Institut für Biochemie und Molekularbiologie III/I
Zentrum für Experimentelle Medizin
Universitätsklinikum Hamburg-Eppendorf
Direktor: Prof. Dr. med. H.J. Seitz / Prof. Dr. med. G. Mayr

Regulation of mGPDH gene expression in human testis

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
dem Fachbereich Medizin der Universität Hamburg

vorgelegt von

Mirjana Rajković, MD, M. Sc.

aus Smederevo, Serbien.

Hamburg 2006

Angenommen vom Fachbereich Medizin
der Universität Hamburg am: 24.07.2006

Veröffentlicht mit Genehmigung des Fachbereichs
Medizin der Universität Hamburg

Prüfungsausschuss, der/die Vorsitzende: Prof. Dr. H. J. Seitz

Prüfungsausschuss: 2. Gutachter/in: Prof. Dr. W. Schulze

Prüfungsausschuss: 3. Gutachter/in: PD Dr. J. M. Weitzel

Die vorliegende Arbeit wurde in der Zeit von Juni 2003 bis April 2006 am Institut für Medizinischen Biochemie und Molekularbiologie in der der Abteilung für Biochemische Endokrinologie am Universitätsklinikum Hamburg-Eppendorf unter Leitung von Prof. Dr. H.J. Seitz und PD Dr. J. M. Weitzel angefertigt.

Diese Arbeit wurde unterstützt durch das Graduiertenkolleg 336 der DFG.

Results of this Doctoral thesis have been presented and published:

Congresses and Conferences:

- Rajkovic M, Seitz HJ and Weitzel JM. Molecular mechanism of activation and repression of the mGPDH gene in male germ cells. 14th European Workshop of the Molecular &Cellular Endocrinology of the Testis, Bad Aibling, Bavaria, Germany, April 22. -26. 2006 (Poster and oral presentation)
- Weitzel JM and Rajkovic M. A critical DNA response element sufficient to maintain post-meiotic gene expression? 14th European Workshop of the Molecular &Cellular Endocrinology of the Testis, Bad Aibling, Bavaria, Germany, April 22. -26. 2006 (Poster)
- Rajkovic M, Middendorff R, Seitz HJ, Weitzel JM. Regulation of mGPDH gene expression in male germ cells, 4thInternational Workshop on Molecular Andrology, Giessen, Germany, October 7.-9. 2005 (Poster)
- Rajkovic M, Iwen KAH, Harneit A, Kröger M, Seitz HJ, Weitzel J M. Two transcription factors in competition for binding at the testis specific promoter of human mGPDH, GBM Annual Fall Meeting Berlin/Potsdam, Germany, September 18.-21. 2005 (Poster)
- Rajkovic M, Weitzel JM. and Seitz HJ. GCNF relieves CREMtau-mediated gene activation. 13th European Workshop of the Molecular &Cellular Endocrinology of the Testis, Dunblane, Scotland, April 24. -28. 2004 (Poster and oral presentation)

Publications:

- Rajkovic M, Middendorff R, Wetzel MG, Frkovic D, Damerow S, Seitz HJ, Weitzel JM. (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 279/50:52493-52499.

Publication in preparation:

- Rajkovic M, Iwen KAH, Kröger M, Seitz HJ, Weitzel JM. (2006) GCNF antagonizes CREMtau-activated gene expression in haploid male germ cells.

Table of contents

1. **Introduction** 1

1.1. **Spermatogenesis** 1

1.1.1. Gene Expression and Transcriptional Regulation during the Process of Spermatogenesis 2

1.2. **CREM** 4

1.2.1. Mechanisms of Signal Transduction 4

1.2.2. CREM Gene 4

1.2.3. CREM Deficient Mouse 5

1.2.4. Clinical Importance 5

1.2.5. CREM Target Genes 6

1.3. **GCNF** 7

1.3.1. Function of GNCF in Signal Transduction 7

1.3.2. GCNF Gene 7

1.3.3. GCNF Target Genes 8

1.3.4. GCNF Deficient Mouse 8

1.4. **mGPDH** 9

1.4.1. A Role in Energy Production 9

1.4.2. Clinical Importance of mGPDH for Sperm Motility 9

1.4.3. mGPDH Gene Expression 10

2. **Background and objective** 11

3. **Material and Methods** 12

3.1. **Material** 12

3.1.1. Permanent Cell Lines 12

3.1.2. Cells and Tissues 12

3.1.3. DNA and Vectors 12

3.1.4. Oligonucleotides and Primers 14

3.1.5. DNA Markers 15

3.1.6. Protein Markers 15

3.1.7. Enzymes 15

3.1.8. Antibodies 16

3.1.9. Kits 16

3.1.10. Media 17

3.1.11. Buffers and Standard Solutions 17

3.1.12. PCR Programs 19

3.2. **Methods for Cloning** 21

3.2.1. Identification of Human Promoter C and Cloning Strategy 21

3.2.2. Construct hu(-106/+105)-luc 21

3.2.3. Construct mut hu(-106/+105)-luc 21

3.2.4. Construct CREwt-rPrl-luc 22

3.2.5. Construct mut CRE-rPrl-luc 22

3.2.6. Polymerase Chain Reaction (PCR) 22

3.2.7. Agarose Gel Electrophoresis 22

3.2.8. Extraction of DNA from Agarose Gels 23

3.2.9. Determination of DNA Concentrations 23

3.2.10. Restriction Endonucleases 23

3.2.11. Ligation of DNA Fragments into Vectors 24

3.2.12. Transformation of Bacteria 24

3.2.13. Growing of Bacteria 24

3.2.14. Plasmid Preparation 24

3.2.15. Sequencing of DNA 25

3.3. **Analysis of Cloned Promoters** 26

3.3.1. Growing of Permanent Cell Lines 26

3.3.2. Transient Transfection Assay 26

3.3.3. Luciferase Activity Assay 27

3.3.4. Determination of Protein Concentration 27

3.3.5. Statistical Evaluation 27

3.4. **Analysis of Protein-DNA interactions** 28

3.4.1. Electrophoretic Mobility Shift Assay (EMSA) 28

3.4.2. Chromatin Immunoprecipitation 29

3.5. **Methods for protein determination** 30

3.5.1. SDS-PAGE and Western Blotting 30

3.6. **Immunohistochemistry** 31

4. **Results** 33

4.1. mGPDH gene is Regulated by Multiple Promoters 33

4.2. Binding of CREM to Promoter C Sequences 35

4.3. Binding of GCNF to Promoter C Sequences 37

4.4. Influence of CREMtau and GCNF on Human Promoter C Activity 40

4.5. Influence of GCNF and GCNF-VP16 Chimeric Protein on Human Promoter C Activity 42

4.6. Influence of CREMtau and GCNF on CREwt/mut-rPrl-Luc Construct

Activity 44

4.7. Effect of Mutated CRE/NR Site on the Interaction with Transcription Factors in Transient Transfection Experiments 46

4.8. Western Blot Analysis 47

4.9. Influence of an Inhibitor of Histone Deacetylases (Trichostatin A) on Promoter C 48

4.10. The Influence of Nuclear Factors on Chromatin Acetylation and Deacetylation 49

4.11. Immunohistochemistry of Human Spermatozoa and Testis-tissue 50

5. **Discussion** 52

5.1. **Tissue-specific Expression of mGPDH Gene** 52

5.2. **Testis-specific Promoter of the Human mGPDH Gene** 52

5.3. **Regulation of the Testis-specific Promoter of mGPDH by CREMtau and GCNF** 53

5.3.1. CREMtau and GCNF are binding to the CRE/NR Site in Promoter C of mGPDH 53

5.3.2. CREM-mediated Transcriptional Activation of Promoter C in Cell Culture Experiments 55

5.3.3. Supression of CREMtau-mediated Activation by GCNF in Cell Culture Experiments 55

5.3.4. Possible Molecular Mechanism of CREMtau/GCNF Regulation of mGPDH Promoter C Activity 56

5.4. **Effect of Transcription Factors on Chromatin Acetylation and Deacetylation** 57

5.5. **Overlapping Expression Profiles of CREM, GCNF and mGPDH** 59

5.5.1. CREM Expression 59

5.5.2. GCNF Expression 60

5.5.3. mGPDH Expression 61

5.6. **Molecular Mechanism of Regulation of mGPDH Expression in Male Germ Cells - General Regulatory Mechanism for Postmeiotically Expressed Genes?** 63

6. **Summary** 65

7. **Abbreviations** 67

8. **References** 69

9. **Acknowledgements** 77

10. **Declaration** 78

11. **Curriculum Vitae** 79

1. Introduction

1.1. Spermatogenesis

Spermatogenesis is a unique process of differentiation of germ cells which occurs in the tubular seminiferous epithelium of testis. The seminiferous epithelium consists of two types of cells: supporting Sertoli cells and cells that constitute the spermatogenic lineage. The germ cells are organized into four to eight layers that occupy the space between the basal lamina and the lumen of the tubule. These cells divide several times and represent various stages during the continuous process of differentiation of male germ cells (Junqueira LC and Carneiro J, 2003).

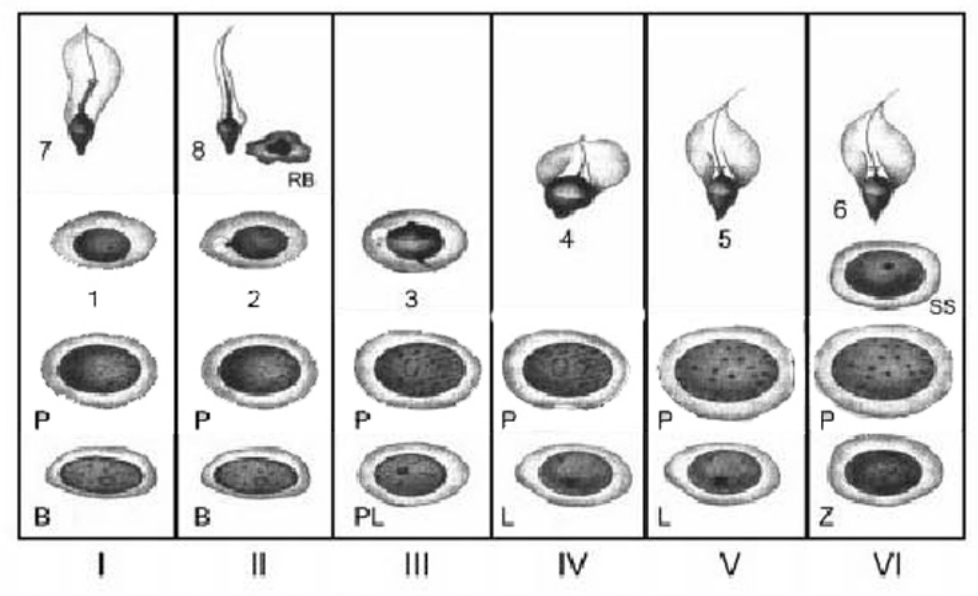

Figure 1. Schematic representation of the six spermatogenic stages in the human testis (modified from Clemont - 1963). Arabic numbers represent steps of spermiogenesis. Roman numbers depict the stage of the spermatogenic cycle.

The process of spermatogenesis can be divided into three phases:

1. Spermatocytogenesis - during which spermatogonia mitotically divide to produce primary spermatocytes.
2. Meiosis - which includes the first meiotic division (during which primary spermatocytes divide to produce secondary spermatocytes) and the second meiotic division without DNA replication, which leads from secondary spermatocytes to haploid (1N) spermatides.

3. Spermiogenesis (post-meiotic phase) during which the haploid round spermatides undergo complex processes of differentiation that includes formation of the acrosome, condensation and elongation of the nucleus, development of flagellum and loss of the majority of the cytoplasm. During this phase the transition proteins and protamines replace histones (Cho C et al. 2001).

The end product of this, about 64 days long process in human, are mature, highly differentiated spermatozoa with haploid number of chromosomes and reduced amount of DNA per cell.

The spermatogenesis occurs in a wavelike fashion in seminiferous tubules, neither simultaneously nor synchronously. Therefore the appearance of tubules is irregular, and each region exhibits a different phase of spermatogenesis.

The spermatogenesis is primary controlled by a genetic program, but it is also a subject of regulation by endocrine, paracrine and autocrine signals. They affect signal transduction pathways and modulate effects of the genetic program (Eddy EM 1998).

The most important effect on spermatogenesis are mediated by endocrine factors, LH and FSH. LH acts on Leydig cells, stimulating production of testosterone which is necessary for normal development of germ cells. More precisely, testosterone acts on Sertoli cells via the androgene receptor, stimulating gene transcription and the secretion of peptides that promote germ cell differentiation (Kimmins S et al. 2004).

FSH acts on Sertoli cells, via receptors on the cell surface, stimulating adenylate cyclases and increasing the concentration of cAMP. Sertoli cells are affecting germ cells by paracrine factors. It is believed that FSH supports spermatogenesis but does not have significant role in regulating gene expression in germ cells.

Effects of other extrinsic factors that influence spermatogenesis (like vitamins, growth factors etc) are mediated mostly by the Sertoli cells. However, this field is still under debate (Eddy EM 1998).

1.1.1. Gene Expression and Transcriptional Regulation during the Process of Spermatogenesis

The genes expressed during spermatogenesis encode proteins necessary for specific structures and functions in different stages of germ cell development, as well as for maintaining the general “house-keeping” functions. Gene expression is stage- and cell-

specific. This is achieved by unique chromatin remodeling, transcription control, and expression of testis specific genes (Kimmins S et al. 2004).
The process of gene expression is strictly regulated at the transcriptional, translational and post-translational levels. The transcriptional regulation plays a particularly important role for proteins synthesized during the postmeiotic phase of spermatogenesis (Eddy EM 1998). The combination of transcription factors that bind to characteristic promoter motifs induces changes of chromatin structure and modulates activity of the transcription machinery.

The tissue-specific gene expression is frequently regulated by unique transcription factors. We were focussed on two testis specific nuclear factors, CREM (cAMP-responsive element modulator) and GCNF (Germ Cell Nuclear Factor) and their influence on regulation of testis-specific promoter C of the postmeiotically expressed mGPDH gene.

1.2. CREM

1.2.1. Mechanisms of Signal Transduction

The cAMP-dependent signal transduction pathway is one of the most important regulatory mechanisms during spermatogenesis. Increased level of cAMP in the cytoplasm causes activation of the catalytic subunit of PKA, its migration into the nucleus, and phosphorylation of target proteins. Signal cascades in the testis include the family of transcription factors (CREB, CREM, ATF) which contain a basic domaine/leucine zipper motif, that enables dimerisation and binding to the cAMP response element (CRE), and regulation of gene expression.

This mechanism is slightly different in somatic (Sertoli) cells and germ cells. After phosphorylation of CREB at Ser 133 CBP is recruited to this site. Activated CREB recruits the basal transcription machinery and activates transcription of genes which are essential for germ cell differentiation. CREB mediated factors produced by Sertoli cells are essential for the survival of germ cells (Don J and Stelzer G, 2002).

Gene activation in germ cells is mainly a result of CREM (cAMP response element modulator) activity. It was shown that the serine residue at position 117 of CREM can be phosphorylated by PKA and by other kinases (Fimia GM and Sassone-Corsi P, 2001). However, in testis-tissue CREM can be activated in a phosphorylation independent manner by the activator of CREM in testis (ACT), a protein which is co-expressed with CREM in round spermatides. ACT has an autonomous activation domain and via binding to CREM it can be activated without Ser-117 phosphorylation and CBP binding (Don J and Stelzer G, 2002). The CREM-ACT complex activates gene expression via interaction with CRE in the gene promoter and recruitment of the general transcription machinery (Kimmins S et al. 2004).

1.2.2. CREM Gene

The CREM gene is localized on the chromosome 10 in humans (localisation 10p11.21). It consists of 14 exons (Blöcher S et al. 2005) and is conserved in mammals (Behr R et al. 2001).

Alternate usage of 4 different promoters and alternative splicing generates repressor and activator forms of CREM in the testis. The repressor variants lacking the transactivation

domain (α, β, γ) are expressed in prepubertal testis and later in premeiotic cells (Foulkes NS et al. 1992). The activator forms are present in postmeiotic haploid germ cells in adult testis and have an important role in regulation of stage and cell specific gene expression (Stelzer G and Don J 2002, Daniel PB et al. 2000, Foulkes NS et al. 1992, Weinbauer GF et al. 1998).

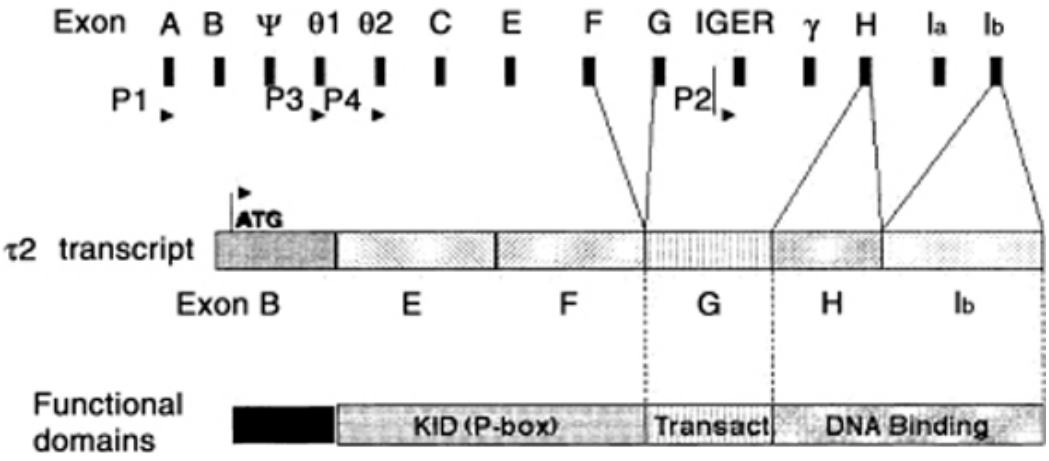

Figure 2. There is a number of CREM transcripts derived from the CREM gene. They encode multiple proteins arising from alternative translation initiation. The middle bar represents the transcription activator τ2 which was used in the current study. The lower bar represents functional domains of CREMτ2 isoform (Modified from Behr R et al. 2001).

1.2.3. CREM Deficient Mouse

CREM is a factor that is necessary for spermatid maturation. In CREM -/- animals spermatogenesis is arrested at the level of round spermatids. In heterozygous animals the number of spermatozoa is reduced as well as a percentage of normal sperms (Nantel F et al. 1996, Blendy JA et al. 1996).

1.2.4. Clinical Importance

CREM mutations can be a causal factor for round spermatid maturation arrest.

The maturation arrest was found in 2-3% of patients with idiopathic infertility. In these patients the lack or reduction of CREM mRNA expression (Steger K et al. 1999) or protein

levels has been observed (Weinbauer GF et al. 1998). It has been reported that the activator/repressor distribution and wrong splicing events could be the reason for infertility (Peri A et al.1998).

1.2.5. CREM Target Genes

Several testis-specific genes contain CRE elements in their promoters: Transition protein 1, Protamine 1 and 2, Calspermin, t-ACE (reviewed by Behr R and Weinbauer GF, 2001). Dimerized CREM binds to a CRE site (8 bp palindromic sequence: 5´-TGACGTCA-3´) in the promoter, usually located 50-250 bp upstream of the transcription start site and subsequently activate gene expression.

The testis-specific promoter C of the mGPDH gene contains a CRE site which is differing from a typical CRE site in one nucleotide (5´- TGA**G**GTCA-3´). It has been shown that CREMtau is able to bind to this nonperfect palindromic sequence and to regulate mGPDH expression in rat (Weitzel JM et al. 2003).

1.3. GCNF

1.3.1. Function of GNCF in Signal Transduction

Germ cell nuclear factor (GCNF) is also known as retinoid acid receptor-related testis-associated receptor (RTR) and neuronal cell nuclear receptor (NCNF). This protein is a member of the nuclear receptor superfamily with the systematic name NR6A1 (Chen F et al. 1994, Hirose T et al. 1995). GCNF is an orphan receptor (without any identified ligands), highly expressed during embryonic development of the nervous system, placental development and embryonal carcinoma cell differentiation (reviewed by Susens U and Borgmeyer U 2000). In adults, transcription is restricted to the developing germ cells of gonads (Chen F et al. 1994, Zhang YL et al 1998).

GCNF is a transcription repressor which binds as a homodimer to DNA, preferentially to a direct repeat of the sequence 5′-AGGTCAAGGTCA-3′ with zero bp spacing (DR-0). The binding of GCNF results in deacetylation of histones in the chromatin of promoter regions and in the repression of gene expression. In promoter C GCNF binds to one half site, at the sequence 5′-AGGTCA-3′ (Cooney AJ et al. 1998).

