cAMP-response element modulator- τ activates a distinct promoter element for the expression of the phospholipid hydroperoxide/sperm nucleus glutathione peroxidase gene

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PHGPx (phospholipid hydroperoxide glutathione peroxidase) is a selenoprotein present in at least three isoforms in testis: cytosolic, mitochondrial and nuclear. All of these derive from the same gene and are structurally related with the exception of the snPHGPx (sperm nucleus-specific form), which differs from the others due to the presence of an arginine-rich N-terminus. It has been demonstrated recently that this N-terminus is encoded by an alternative exon located in the first intron of the *PHGPx* gene. The expression of snPHGPx has been attributed either to an alternative pre-mRNA splicing or to the presence of a distinct promoter region. Nevertheless, the exact molecular mechanism by which the expression of snPHGPx occurs has not been demonstrated so far. Preliminary sequence analysis of the region located upstream of the alternative exon revealed some potential DNA-binding sites, one of which is specific to the binding of CREM (cAMP-response element modulator) transcription factors. By using electrophoretic mobility-shift assays, we demonstrated that both nuclear protein

extract from highly purified rat spermatid cells and recombinant CREM- τ protein can specifically bind to this element. Furthermore, we cloned a 1059 bp comprising the intron and the alternative exon for snPHGPx in the pCAT[®]3 reporter vector. By transient transfection experiments, we demonstrated that the expression of the transcription factor CREM- τ can induce the activation of the reporter gene in NIH-3T3 cell line. These results were confirmed by chromatin immunoprecipitation experiments performed on highly purified rat spermatid cells. On the basis of these results, we demonstrate that snPHGPx expression is mediated by the transcription factor CREM- τ , which acts as a *cis*-acting element localized in the first intron of the *PHGPx* gene.

Key words: cAMP-response element modulator (CREM), selenoprotein, sperm nuclei phospholipid hydroperoxide glutathione peroxidase (snPHGPx), spermatogenesis, transcription factor.

INTRODUCTION

During male germ-cell differentiation, important changes in gene expression occur. The genes specifically transcribed during this event are very often the genes whose products are required for sperm structure and motility [1–4]. Accumulating results clearly indicate that the cAMP-dependent signal transduction pathway is a major regulatory mechanism that operates at different stages of spermatogenesis [5–9]. Regulation of gene expression occurs by means of a family of nuclear transcription factors that contain a bZIP (basic domain/leucine zipper motif), which enables dimerization and binding to a specific DNA *cis* element, designated CRE (cAMP-response element). Two predominant members of this family, which have been shown to be active during spermatogenesis, are CREB (CRE-binding protein) and CREM (CRE modulator) [5,10]. Several alternatively spliced forms of these factors act as transcription activators (CREM- τ , - τ_1 and - τ_2), whereas other isoforms function as transcription suppressors (CREM- α , - β and - γ) [5]. In particular, it has been observed that a differential regulation of expression of the *CREM* gene occurs in the testis during spermatogenesis. Pre-meiotic germ cells express the repressor isoforms of CREM at low levels, whereas a high level of the activator isoform CREM- τ is observed from the round spermatid [5,9].

In male germ cells and spermatozoa, normal spermatogenesis is undoubtedly linked to a proper intake of selenium [11–13], whose role and function are fulfilled by a family of selenoproteins. The group of glutathione peroxidases is made up of the best-known selenoenzymes and, among these, phospholipid hydroperoxide glutathione peroxidase (referred to as PHGPx) or GPx-4 (EC 1.11.1.12) is of particular relevance for testicular and sperm-cell functions. The presence, distribution and role of PHGPx during spermatogenesis and spermiogenesis have been and still are actively considered in the male reproductive cells. This interest has its roots not only in the specific detoxification role of the enzyme towards membrane phospholipid hydroperoxides, but also in its additional functions. One function concerns its structural role in the integrity of sperm-cell mid-piece mitochondria [14] and another function is based on its alternative redox activity towards the -SH groups other than those of glutathione, namely the thiol group of protamines [15,16]. Moreover, all this seems to be connected with alterations of the enzyme's activity and expression in sperm cells of infertile human subjects [17,18]. It is important to recall that the *PHGPx* gene originates in at least three isoforms, whose N-terminal peptide shows a considerably different length: cytosolic, mitochondrial and nuclear [snPHGPx (sperm nuclei PHGPx)] [16]. The snPHGPx seems to play an important role in chromatin condensation and in determining the

Abbreviations used: CAT, chloramphenicol acetyltransferase; ChIP, chromatin immunoprecipitation; CRE, cAMP-response element; CREM, CRE modulator; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; PHGPx, phospholipid hydroperoxide glutathione peroxidase; snPHGPx, sperm nuclei PHGPx; 5'-RACE, rapid amplification of cDNA ends; RFX1, regulatory factor X1; 5'-UTR, 5'-untranslated region.

