Cell Biol.*, **19**, 690–703.
47. Iannello,R.C., Young,J., Sumarsono,S., Tymms,M.J., Dahl,H.H., Gould,J., Hedger,M. and Kola,I. (1997) Regulation of Pdha-2 expression is mediated by proximal promoter sequences and CpG methylation. *Mol. Cell Biol.*, **17**, 612–619.
48. Kroft,T.L., Jethanandani,P., McLean,D.J. and Goldberg,E. (2001) Methylation of CpG dinucleotides alters binding and silences testis-specific transcription directed by the mouse lactate dehydrogenase C promoter. *Biol. Reprod.*, **65**, 1522–1527.
49. Feldman,N., Gerson,A., Fang,J., Li,E., Zhang,Y., Shinkai,Y., Cedar,H. and Bergman,Y. (2006) G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis. *Nat. Cell Biol.*, **8**, 188–194.
50. Halperin,Y., Linhart,C., Ulitsky,I. and Shamir,R. (2009) Allegro: analyzing expression and sequence in concert to discover regulatory programs. *Nucleic Acids Res.*, **37**, 1566–1579.
51. Zambrowicz,B.P., Harendza,C.J., Zimmermann,J.W., Brinster,R.L. and Palmiter,R.D. (1993) Analysis of the mouse protamine 1 promoter in transgenic mice. *Proc. Natl Acad. Sci. USA*, **90**, 5071–5075.
52. Howard,T., Balogh,R., Overbeek,P. and Bernstein,K.E. (1993) Sperm-specific expression of angiotensin-converting enzyme (ACE) is mediated by a 91-base-pair promoter containing a CRE-like element. *Mol. Cell Biol.*, **13**, 18–27.
53. Charron,M., Shaper,N.L., Rajput,B. and Shaper,J.H. (1999) A novel 14-base-pair regulatory element is essential for in vivo expression of murine beta4-galactosyltransferase-I in late pachytene spermatocytes and round spermatids. *Mol. Cell Biol.*, **19**, 5823–5832.
54. Abhyankar,M.M., Urekar,C. and Reddi,P.P. (2007) A novel CpG-free vertebrate insulator silences the testis-specific SP-10 gene in somatic tissues: role for TDP-43 in insulator function. *J. Biol. Chem.*, **282**, 36143–36154.