1.3.2. GCNF Gene

The GCNF gene is located on chromosome 9 at the locus q33-q34.1 (Agoulnik IY et al. 1998). The coding region of this gene contains 11 exons and 10 introns and is highly conserved between mouse, rat and human. Unlike somatic cells where a single transcript of 7.4 kb is detectable, the spermatogenic cells express two transcripts of 7.4 and 2.1 kb. The 7.4 kb transcript is expressed within pachytene spermatocytes. In rodents, the smaller GCNF transcript is expressed predominantly in haploid round spermatids (Katz D. et al. 1997, Yang G et al. 2003). Interestingly, the highest level of GCNF expression in human is detectable in the late stage spermatocytes and less in round spermatids (Agoulnik IY et al. 1998).

In human, three alternatively spliced transcript variants code for three different protein isoforms (GCNF-I, GCNF-2a and GCNF-2b - Susens U and Borgmeyer U, 2001). They are particularly abundant in heterochromatin regions of pachytene spermatocyte and round spermatid nuclei and additionally in condensed chromosomes during meiotic divisions.

GCNF expression profiles in germ cells suggest that this protein plays a critical role in transcriptional regulation of gametogenesis.

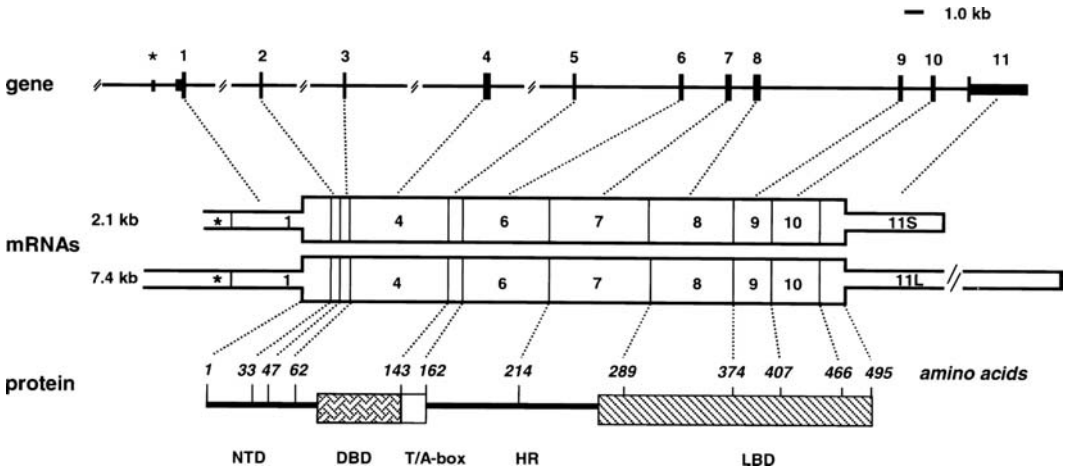

Figure 3. Diagram showing the correlation of the GCNF gene with the two mRNAs (2.1 and 7.4 kb) and the protein which is encoded by this gene. The GCNF mRNAs are shown (the two transcripts share the same open reading frame). The GCNF protein diagram denotes amino acids corresponding to each exon and functional domains (modified from Yang G et al. 2003).

1.3.3. GCNF Target Genes

GCNF plays a role during spermatogenesis, from the pachytene stage of meiotic prophase through the early phase of spermatid differentiation.

This nuclear factor is able to bind to testis-specific promoters of different genes: Oct 4 (Fuhrmann G et al. 2001), tACE (Zhou Y et al. 1996), RT7 (van der Hoorn FA et al. 1990). Protamine 1 and protamine 2, which have DR0 response elements in their promoters are also targets for GCNF regulation (Hummelke GC et al. 1998 and 2004).

1.3.4. GCNF Deficient Mouse

Disruption of GCNF expression is embryonically lethal, with defects in anteroposterior development, including failures in neural tube closure (Chung AC et al. 2001).

1.4. mGPDH

1.4.1. A Role in Energy Production

In cells ATP is mainly synthesized through two sequential processes: glycolysis/fructolysis and mitochondrial oxidative phosphorylation. The H^+ which is produced in the cytoplasm is transferred to the mitochondria through two NADH shuttles, the glycerol-phosphate and the malat-aspartate shuttle (Eto K et al. 1999).

FAD-dependent glycerol-3-phosphate dehydrogenase (mGPDH) (EC 1.199.5) is located on the outer surface of the inner mitochondrial membrane. It catalyses the oxidation of glycerol-3 phosphate to dihydroxyacetone phosphate. The combined action of this enzyme with the cytoplasmic GPDH (EC 1.1.1.8) forms the glycerol-phosphate shuttle (Dawson AG, 1979). This shuttle is forming functional connection between glycolysis/fructolysis in cytoplasm and the respiratory chain in mitochondria (Gong DW et al. 1998).

The activity of this enzyme is highly important in tissues with high energy consumption rate such as muscle and brown adipose tissue (Koza RA et al. 1996, Gong DW et al. 1998), pancreatic islets β-cells (Ferrer J et al. 1996) and spermatozoa (Mietkiewski K and Lukaszyk A, 1966, Weitzel JM at al. 2003).

1.4.2. Clinical Importance of mGPDH for Sperm Motility

The appropriate function of mGPDH is necessary for normal sperm motility. Common problems in subfertile and infertile human patients include low sperm number, low number of morphologically normal sperm, and low number of motile sperm (Turner RM, 2003). Curi SM et al. (2003) reported that approximately 80 % of infertile men had altered sperm motility.

During the process of spermatogenesis mitochondria aggregate around the proximal part of the flagellum, forming a thickened region (middle piece) where the movements of spermatozoa are generated (Junqueira LC and Carneiro J, 2003). Since cell movement is correlated with high energy consumption, a normal expression and function of mGPDH and other components of the respiratory chain is of great importance.

1.4.3. mGPDH Gene Expression

The mGPDH gene is a single-copy gene localized on the chromosome 2 (location 2q24.1) in the human, and consists of 17 exons. The alternative first exon (1a, 1b,1c) is spliced to a common second exon (Gong DW et al. 1998). The translation start codon is localized in the common second exon and the coding sequence is identical in all tissues.
The expression of mGPDH gene is regulated in a tissue-specific manner (Gong DW et al. 1998, Gong Q et al. 2000, Urcelay E et al. 2000, Weitzel JM et al. 2000 and 2001).
Activity of three alternate promoters results in three transcripts with alternate first exons in the 5´- nontranslated region. Promoter A is used in the brain, brown adipose tissue and the pancreas while promoter B is used ubiquitously. The expression of rat mGPDH is additionally regulated by the third testis-specific promoter C (Weitzel JM et al. 2000 and 2003).

The ubiquitous promoter B appears to provide the basic expression level in all tissues. Usage of alternative promoters enables enrichment of mGPDH transcripts in tissues with high energy consumption rates. It was shown, that besides exon 1b containing transcripts also exon 1c containing transcripts are present in the testis (Weitzel JM et al. 2000).

2. Background and objective

Mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) is the rate limiting enzyme of the glycerol-phosphate shuttle which is responsible for proper energy production in a cell. The glycerol-phosphate shuttle connects glycolysis and fructolysis in the cytoplasm with the respiratory chain in the mitochondria.

The expression of the mitochondrial glycerol-3-phosphate dehydrogenase is regulated by multiple promoters in a tissue specific manner. Recently, a third testis-specific promoter C has been described in rat (Weitzel JM et al. 2003).

The usage of alternative promoters enables enrichment of mGPDH transcripts in tissues with high energy consumption rates. In sperm mGPDH contributes to the aerobic metabolism powering its motility. Therefore appropriate expression of mGPDH is necessery for normal sperm function. We demonstrated a reduced motility of spermatozoa and reduced fertility of mGPDH -/-mice. The mGPDH can also be a cause of altered sperm motility in infertile men. However, this topic is until now not well understood.

Therefore, the aim of this study is to characterise the molecular mechanisms of regulation of the mGPDH gene expression in human testis and to prove the importance of two testis-specific transcription factors, CREMtau (cAMP responsive element modulator tau) and GCNF (germ cell nuclear factor) for mGPDH expression and sperm function. The aim of this study was to characterize the binding sites of CREMtau and GCNF within promoter C of mGPDH gene. Further, to investigate the functional consequences of CREMtau and GCNF action in cell culture and their impact on chromatin structure and finally, detection of cellular localization of mGPDH in post-meiotic germ cells.

The developmental program of spermatogenesis is regulated by several testis-specific transcription factors. The cross-talk between CREMtau and GCNF signalling pathways may play an important role in the control of mGPDH gene expression and probably expression of other factors during spermatogenesis. We believe that this study can elucidate the importance of mGPDH for sperm motility in men and bring better insights into the problem of male infertility.

3. Material and Methods

3.1. Material

3.1.1. Permanent Cell Lines:

Human hepatocyte carcinoma cells, HEP G2, ATCC Number: HB-8065
Human embryonic kidney cells, HEK 293, ATCC Number: CRL-1573

3.1.2. Cells and Tissues:

Human testis tissue (from fertile patients who were investigated to exclude a testicular tumor)
Human spermatozoa (from fertile patients)
E. coli, Nova Blue strain (Novagen)

3.1.3. DNA and Vectors:

Human genomic DNA (from healthy volunteer)
Salmon-sperm DNA (Stratagene)
211bp long sequence of human promoter C (-106/+105) - PCR product (see Methods section)

Plasmids used for cloning:
pGEM T- easy (Promega)
pGL3 basic (Promega)

Reporter Vectors used for transient transfections

- **hu(-106/+105)-luc construct:** Human promoter C fragment, subcloned into pGL3-basic luciferase reporter vector (Promega)
- **rCREwt/mut-rPRL-Luc 3 and hCREwt/mut-rPRL-Luc 3:** Flanking CRE site region (-57/-38), wild type (5´- tgaggtca-3´or mutated (5´- t**ct**gg**ag**a-3´), was cloned into pGL3-basic luciferase reporter vector with the minimal prolactin promoter (position -38/+36) **(**gift of Birgit Gellersen, IHF - Gellersen B. 97)

Reporter constructs with point mutation within the CRE site:

- **hCRE 4C-rPRL-Luc 3 construct** (see Methods section)

- **hCRE 5T-rPRL-Luc 3 construct** (see Methods section)

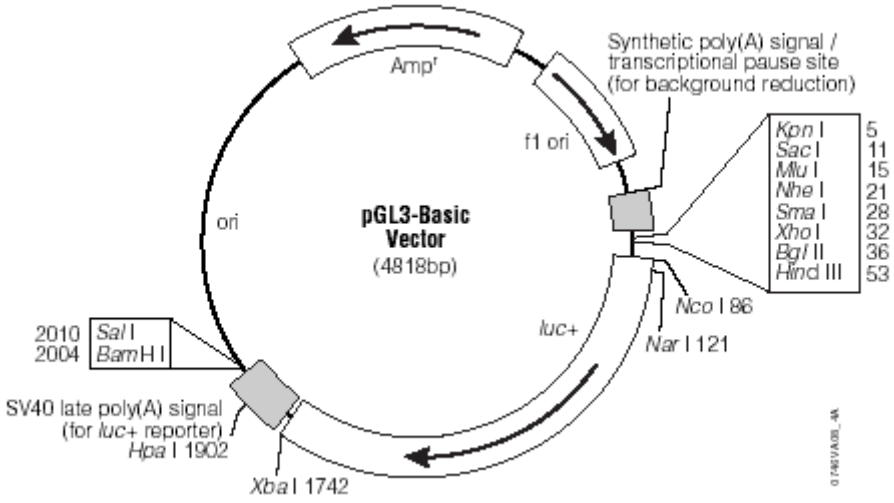

Figure 4. pGL3-Basic Vector circle map. Additional description: luc+, cDNA encoding the modified firefly luciferase; Amp^r, gene conferring ampicillin resistence in E. coli; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in E. coli. Arrows within luc+ and Amp^r gene indicate the direction of transcription; the arrow in the f1 ori indicates the direction of ssDNA strand synthesis.

Expression vectors used for Transient Transfections:

- **mGCNF in pCMX vector**
- **HA-GCNF in pCMX vector**
- **GCNF-VP16 in pCMX vector**
- **VP16 in pAASV vector, "VP16 only" (Nova Blue Company)**
- **hCREM-τ2α in pRc/CMV**
- **CREM-α in pRc/CMV**

Receptors and Vectors used for EMSA, for in vitro transcription/translation:

- **FLAG-hCREM-τ2α in pSPUTK**
- **HA-mGCNF in pSPUTK**

** GCNF expression vectors were a gift from Uwe Borgmeyer (ZMNH)*

** CREM expression vectors were a gift from Birgit Gellersen (IHF)*

3.1.4. Oligonucleotides and Primers

• Oligonucleotides for cloning in repoter vectors

Oligonucleotides for flanking rCRE site region (-57/-38):

CRE3: 5´-gcgtgctagccccttttgtgaggtcatgaatgtccctcgaggcga-3´

Oligonucleotides for flanking hCRE site region, wild type/with point mutation (-57/-38):

Forward oligonucleotide hCREwt 5´-ctagcggtatcctttgtgaggtcaacaatgac-3´

Forward oligonucleotide hCRE 4C mutation: 5´-ctagcggtatcctttgtga**c**gtcaacaatgac-3´

Forward oligonucleotide hCRE 5T mutation: 5´-ctagcggtatcctttgtgag**t**tcaacaatgac-3´

• Oligonucleotides for EMSA

hCRE wt, for competition experiment:

Forward oligonucleotide K13: 5´- gtatcctttgtgaggtcaagaatgacatta -3´

hCRE wt, ^{32}P-labeled:

Forward oligonucleotide K17: 5´- ggtatcctttgtgaggtcaacaatga -3´

hCRE with 4 mutated nucleotides:

Forward oligonucleotide K23: 5´- gtatcctttgt**ct**gg**ag**aagaatgacatta-3

hCREwt/with point mutation within CRE site:

Forward oligonucleotide hCREwt: 5´-ggtatcctttgtgaggtcaacaatgac-3´

Forward oligonucleotide hCREmut 1A: 5´-ggtatcctttg**a**gaggtcaacaatgacat-3´

Forward oligonucleotide hCREmut 4C: 5´-ggtatcctttgtga**c**gtcaacaatgacat-3´

Forward oligonucleotide hCRE mut 5T: 5´-ggtatcctttgtgag**t**tcaacaatgacat-3´

Forward oligonucleotide hCREmut 7T: 5´-ggtatcctttgtgaggt**t**aacaatgacat-3´

Forward oligonucleotide hCREmut 8C: 5´-ggtatcctttgtgaggtc**c**acaatgacat-3´

Primers:

Forward mutations primer for CRE site within promoter C, K25:

5´-gtgtggtatcctttgt**ct**gg**ag**aagaatgacattatcaac-3´

Reverse mutations primer for CRE site within promoter C, K26:
5´-gttgataatgtcattctt**ct**cc**ag**acaaaggataccacac-3´

Forward mutations primer for CRE site, K27:

5´-cgtgctagccccttgtctggagatgaatgtccctcgagg-3´

Reverse mutations primer for CRE site, K28:
5´-cctcgagggacattcatctccagacaaaggggctagcacg-3´

Forvard primer for pGL3 basic:

5'-catgcaaaataggctgtccc-3'

Reverse primer for pGL3 basic:

5´-ctttatgtttttggcgtcttcca-3`

Hum 1: 5`-actgtgttgtatataacttcc-3`

Hum 2: 5`-gaagacagagaataaagtcc-3`

Sp6 primer, 25 pmol/mL (Ambion)

T7 primer, 10 pmol/mL (Ambion)

**All oligonucleotides and primers were ordered from MWG.*

3.1.5. DNA Markers

100 Base pair Ladder (Amersham Biosciences)

DRIgest III (Amersham)

3.1.6. Protein Markers

Bench Mark Prestained Protein Lader (Invitrogen)

3.1.7. Enzymes

Sac II, Sal I, Kpn I, Xho I (BioLabs)

Nhe I (BioLabs)
Hind III (Pharmacia Biotech)
Klenow fragment DNA polymerase I (Usb)
T7 Polymerase (Ambion)
SP6 Polymerase (Ambion)
Calf intestinal alkaline phosphatase (Roche)
T4 DNA ligase (Usb)

3.1.8. Antibodies

- **For Western Blot:**

Anti FLAG-M2 Monoclonal Antibody (Sigma)
Rabbit polyclonal IgG (HA-Probe (y-11): sc-805, Santa Crus)
Peroxidase conjugated Goat Anti rabbit IgG (Jackson Immunoresearch)
Peroxidase conjugated Sheep Anti mouse IgG (Jackson Immunoresearch)

- **For Immunohistochemistry:**

Rabbit polyclonal anti-mGPDH antibody directed against amino acids 42-206 (Weitzel JM et al 2001)
Biotinylated swine anti-rabbit secondary antibody (DAKO)
Anti-rabbit IgG, Alexa 488 nm, 1:1000 (Molecular Probes)
Monoclonal anti-rabit PAP complex (DAKO)
ABC (Vector, Burlingame)

- **For EMSA**

Anti FLAG-M2 Monoclonal Antibody (Sigma)
HA-tag 2367 Monoclonal Antibody (Cell Signaling)

- **For ChIP**

Anti-Acetyl-Histone H3 Antibody, rabbit polyclonal IgG (UPSTATE)

3.1.9. Kits

"Quick Change Site Directed Mutagenesis Kit (STRATAGENE)

QIAquick Gel Extraction Kit (Qiagen)

Quantum Prep Plasmid MiniPrep (Bio Rad)

Qiagen Plasmid Midi Kits (QIAGEN)

BigDye terminator sequencing kit (Perkin-Elmer Applied Biosystems)

TnT Coupled Reticulocyte Lysate Systems (Promega)

Maxi Script SP 6 (Ambion)

QIAamp DNA Blood Mini Kit (QIAGEN)

ChIP Assay Kit (Upstate)

3.1.10. Media

LB Agar:	LB Medium, 1.5% Bacto-Agar (Difco)
LB Medium:	1% Bacto Tryptone (Difco); 0.5% Bacto Yeast Extract (Difco); 1% NaCl; pH 7.0
DMEM:	Gibco
Cell Culture medium:	Dulbeccos modified Eagle´s medium plus Glutamax (Gibco), 10% Foetal calf serum (Cibco), 40 U/mL Penicillin, 40 U/mL Streptomycin

3.1.11. Buffers and Standard Solutions

HBS	280 mM NaCl, 3 mM Na_2HPO_4, 50 mM HEPES, pH 7,2
$CaCl_2$	250 mM
Luciferase-Assay-Buffer:	25mM Glycylglycine; 15mM $MgSO_4*7H_2O$; 4mM EGTA; 1mM DTT; 15mM K_2HPO_4/KH_2PO_4; 2mM ATP; pH 7.8
Luciferin (Roche)	
Luciferin-Solution:	25mM Glycylglycine; 10mM DTT; 0.2mM Luciferin
Lysis Solution with DTT:	Galacto-Light Plus Lysis Solution (Applied Biosystems), 0.5mM DTT
PBS:	137mM NaCl; 2.7mM KCl; 4.3mM $Na_2HPO_4*7H_2O$; 1.4mM KH_2PO_4
PBS for Cell Culture:	KH_2PO_4 1,05g; NaCl; 45g; $Na_2HPO_4*2H_2O$; add 5L H2O

TSA (Calbiochem)

DTT (Roche)

Trypsin/EDTA-solution (0.05%/0.02%, w/v in PBS, Biochrom AG)

EDTA

NaCl 180mM

Sodium acetate (0.3M)

Isopropanol (Merck)

Phenol

Chloroform

Ethanol (100%, 80%, 75%,70%)

2x Rapid Ligation Buffer (Promega)

RNasin ribonuclease inhibitor (Promega)

NEB restriction Buffers 1, 2, 3, 4 (BioLabs)

Buffer H (Pharmacia Biotech)

10X filling-in buffer (Klenow), (Usb)

SOC Medium (Novagen)

Ampicilin (Roche)

Agarose (Sigma)

Ethidium bromide (Pharmacia Biotech)

Gel Loading Buffer, 6x: 50% Glycerol; 1mM EDTA; 0.4 Bromophenol blue; 0.4% Xylencyanol

dNTPs, 0.2 mM final concentration (Invitrogen)

^{32}P dCTP , 50 µCi (Amersham)

Taq DNA polymerase (2.5 U, Amersham Pharmacia Biotech).