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differentiation of spermatogenic cells into spermatozoa [16]. Its gene is, therefore, one of those specifically activated during the late stages of spermatogenesis. Despite the important role played by snPHGPx, the molecular processes by which its transcription activation is controlled are still unknown.

In the present study, we provide evidence that the snPHGPx expression is controlled by the presence of an alternative promoter localized in the first intron (intron I1a) of the *PHGPx* gene and that its expression is mediated by the interaction of the CRE, CREM- τ .

EXPERIMENTAL

Cloning of intron I1a of a *PHGPx* gene

Human genomic DNA was extracted as described by Alijanabi and Martinez [19] and 10 μ l of the supernatant was used for PCR analysis. PCR was performed in a total volume of 100 μ l containing 0.5 unit of *Taq* DNA polymerase (EuroClone, Paignton, Devon, U.K.), 1 $\times$ standard PCR buffer, 0.1 mM of each dNTP and 50 pmol of each primer, under the following cycling conditions: 94 °C, 40 s; 64 °C, 45 s; and 74 °C, 90 s for 40 cycles in a Progene DNA Thermal Cycler.

Oligonucleotide primers used for cloning were: forward primer, 5'-TTGGTACCTGGTGAGCTAGCGCCGC-3' (*Kpn*I site is underlined); reverse primer, 5'-GCGAGCTCTGCAAGGCAAA-GGAAGCTG-3' (*Sac*I site is underlined).

The sequence was cloned using *Kpn*I and *Sac*I restriction sites in pCAT[®]3 Basic reporter vector (Promega) according to standard methods [20].

Cell lines

Round spermatids were obtained from 36-day-old Wistar rat testis as described previously [21]. Our preparations were steps 1–8 round spermatids with over 95 % purity with less than 2 % steps 9–10 spermatids, according to the histological classification of the codified stages of the spermiogenesis [21a].

The fibroblast NIH-3T3 cells were obtained from A.T.C.C. and were grown in Dulbecco's modified Eagle's medium, supplemented with 10 % (v/v) foetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin at 37 °C under 5 % CO₂.

Construction of mutant intron I1a construct

Regulatory region in the construct pCAT-I1a was mutated in the CRE consensus sequence, using the GeneTailor site-directed mutagenesis system kit (Invitrogen) according to the manufacturer's instructions. Primers used in the mutagenesis reaction were 5'-TAGCGCGGTGGAGATCTAGTCGCGCA-3' (forward) and 5'-CCACCGCGCTAGGCCAAGGTGC-3' (reverse). Mutated bases are underlined.

Cell culture and transfection

NIH-3T3 cells were plated 24 h before transfection on 12-well plates at a density of 1.5 $\times$ 10⁵ cells/well and transfected by LIPOFECTAMINE[™] (Gibco, Invitrogen) according to the standard conditions for mammalian cells described by the manufacturer. The amount of each expression or reporter plasmid used in transfection was 570 ng/well and the final 1.6 μ g of DNA was equilibrated with empty pCAT[®]3 vector. To test co-transfection efficiency and to normalize the CAT (chloramphenicol acetyltransferase) activity of the experimental construct, cells were co-transfected with internal control vector pSV- β -galactosidase (Promega), encoding for the β -galactosidase enzyme.

Cells were harvested 48 h after transfection and extracts were assayed for β -galactosidase activity using the β -galactosidase enzyme assay system (Promega) and for CAT activity according to the manufacturer's instructions.

Preparation of nuclear extracts

Nuclear proteins were prepared as described elsewhere [22]. The nuclei from spermatid cells were isolated by means of cell disruption in lysis buffer [10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM MgCl₂, 0.1 mM EDTA, 0.1 mM DTT (dithiothreitol) and 0.5 mM PMSF]. The nuclear pellet was resuspended in cold nuclear extraction buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.1 mM DTT and 0.5 mM PMSF), centrifuged at 10 000 *g* for 5 min at 4 °C and the supernatant 'nuclear extract' was stored at – 80 °C.