PCR Buffer, 10x (Amersham Pharmacia Biotech)

BigDye (Perkin-Elmer Applied Biosystems)

HT 2.5x Buffer (Perkin-Elmer Applied Biosystems)

NaN_3 0,1%

Bouin solution

Paraformaldehyde 4%

Formaldehyd 1%

Elution buffer for ChIP: 1% SDS, 0,1M $NaHCO_3$

Normal swine serum (DAKO)

Eukitt (Kindler)

Protein Assay (Bio Rad)

Milk powder (Roth)

Poyacrylamide (Roth)

AEBSF (Calbiochem)

SDS Loading Buffer, 6x: 6% SDS, 62.5 mM Tris-HCl, 10% Glycerin, 2% Mercaptoethanol, 0.01% Bromphenolblau, pH 6.8

Electrophoresis buffer: 25mM Tris, 192 mM Glycin, 0,1% SDS

Transfer Buffer with Methanol: 48mM Tris, 39 mM Glycin, 20% Methanol, pH 9.2

Stacking Gel Buffer: 125 mM Tris-HCl, 0.1% SDS, pH 6.8

Resolving Gel Buffer: 375 mM Tris-HCl, 0.1% SDS, pH 6.8

Acetic acid/Methanol solution

Nuclear extraction Buffer:

Buffer A: 10 mM HEPES pH 7.9, 10 mM KCl, 0,1 mM EDTA pH 8.0
10 mM EGTA pH 8.0, 0.1 mM DTT, 0.05 mM AEBSF

Buffer B: 20 mM HEPES pH 7.9, 0.1 mM NaCl, 0,1 mM EDTA pH 8.0,
10 mM EGTA pH 8.0, 0.1 mM DTT, 0.1 mM AEBSF

Triton X-100 (Sigma)

Bovine serum Albumin fraction V (Serva)

Detection reagent 1 and 2 (Amersham)

poly(dA-dT) poly(dA-dT) (Amersham Biosciences)

Band-Shift-Buffer (10x): 100mM Tris-HCl; 500mM NaCl; 1mM EDTA; 10mM DTT; 5mM $MgCl_2$;1mg/ml BSA; 50% Glycerol

Nick-Buffer: 500mM TrisHCl; 100mM $MgCl_2$; 1mM DTT; 500 µg/ml BSA

Ultima Gold LSC-coctail (Pickard)

Qiagen Buffers: See manufacturers manuals

TBE (10x): 890mM Tris base; 890mM Boric Acid; 20mM EDTA

TE: 10mM Tris-HCl; 1mM EDTA

TnT Reaction Buffer (Promega)

**All chemicals were obtained from Sigma, unless specified differently*

3.1.12. PCR Programs

Program 0 for sequencing:

Cycle 1:	95°C	1 min
	96°C	30 sec
	50°C	15 sec
	60°C	4 min
Cycles 2-35:	96°C	30 sec
	50°C	15 sec
	60°C	4 min

PCR program for amplification of promoter C fragment (using primers hum 1 and hum 2) and for amplification of CRE-PRL-Luc3 construct (using forvard and reverse primers for pGL3 basic vector):

Cycle 1:	95°C	5 min
	53°C	30 sec
	72°C	30 sec
Cycles 2-40:	95°C	30 sec
	53°C	30 sec
	72°C	30sec
Cycle 41:	95°C	30 sec
	53°C	30 sec
	72°C	10 min

PCR for ChIP (No 73):

Cycle 1:	95°C	2 min
	57°C	60 sec
	74°C	60 sec
Cycles 2-30:	95°C	60 sec
	57°C	60 sec
	74°C	60sec
Cycle 31:	95°C	60 sec
	57°C	60 sec
	74°C	2min

3.2. Methods for Cloning

3.2.1. Identification of Human Promoter C and Cloning Strategy

A BLAST search was performed using the rat promoter C sequence (Weitzel JM et al. 2003) as a bait. This sequence was compared with human genomic DNA and a homologous sequence was found within the human mGPDH gene.

3.2.2. Construct hu(-106/+105)-luc

The sequence of promoter C of the human mGPDH was identified and amplified by a polymerase chain reaction technique (Biometra) using human genomic DNA as a template (from a healthy volunteer) and primers hum1 (5`-actgtgttgtatataacttcc-3`) and hum 2 (5`-gaagacagagaataaagtcc-3`) (MWG). The resulting 211 bp long PCR fragments were gel purified (using QIA Quick Kit, according to the manufacturers instructions), ligated into pGEM t-easy (Promega) and sequenced. pGEM t-easy was cut with Sac II (with addition of Klenow fragment DNA Polymerase I) and Sal I and human promoter C fragment was subcloned into pGL3-basic luciferase reporter vector (Promega) between Kpn I and Xho I restriction sites.

3.2.3. Construct mut hu(-106/+105)-luc

A promoter C sequence with a mutated CRE site was prepared using the 211 bp wt construct cloned in pGEM T-easy as template. Primers K25 and K26 were used with the "Quick Change Site Directed Mutagenesis Kit (STRATAGENE) and subcloned as described above.

Original CRE-site sequence was: 5`-tgaggtca-3`

3`-actccagt-5`

CRE-site sequence with 4 base pairs mutated was: 5`-t**ct**gg**ag**a-3`

3`-a**ga**cc**tc**t-5`

The sequence of promoter C, “211 bp long wild type fragment”:
5´- ACTGTGTTGTATATAACTTCCCTGAGTTGGCCATGTTGATGTGTGGTATC
CTTTG**TGAGGTCA**ACAATGACATTATCAACAAGACAGAAGCAATGATAAG
TCAATCAGTCACAACACTCATATCCTAGGGACCACCACACTGCTATGAGT
CAGGCATGTGCGTTGTTTAAGGTAAATAGTAAATAGAACTAGGACTTTAT
TCTCTGTCTCC -3´

3.2.4. Construct CREwt-rPrl-luc

Two 44 bp long oligonucleotides: CRE 3 (5´-cgtgctagccccttttgtgaggtcatgaatgtccctcgaggcga-3´) and CRE 4 (5´-tcgcctcgagggacattcatgacctcacaaaggggctagcacgc-3´) were annealed and cloned into pGL3-basic luciferase reporter vector upstream of the minimal prolactin promoter (-38/+36, between Xho I and Hind III restriction sites**,** gift from IHF). pGL3-basic was previously cut with Nhe I and XhoI.

3.2.5. Construct mut CRE-rPrl-luc

Construct CREwt-rPrl-luc was mutated using primers K27 and K28 and “Quick Change Site Directed Mutagenesis Kit (STRATAGENE).
Original CRE-site sequence was mutated in the same way like for generating construct mut hu(-106/+105)-luc.

3.2.6. Polymerase Chain Reaction (PCR)

A PCR sample was prepared with the following components: Template DNA (either plasmids or whole cells), two primers (1mM final concentration each), PCR Buffer (1x final concentration, Amersham Pharmacia Biotec), dNTPs (0.2 mM final concentration), and Taq DNA polymerase (2.5 U, Amersham Pharmacia Biotech). The reaction was performed in an automated thermal cycler (T3 Thermocycler, Biometra). The different PCR programs are listed.

3.2.7. Agarose Gel Electrophoresis

1% to 2% agarose (Sigma) gels containing 0.25 μg/ml ethidium bromide (Pharmacia Biotech) were prepared. 5 μl of DNA solution were added to 1 μl of 6x loading buffer. To

perform electrophoresis, the voltage was set to 10 V/cm. BioRad Mini Sub Cell GT electrophoresis tanks and the BioRad Power Pac 300 power supply were used. 0.5x TBE was used as electrophoresis buffer. After electrophoresis, the gels were placed on the IL-305-M UV transilluminator (Bachofer) and photographed with a Polaroid CU-5 camera (Bachofer).

3.2.8. Extraction of DNA from Agarose Gels

The QIAquick Gel Extraction Kit (Qiagen) was used to extract DNA from agarose gels. The DNA fragment was excised from agarose gel. To one volume of agarose slice (100 mg equal 100 µl), three times the volume of buffer QG was added. The gel slice was dissolved at 50°C, and one gel slice volume of isopropanol (Merck) was added to the sample. The sample was then applied to a QIAquick column and centrifuged for 1 minute at 10,000g. Wash buffer PE (0.75 ml) was added to the column and again centrifuged for 1 minute at 10,000g. To remove residual ethanol, the column was centrifuged for an additional 1 minute. DNA was eluted from the silica-gel membrane with 50 µl of water.

3.2.9. Determination of DNA Concentration

The spectrophotometer (Eppendorf BioPhotometer) was set to zero by reading the absorption of a cuvette filled with blank at the particular wavelength. A DNA sample was diluted in an adequate volume of water. A_{260} and A_{280} were read and the concentration and purity of the sample was calculated.

3.2.10. Restriction Endonucleases

NEB restriction buffer (at a final concentration of 1x) and 20 through 40000 units of restriction endonuclease (2 µL, 10000-20000 U/mL) were added to the DNA samples. This reaction mixture was incubated at 37°C for 2 hours. If cohesive ends were generated by the endonuclease but blunt ends were needed, 5 units of Klenow fragment and 5 nmol of dNTPs were added and incubated at 37°C for 15 minutes. Inactivation of the Klenow fragment was achieved by heating the sample to 65°C for 20 minutes.

To prevent re-closure of a cleaved vector on itself, 1 unit of calf intestinal alkaline phosphatase (Roche) was added and incubated for 20 minutes at 37°C.

3.2.11. Ligation of DNA Fragments into Vectors

The pGEM-T easy Vector Kit (Promega) and the pGL3 basic Vector (Promega**)** were used for cloning PCR products and generating luciferase reporter gene vector constructs, respectively.

50ng of pGEM-T easy vector or pGL3 basic vector, insert DNA (at a insert:vector molar ratio of 3:1), Ligation Buffer at a final concentration of 1x (Promega), and 1 unit of T4 DNA Ligase (Usb) were incubated overnight at 4°C.

3.2.12. Transformation of Bacteria

Plasmids were introduced into competent E. coli cells Nova Blue strain (Novagen).

0.5 µl of a ligation reaction or plasmid DNA was added to a 20 µl aliquot of competent cells and incubated on ice for 5 minutes. The sample was heated to 42°C for 30 seconds and then incubated on ice for another 2 minutes. 80 µl of SOC medium (Novagen) was added and the sample was plated on LB medium. The plate was incubated overnight at 37°C.

3.2.13. Growing of Bacteria

LB medium with Ampicillin (Roche) at a concentration of 25 µg/ml, was used to grow E. coli cells in liquid cultures as well as on solid plates. The cells were inoculated onto or into the medium and incubated overnight at 37°C.

3.2.14. Plasmid Preparation

The Qiagen Plasmid Midi Kits were used for plasmid purification.

An over night bacterial culture was grown in 100 ml LB medium at 37°C with vigorous shaking. The bacterial cells were harvested by centrifugation at 6000g for 15 minutes at 4°C (RC-5B Refrigerated Superspeed Centrifuge, Sorvall). The bacterial pellet was re-suspended in 4 ml buffer P1. 4 ml of buffer P2 were added and incubated at room temperature for 5 minutes to lyse the cells. For neutralisation, 4 ml of buffer P3 were added and incubated on ice for 20 minutes. The sample was centrifuged at 20,000g for 30 minutes at 4°C and the supernatant was re-centrifuged at 20,000g for 15 minutes at 4°C. After equilibration of a Qiagen-tip 100 with 4 ml of buffer QBT, the supernatant was

applied to the Qiagen-tip. The resin was washed twice with 10 ml of buffer QC. The plasmid DNA was then eluted with 5 ml of buffer QF. Precipitation of plasmid DNA was achieved by adding 3.5 ml of isopropanol and centrifuging at 15,000xg for 30 minutes at 4°C. The pellet was washed with 2 ml of 70% ethanol, centrifuged at 15,000xg for 10 minutes and air-dried. The DNA pellet was re-dissolved in a appropriate volume of water.

3.2.15. Sequencing of DNA

700 to 1200 ng of DNA, 8 µl of HT 2.5x Buffer (Perkin-Elmer Applied Biosystems), 2 µl of BigDye reaction mixture, 12.5 pmol of primer, and water to a finale volume of 20 µl were assembled. The PCR program for sequencing was set up and performed in an automated thermal cycler (T3 Thermocycler, Biometra). After PCR, 80 µl of sodium acetate (0.3M) and 300 µl of ethanol (100% vol.) were added to the sample and incubated at room-temperature for 10 minutes. The samples were then centrifuged at 14,000xg and 4°C for 30 minutes. The supernatant was decanted and 200 µl of ethanol (75%) was added. The sample was first centrifuged at 14,000xg and 4°C for 10 minutes and then air dried.

Sequencing of DNA was performed at the DNA Sequencing Unit at the Institute of Cellular Biochemistry and Clinical Neurobiology, Hamburg. The BigDye terminator sequencing kit (Perkin-Elmer Applied Biosystems) was used.

3.3. Analysis of Cloned Promoters

3.3.1. Growing of Permanent Cell Lines

The cells (Hep G2 and HEK 293) were cultured with DMEM (Gibco) supplemented with 10% (v/v) delipidated foetal calf serum (Gibco) in an incubator (B 50 60 EC-CO2, Heraeus Christ) at 37°C under a humidified atmosphere with 5% CO_2. To separate adherent cells, medium was removed and the cells were washed with PBS. For dissociating cells, a suitable volume of trypsin/EDTA-solution (0.05%/0.02%, w/v in PBS, Biochrom AG) was added and incubated in the incubator for 3-5 minutes. To stop trypsin activity, an appropriate volume of medium was added and pipetted up and down to separate the cells. Cells were centrifuged for 5 minutes at 900xg, supernatant was removed and new medium was added.

For transfection, the dissociated cells were counted and seeded at a density of 10^5 cells per 35 mm culture dish. After 24 hours, medium was changed and the cells were transfected.

3.3.2. Transient Transfection Assay

Transient Transfection experiments were performed using a modified calcium-phosphate method.

For each 35mm diameter culture dish, 1400 ng of promoter-containing pGL3-basic luciferase reporter plasmid was mixed with 800 ng of CGNF-VP16, VP-16 or CREMtau expression vectors and 800 ng, 1600 ng or 2400 ng of GCNF expression vectors in 250 mM $CaCl_2$. This solution was mixed with the same volume of 280 mM NaCl, 3 mM Na_2HPO_4, 50 mM HEPES, pH 7,2. The total DNA amount was kept constant by the addition of salmon-sperm-DNA.

Prepared DNA/calcium phosphate mixture (230 µl) was added drop by drop to a culture dish and cells were harvested after 18 to 20 hours of incubation. Expression plasmids were listed in Material section.

In some experiments TSA was added to a final concentration 100 ng/mL after 24 hours.

3.3.3. Luciferase Activity Assay

A 35 mm culture dish with transfected cells was washed two times with ice-cold PBS and the cells were lysed and solubilised in Lysis Solution (Galacto-Light, Applied Biosystems) with 0.5 µl/mL DTT. The cells were centrifuged for 3 minutes at 4°C and 14,000xg. The supernatant was used for the assay. Cell lysate (10 µl) and 100 µl of luciferase-assay-buffer were mixed and placed in the luminometer chamber (Lumat LB 9501, Berthold). Luciferin-solution (100 µl) was injected into the sample and light output was measured for 10 seconds at room temperature. Two samples from each cell lysate were analysed and average values were calculated.

3.3.4. Determination of Protein Concentration

Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad).
Protein solutions of unknown concentration (2 µl) were diluted in 98 µl of water. Bio-Rad Protein Assay solution (900 µl) was added. The sample was incubated for 10 to 20 minutes at room temperature and the extinction was measured in a spectrophotometer (Eppendorf BioPhotometer).

3.3.5. Statistical Evaluation

The results obtained from luciferase activity assays were evaluated with the program Excel (Microsoft Office XP).
Luciferase activity assay results were normalised to protein concentration to determine transfection efficiencies. Activities of promoter/reporter gene constructs were additionally normalised to CREwt or 211 bp wt construct activities. Each construct was tested in 3 to 5 independent transfections with three culture dishes per experiment. The results are means ±S.D.

3.4. Analysis of Protein-DNA Interactions

3.4.1. Electromobility Shift Assay (EMSA)

The labelled DNA probe, band shift buffer (at a final concentration of 1x), the unlabeled DNA probe as specific competitor (100x molar excess relative to the labelled probe), poly(dA-dT) as non-specific competitor, and *in vitro* translated receptors were assembled for DNA-protein binding reaction and incubated at room-temperature for 30 minutes. A non-denaturing 5% polyacrylamide gel was prepared, 0.5x TBE was used as electrophoresis buffer. The gel was prerun for 30 minutes at 200 V (electrophoresis tank: Sequencing Electrophoresis System Model S1, BRL Bethesda Research Laboratories Life Technologies; power supply: Desatronic 500/400, DESAGA Heidelberg). The binding reactions were inserted into the wells of the prerun gel and the gel was run for 2 hours at 200 V. To analyse the gel, it was transferred onto a sheet of 3MM Whatman paper and dried under vacuum and heat (Model 483 Slab Dryer, BioRad). Autoradiography was performed by placing the membrane onto a X-ray film (Super RX, Fuji Film) for 1 to 7 days, at -80°C.

In vitro transcription /translation

TnT Coupled Reticulocyte Lysate Systems (Promega) were used for in vitro translation.
The following reaction components were assembled: 25 µl of TnT rabbit reticulocyte lysate, 2 µl of TnT reaction buffer, 1 µl of TnT RNA polymerase (SP6 or T7, depending on the promoter in the vector for in vitro translation), 2 µl of amino acid mixture (1nM), 40 U of RNasin ribonuclease inhibitor (Promega), 1 µg of DNA template, and water to a final volume of 50 µl. The translation reaction was incubated at 30°C for 90 minutes.

Preparation of DNA probes for EMSA

The forward and reverse oligonucleotides (described in the Material section) were annealed to generate an EMSA probe which contains the wild type or mutated CRE sequence of promoter C, respectively.
Two oligonucleotides (200 pmol of each) and NaCl (at a final concentration of 180 mM) were assembled in a microcentrifuge tube. The tube was placed in hot water (95°C) and was allowed to cool down to room-temperature. Annealed oligonucleotides (5 pmol), nick

buffer (final concentration: 1x), dGTP, dATP, dTTP (10nmol of each), 5 µl of ^{32}P dCTP (50 µCi), and 5 U of Klenow fragment DNA Poymerase I (Usb) were incubated for 20 minutes at 37°C. The reaction was stopped by adding 2 µl of 0.5 mol/L EDTA (pH 8.5). The DNA probes were purified using G-50 Micro-columns (Amersham Biosciences). To determine specific activity of the DNA probe, 200 pg (1 µL) of DNA were diluted in 2 ml of Ultima Gold LSC-cocktail (Packard) and read for Cerenkov counts in a Wallac 1409 (Wallac) scintillation counter.

3.4.2. Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) is an important technique to study protein-gene interactions within the context of a cell. With this method it is possible to analyse DNA fragments which are directly bound by specific transcription factors.

The transient transfection was performed in HEP G2 cells, with CREwt construct only and cotransfection with expression constructs for CREMtau and 3 fold higher amount of GCNF. After 20 hours incubation cells were washed with ice-cold PBS containing 1mM AEBSF, and protein/DNA interactions cross-linked with 1% Formaldehyde dissolved in serum-free medium for 4 min at 37°C. The cells were washed once again, harvested, and centrifugated for 1 min at 14000 rpm at 4°C.

Pellet was resuspended in the lysis buffer with 1 mM AEBSF, and incubated for 10 min on ice. DNA was then sheared by sonification for 60 s. After centrifugaton supernatant was mixed with the dilution buffer and divided into 2 portions. One was used as an input control. The other was mixed with Salmon sperm DNA/Protein A agarose for 30 min at 4°C. After the centrifugation supernatant was incubated overnight with anti-acetyl-histone H3 antibody in a dilution of 1:500 (UPSTATE). After incubation with Salmon sperm DNA/Protein A agarose for 1 hour at 4°C and centrifugation, beeds were sequentially washed in low salt, high salt, LiCl, and in TE buffer. The elution buffer (1% SDS, 0,1 M $NaHCO_3$) was applied twice, eluate was mixed with 5 M NaCl (to a final concentration of 192 mM) and incubated for 4 hours at 65°C.

After reverting the crosslinks in both portions, the DNA was purified by Phenol/Chlorophorm/Ethanol procedure and dissolved in water. PCR analyses were performed to determine which genes were bound to the protein of interest and to quantify the amount of precipitated DNA.

The forward and reverse PGL_3 basic primers were used and PCR program No73.

3.5.Methods for Protein Determination

3.5.1. SDS-PAGE and Western Blotting

The extraction of nuclear proteins

Transient Transfection was performed as described above (section 3.3.2.), using CREMtau and GCNF expression vectors. HEP G2 cells (approximately 30 million per experiment) were washed with ice cold PBS and harvested and diluted in 40 ml PBS. Centrifugation at 2500 rpm at 4˚C was performed for 10 minutes. Pellets were resuspended in 1.5 mL of cold Buffer A and left for 15 min on ice, with slow shaking. 10% Triton X-100 in 100 µL Buffer A was added in drops, and left for additional 5 minutes on ice. A next centrifugation was performed for 5 min, 1500 rpm at 4˚C. Pellet was washed in cold Buffer A and transferred to a new tube. Buffer B (100 µL) was added and samples were left for 30 minutes at 4˚C with shaking. Samples were centrifuged for 10 minutes, 14000 rpm at 4˚C. Concentration of proteins were detected in the supernatant by the Bradford method.

SDS-PAGE and Western Blot

For Western Blot Assay, 50 or 70 µg of the proteins were prepared from each sample. The Gel Loading Buffer (6x) and water was added to the probes to reach a volume of 30 µL. Proteins were denatured for 5 min at 95˚C. The samples and the protein marker (Bench Mark Prestained Protein Lader, Invitrogen) were then loaded on 10% SDS-polyacrylamide gel. The gel was run in 1x electrophoresis buffer with 0,1% SDS, at 70 V for 30 min and then at 100 V for 2 hours (Electrophoresis set Biometra). The gel, the Whatman 3 mm filter paper and nitrocellulose membrane (0,45 µm Schleicher&Schnell) were equilibrated for 10 minutes in 1x Transfer Buffer with Methanol. The proteins from the gel were then transferred onto nitrocellulose membrane using a semi-dry electroblotting system (Phase). The transfer was conducted at 70-80 mA for 75 min. Gel was stained by Coomassie-Blue solution for 5-10 minutes to proof quality of transfer and then unstained overnight at 4 ˚C in Acetic acid/Methanol solution.

Immunological detection of specific proteins

Membrane was blocked in 5 % milk solution (Milchpulver, Roth) and 1% BSA (Bovine serum Albumin fraction V, Serva) in Tris-Tween wash Buffer for 2 hours and incubated at 4 ˚C with the primary Antibody overnight. To detect FLAG-tagged CREMtau, Anti

FLAG-M2 Monoclonal Antibody (Sigma) was used in a dilution of 1:2000. To detect HA-Tagged GCNF, Rabbit polyclonal IgG was used (HA-Probe (y-11): sc-805, Santa Crus), in a dilution of 1:1000.

After washing in Tris-Tween Wash Buffer for 45 minutes (solution was refreshed every 15 min), the membrane was incubated for 2 hours with Peroxidase conjugated secondary antibody dissolved in Dilutions Buffer with 5 % Milk and 1% BSA.

Peroxidase conjugated Goat Anti rabbit IgG (Jackson Immunoresearch) was used in a dilution of 1:5000 while Anti-mouse-POX Antibody was used in dilution of 1:10000.

The membrane was washed two times in Dilution buffer and then briefly in PBS. All incubation and washing steps were performed with slow shaking.

Mixture of Detection reagent 1 and 2 (Amersham) was applied for 1 min. The membrane was placed onto an autoradiographic film (Super RX Fuji Film) and exposed for 2, 5, 30 or 60 minutes. Film was developed using Curix 60 film developer.

3.6. Immunohistochemistry

Immunohistochemistry of human testis tissue

Immunohistochemistry experiments of human testis tissue (from fertile patients) were performed using a rabbit polyclonal anti-mGPDH antibody directed against a protein fragment from amino acids 42-206 of rat mGPDH (Weitzel JM et al. 2001).

Immunohistochemistry experiments were performed on 6 µL paraffin-embedded sections of human testis fixed in Bouin solution. After dewaxing and hydrating in descending ethanol solutions (100%, 96%, 80% and 70%), sections were washed in PBS and incubated for 30 minutes with 2% normal swine serum dissolved in PBS. Sections were incubated overnight at 4˚C with primary antibody (dilution 1:600), 0,2%BSA and 0,1% NaN_3 in PBS, then washed in PBS and incubated for 1 hour at room temperature with biotinylated swine anti-rabbit secondary antibody (dilution 1:250 in PBS, DAKO). Sections were again washed in PBS and incubated for 30 minutes with a monoclonal anti-rabbit PAP complex (DAKO), at a 1:200 dilution. After a new washing step sections were incubated for 30 minutes with ABC (Vector, Burlingame) at a 1:250 dilution, washed in PBS 100 mM sodium phosphate buffer (pH 7.4) and peroxidase reaction was developed for 15 min. After final incubation in PBS and dehydrating in ascending ethanol solutions sections were

mounted with Eukitt (Kindler). Control sections were incubated without anti-mGPDH antibody or with preimmune serum.

Immunohistological analysis of mGPDH in spermatozoa

Sperm samples were centrifuged for 1 min at 650xg, the supernatant discarded and the pellet fixed by administration of 4% paraformaldehyde in PBS followed by application to BD Falcon culture slides. After sedimentation (40 min at 4˚C) supernatant was discarded and chambers were washed with PBS followed by drying of spermatozoa at 36˚C to improve adhesion. Chambers were again washed with PBS and incubated with 2% normal swine serum (DAKO) in PBS containing rabbit polyclonal anti-mGPDH antibody (1:600). Spermatozoa were incubated overnight at 4˚C with primary antibody and washed with PBS. Secondary antibody (anti-rabbit IgG, Alexa 488 nm, 1:1000, Molecular Probes) was applied in PBS with 0,5%Triton X-100 for 60 minutes in dark chamber. Slides were washed with PBS three times and covered for fluorescence microscopy (Zeiss Axioskop, Zeiss). For negative controls primary antibody was omitted or preimmune serum (1:600) was used instead of the antibody.

4. Results

4.1. mGPDH Gene is Regulated by Multiple Promoters

The human mGPDH gene is located on chromosome 2 (location 2q24.1.). This gene consists of 17 exons and spans approximately 100 kb. The first exon is an alternative one (labeled as 1a, 1b and 1c), whereas the second and other exons are common for all tissues.

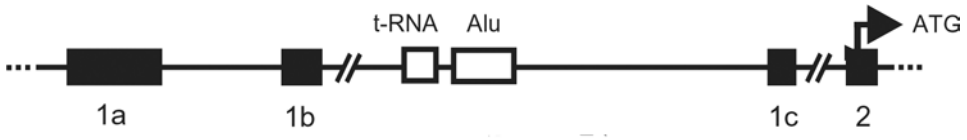

Figure 5. Exon-intron structure of the mGPDH gene. The first exon is alternatively used in different tissues (1a, 1b and 1c). Translational start site (ATG) is situated in the second exon which is common to all transcripts in all tissues.

The expression of the rat mGPDH gene is regulated by three different promoters in a tissue-specific manner. Promoter A is used in brain, brown adipose tissue and pancreas while promoter B is used ubiquitously (Weitzel JM et al. 2000). Recently, it has been shown that promoter C activity is testis-specific in rats (Weitzel JM et al. 2003).

To test whether human mGPDH gene can be regulated in a similar manner BLAST search was performed, using the rat promoter C sequence as bait against human genomic DNA.
A highly conserved sequence with a sequence identity of 87% between rat and human was identified (Fig. 6). This sequence is situated downstream of exon 1b and upstream of common exon 2, suggesting a human orthologous sequence of the rat promoter C. The usage of promoter C results in an alternative transcript of 2,4 kb which contains the sequence of exon 1c at the 5´end. Shortened mGPDH transcripts (2,4 kb) were solely detectable from testis-tissue RNA in human and rat (Rajkovic M et al. 2004, Weitzel JM et al. 2003).

Since promoter C is testis-specific in the human, we were interested in mechanisms that are responsible for regulation of promoter C activity. The current work examines the effects of CREMtau and GCNF on mGPDH gene expression in testis, as well as how these factors affect each other.

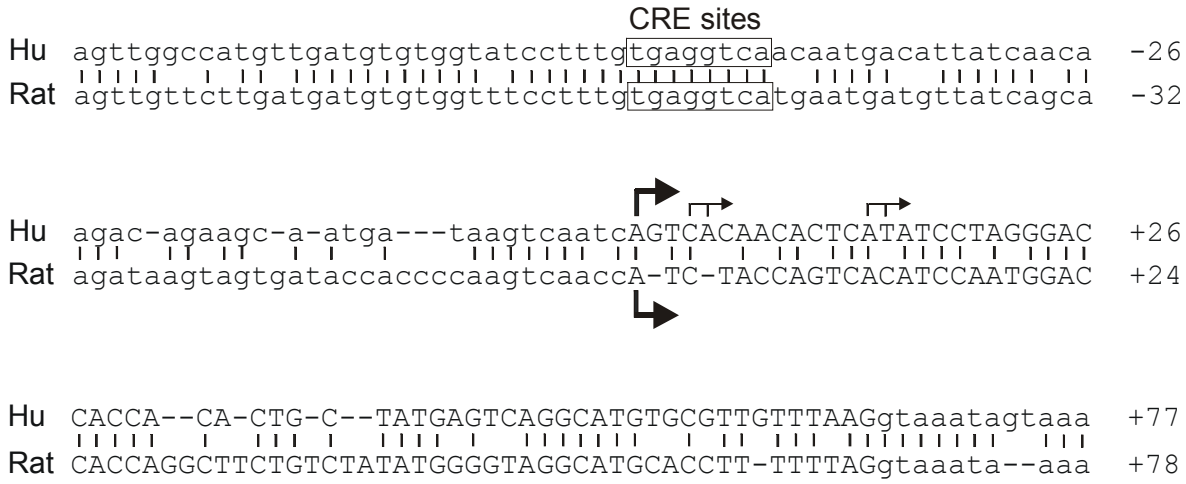

Figure 6. The alignment of human and rat promoter C sequences showed a sequence identity of 87% and presence of a putative CRE site indicated by a box.

4.2. Binding of CREMtau to Promoter C Sequence

To identify whether a putative CRE site which is present in the promoter C sequence could be a binding site for CREMtau we performed EMSA. For DNA-protein binding reaction the ^{32}P-labelled DNA probe (-62/-32) was incubated with FLAG-tagged CREMtau. This transcriptional factor was synthesized *in vitro* using a reticulocyte lysate transcription/translation system.

EMSA showed that CREMtau was able to bind to the labelled promoter C sequence portion (-62/-32) which includes the putative CRE site, and to generate a slower migrating bands indicated by arrows (lane 2, Fig. 8). The data presented in this figure demonstrate that protein-DNA complexes observed using the oligonucleotide probe were partially inhibited by 100-fold molar excess addition of its unlabeled sequences (lane 4).
Anti-FLAG-Antibody added in the binding reaction interacted with CREMtau and supershifted the DNA-protein complex in the same assay (lane 3).
Taken together, this data confirmed a specific interaction of CREMtau with the putative CRE site within promoter C of mGPDH gene, which was confirmed in competition and supershift experiments.

The importance of the CRE site was further assessed by generating point-mutations in CRE site, followed by competition analysis in EMSA.
The formation of these complexes was markedly affected in a probe containing the CRE 4C mutation, which generated consensus CRE sequence (lane 6).

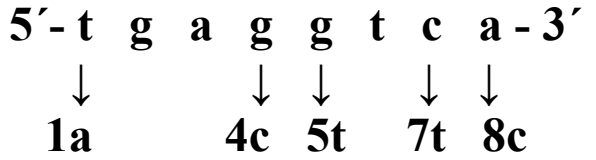

Figure 7. It demonstrates putative CREwt sequence and five different point-mutations within CRE site which were used in competition experiments in EMSA.

However, 1A mutation within CRE sites (lane 5) demonstrated relatively weak competition. On the other hand, most of other mutations applied were not able to compete with these complexes, suggesting the greater importance of the intact CRE site for CREM-DNA binding.

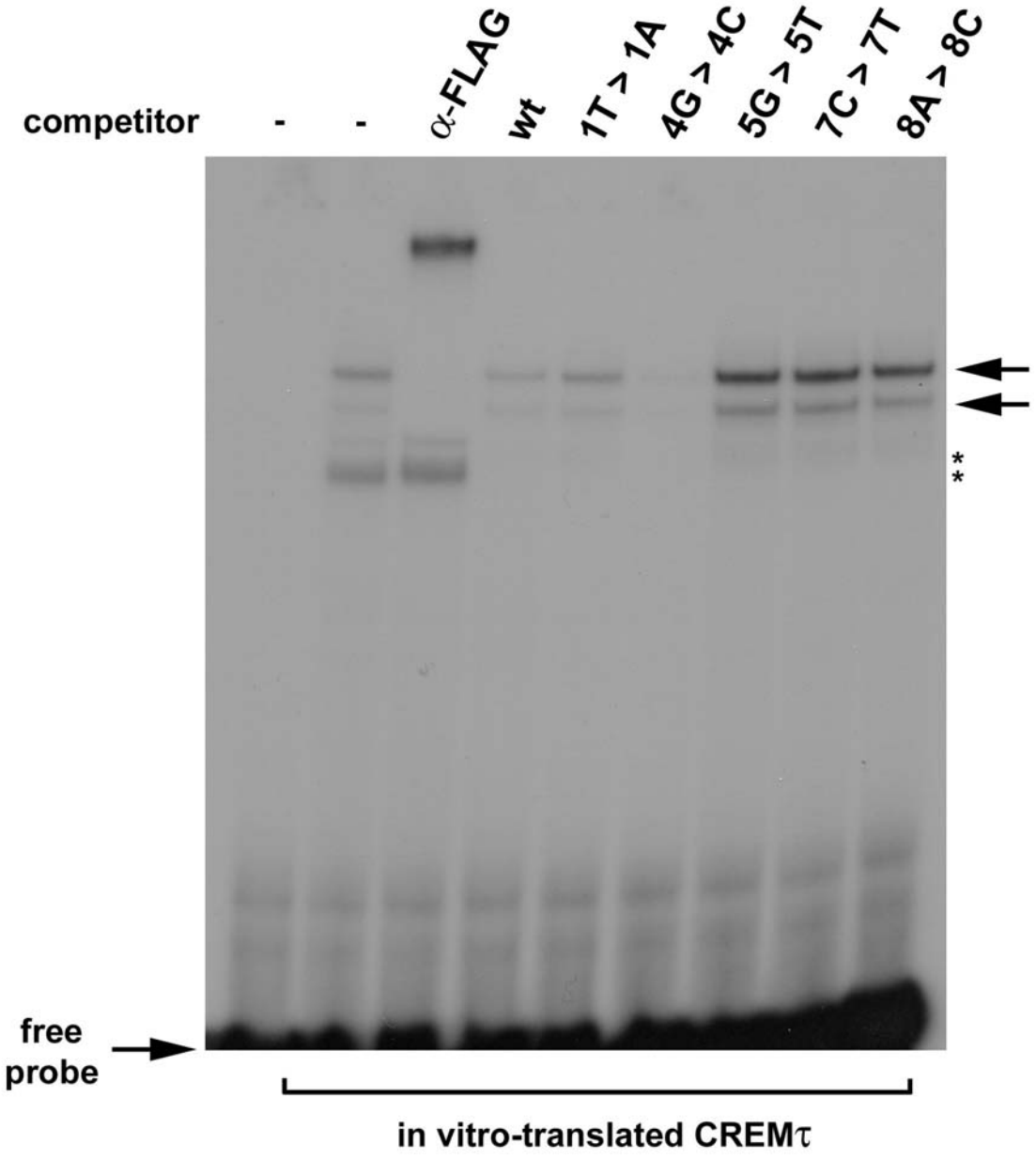

Figure 8. Binding of CREMtau to a sequence portion of promoter C in EMSA. *In vitro* translated FLAG-tagged CREMtau was incubated with double-stranded oligonucleotide containing CRE site (-62/-32). For competition experiments 100 fold molar excess of different oligonucleotides (with wild type or mutated CRE site within the CRE sequence) were added to the reaction mixture. Anti-FLAG antibody was part of reaction mixture in the third line. Specific protein-DNA complexes are indicated by arrows. Nonspecific complexes are indicated by asterisks.

4.3. Binding of GCNF to Promoter C Sequence

To identify whether GCNF can also bind to the NR half-site which is present in promoter C we performed EMSA. For DNA-protein binding reaction the ^{32}P-labelled DNA probe (-62/-32) was assembled with HA-tagged GCNF which was synthesized *in vitro,* using a reticulocyte lysate transcription/translation system.

The data demonstrate that GCNF was able to bind to the labelled oligonucleotide sequence containing the 5'-AGGTCA-3' sequence and to generate a slower migrating band (lane 2, Fig. 9 and lane 3, Fig. 10). The protein-DNA complexes observed were effectively inhibited by 100-fold molar excess addition of its unlabeled sequences (lane 4, Fig. 9 and lane 5, Fig. 10). An excess of unlabeled promoter C sequence, was also able to compete DNA-protein binding reaction (lane 3, Fig. 9). The addition of anti-HA-antibody in the binding reaction supershifted the DNA-protein complex in EMSA (lane 6, Fig. 9 and lane 4, Fig. 10).
Thus, GCNF binds to the NR half-site within the promoter C. The specificity of this interaction was confirmed in competition and supershift experiments.
Since CREMtau and GCNF are able to bind specifically to the same response element within mGPDH promoter, we renamed this sequence as CRE/NR site.

To assess the importance of the CRE/NR sequence for interaction with GCNF, the protein-DNA complexes were competed with 100-fold molar excess oligonucleotides bearing different point-mutations within CRE/NR site (Fig. 10). The formation of protein-DNA complexes was markedly affected in a probe containing the 1A and 5T mutation (lane 6 and 8). The formation of these complexes was not affected in lanes containing the other mutations.

These results confirmed that GCNF is not able to bind to the sequence of a typical consensus CRE sequence. Interestingly, the sequence of the putative CRE site (5'-TGAGGTCA-3') present within promoter C makes interaction between testis-specific promoter and both nuclear factors possible, although different parts of the critical sequence are of importance for CREMtau-DNA and GCNF-DNA interaction.

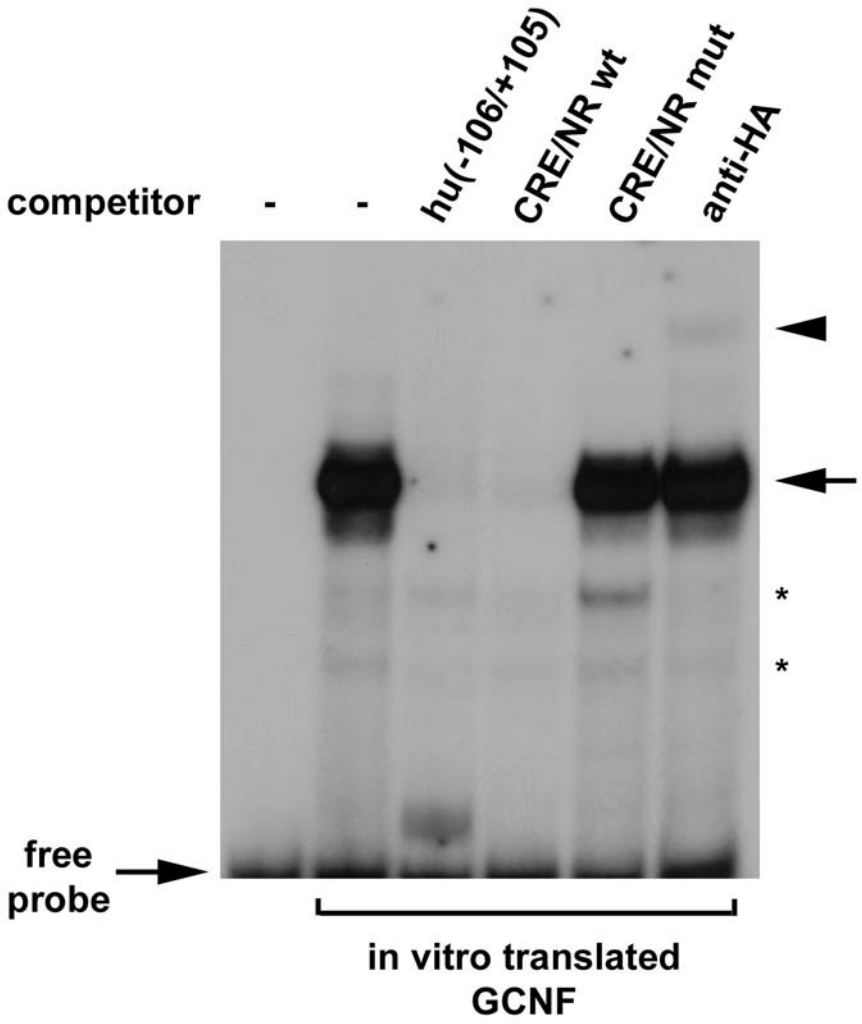

Figure 9. Binding of GCNF to a sequence portion of promoter C in EMSA. *In vitro* translated HA-tagged GCNF was incubated with double-stranded oligonucleotide (-62/-32). For competition experiments 100 fold molar excess of promoter C sequence (-106/+105), or oligonucleotides bearing wild type or mutated CRE/NR site were incubated with the reaction mixture. Anti-HA antibody was part of the reaction mixture in the last lane. The protein-DNA complexes are shown by an arrow and supershift is indicated by an arrowhead.