EMSA (electrophoretic mobility-shift assay)

Wild-type and mutated CRE-specific oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). The sequences were as follows: 5'-GGCCTAGCGCGGTGGCGTCAC-AGTCGCGCA-3' (wild-type CRE sequence is underlined) and 5'-TAGCGCGGTGGAGATCTAGTCGCGCA-3' (mutated nucleotides of the CRE sequence are in italics). The oligonucleotide O-r used in competition assays was 5'-ATAAGGCTAGGCTATCGGATCCTAGCTGATTGCAATGCCCTGAATTCCTTAA-3'.

Oligonucleotides were 5'-end-radiolabelled with [γ -³²P]ATP (PerkinElmer) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.) according to standard procedures [20]: routinely, 4 pmol of each DNA strand was labelled with 10 μ Ci of high-activity ATP. Labelled probes were purified from free [γ -³²P]ATP using Microspin S-300 HR columns (Amersham Biosciences). A binding reaction mixture containing 10 % (v/v) glycerol, 20 mM Tris/HCl (pH 7.5), 75 mM KCl, 0.5 mM DTT, 0.05 μ g/ μ l BSA, excess of the indicated competitor DNA and 0.5 μ g of nuclear protein extract or 0.5 μ g of *in vitro* expressed CREM- τ was incubated at room temperature (22–24 °C) for 30 min. Approx. 100 fmol of the probe was added for an additional 30 min. Protein–DNA complexes were resolved by electrophoresis on non-denaturing 7 % (w/v) polyacrylamide gel in 0.5 $\times$ TBE buffer (44 mM Tris/44 mM borate/1 mM EDTA) at 10 V/cm for 6 h at 4 °C. The gel was then dried and autoradiographed.

For the supershift assay, 8 μ g of anti-CREM- τ (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was added to the mixture before the DNA probes and preincubated with proteins for 30 min. DNA probes were then added to the whole mixture, which was incubated at room temperature (1 h). Complexes were analysed on a 5 % polyacrylamide gel in 0.5 $\times$ TBE buffer.

In vitro expression of CREM- τ

Recombinant CREM- τ from the expression vector pET-CREM- τ was produced as described in [23].

ChIP (chromatin immunoprecipitation) assay

Highly purified rat spermatid cells (3 $\times$ 10⁷) were washed once with PBS at room temperature and fixed with 1 % formaldehyde in PBS at 25 °C for 10 min. Cells were quickly rinsed twice in ice-cold PBS. After centrifugation at 1000 *g* at 4 °C, the cell pellet was resuspended in 3.5 ml of lysis buffer [1 % SDS, 5 mM EDTA and 50 mM Tris/HCl (pH 8.1), containing a protease inhibitor cocktail] and incubated on ice for 10 min. After lysis, the sample was sonicated in ice three times for 15 s each (Sonifier Power Supply, Branson, Danbury, CT, U.S.A.) and then centrifuged at 15 000 *g* for 10 min at 4 °C. After centrifugation, 100 μ l of

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Figure 2 EMSA of DNA-protein complex with CRE-containing oligonucleotide

(A) The labelled oligonucleotide dimer (5'-GGCCTAGCGCGGTGGCGTACAGTCGCGCA-3') containing the CRE consensus sequence for CREM- τ transcription factor was incubated with 0.5 μ g of nuclear extract from round spermatids as described in the Experimental section. A clearly visible complex is formed (arrow). Lane 1, no competitor; lane 2, 1 μ g of salmon sperm DNA plus 1 μ g of poly(dI-dC) (dl-dC) added to the labelled oligonucleotides; lane 3, 100-fold molar excess of a non-related double-stranded competitor oligonucleotide (O-r); lane 4, unlabelled CRE-containing oligonucleotide at 30-fold molar excess of the labelled one. (B) A specific binding complex is detected by an antibody (α CREM- τ) directed against CREM- τ transcription factor. Labelled CRE-containing oligonucleotide was incubated with nuclear extract from round spermatids and the complex formed is reported in lane 2. The antibody was incubated with nuclear extract before the addition of the labelled probe (lane 1). Supershifted complex is indicated by the arrow. (C) The labelled CRE-containing oligonucleotide was incubated with 0.5 μ g of the recombinant form of CREM- τ transcription factor obtained as described in the Experimental section. A specific complex is present (lane 1). In lanes 2 and 3, the indicated antibodies were incubated in the binding mixture with the recombinant protein before the addition of the labelled probe. (D) The labelled mutated oligonucleotide dimers (5'-TAGCGCGGTGGAGATCTAGTCGCGCA-3') containing a mutation in the consensus sequence for CREM- τ transcription factor and the wild-type one (5'-GGCCTAGCGCGGTGGCGTACAGTCGCGCA-3') were incubated with the recombinant form of CREM- τ or with nuclear extract from round spermatids as described in the Experimental section. Lane 1, wild-type nucleotide plus 0.5 μ g of recombinant CREM- τ ; lane 2, wild-type nucleotide plus 0.5 μ g of nuclear extract from round spermatids; lane 3, mutated nucleotide plus 0.5 μ g of recombinant CREM- τ ; lane 4, mutated nucleotide plus 0.5 μ g of nuclear extract from round spermatids; lanes 5 and 6, free oligonucleotides.