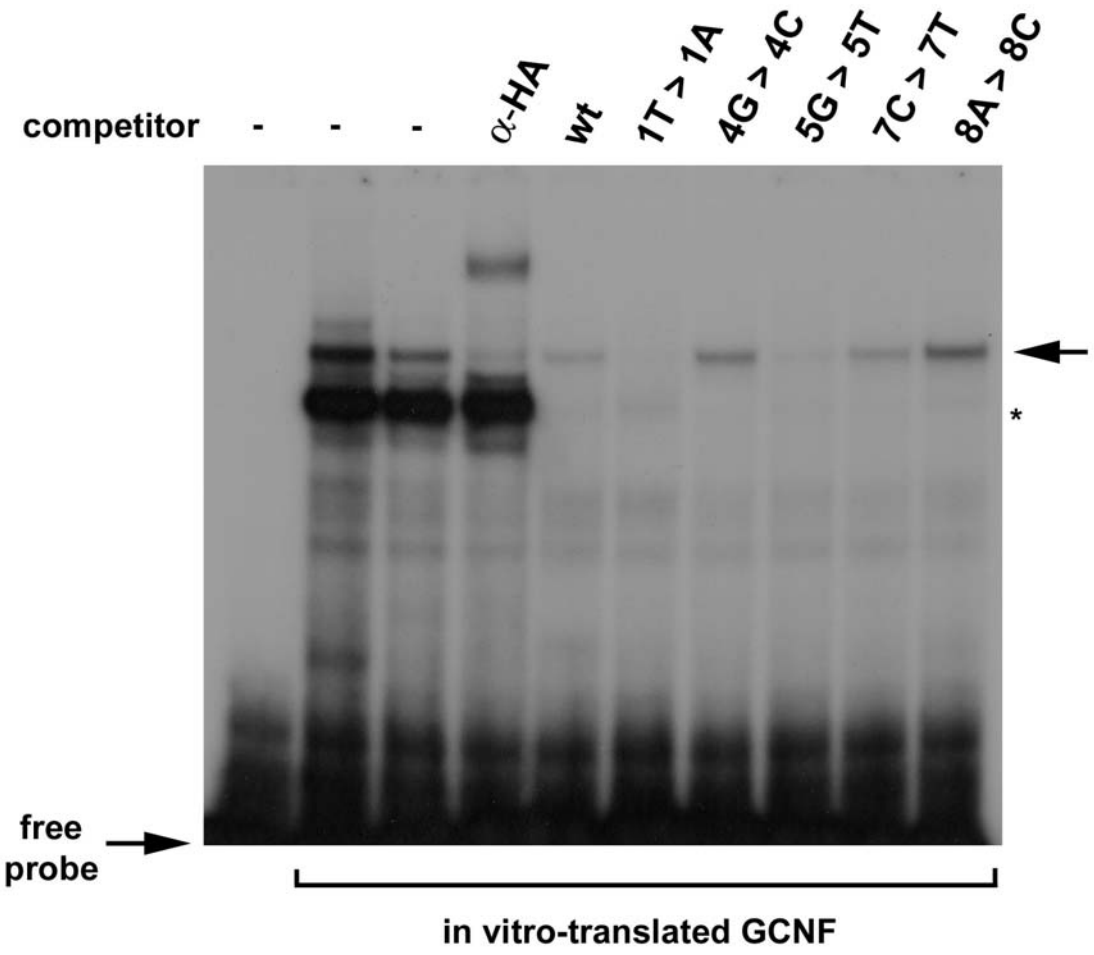

Figure 10. Binding of GCNF to a sequence portion of promoter C in EMSA. *In vitro* translated HA-tagged GCNF was incubated with double-stranded oligonucleotide (-62/-32) bearing CRE/NR site. For competition experiments 100 fold molar excess of wild type or mutated oligonucleotides was added to the reaction mixture. Anti-HA antibody was part of reaction mixture in forth lane. Specific protein-DNA complexes are incubated by an arrow. Nonspecific complexes are indicated by an asterisk.

4.4. Influence of CREMtau and GCNF on Human Promoter C Activity

The potential role of CREMtau and GCNF in the regulation of mGPDH gene expression was determined performing reporter assays in cell culture.

The promoter C sequence of human mGPDH gene (-106/+105) was subcloned into pGL3-basic luciferase reporter vector. Transient transfections were performed with this hu(-106/+105)-luc reporter construct and CREMtau and GCNF expression constructs. The transcriptional activities of the reporter constructs were measured approximately 20 hours later.

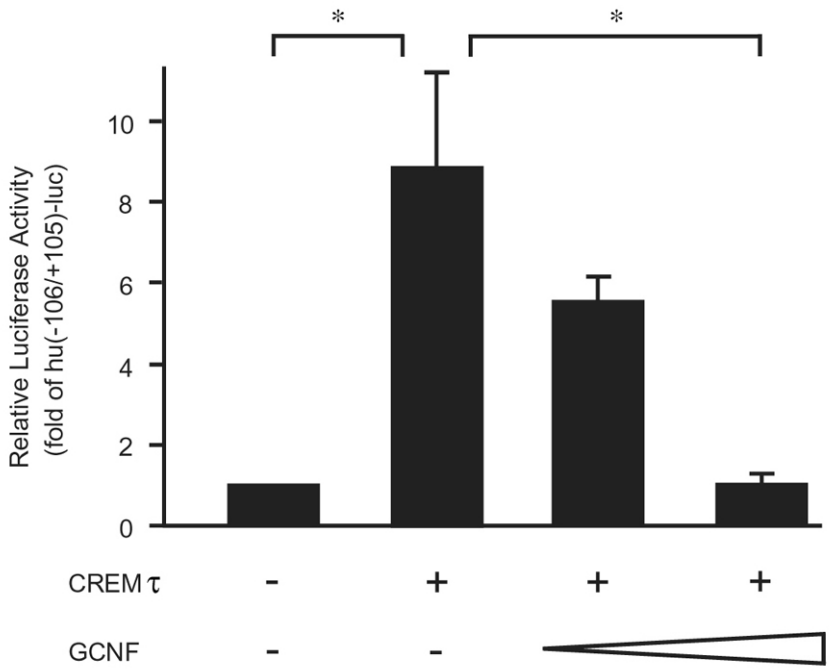

Figure 11. CREMtau activates promoter C-containing reporter construct while GCNF downregulates this activity. Relative luciferase activity was measured after transient transfection with hu(-106/+105)-luc reporter construct (promoter C) and CREMtau and GCNF expression constructs. Transient transfection experiments were performed in HEP G2 cells. The results presented are averages from 3 separate experiments, each carried out in triplicate. Error bars represent SD. Significant values estimated by Student's t test are given (*, p< 0.05).

Cotransfection with CREMtau expression construct increased luciferase activity nine-fold in comparison to basal luciferase activity measured in control group, transfected only with the reporter construct hu(-106/+105)-luc. Additional cotransfection with GCNF expression

construct partially relieved CREMtau mediated activation, while the increased amount of GCNF completely blocked CREMtau activity.

These data indicate that CREMtau and GCNF play an important role in the regulation of promoter C. CREMtau activates promoter C-containing reporter construct while GCNF downregulates this activity, suggesting an interference of CREMtau and GCNF signaling pathways.

4.5. Influence of GCNF and GCNF-VP16 Chimeric Protein on Human Promoter C Activity

Most of nuclear receptors influence transcription process by acting through specific DNA elements. GCNF is a transcription repressor which acts through 5'-AGGTCA-3' target sequences (Cooney AJ et al. 1998). In order to check whether this sequence present within promoter C of mGPDH can be a functional binding site for GCNF, we cloned annealed oligonucleotides -57/-38 (flanking CRE/NR sequence) into pGL3-basic luciferase reporter vector, in front of the minimal prolactin promoter.

We performed transient transfection experiments with reporter vector CREwt-rPrl-Luc and GCNF and GCNF-VP16 expression constructs. GCNF-VP16 is a fusion protein consisting of GCNF and a strong viral activator domain. The transcriptional activities of reporter constructs were measured approximately 20 hours later and corrected for protein concentrations determinated by the Bradford method.

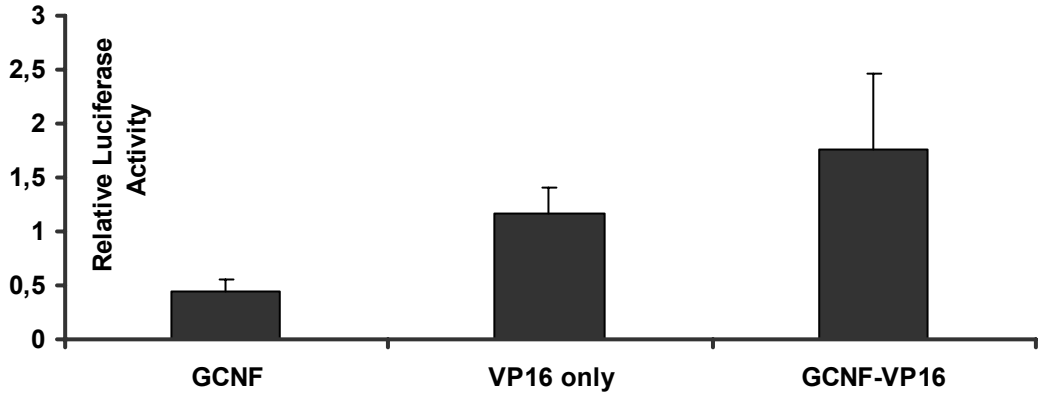

Figure 12. The fusion protein GCNF-VP16 binds and activates reporter gene expression through 5'-AGGTCA-3' element while GCNF alone represses basal activity. Relative luciferase activity was measured after transient transfection with CREwt-rPrl-luc reporter construct and GCNF and GCNF-VP16 expression constructs. Transient transfection experiments were performed in HEK 293 cells. The results presented are averages from 4 separate experiments, each carried out in triplicate. Error bars represent SD.

The results shows that -57/-38 sequence contains a response element which is critical for GCNF binding and function. The fusion protein GCNF-VP16 was able to bind to this sequence and to activate reporter gene expression through this element. GCNF alone was able to repress basal activity of the reporter construct in cellular context.

Thus, GCNF binds in a functional manner with 5'-AGGTCA-3' sequence and represses promoter C activity.

4.6. Influence of CREMtau and GCNF on CREwt/mut-rPrl-Luc Construct Activity

In order to check whether response element within -57/-38 sequence of promoter C can be a functional binding site for both CREMtau and GCNF, transient transfections of HEP G2 as well as HEK 293 cells were performed. We used CREwt/mut-rPrl-luc reporter construct and CREMalpha/tau and GCNF expression constructs. The transcriptional activity of reporter construct was measured approximately 20 hours later.

In the control group of HEP G2 cells (which were transfected with the reporter construct CREwt-rPrl-luc) only background luciferase activity was detected, while in cotransfection experiment CREMtau was able to activate reporter construct (third bar, Fig.13). Cotransfection of a CREM protein lacking the activator domain (CREMalpha) had no significant influence on promoter activity.

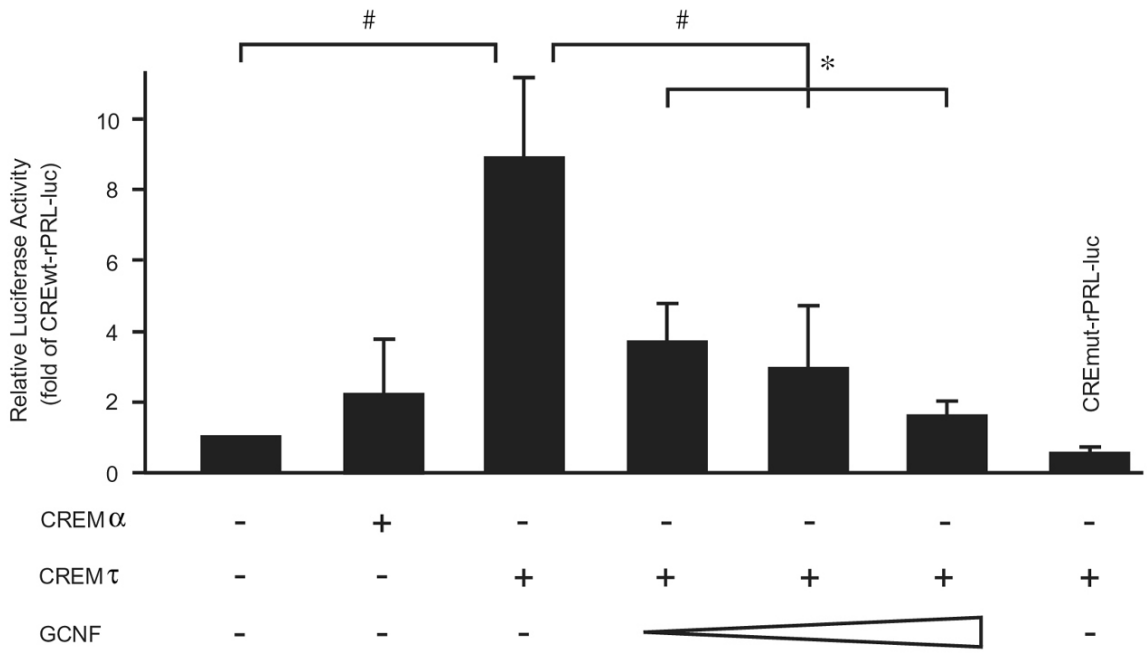

Figure 13. The promoter C activity was conserved when flanking CRE site-sequence was introduced into a heterologous reporter construct. Relative luciferase activity measured after transient transfection with CREwt/mut-rPrl-luc reporter construct and CREMalpha/tau and GCNF expression constructs are shown. Transient transfection experiments were performed in HEP G2 cells. The data are averages from 5 independent experiments, each carried out in triplicate, with standard deviations shown.
Significant values estimated by Student's t test are given (*, p< 0.05; #, p< 0.01).

The additional cotransfection with GCNF expression construct partially relieved CREMtau mediated activation while three fold increased amount of GCNF construct completely blocked CREMtau mediated activation (sixth bar).
To check whether this is also true for another cell line, we performed similar experiment in HEK 293 cells. Here, GCNF was able to cause repression of spontaneous activity of CREwt-rPrl-luc construct. Overall, the pattern of response to two transcription factors was similar (see Fig. 14).

Thus, CREMtau is able to bind to flanking CRE site-sequence (-57/-38) which was introduced into a heterologous reporter construct, and to activate transcription. Mutation of the CRE site prevented CREMtau activation, confirming that this part of the sequence is critical for CREMtau-DNA interaction (the last bar, Fig. 13.).
GCNF repressor activity is also preserved within -57/-38 sequence. CREMtau-mediated activation is relieved or completely blocked, after additional cotransfection of GCNF.

This results confirmed a critical role for the CRE/NR site (5'-TGAGGTCA-3') within the promoter C sequence for interaction and function of both nuclear factors, CREMtau and GCNF.

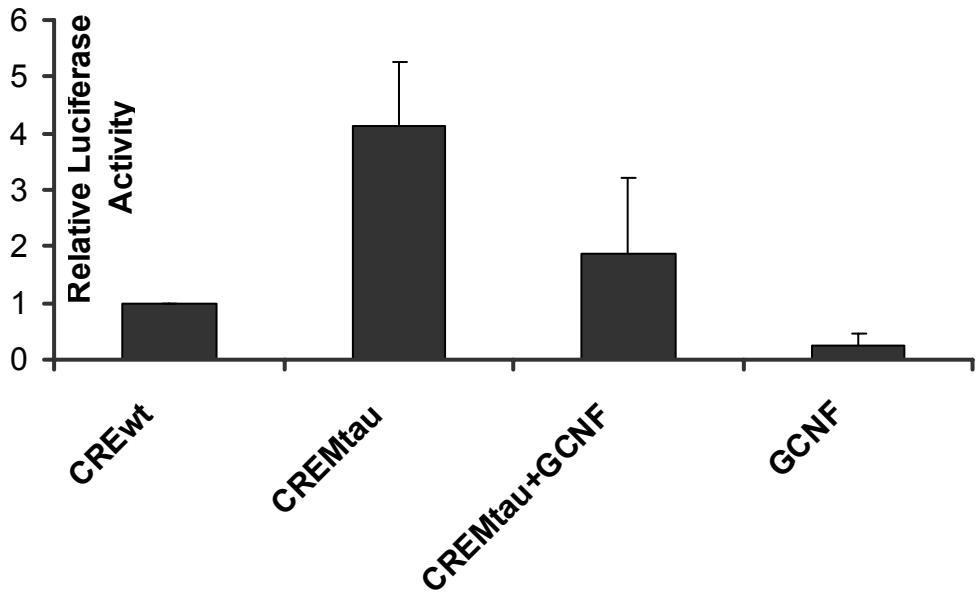

Figure 14. The promoter C activity was conserved when flanking CRE site-sequence was introduced into a heterologous reporter construct and transient transfections were performed in HEK 293 cells. Relative luciferase activity was measured after transient transfection with CREwt-rPrl-luc reporter construct and CREMtau and GCNF expression constructs. The data are averages from 6 independent experiments, each carried out in triplicate, with standard deviations shown.

4.7. Effect of Mutated CRE/NR site on the Interaction with Transcription Factors inTtransient Transfection Experiments

To verify in vivo efect of mutated CRE/NR site on binding and function of CREMtau and GCNF, we investigated 4C and 5T mutated constructs (see EMSA, Fig. 8 and Fig. 10) in cell culture. HEK 293 and HEP G2 cells were transfected using mutated reporter constructs hCRE 4C-rPRL-Luc or hCRE 5T-rPRL-Luc and expression constructs for CREMtau and GCNF.

In HEK 293 cells we could not confirm findings from EMSA (data not shown).
Interestingly, in HEP G2 cells CREMtau was able to bind to 4C mutated reporter construct causing activation (Fig. 15), while GCNF was able to down-regulate activity of mutated reporter construct (data not shown).

These data indicate that there is no correlation between results of EMSA and of the cell culture experiments.

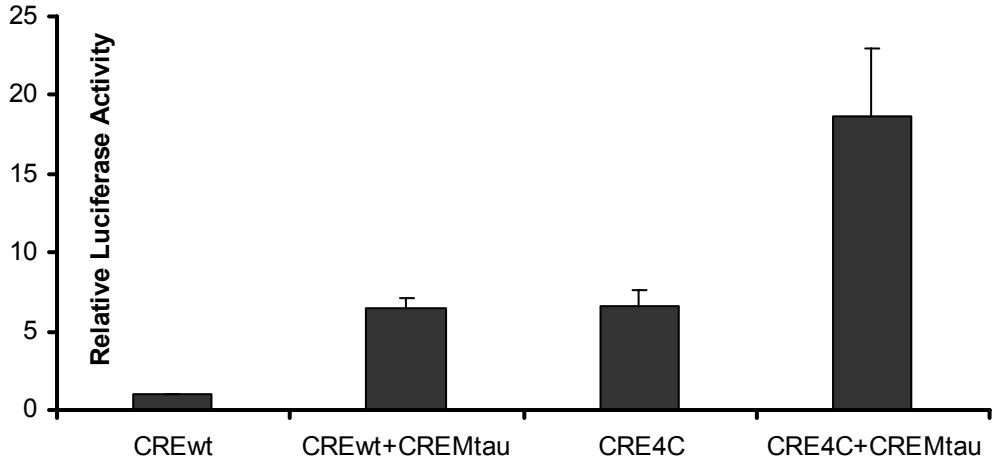

Figure 15. In HEP G2 cells CREMtau binds to 4C mutated reporter construct causing activation. Relative luciferase activity was measured after transient transfection with CREwt-rPrl-luc (control) or CRE 4Cmut-rPrl-luc and CREMtau expression construct. The data are averages from 3 independent experiments, each carried out in triplicate, with standard deviations shown.

4.8. Western Blot Analysis

To exclude the possibility that expression levels of CREMtau is influenced by the presence of GCNF we performed transient transfections with CREMtau expression construct, with or without GCNF expression construct. Applied concentrations of expression plasmids were the same as in all previous experiments. After cell lysis, the protein concentration was measured and equal amounts of proteins (70 μg) were loaded to SDS-PAGE.

We detected a strong signal after the application of an anti-FLAG-M2 monoclonal antibody (Fig.16). *In vitro* translated FLAG-tagged CREMtau was used as a positive control.

Expression level of CREMtau was equal in the presence and in the absence of GCNF. Thus, downregulation of the promoter C activity in the presence of GCNF was not the result of a disturbed expression level of CREMtau but rather the result of a direct influence of GCNF on promoter activities.

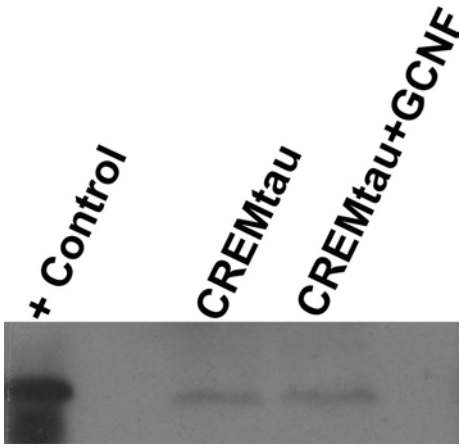

Figure 16. Expression levels of CREMtau in the presence or in the absence of GCNF.
Transient transfections were performed in HEP G2. Western blot was performed using anti FLAG-M2 monoclonal antibody. Positive control was *in vitro* translated FLAG-tagged CREMtau.

4.9. Influence of an Inhibitor of Histone Deacetylases (Trichostatin A) on Promoter C

To check the hypothesis that histone deacetylation is involved in repression of the transcription of promoter C by GCNF, we tested the effect of the HDAC inhibitor Trichostatin A (in final concentration of 100 ng/mL) on the transcription of reporter construct CREwt-rPrl-luc, in the presence of both examinated transcription factors. Transient transfections were performed in HEK 293 cells. After 48 hours incubation the transcriptional activity of reporter construct were measured and corrected for protein concentrations which were determinated by the Bradford method.

As shown in Fig. 17, luciferase activity was 1,7 fold higher in cells transfected with CREMtau and GCNF expression constructs in the presence of trichostatin A, compared to the group transfected with the same constructs without Trichostatin A.

Thus, the increased level of histone acetylation prevented GCNF down-regulation of reporter gene. It seems that histone deacetylation and changes in chromatin structure are involved in the GCNF-mediated repression of the transcription.

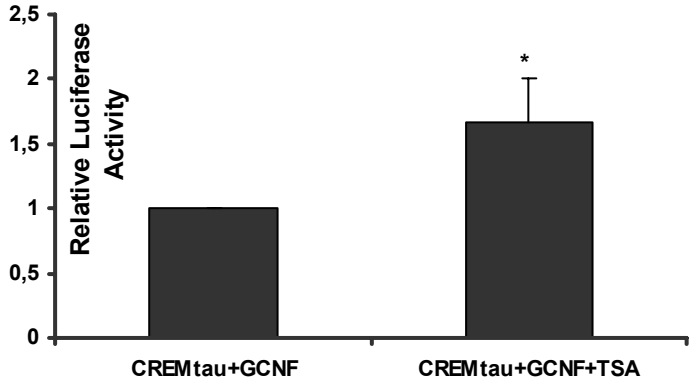

Figure 17. Effect of an Inhibitor of Histone Deacetylases (TSA) on the transcriptional activity of promoter C. HEK 293 cells were transfected with CREMtau and GCNF expression constructs in the presence or in the absence of trichostatin A. The data are averages from 5 independent experiments, each carried out in triplicate, with standard deviation shown.

4.10. Influence of Nuclear Factors on Chromatin Acetylation and Deacetylation

For ChIP analysis the transient transfection was performed in HEP G2 cells, as described above. The reporter construct CREwt-rPrl-Luc and expression constructs for CREMtau and GCNF were used. We compared the histone acetylation levels in the promoter region in the presence of CREM alone or in the presence of both transcription factors.

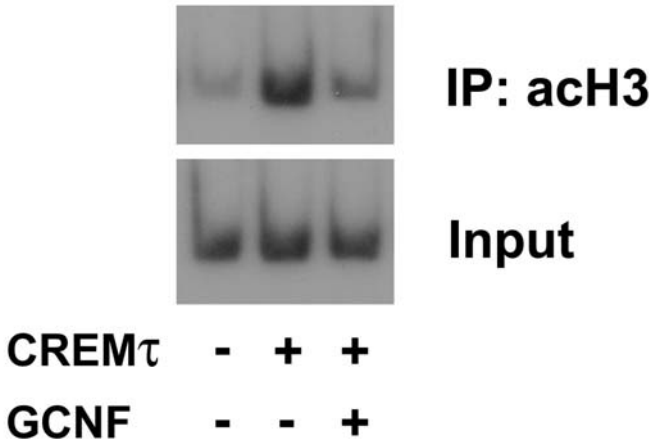

Figure 18. ChIP analyses confirmed an impact of histone modification on activation/repression by CREM and GCNF. The histone acetylation levels in DNA obtained from cells which were cotransfected with CREMtau was clearly higher than in the presence of CREM and GCNF (IP-s). Inputs are cell lysate samples before application of antibody.

As shown in Fig. 18, the histone acetylation level in DNA obtained from cells which were cotransfected with CREMtau alone was clearly higher than in cells cotransfected with CREMtau and GCNF simultaneously. The acetylation could be reversed by GCNF. These results are in correlation with activation and downregulation of promoter C under the influence of these two transcription factors, described in sections 4.4. and 4.6.

The results of ChIP experiments indicate that the expression mGPDH gene is related to the acetylation/deacetylation of histone in the promoter region.

4.11. Immunohistochemistry of Human Spermatozoa and Testis-tissue

To localise mGPDH protein within germ cells, human testis cross-sections were incubated with an anti-mGPDH polyclonal antibody. As showed in Fig. 19 (arrow) late postmeiotic germ cells were immunostained. This staining was not detectable in negative controls where preimmune serum was used instead of the antibody.

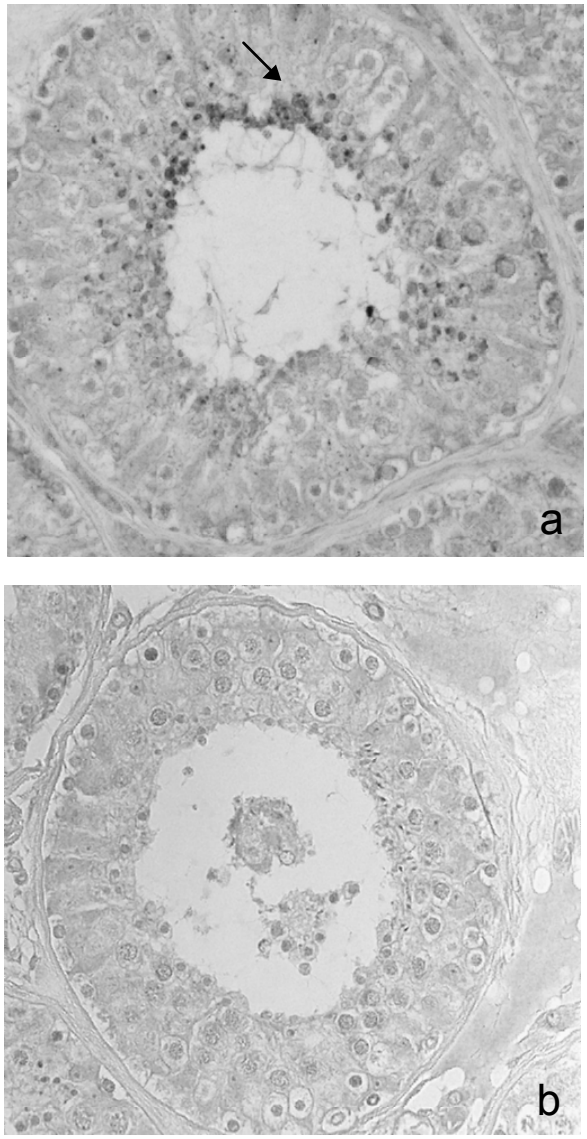

Figure 19. Immunohistochemical staining of mGPDH in human testis. Cross sections (6µm) were stained with an anti-mGPDH polyclonal antibody (a) or preimmune serum instead of antibody (b). Late postmeiotic germ cells were immunostained (arrow).

To get more precise information about the localisation of mGPDH within mature cells, spermatozoa were incubated with an anti-mGPDH antibody.
Green fluorescence signal was clearly visible in the midpiece of spermatozoa which are rich in mitochondria. This staining was not detectable in negative controls, when preimmune serum was used instead of the antibody or when the primary antibody was omitted.

Taken together, this data indicate that mGPDH is expressed in elongated spermatids and mature spermatozoa (mitochondria-rich midpiece) which confirms importance of mGPDH for energy production and motility of mature spermatozoa.

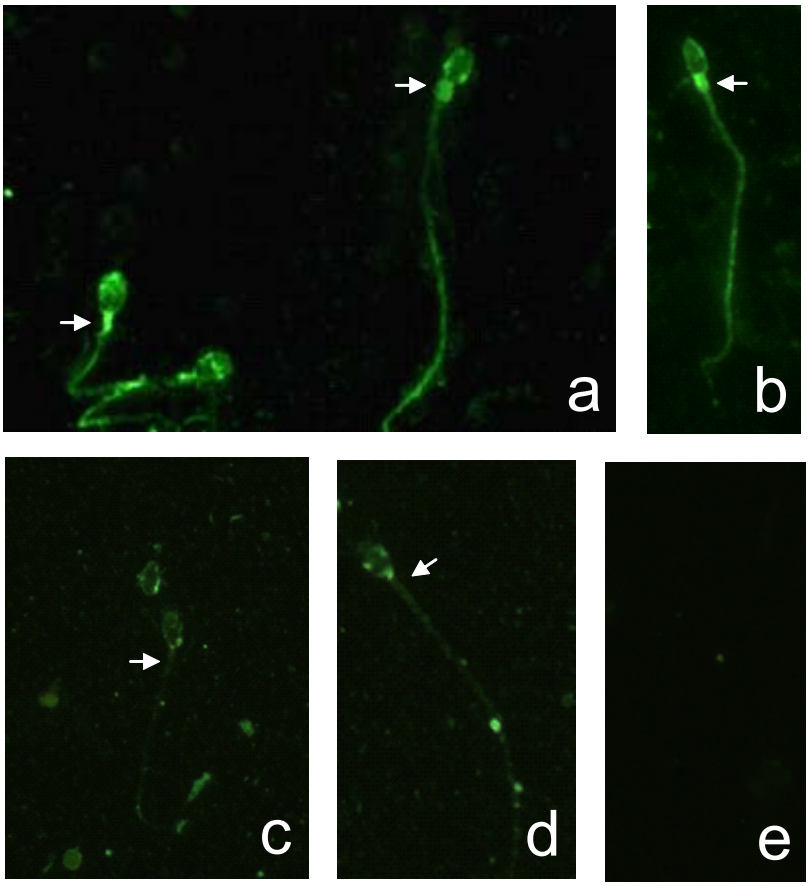

Figure 20. Immunohistochemical staining of mGPDH in human spermatozoa.
Cells were incubated with an anti-mGPDH polyclonal antibody (a, b), with preimune rabbit serum (c, d) or without first antibody. It was shown that mGPDH is expressed in the midpiece of mature spermatozoa (a, b - arrowheads).

5. Discussion

5.1. Tissue-specific Expression of mGPDH Gene

The expression of mGPDH is high in tissues with high ATP consumption, suggesting an important role of mGPDH in appropriate energy production (Urcelay E et al. 2000).

The regulation of human mGPDH gene expression in somatic tissues is a result of the combinatorial effect of transcription factors on at least two promoters. The promoter B is ubiquitously active, while both promoters are active in tissues with particularly high mGPDH expression such as brain, brown adipose tissue and endocrine pancreas (Gong DW et al. 1998 and Gong Q et al. 2000, Koza RA et al.1996, Ferrer J et al. 1996, Urcelay E et al. 2000, Weitzel JM et al. 2000).

In addition, Gong et al. (1998) showed that a third mGPDH promoter (which contains exon 1c) was used in testis while, Weitzel JM at al. showed that both promoter B and promoter C were active in testis (2000). This correlates with a high level of mGPDH expression in this tissue.

5.2. Testis-specific Promoter of Human mGPDH Gene

Specific and complex regulation of gene expression in testis includes use of specific transcription factors, alternative promoters and start sites and alternative exon utilization (reviewed by Eddy EM, 1998).

The list of genes which are additionally regulated by testis-specific promoters include Protamine 1 and Protamine 2 (Hummelke GC and Cooney AJ, 2004), t-ACE (Zhou Y et al. 1996), SP10 (Reddi PP et al.1999), ELP (Valentin M et al. 2000), LacDH (Kroft TL et al. 2001). These promoters can drive transcription during specific developmental stages or in a particular cell type.

Since testis specific promoter of mGPDH in rat was already described (Gong DW et al. 1998, Weitzel JM et al. 2003), we made an alignment of the rat promoter C sequence with human genomic sequences and identified a 48-bp human sequence portion, which shows an 87% sequence identity. This sequence is situated downstream of the exon 1b and upstream of the common exon 2, suggesting an orthologous position of the promoter C in the human mGPDH gene (Rajkovic M et al. 2004).

There are additional evidences that mGPDH is additionally regulated by a third testis-specific promoter C in rodent and human. Usage of promoter C results in an alternative transcript which contains the sequence of exon 1c directly adjacent to exon 2 at 5´end in rat (Gong DW et al 1998**,** Weitzel JM et al. 2003) and which is also conserved in human (Rajkovic M et al. 2004).

Different mGPDH transcripts are detectable in different tissues. Most of rodent tissues expressed 6.5 kb transcripts including BAT (Gong DW et al. 1998), brain (Nguyen NH et al. 2003) and isolated pancreatic islets (Ferrer et al.1996). From muscle tissue an additional 4.5 kb transcript was detectable (Gong DW et al. 1998, Ferrer J et al. 1996, Rajkovic M et al. 2004). Brown adipose tissue expresses predominantly a 6.5 kb transcript along with some smaller (probably unspecific) bands (Gong DW et al. 1998, Koza RA et al. 1996).
A shortened mGPDH transcript of 2.4 kb was detectable in mouse (Koza RA et al. 1996) and rat only in testis tissue (Gong DW et al.1998, Weitzel JM et al. 2003). The transcripts of the same length are detectable in human. Out of 23 human tissues, a 2.4 kb transcript was solely detectable from testis-tissue (Rajkovic M et al. 2004).

5.3. Regulation of the Testis-specific Promoter of mGPDH by CREMtau and GCNF

5.3.1. CREMtau and GCNF are binding to the CRE/NR site in Promoter C of mGPDH

- **CREMtau**

The consensus CRE site (5'-TGACGTCA-3'), or a variation of it, has previously been found in promoter regions of a number of CRE-responsive genes in testis (tACE-Zhou Y et al. 1996, Protamine 1-Hummelke GC and Cooney AJ, 2004, ACP -Hurst S et al 1998, Transition protein-1 -Steger K et al. 1999, mGPDH in rat-Weitzel JM et al. 2003).

In addition, a large family of CRE-binding factors (CREB, CREM and ATF1) has been reported to interact with this sequence and mediate transcriptional activation (Foulkes NS and Sassone-Corsi P, 1996).

By sequence inspection of the testis-specific promoter of human mGPDH we identified the putative CRE site 5'-TGAGGTCA-3' at position -51 to -44, which differs from a typical CRE consensus sequence by one nucleotide.
Thus, CRE site in the upstream sequence of human mGPDH gene could be potential target for CREMtau, an important factor in spermatogenesis.

The electrophoretic mobility shift analysis of flanking CRE site of promoter C (-62/-32 bp) identified the presence of CREMtau-DNA complexes. The protein-DNA complexes were essentially abolished and supershifted in the presence of anti-FLAG-antibody. The competition experiments have additionally confirmed specificity of this interaction.

The importance of the CRE site was further assessed by generating different point-mutations within CRE site, followed by competition analysis in EMSA. The formation of protein-DNA complexes was markedly affected in a probe containing the 4C mutation which generated consensus CRE sequence (lane 6, Fig.8).
On the other hand, most of other mutations applied were not able to compete with these complexes, suggesting the greater importance of the intact CRE site in CREM protein-DNA binding.

Taken together these results have confirmed that CRE site within promoter C is the target for CREMtau. It suggests a role of CREMtau in the regulation of promoter C activity.

- **GCNF**

Previous studies have demonstrated that GCNF is able to bind to the response elements to direct repeats of 5'-AGGTCA-3', referred as DR0, which is conserved in mammals (Chen F et al.1994, Schmitz TP et al. 1999).
Interestingly, sequence of described putative CRE site differs from a typical CRE consensus sequence by one nucleotide. This difference generates a nuclear receptor-binding site (5'-AGGTCA-3' at -49 to -44) within promoter C.

In our study, in electrophoretic mobility shift assays GCNF is binding to NR-binding site. Our data presented in Fig. 9 and 10, clearly showed that oligonucleotide probe containing CRE/NR site (-62/-32) resulted in the formation of specific protein-DNA complexes with GCNF, which were supershifted by addition of anti-HA-antibody. The protein-DNA complexes observed were effectively inhibited by 100-fold molar excess addition of its unlabeled sequences (lane 4 in Fig. 9 and lane 5 in Fig. 10).

When competed with oligonucleotides bearing different point-mutations in CRE, the formation of protein-DNA complexes was markedly affected in a probe containing the 1A and 5T mutation (lane 6 and 8). The formation of specific complexes was not affected in a probe containing 4C mutation as well as other point-mutations, indicating that these mutations did not affect GCNF binding.

Thus, in electrophoretic mobility shift assays both the testis-specific transcriptional activator CREMtau and the testis-specific transcriptional repressor GCNF are able to bind to CRE/NR site in testis-specific promoter C of mGPDH gene.

5.3.2. CREM-mediated Transcriptional Activation of Promoter C in Cell Culture Experiments

It appears that CREMtau, the predominant transcription factor in adult testis (Foulkes NS et al. 1992, Delmas V et al. 1993), is able to bind to the CRE site in promoter C of mGPDH.

The potential role of CREMtau in the regulation of mGPDH gene expression was determined performing reporter assays in cell culture. Under CREMtau stimulation we observed a significant increase of promoter C activity when compared with that seen in control group transfected only with reporter construct -106/+105-Luc (Fig. 11).
This activation capacity is preserved within the CRE/NR motif as indicated by introducing this response element into a heterologous reporter construct (Fig. 13 and 14). The cotranfection of CREwt-rPrl-Luc with CREMtau expression construct yielded a similar degree of luciferase response induction as above, indicating that the regulatory element remained present within this region.

These results clearly demonstrate that CREMtau is involved in the activation of mGPDH gene expression in human testis via CRE site in promoter C.

5.3.3. Suppression of CREMtau-mediated Transcriptional Activation by GCNF in Cell Culture Experiments

The electrophoretic mobility shift assays demonstrated that CREMtau, as well as GCNF, were able to bind to CRE/NR within promoter C (Fig. 8, 9, 10) and to form CREMtau- and GCNF-DNA complexes. Therefore, we also investigated functional consequences of GCNF-DNA interaction in cell culture experiments.

In transient transfections a chimeric GCNF-VP16 protein activates the CREwt-rPrl-Luc reporter construct confirming that the CRE/NR site is also a functional binding site for this transcription repressor.

Since GCNF and CREM were shown to be co-expressed in germ cells (Behr R and Weinbauer GF, 2001, Yang G et al 2003), we analyzed whether GCNF would interfere with CREMtau-induced transcriptional activation. We examined the effect of GCNF expression on the transcriptional activation of a hu(-106/+105)-Luc, and of CREwt-rPrl-Luc reporter construct, in the presence of CREMtau, respectively (Fig. 11 and 14). GCNF was able to partially relieve CREMtau mediated activation of promoter C. In HEK 293 cells, GCNF reduced basal activity of promoter construct to a level several fold lower than that of the basal activity. These observations indicate that GCNF can function as an active suppressor of gene transcription.
To analyze this in more detail, we examined the effect of increasing levels of GCNF expression on the transcriptional activation of a CREwt-rPRL-Luc reporter construct by CREMtau. As shown in Fig. 13, GCNF strongly inhibited CREMtau mediated activation in HEP G2 cells in a dose-dependent manner.

The observations from cell culture experiments indicate that CREMtau acts as an activator while GCNF acts as a repressor, which can interfere with the transcriptional activation induced by CREMtau. GCNF repressor activity likely involves interactions of GCNF with various co-repressors, such as SMRT and N-CoR (Yan Z and Jetten AM, 2000) and consequently interactions with the general transcriptional machinery.

5.3.4. Possible Molecular Mechanism of CREMtau/GCNF Regulation of mGPDH Promoter C Activity

The cross-talk between nuclear receptors can occur at any step of transcriptional control, including competition for the same response element, competition for the same co-repressors or co-activators, or direct protein-protein interactions.
Perhaps the differences in the expression level of the nuclear receptors in the affinity for the respective DNA element and the presence of ligand are contributing factors for the activation and repression.