reporter vector. The recombinant vector pCAT-IIa was used for functional promoter assays.

Characterization of nuclear proteins binding to I1a

On the basis of the above results, we focused our attention on the possible involvement of CREM- τ in the activation of snPHGPx. Therefore we performed EMSAs using a 30 bp radiolabelled probe including the consensus domain of CREM. Nuclear extracts from highly purified spermatid cells generated one major DNA-protein complex (Figure 2A, lane 1). The addition of 1 μ g of salmon sperm DNA and poly(dI-dC) or a 100-fold molar excess of a non-related double-stranded competitor oligonucleotide (O-r) does not induce the disappearance of the band (Figure 2A, lanes 2 and 3). The specificity of DNA-protein interaction was examined by preincubating the extracts with an excess of unlabelled, specific double-stranded competitor DNA (Figure 2A, lane 4).

Identification of the factor that binds to intron I1a

To determine whether CREM- τ is capable of interacting with the CRE consensus sequence of the intron I1a regulatory element, the DNA-protein complex generated in gel shift assays was analysed in the presence of an antibody against human CREM- τ (Figure 2B). The antibody against CREM- τ recognized protein in the complex, as shown by the supershift of the corresponding band in Figure 2(B) (arrow). The experiments were performed in the presence of unlabelled DNA competitor, poly(dI-dC). The results clearly indicated that a specific recognition occurred in complex formation. On the contrary, the binding to the probe was not affected by antibodies directed against a non-correlated protein (human IgG; results not shown).

The binding of CREM- τ to the CRE region of the I1a regulatory element was confirmed by gel shift assays using recombinant CREM- τ protein. As shown in Figure 2(C), CREM- τ , in the presence of 1 μ g of poly(dI-dC) DNA, binds to the probe forming the protein-DNA complex.

More information about the nature of the observed protein-DNA complex was obtained from supershift experiments using anti-CREM- τ antibody. As shown in Figure 2(C), lane 2, the addition of the anti-CREM- τ antibody again influenced the supershift of the retarded complex.

Effect of mutation of the CRE consensus sequence

Major insights regarding the specific role of CRE were obtained from EMSAs performed with oligonucleotides bearing a specific mutation in the consensus sequence for CREM- τ . In all these experiments, both nuclear extracts from highly purified spermatid cells and the recombinant form of CREM- τ failed to form complexes (Figure 2D).

CREM- τ binds *in vivo* to the I1a regulatory promoter region

The EMSAs indicated that CREM- τ binds *in vitro* to the regulatory element GTCA of intron I1a. To demonstrate that CREM- τ binds to the promoter regulatory element also *in vivo*, a ChIP assay using extracts from highly purified round spermatid cells was performed. For this purpose, DNA-binding proteins of spermatids were covalently linked to genomic DNA by treatment with formaldehyde. The DNA-protein complexes were then sheared by sonication and specific protein-DNA complexes were immunoprecipitated with antibody against CREM- τ . Covalent linkage was reversed and the purified double-stranded DNA was amplified by PCR using primers that specifically amplified for a 280 bp promoter region. This region specifically covers the

Figure 3 ChIP for CREM- τ transcription factor

ChIP was performed as described in the Experimental section. The ChIP assay detects CREM- τ transcription factor bound to the endogenous regulatory region in intron 11a of the *PHGPx* gene. After the precipitation of protein–DNA complexes with anti-CREM- τ antibody, PCR amplification was performed using primers that amplify a 280 bp promoter region that specifically covers the consensus sequence for CREM. A positive signal was observed after immunoprecipitation with anti-CREM- τ antibody, but not in the control, in which immunoprecipitation was performed without a specific antibody. Input fraction refers to DNA from round spermatid cells that were not immunoprecipitated.