Because of the sequence overlap between CRE and NR in promoter C, both transcription factors are able to bind to this sequence and to change gene expression. Thus, repression by GCNF could be due to competition between the two receptors for binding to the same response element. The GST-pull-down analysis of CREMtau and GCNF indicates that these two proteins physically interact with each other (our unpublished data).

These results suggest that the repression of CREM-induced activation by GCNF is at least in part due to the competition for the same binding site, although other mechanisms could not be excluded. To clarify this question further investigations are required.

5.4. Effect of Transcription Factors on Chromatin Acetylation and Deacetylation

The results of ChIP experiments indicate that the expression of mGPDH gene is related to the acetylation of histones in the promoter region. We compared the histone acetylation levels in the promoter region in the presence of CREMtau or in the presence of both transcription factors. The PCR analysis showed that histone acetylation level in the promoter region in the presence of CREM was clearly higher than in the presence of CREM and GCNF simultaneously (Fig. 18).

CREMtau may be involved in the regulation of postmeiotic transcription by recruitment of histone acetyl-transferases and acetylation of the nucleosomes in the promoter region. An open chromatin structure then allows approach of the transcription machinery and different co-activators.

GCNF may be involved in the shut-down of transcription at the end of spermiogenesis by recruitment of histone deacetylase activity to promoters, deacetylation of the nucleosome and an induction of a closed chromatin conformation thereby inducing gene silencing. The different co-repressors (SMRT and N-CoR) are already known to act in similar manner (McKenna NJ et al. 1999).

Trichostatin A, a potent inhibitor of histone deacetylase activity, was able to increase level of histone acetylation, to inhibit cell proliferation, and to induce cell-cycle arrest and apoptosis in HEP G2 cells (Yamashita Y et al. 2003, Herold C et al. 2002).

The treatment of HEK 293 cells with TSA has increased level of histone acetylation and prevented GCNF down-regulation of reporter gene in cell culture. These results are in correlation with the ChIP experiments performed in this study.

These findings support the conclusion that histone deacetylaton and changes in the chromatin structure are involved in repression of the transcription by GCNF.

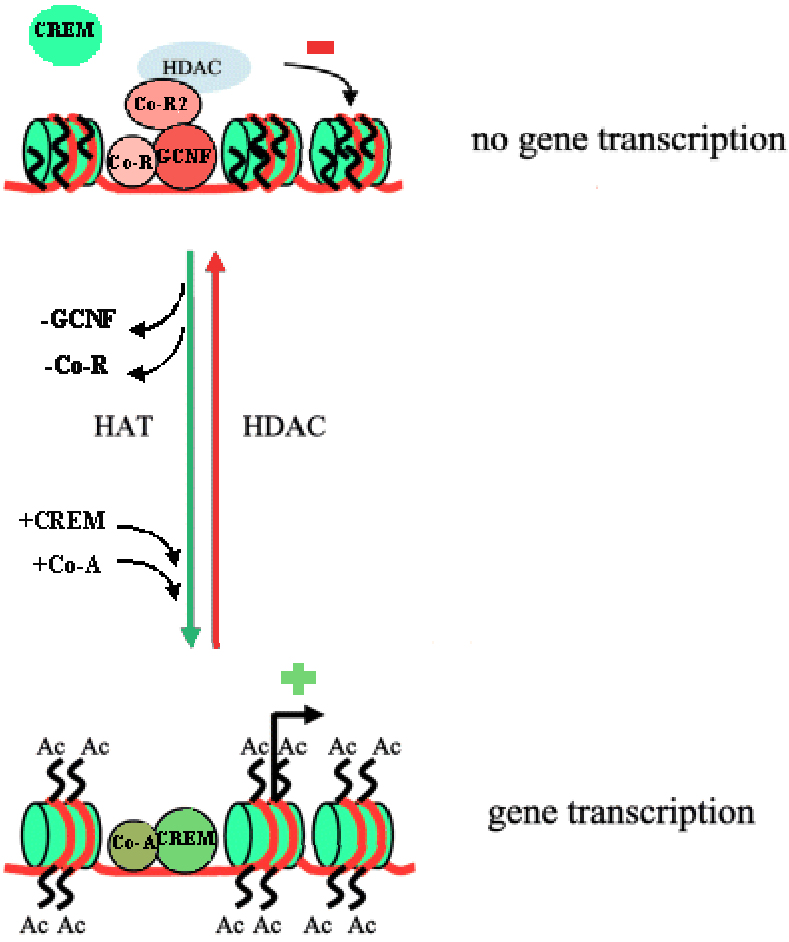

Figure 21. The repression of mGPDH gene expression by GCNF could be due to competition between the two nuclear factors for binding to the same response element. GCNF recruits different co-repressors (Co-R) and consequently HDAC (histone deacetylases) which results in the deacetylation of promoter region and more compact chromatin structure that is not easily accessible for the transcriptional machinery.
If GCNF is not present, CREM binds to the CRE/NR site and recruits co-activators (Co-A). After acetylation (under the influence of HAT- histone acetyltransferse) the N-termini of histones are displaced, the nucleosome unfolds and provides access for transcription machinery.

5.5. Overlapping Expression Profiles of CREM, GCNF and mGPDH

5.5.1. CREM Expression

CREM is essential for spermatid maturation. Analysis of testicular CREM expression in rodents, monkeys and men showed a highly comparable pattern, suggesting that CREM is of general importance for spermatid development in mammals (Behr R and Weinbauer GF, 2001).

Alternative exon splicing and alternative starting sites for translation result in CREM isoforms with either activating: τα, τ, τ1, τ2 (Foulkes N.S. et al 1992), CREM θ1 and CREM θ2 (Daniel PB et al. 2000, Don J and Stelzer D 2002) or repressing activity: α, β, γ, S-CREM (Foulkes NS et al. 1991, Delmas V et al. 1993).

Testicular CREM expression exhibits a switch from CREM repressor to CREM activator isoforms during testicular development (Foulkes NS et al. 1992; Delmas V et al. 1993, Daniel PB et al. 2000). It was described that CREM repressors were only detectable in prepubertal testis, in premeiotic male germ cells, and CREM activators are abundantly expressed in pachytene spermatocytes and round spermatids. In rodents CREMtau protein was found only in round spermatids (Delmas V et al. 1993, Behr R and Weinbauer GF, 2001).
Analysis of CREM in human testes with complete spermatogenesis revealed a similar cell-type and stage-specfic expression pattern (Weinbauer GF et al. 1998; Steger K et al. 1999). The analysis of human and monkey testicular protein by Western blotting revealed only one major band representing the CREMtau2 activator in adults (Weinbauer GF et al. 1998, Behr R and Weinbauer GF, 1999).

In contrast, Zhou Y et al. (1996) identified CREM activators (tau, τ1, τ2) as well as repressors (α, β and S-CREM) in adult testis extract. Behr R et al. (2000) also demonstrated the presence of repressors in human and monkey adult testis. By more precise methods Blöcher S et al. (2005) demonstrated CREM activator and repressor isoforms in all germ cell types. However, the percentage of germ cell samples that expressed CREM activators was higher in spermatocytes and round spermatids than in spermatogonia and elongated spermatids.

- **Altered expression of CREM in human spermatogenic disorders**

Together with comparable expression pattern, the similarity of spermatogenic lesions in mice lacking a functional CREM gene and patients with altered testicular CREM expression indicate that CREM protein is relevant for spermatid maturation in human.

Studies in men with spermatogenic disturbance and spermatid maturation arrest demonstrated abnormal CREM expression, activator/repressor distribution and altered splicing events. The findings of Weinbauer GF et al. (1998) and Steger K et al. (1999) revealed a lack or reduction in testicular CREMtau expression at the level of mRNA or protein. Blöcher S at al. (2005) demonstrated drastically decreased CREM transcripts with leader exons θ1 and θ2 in patients exhibiting impaired spermatogenesis. Unusual and inacurate CREM splicing, giving rise to inactive transcripts (Behr R and Weinbauer GF 2000) or a new putative repressors (Peri A et al. 1998, Blöcher S et al. 2005) were also observed.

- **Altered expression of CREM and apoptosis in germ cells**

The necessity of CREM is manifested not only by the lack of maturation of the germ cells but also by their entering to cell death pathway. A portion of germ cells that undergo apoptosis in human increases dramatically in idiopathic infertility including spermatogenic arrest (Sassone-Corsi P. 2000, Takagi S et al. 2001).

The data from CREM -/- mice also suggest that CREM may be necessary for the balance between differentiation and apoptosis. Together with the complete absence of spermatozoa, CREM deficient mice showed ten-fold increase in the apoptosis of the germ cells (Nantel F et al. 1996).

The anti-apoptotic genes (Bcl2 and BAZF) are found to be down-regulated. The expression of these apoptotic suppressors seems to be CREMtau dependent. Thus, CREM may be a signal necessary for spermatid survival (Beißbarth T et al 2003).

5.5.2. GCNF Expression

Although GCNF is generally distributed throughout the nucleus, it is particularly prominent in heterochromatic regions and in condensed chromosomes undergoing the meiotic divisions. In male rodents the expression of shorter GCNF transcript occurs postmeiotically with maximal levels in stage VI–VIII spermatids (Katz D et al. 1997,

Zhang YL et al. 1998), while GCNF protein was detected in the nuclei of pachytene spermatocytes and round spermatids, until spermatids begin to elongate (Yang G et al. 2003). In human testis GCNF is expressed in late stage spermatocytes and round spermatides (Agoulnik IY et al. 1998).

This expression profile suggests that GCNF plays a role in transcriptional regulation during meiosis and the early haploid phase of spermatogenesis, prior to the initiation of nuclear elongation and condensation (Yang G et al. 2003, Lan Z J et al. 2003).

5.5.3. mGPDH Expression

In rat the expression of mGPDH has been detected in postmeiotic germ cells restricted from round spermatid up to early elongating spermatid in a temporal expression peak (step 2 to 11) whereas mGPDH protein was seen in late elongated spermatides to mature spermatozoa (step 16 to 19, Weitzel JM et al. 2003). These expressions profiles suggest that mGPDH is of importance for appropriate energy production necessary for sperm motility.

Findings of Brown et al. (2002) showed that cGPDH and mGPDH mice have minor metabolic changes, suggesting that compensatory mechanisms are sufficient. cGPDH-deficient animals are viable and fertile. In contrast, the mGPDH knockout mice have decreased body weight and reduced viability. Interestingly, differences in blood glucose were seen only in the male mGPDH -/- mice, as well as reduced fertility (proportionately with the number of knockout alleles in the cross).
In addition, our work demonstrated a reduced motility of spermatozoa of mGPDH in -/- mice and reduced fertility. Thus, mGPDH contributes to the aerobic metabolism in sperm powering its motility. Therefore, appropriate expression of mGPDH is necessary for normal sperm function.

To get better insight into mGPDH expression in human, we performed Immunostaining of human spermatozoa and testis-tissue sections. Correspondingly to the findings in rodents, mGPDH was localized to late spermatids and to the mitochondria-rich midpiece of spermatozoa.

All results presented in this work suggest a major importance of mGPDH for sperm motility in human. We think that inappropriate gene expression and function of mGPDH

can be an important cause of altered sperm motility in infertile man. However, further clinical studies are necessary to confirm this idea.

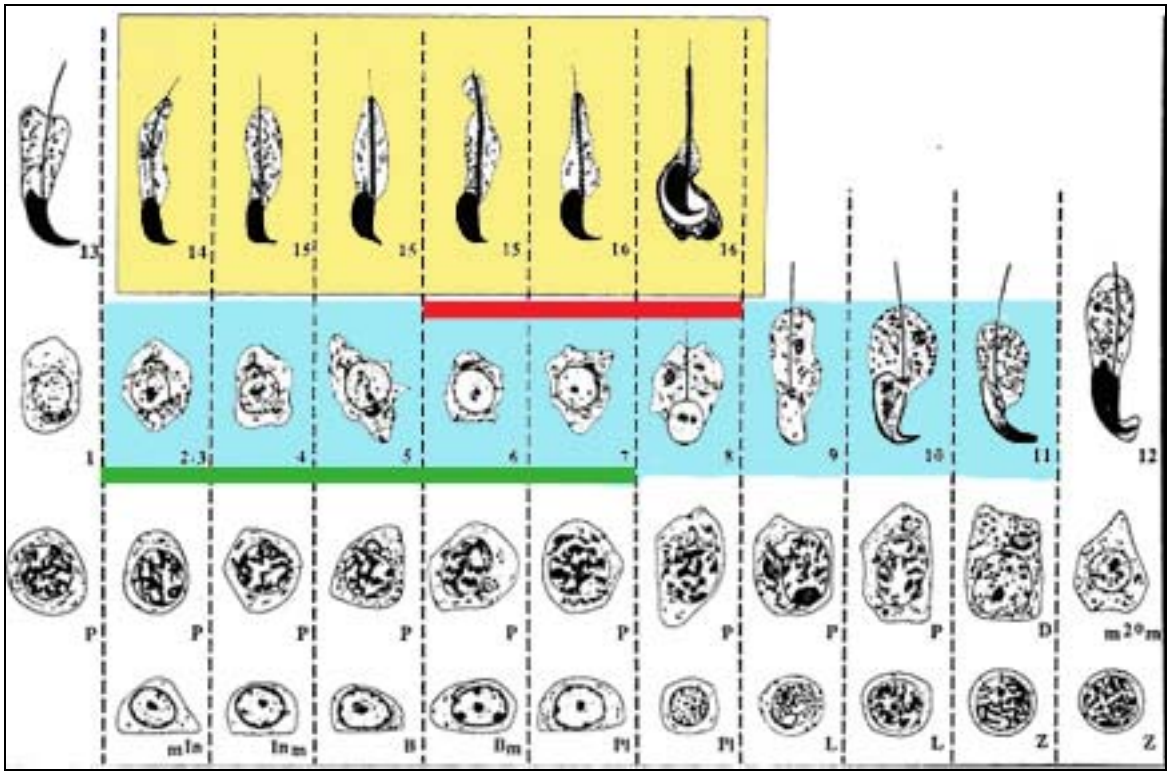

Figure 22. The comparison of expression profiles of CREM, GCNF and mGPDH in rat germ cells.
The blue area depicts expression of mRNA, yellow area presence of mGPDH protein (Weitzel et al. 03). The green bar depicts presence of CREM protein (Delmas et al. 1993, Behr et al. 2001) while red bar depicts maximal levels GCNF protein (Jetten et al. 2003). We speculate that CREM may serve as a critical regulator for mGPDH up-regulation in round spermatids, whereas GCNF is responsible for mGPDH down-regulation during spermatid elongation. (Modiffied from Russell et al. 1990).

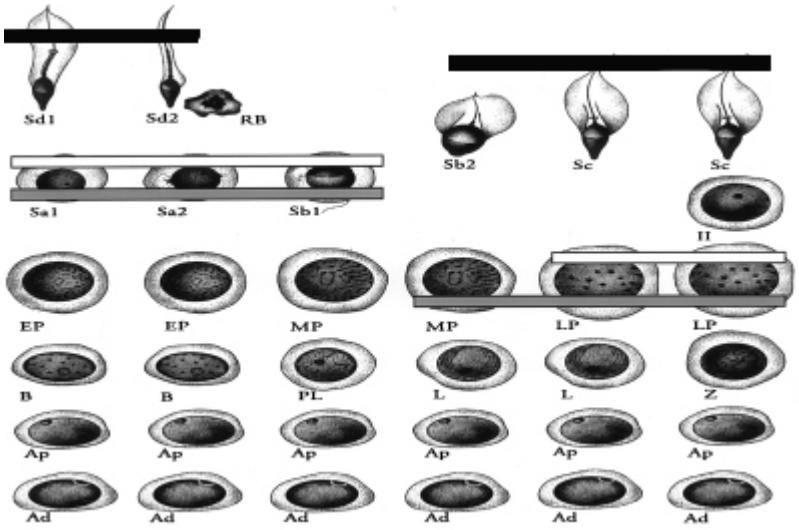

Figure 23. The comparison of the expression profiles of CREM and mGPDH in human germ cells.
The black bar depict presence of mGPDH protein (Rajkovic et al. 2004). The gray and white bars depict expression of CREM mRNA and protein (Behr et al. 2001). The data for GCNF expression in human have not been published yet (modified from Clermont, 1963).

5.6. Molecular Mechanism of Regulation of mGPDH Expression in Male Germ Cells - General Regulatory Mechanism for Postmeiotically Expressed Genes?

The structure and the function of all three proteins studied (mGPDH, CREMtau and GCNF) are conserved between rodents and humans.
CREM, GCNF and mGPDH deficient animals showed that all three studied proteins are of major importance for fertility. In CREM -/- animals spermatogenesis was arrested at the level of round spermatids (Nantel F et al. 1996, Blendy JA et al. 1996). Disruption of GCNF expression results in embryonic lethality (Chung AC et al. 2001), while mGPDH deficient mice showed reduced fertility (Brown LJ et al. 2002).

Additionally, overlapping in postmeiotic expression of CREMtau, GCNF and mGPDH suggests a relevance of these two transcription factors for regulation of postmeiotic mGPDH gene expression. In this work we show for the first time that GCNF and CREMtau bind to the same DNA response element (CRE/NR site) within promoter C of human mGPDH gene in testis. We present evidence that CREMtau is involved in the activation of mGPDH gene, while GCNF functions as an active repressor of mGPDH gene expression via CRE/NR site. The mechanism of regulation of mGPDH gene was found to be predominantly mediated through the competition of CREMtau and GCNF for binding to the CRE/NR site.

We show that histone acetylation/deacetylation and associated changes in chromatin structure are involved in the activation of the transcription by CREMtau and repression by GCNF, respectively. Therefore, cross-talk between CREMtau and GCNF signalling pathways may play an important role in the control of mGPDH gene expression during spermatogenesis.

Since CREMtau protein is highly detectable in round spermatids, we speculate that it may serve as a critical regulator for mGPDH up-regulation in round spermatids, whereas maximal level of GCNF (stage VI-VIII of the cycle) is responsible for mGPDH down-regulation during spermatid elongation. A tight regulation of the testis-specific promoter of mGPDH may be responsible for proper motility of sperm and for fertility in mammals.

In addition, there are several genes that contain CRE or CRE-like elements that were shown to be specifically activated during the haploid phase of spermatogenesis at the time

of appearance of CREMtau protein. The examples are a testis-specific form of an actin-capping protein (ACP) in developing acrosome (Hurst S et al. 1998), transition protein-1 (Steger et al. 1999), t-ACE (Zhou Y et al. 1996), protamine 1, protamine 2 (Hummelke GC et al 2004), RT7, CYPS1, and calspermin (reviewed by Peri A and Serio M, 2000; Don J and Stelzer G, 2002). In correlation with these data many of these genes expressed at late spermatogenesis are found to be affected by the CREM mutation including proacrosine, protamine, TP1, Krox 20, RT7, and others (Blendy JA et al. 1996 and Nantel F et al. 1996).

The targets for GCNF regulation are testis-specific promoters of different genes which contain DR0 response elements in their promoters. Some examples are: Oct 4, tACE, RT7, protamine 1 and protamine 2. Since many of them are already listed as CREM regulated genes, we belive that the reciprocal CREM/GCNF regulation may be assumed as a general regulation mechanism during spermatid development. Further investigations of CREMtau and GCNF-regulated target genes should clarify this question.

6. Summary

Mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) is the rate limiting enzyme of the glycerol-phosphate shuttle which is responsible for proper energy production in a cell. The expression of human and rat mGPDH gene is regulated by two somatic promoters. The rat mGPDH gene is additionally regulated by the third, testis-specific promoter C.

The usage of alternative promoters enables enrichment of mGPDH transcripts in tissues with high energy consumption rates. In sperm mGPDH contributes to the aerobic metabolism powering its motility. Therefore a normal expression and function of mGPDH is of great importance for male fertility.

To test whether human mGPDH might also be regulated by a testis-specific promoter we screened the data bases and found a homologous sequence within the human mGPDH gene. The aim of this study was to characterise the regulation of the testis specific promoter C of the human mGPDH gene and to prove the importance of two testis-specific transcription factors, CREMtau (cAMP responsive element modulator tau) and GCNF (germ cell nuclear factor) for mGPDH expression and normal sperm function.

By sequence inspection of the testis-specific promoter of the human mGPDH we identified the putative cAMP response element (CRE site: 5'-TGAGGTCA-3') at position -51 to -44 (relative to the transcription start site), which differs from a typical CRE consensus sequence by one nucleotide. Interestingly, this difference generates a nuclear receptor-binding site (5'-AGGTCA-3' at -49 to -44). Thus, a CRE site overlaps with a nuclear receptor binding site (NR).

We show that GCNF and CREMtau bind the same DNA response element (which we named CRE/NR site) within promoter C in EMSA. Competition and supershift experiments confirmed the specificity of the binding of the two transcription factors to their target sequence.

In cell culture experiments, we present evidence that CREMtau is involved in the activation of mGPDH gene, via CRE/NR site within promoter C. CREMtau stimulates promoter C activity when compared with control cells. Interestingly, this activation capacity is preserved within the CRE/NR motif as indicated by introducing this response element into a heterologous reporter construct.

Cell culture experiments show that a chimeric GCNF-VP16 fusion protein activates the CREwt-rPrl-Luc reporter construct, confirming that this sequence includes a functional binding site for GCNF.

Further cell experiments demonstrated that GCNF functions as an inhibitor of transcriptional activation mediated by CREMtau in a dose-dependent manner and can act as an active repressor of mGPDH gene expression via CRE/NR site.

Thus, the mechanism of regulation of mGPDH gene was found to be predominantly mediated through the competition of CREMtau and GCNF for binding to the CRE/NR site. Therefore, we show for the first time that cross-talk between CREMtau and GCNF signalling pathways may play an important role in the control of mGPDH gene expression during spermatogenesis.

Cell culture experiments using an inhibitor of histone deacetylases (Trichostatin A) suggested that histone deacetylation and associated changes in chromatin structure are involved in the GCNF mediated transcription repression. These results were confirmed by chromatin immunoprecipitation experiments. In the presence of CREMtau nucleosomes in the promoter region were acetylated, which allows a better approach for the transcription machinery. In the presence of GCNF nucleosomes are deacetylated and mGPDH gene is silenced.

Immunohistochemical staining detected human mGPDH protein in postmeiotic germ cells restricted from late elongated spermatides to mature spermatozoa. The overlap in postmeiotic expression of CREMtau proteins, GCNF proteins and mGPDH transcripts suggests a relevance of this transcription factors for regulation of postmeiotic mGPDH gene expression. Presumably, CREMtau may be a positive regulator of mGPDH gene expression, by turning-on the gene expression at round spermatid phase. GCNF could play a role in repressing CREMtau-activated transcription of this gene at the beginning of spermatid elongation.

Several genes in testis that contain CRE or CRE-like elements are activated by CREMtau and some of them are also shown to be targets for GCNF. This implies that the elucidated reciprocal CREM/GCNF regulation reflects a general regulation mechanism during spermatid development. Additional investigations should confirm this hypothesis.

Further elucidation of interactions between the two transcription factors are certainly of importance for better understanding of male infertility and should give further answer to the control of fertility in men.

7. Abbreviations

A	adenine
ACT	activator of cAMP-responsive element modulator in testis
ATP	adenosin-5'- triphosphate
AEBSF	4-(2-Aminoethyl)benzenesulphonyl fluoride
ATF	activation transcription factor
BAT	brown adipose tissue
bp	base pair
BSA	bovine serum albumin
˚C	Celsius degree
cAMP	cyclic adenosin-3',5'-monophosphate
cGPDH	cytosolic glycerol-3-phosphat dehydrogenase
CBP	CREB binding protein
C	cytosine
ChIP	chromatin immunoprecipitation
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
CREM	cAMP-responsive element modulator
C-terminus	carboxy-terminus
Da	Dalton
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	desoxyribonukleosidtriphosphat (dATP, dGTP, dCTP, dTTP)
DTT	dithiothreitol
E. coli	Escherichia coli
EtBr	ethidiumbromid
EDTA	ethylenediamintetraacetate
EGTA	[ethylenebis(oxyethylenenitrilo)]tetraacetate
ELP	endozepine-like peptide
EMSA	electrophoretic mobility schift assay
f.c.	final concentration
FAD	flavine adenine dinucleotide
FCS	fetal calf serum
Fig	figure
FSH	follicle stimulating hormone
G	guanine
GCNF	germ cell nuclear factor (RTR, NCNF, NR6A1)
GCNF-VP16	germ cell nuclear factor/ viral activator domain
g	gravity force
h	hour
HAT	histone acetyltransferase
HBS	hepes-buffered saline
HDAC	histone deacetylase

HEK 293	human embryonic kidney cells
HEP G2	human hepatocyte carcinoma cells
kb	kilobase
kD	kilodalton
LacDH	lactate dehydrogenase
LH	luteinizing hormone
M	molar
mg	milligram
mGPDH	mitochondrial glycerol-3-phosphat dehydrogenase
min	minute
ml	millilitre
mM	millimolar
mRNA	messenger RNA
MW	molecular weight
NADH	nicotinamide adenine dinucleotide hydride
NAD	nicotinamide adenine dinucleotide
NIDDM	noninsulin-dependent diabetes mellitus (Type 2 diabetes)
NR	nuclear receptor
N-terminus	amino-terminus
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
T	thymine
tACE	testis angiotensin-converting enzyme
TBE	tris-borat-EDTA-Buffer
TE	tris-EDTA-Buffer
TFIIA	general transcription factor
TP1	transition protein 1
Tris	tris(hydroximethyl)aminoethane
TSA	Trichostatin A
U	unit
UTR	untranslated region
V	Volt
µL	microlitre

8. 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

Behr R, Weinbauer GF (1999) Germ cell-specific cyclic adenosine 3',5'-monophosphate response element modulator expression in rodent and primate testis is maintained despite gonadotropin deficiency. Endocrinology 140:2746-2754

Behr R, Hunt N, Ivell R, Wessels J, Weinbauer GF (2000) Cloning and expression analysis of testis-specific cyclic 3', 5'-adenosine monophosphate-responsive element modulator activators in the nonhuman primate (Macaca fascicularis): comparison with other primate and rodent species. Biol Reprod 62:1344-1351

Behr R, Weinbauer GF (2000) CREM activator and repressor isoforms in human testis: sequence variations and inaccurate splicing during impaired spermatogenesis. Mol Hum Reprod 6:967-972

Behr R, Weinbauer GF (2001) cAMP response element modulator (CREM): an essential factor for spermatogenesis in primates? Int J Androl 24:126-135

Beissbarth T, Borisevich I, Horlein A, Kenzelmann M, Hergenhahn M, Klewe-Nebenius A, Klaren R, Korn B, Schmid W, Vingron M, and Schutz G (2003) Analysis of CREM-dependent gene expression during mouse spermatogenesis. Mol Cell Endocrinol 212:29-39

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

Blocher S, Fink L, Bohle RM, Bergmann M, Steger K (2005) CREM activator and repressor isoform expression in human male germ cells. Int J Androl 28:215-223

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

Brown LJ, Koza RA, Marshall L, Kozak LP, MacDonald MJ (2002) Lethal hypoglycemic ketosis and glyceroluria in mice lacking both the mitochondrial and the cytosolic glycerol phosphate dehydrogenases. J Biol Chem 277:32899-32904

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 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

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

Clermont Y (1963) The cycle of the seminiferous epithelium in man. Am J Anat 112:35-51

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

Curi S M, Ariagno J I, Chenlo P H, Mendeluk G R, Pugliese M N, Sardi Segovia L M, Repetto H E, and Blanco A M (2003) Asthenozoospermia: analysis of a large population. Arch Androl 49:343-349

Daniel PB, Rohrbach L, Habener JF (2000) Novel cyclic adenosine 3',5'-monophosphate (cAMP) response element modulator theta isoforms expressed by two newly identified cAMP-responsive promoters active in the testis. Endocrinology 141:3923-3930

Dawson AG (1979) Oxidation of citosolic NADH formed during aerobic metabolism in mammalian cells. Trends Biochem.Sci. 4:171-176

Delmas V, van der Hoorn F, Mellstrom B, Jegou B, 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

Don J, Stelzer G (2002) The expanding family of CREB/CREM transcription factors that are involved with spermatogenesis. Mol Cell Endocrinol 187:115-124

Eddy EM (1998) Regulation of gene expression during spermatogenesis. Semin Cell Dev Biol 9:451-457

Eto K, Tsubamoto Y, Terauchi Y, Sugiyama T, Kishimoto T, Takahashi N, Yamauchi N, Kubota N, Murayama S, Aizawa T, Akanuma Y, Aizawa S, Kasai H, Yazaki Y, and Kadowaki T (1999) Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion. Science 283:981-985

Ferrer J, Aoki M, Behn P, Nestorowicz A, Riggs A, Permutt MA (1996) Mitochondrial glycerol-3-phosphate dehydrogenase. Cloning of an alternatively spliced human islet-cell cDNA, tissue distribution, physical mapping, and identification of a polymorphic genetic marker. Diabetes 45:262-266

Fimia GM, Sassone-Corsi P (2001) Cyclic AMP signalling. J Cell Sci 114:1971-1972

Foulkes NS, Borrelli E, Sassone-Corsi P (1991) CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. Cell 64:739-749

Foulkes NS, Mellstrom B, Benusiglio E, Sassone-Corsi P (1992) Developmental switch of CREM function during spermatogenesis: from antagonist to activator. Nature 355:80-84

Foulkes NS, Sassone-Corsi P (1996) Transcription factors coupled to the cAMP-signalling pathway. Biochim Biophys Acta 1288:F101-121

Fuhrmann G, Chung A C, Jackson K J, Hummelke G, Baniahmad A, Sutter J, Sylvester I, Scholer H R, and Cooney A J (2001) Mouse germline restriction of Oct4 expression by germ cell nuclear factor. Dev Cell 1:377-387

Gong DW, Bi S, Weintraub BD, Reitman M (1998) Rat mitochondrial glycerol-3-phosphate dehydrogenase gene: multiple promoters, high levels in brown adipose tissue, and tissue-specific regulation by thyroid hormone. DNA Cell Biol 17:301-309

Gong Q, Brown LJ, MacDonald MJ (2000) Functional analysis of two promoters for the human mitochondrial glycerol phosphate dehydrogenase gene. J Biol Chem 275:38012-38021

Herold C, Ganslmayer M, Ocker M, Hermann M, Geerts A, Hahn E G, and Schuppan D (2002) The histone-deacetylase inhibitor Trichostatin A blocks proliferation and triggers apoptotic programs in hepatoma cells. J Hepatol 36:233-240

Hirose T, O'Brien DA, Jetten AM (1995) RTR: a new member of the nuclear receptor superfamily that is highly expressed in murine testis. Gene 152:247-251

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

Hummelke GC, Cooney AJ (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

Hurst S, Howes EA, Coadwell J, Jones R (1998) Expression of a testis-specific putative actin-capping protein associated with the developing acrosome during rat spermiogenesis. Mol Reprod Dev 49:81-91

Junqueira LC and Carneiro J (2003) Basic Histology text & atlas. In, tenth edition edn. Mc Graw Hill Companie Inc

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

Kimmins S, Kotaja N, Davidson I, Sassone-Corsi P (2004) Testis-specific transcription mechanisms promoting male germ-cell differentiation. Reproduction 128:5-12

Kimmins S, Kotaja N, Fienga G, Kolthur U S, Brancorsini S, Hogeveen K, Monaco L, and Sassone-Corsi P. (2004) A specific programme of gene transcription in male germ cells. Reprod Biomed Online 8:496-500

Koza RA, Kozak UC, Brown LJ, Leiter EH, MacDonald MJ, Kozak LP (1996) Sequence and tissue-dependent RNA expression of mouse FAD-linked glycerol-3-phosphate dehydrogenase. Arch Biochem Biophys 336:97-104

Kroft TL, Jethanandani P, McLean DJ, 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

Lamas M ML, Zazopoulos E, Lalli E, Tamai K, Penna L, Mazzucchelli C, Nantel F, Foulkes NS, Sassone-Corsi P. (1996) CREM: a master-switch in the transcriptional response to cAMP. Philos Trans R Soc Lond B Biol Sci. 351:561-567

Lan ZJ, Gu P, Xu X, Cooney AJ (2003) Expression of the orphan nuclear receptor, germ cell nuclear factor, in mouse gonads and preimplantation embryos. Biol Reprod 68:282-289

McKenna NJ, Lanz RB, O'Malley BW (1999) Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 20:321-344

Mietkiewski K, Lukaszyk A (1966) [Determination of alpha-glycerolphosphate dehydrogenase activity during spermatogenesis in the testis of the rat]. Histochemie 7:28-38

Nantel F ML, 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

Nguyen NH, Brathe A, Hassel B (2003) Neuronal uptake and metabolism of glycerol and the neuronal expression of mitochondrial glycerol-3-phosphate dehydrogenase. J Neurochem 85:831-842

Peri A, Krausz C, Cioppi F, Granchi S, Forti G, Francavilla S, and Serio M (1998) Cyclic adenosine 3',5'-monophosphate-responsive element modulator gene expression in germ cells of normo- and oligoazoospermic men. J Clin Endocrinol Metab 83:3722-3726

Peri A, Serio M (2000) The CREM system in human spermatogenesis. J Endocrinol Invest 23:578-583

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

Reddi PP, Flickinger CJ, Herr JC (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

Russell LD ER, Sinha Hikim AP, Clegg ED (1990) Histological and histopathological evaluation of the testis.

Sassone-Corsi P (2000) CREM: a master-switch regulating the balance between differentiation and apoptosis in male germ cells. Mol Reprod Dev 56:228-229

Schmitz TP, Susens U, Borgmeyer U (1999) DNA binding, protein interaction and differential expression of the human germ cell nuclear factor. Biochim Biophys Acta 1446:173-180

Steger K, Klonisch T, Gavenis K, Behr R, Schaller V, Drabent B, Doenecke D, Nieschlag E, Bergmann M, and Weinbauer G F (1999) Round spermatids show normal testis-specific H1t but reduced cAMP-responsive element modulator and transition protein 1 expression in men with round-spermatid maturation arrest. J Androl 20:747-754

Steger K, Behr R, Kleiner I, Weinbauer GF, Bergmann M (2004) Expression of activator of CREM in the testis (ACT) during normal and impaired spermatogenesis: correlation with CREM expression. Mol Hum Reprod 10:129-135

Susens U, Borgmeyer U (2000) Genomic structure of the gene for mouse germ cell nuclear factor (GCNF). Genome Biol 1:RESEARCH0006

Susens U, Borgmeyer U (2001) Genomic structure of the gene for mouse germ-cell nuclear factor (GCNF). II. Comparison with the genomic structure of the human GCNF gene. Genome Biol 2:RESEARCH0017

Takagi S, Itoh N, Kimura M, Sasao T, Tsukamoto T (2001) Spermatogonial proliferation and apoptosis in hypospermatogenesis associated with nonobstructive azoospermia. Fertil Steril 76:901-907

Turner RM (2003) Tales from the tail: what do we really know about sperm motility? J Androl 24:790-803

Urcelay E, Jareno MA, Menaya J, Parrilla R, Ayuso MS, Martin-Requero A (2000) Cloning and functional characterization of the 5' regulatory region of the human mitochondrial glycerol-3-phosphate dehydrogenase gene. Lack of 3,5,3'-triiodothyronine responsiveness in adipose tissue. Eur J Biochem 267:7209-7217

Valentin M, Balvers M, Pusch W, Weinbauer GF, Knudsen J, 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

van der Hoorn FA, Tarnasky HA, Nordeen SK (1990) A new rat gene RT7 is specifically expressed during spermatogenesis. Dev Biol 142:147-154

van der Hoorn FA, Tarnasky HA (1992) Factors involved in regulation of the RT7 promoter in a male germ cell-derived in vitro transcription system. Proc Natl Acad Sci U S A 89:703-707

Weinbauer GF, Behr R, Bergmann M, Nieschlag E (1998) Testicular cAMP responsive element modulator (CREM) protein is expressed in round spermatids but is absent or reduced in men with round spermatid maturation arrest. Mol Hum Reprod 4:9-15

Weitzel JM, Grott S, Radtke C, Kutz S, Seitz HJ (2000) Multiple promoters direct the tissue-specific expression of rat mitochondrial glycerol-3-phosphate dehydrogenase. Biol Chem 381:611-614

Weitzel JM, Kutz S, Radtke C, Grott S, Seitz HJ (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

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

Yamashita Y, Shimada M, Harimoto N, Rikimaru T, Shirabe K, Tanaka S, and Sugimachi K (2003) Histone deacetylase inhibitor trichostatin A induces cell-cycle arrest/apoptosis and hepatocyte differentiation in human hepatoma cells. Int J Cancer 103:572-576

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

Yang G, Zhang YL, Buchold GM, Jetten AM, O'Brien DA (2003) Analysis of germ cell nuclear factor transcripts and protein expression during spermatogenesis. Biol Reprod 68:1620-1630

Zhang Y L, Akmal K M, Tsuruta J K, Shang Q, Hirose T, Jetten A M, Kim K H, and O'Brien D A (1998) Expression of germ cell nuclear factor (GCNF/RTR) during spermatogenesis. Mol Reprod Dev 50:93-102

Zhou Y, Sun Z, Means AR, Sassone-Corsi P, Bernstein KE (1996) cAMP-response element modulator tau is a positive regulator of testis angiotensin converting enzyme transcription. Proc Natl Acad Sci U S A 93:12262-12266

9. Acknowledgements

I would like to thank the team from the Institute of Biochemistry and Molecular Biology III and I.
I gratefully acknowledge continuous encourage and helpful discussions from Prof. Dr. med. H.J. Seitz and support from Prof. Dr. med. G. Mayr.
In particular, I would like to thank PD Dr. J. M. Weitzel for the support and numerous helpful discussions.
I would like to thank A. Harneit and M. Kröger for their excellent assistance in experiments, M. Wetzel and B. Henkel for their technical help at the beginning of my work and also Dr. med. A. Iwen, A. Wulf and S. Damerow for their kind help.

I am very grateful to Prof. Dr. R. Middendorff for support in Immunohistochemistry and to A. Blaszczyk-Wewer for sequencing some of the PCR products.

This work was generously financed by DFG – Graduiertenkolleg 336.

10. Declaration

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Unterschrift: .....
(Mirjana Rajkovic)

11. Curriculum Vitae

Personal data:

Date/Place of birth: 11.10.1970, Smederevo, Serbia

Education:

1977-1989	**Elementary School** " R. Rankovic", Lozovik, Serbia Marks: 5,0 from 5,0 **High School** " S. Markovic", Velika Plana, SerbiaMarks: 5,0 from 5,0 ; Matura 1989 (Mark 5) (Students in Elementary and High school are graded with marks: 1 (one) to 5 (five), where 2 (two) is the lowest passing mark and 5 (five) is the highest mark!)
1989 – 1996	**School of Medicine, University of Belgrade**, Belgrade, Serbia Total number of lessons is 6648 (6 years). **Marks: 9.03 from 10.00** *(Students at the University are graded with marks: 5 (five) to 10 (ten), where 6 (six) is the lowest passing mark and 10 (ten) is the highest mark)*
1996	**Diploma Doctor of Medicine (MD), School of Medicine, University of Belgrade, Serbia**
28.04.1998	**License to practice Medicine in FR Yugoslavia**
1998 – 2000	**Postgraduate multidisciplinary studies in Biochemistry,** School of Medicine, University of Belgrade, Belgrade, Serbia, finished with **Master of Science degree in Medicine (M.Sc.)** Thesis title: "[^{3}H] 8-Cl-cAMP transport through the biological membranes"
1999 –2003	**Specialisation in Clinical Biochemistry** (3 years) University of Belgrade, School of Medicine, Institute of Biochemistry, Belgrade, Serbia
2005	**Diploma Specialist in Clinical Biochemistry**
2003-2006	**PhD Work at the University of Hamburg** **Thesis: Regulation of mGPDH gene expression in human testis** Institute of Biochemistry and Molecular Biology University Hospital Hamburg-Eppendorf **Financed by DFG - Graduiertenkolleg 336**

Employment:

10.11.1998 - 10.02.2005

Assistant for Biochemistry
School of Medicine, University of Belgrade, Serbia

Teaching two-semester laboratory course in biochemistry at the 2nd year of undergraduate studies including all fields in biochemistry

Co-author of students booklet: Metabolism, questions and answers

Actively involved in the research program of Institute of Biochemistry in Belgrade, especially in project "Molecular mechanisms of apoptosis regulation in normal and malignant cells"

Professional skills:

List of knowledge and skills of a specialist of Clinical Biochemistry

Research skills:

Experience in cell biology and molecular biology techniques: DNA and mRNA extraction, PCR, DNA and RNA-electrophoresis, Northern blot, Plasmide isolation, Restriction, Ligation and transformation of bacteria, Mutagenesis, *In vitro* Transcription/Translation, Electrophoretic Mobility Assay Shift, Chromatin Immunoprecipitation, Preparation procedures for DNA sequencing, Western blotting

Cell culture technique: Transient Transfections, Luciferase Reporter Assay

Immunocytochemistry

Extensive hands-on experience in working with breathing animals: surgical techniques, in situ vascular perfusion, tissues preparation

Extensive hands-on experience in use of radioisotopes

Other skills:

Fluent in English, German and Russian

Proficient in Windows software, including MS Word, MS Power Point, MS Excel, Origin, Adobe Acrobat and other