5'GGGGGGGGGGTCCCGCGCTGTGCTTGGGACGCACAGACGGGCGGGCATGGGCCGG
GCGGCCGCCGCAAGCGGGGACGTGCAGACAGCGCGCGGTCCCCGGGAGGCCGG
CGACGGCGTGAACCTGGACGCCAAAGTCTAGGAAGCGCCAGGCCCTCGGAGGAG
GAGAGCTCGCGCGCGCCGCCGAGGAGGGCGCGCCCTCGCCGG 3'

Figure 4 5'-UTR of snPHGPx transcript

The 5'-end of the snPHGPx was determined by RACE of the corresponding mRNA. The 38 bp sequence determined is shown in italics. The translation start of the transcript is shown in boldface.

consensus sequence for CREM. As shown in Figure 3, in a 1.5 % agarose gel, a band of the expected amplified product can be observed both in the input fraction used as positive control and in the chromatin fraction obtained after immunoprecipitation with anti-CREM- τ antibody. In contrast, the corresponding control, in which immunoprecipitation was performed without specific antibodies, did not show any PCR signal.

5'-RACE

The RACE approach applied to mRNA from spermatids using, for retrotranscription, a primer specific to snPHGPx detected only one transcript, which extended 38 bp into 5'-UTR (5'-untranslated region; Figure 4).

Transactivation of the 11a promoter by CREM- τ

We examined the ability of CREM- τ to activate the transcription of the *snPHGPx* gene by transiently co-transfecting NIH-3T3 cells with mammalian expression vector pSV-CREM- τ , which expresses mouse CREM- τ and with the construct pCAT-11a. pCAT-11a vector was generated using a reporter *CAT* gene fused 3' with 1.06 kb of exon 1 of the *PHGPx* gene.

NIH-3T3 cells, which do not express CREM- τ , were transfected either with pCAT-11a or pSV-CREM- τ vectors alone or with pSV-CREM- τ expression vector and construct pCAT-11a.

Figure 5 Transactivation of 11a promoter by CREM- τ expression construct

NIH-3T3 cells were transfected with either pCAT-11a reporter vector alone or the pSV-CREM- τ expression vector as described in the Experimental section. CAT activity was normalized with respect to the activity of β -galactosidase.

The transfection efficiency was checked by co-transfection with pSV- β -galactosidase as internal control vector. CAT activity in cell extracts was normalized with respect to β -galactosidase activity.

As shown in Figure 5, co-transfection with pSV-CREM- τ and pCAT-11a activates reporter gene expression significantly, suggesting that CREM- τ can activate reporter gene transcription by stimulating a promoter element. Cells that were transfected with pCAT-11a or pSV-CREM- τ alone, similar to non-transfected cells, did not show any stimulation in CAT reporter expression.

On the basis of the results obtained from EMSAs, transactivation assays of mutated oligonucleotides were performed using a pCAT-11a mutated in the consensus sequence for CREM- τ . The construct was generated by PCR-based site-directed mutagenesis of the parental pCAT-11a construct and the mutation was the same as in the mutated oligonucleotide used in EMSAs. The construct was examined for promoter activity by transient co-transfection of NIH-3T3 cells with pSV-CREM- τ as described above. No CAT activity was detected after the removal of the CRE consensus sequence (results not shown).

DISCUSSION

Different PHGPx isoforms perform different roles in various cellular localizations. During spermatogenesis and spermiogenesis, it has been demonstrated that PHGPx catalytic activity and protein expression vary from diploid to mature epididymal spermatozoa [14,26], as well as a function of the age of the animal model tested from prepubertal status to old age [26]. Therefore fine regulation in the expression of the different isoforms should be considered as a must for the correct differentiation of germ cells.

Despite the pivotal role played by snPHGPx, very little is known about the molecular mechanisms by which its expression is controlled. Two alternative mechanisms were recently suggested. Moreno et al. [27], using a combination of techniques, including cloning of mRNA 5'-ends, reverse transcriptase–PCR and S1 analyses, suggested that the transcript encoding for snPHGPx is generated by transcription initiation at an alternative promoter. No information about the possible involvement of the transcription factor is reported [27].

In contrast with these results, Borchert et al. [28] reported the presence of negative regulatory elements in the first intron

of the *PHGPx* gene, suggesting that transcription of snPHGPx may be carried out by alternative splicing of a joint primary transcript. Furthermore, these authors analysed *in silico* the sequence of the first intron of the *PHGPx* gene for the presence of putative transcription factor-binding sites. They found consensus sequences for different transcription factors, including CRE(B). EMSAs with testis extract demonstrated the presence of a specific complex. When functional promoter assays were performed using a β -galactosidase-based reporter gene construct containing the 5'-flanking region of exon E1b that comprises the CRE-specific sequence, Borchert et al. observed a minimal promoter activity. These results are not surprising; they performed experiments in a somatic cell line and the expression of CRE in a somatic cell line is switched mainly towards the expression of transcription suppressors. This could explain the absence of snPHGPx expression from most somatic tissues and its very low-level expression in the HEK-293 cell line (human embryonic kidney 293 cell line) [28].

Maiorino et al. [29], using a similar approach, obtained opposite results. They used various reporter gene constructs with a different portion of intron I1a and with intron I1a and exon E1b. In all experiments, they found a reporter activity, with the best results obtained for the construct bearing intron I1a and exon E1b. They concluded that the formation of snPHGPx is due to alternative transcription and not due to alternative splicing.

The results reported in the present study are in agreement both with those reported by Moreno et al. [27] and with those of Maiorino et al. [29]. Our results support the hypothesis of the existence of an alternative promoter, but we give more insight on the direct involvement of CREM- τ in the transcription event of snPHGPx. By an *in silico* analysis for the search of a putative transcription factor-binding site, we identified the presence of consensus sequences for RFX1 and CRE. Our attention was directed towards the latter, owing to the important role played by cAMP-response factors in spermatogenesis being well known [8–10].

The CREM gene presents many remarkable characteristics. By alternative splicing, the CREM gene may encode both activators (CREM- τ) and repressors (CREM- α , - β and - γ). Levels of CREM transcripts are low in prepubertal testis and only the repressor isoforms are detected. However, during puberty, transcripts encoding the activator form accumulate to a high level in pachytene spermatocytes and spermatids [6]. Many haploid genes have been identified as potential CREM target genes since they contain CRE consensus sequences in their promoters [6,30,31].

The CREM gene is highly expressed in the adult testis [5]. Levels of CREM transcripts are low in prepubertal testis and only the repressor isoforms are detected. However, during puberty, transcripts encoding the activator form, CREM- τ , accumulate to high levels in pachytene spermatocytes and spermatids [5]. Interestingly, the CREM- τ protein is not detected in spermatocytes but only in haploid spermatids [6]. The switch in CREM expression pattern during spermatogenesis is under the control of the gonadotropin, follicle-stimulating hormone [32]. Surgical removal of the pituitary gland from adult rats leads to loss of CREM- τ expression in the testis, and hypophysectomy of prepubertal rats prevents the developmental switch in CREM expression [32]. Interestingly, there is a similar hormonal [33,34] and temporal [35] pattern for the expression of PHGPx.

According to that, we developed a research strategy aiming to demonstrate the involvement of CREM- τ in the activation of expression of snPHGPx. All data are in agreement with that hypothesis. In fact, EMSAs performed using nuclear protein extracts from highly purified spermatids or recombinant protein and an oligonucleotide bearing the consensus sequence for CREM- τ

demonstrated the formation of a specific complex that can be supershifted by the addition of a specific antibody, anti-CREM- τ . Furthermore, we also reported functional results. In fact, transfection of NIH-3T3 cells with the constructs pCAT-I1a and pSV-CREM- τ demonstrated the existence of a reporter activity only when they were co-transfected and only when CREM- τ consensus sequence is present.

The 5'-RACE assay detected a 5'-UTR sequence of 38 bp, which is in agreement with the hypothesis of a new transcription start, in general, and with the involvement of CREM in the activation of expression of snPHGPx on the basis of the more upstream localization of the consensus sequence.

Finally, ChIP experiments demonstrated and confirmed the existence *in vivo* of a complex between CREM- τ and the consensus sequence identified by *in silico* analysis.

In the light of these findings, we can conclude that (i) a *cis*-regulatory element is present in intron I1a of the *PHGPx* gene and (ii) CREM- τ is the functional modulator responsible for the activation.

We thank Professor P. Sassone-Corsi for kindly providing the expression vectors pSV-CREM- τ and pET-CREM- τ , containing the mouse CREM- τ full-length cDNA. We also thank Professor S. Cattermole (University of Trieste) for a critical reading of this paper. This work was supported by the MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca Scientifica) PRIN (progetti di ricerca di interesse nazionale) grant no. 2001062394.

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Received 9 June 2004/24 June 2004; accepted 30 June 2004

Published as BJ Immediate Publication 30 June 2004, DOI 10.1042/BJ20040974