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Assessment of Promoter Elements of the Germ Cell-Specific Proacrosin Gene

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Abstract The testis-specific proacrosin gene encodes for a fertilization-promoting protein. In mouse and rat it is first transcribed in late pachytene spermatocytes and revealed to be translationally regulated. Former proacrosin promoter studies demonstrated that elements necessary for conducting a stage and temporal-specific expression of the gene are located within 0.9 kb upstream of the translational start codon. In the present study we analyzed putative *cis*-acting elements located in this promoter region for their specific binding properties to nuclear factors assumed to be involved in proacrosin gene regulation. Supplement of specific antibodies in electrophoretic mobility shift assays (EMSA) revealed that two Y-box proteins and the transcription factors CREM and YY1 interact with proacrosin promoter elements. The Y-box proteins, antigenically related to the frog Y-box proteins FRGY1 and FRGY2, bound to the Y-box (55–66 bp upstream of the ATG initiation codon) in brain and testis nuclear extracts, respectively. CREM bound to three elements (30–37, 252–259, and 717–724 bp upstream of ATG). The ubiquitous transcription factor YY1 bound to a conserved element in the central proacrosin promoter (457–473 bp upstream of ATG) and showed almost germ cell-specific truncates in EMSA. These results suggest that the identified factors are involved in proacrosin gene regulation. J. Cell. Biochem. 83: 155–162, 2001.

Key words: Y-box proteins; CREM; YY1

The development of diploid germ cells to morphologically highly differentiated haploid spermatozoa requires a stage-specific expression of the genes involved in spermatogenesis. The proacrosin gene itself belongs to the germ cell-specific genes that show in mouse and rat a delay of translation in relation to the appearance of the transcript. In both species the proacrosin mRNA is first detectable in late pachytene spermatocytes [Kashiwabara et al., 1990; Nayernia et al., 1994a]. However, the protein is not detectable earlier than in the stage of haploid round spermatids, as revealed by immunochemical experiments [Flörke et al., 1983] and transgenic in vivo studies [Nayernia et al., 1994b]. Finally, proacrosin is stored as a zymogen protein in the acrosomal vesicle of the developing spermatozoa and is activated and released as a consequence of the acrosome reaction. It serves to facilitate the penetration of sperm through the zona pellucida [Adham et al., 1997; Yamagata et al., 1998].

Previous studies, that utilized proacrosin promoter variants in transgenic mice demonstrated that fusion genes containing either a 2.3 kb (45–2.3 kb upstream of ATG) or a 0.9 kb promoter fragment (45–922 bp upstream of ATG) linked to the CAT reporter gene showed the same developmental and stage-specific expression pattern as the endogenous proacrosin gene [Nayernia et al., 1992, 1994b]. However, a shorter promoter fragment of about 0.4 kb (45–442 bp upstream of ATG) conferred only basal CAT transcription, and CAT enzyme activity was no longer detectable [Nayernia et al., 1994b]. It was therefore concluded that functional promoter elements of the proacrosin gene are located in the 0.9 kb 5′-flanking region. By employing the yeast one-hybrid assay we identified the ubiquitous transcription factor YY1 binding to a conserved DNase I footprint element located in the mouse proacrosin

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promoter (474–490 bp upstream of ATG) [Schulten et al., 1999].

In this study we made use of online databases to recognize *cis*-acting elements in the 0.9 kb promoter region that could be involved in the testis-specific regulation of the proacrosin gene. Besides the YY1 element, one putative *cis*-acting element shared common sequences with a previously assayed DNase I footprint [Kremling et al., 1995]. Binding of *trans*-acting factors to the elements was verified with specific antibodies.

MATERIALS AND METHODS

Electrophoretic Mobility Shift Assays (EMSA)

Double-stranded oligonucleotides generated by annealing single stranded oligonucleotides (Table I) and a proximal promoter fragment of the rat proacrosin gene (F45 fragment) were used as probes. The F45 fragment that contains the CRE (cAMP response element) site 1 (5′-TGAGGTCA-3′) was excised with *Pst*I (45 bp upstream of ATG) and *Xba*I from a fragment of the proximal 127 bp upstream of ATG initiation codon of the rat proacrosin gene that contained an overhanging *Xba*I restriction site at the 3′ end. The F45 fragment and the double-stranded oligonucleotides were filled in with Klenow enzyme (MBI Fermentas, St. Leon-Rot, Germany) and [^{32}P]dCTP for labeling. Nuclear extracts were isolated from testis and brain of SIV 50 rats, wild-type mice (NMRI strain), and the mutant mice W/W^{v} [Coulombre and Russell, 1954] by a minipreparation technique [Deryckere and Gannon, 1994]. All further preparation steps for EMSA were performed as described [Schulten et al., 1999]. Ten-microgram nuclear extracts were used for each reaction mixture except for testis extracts (20 μg) used with the probe of the CRE site 1. Various amounts of poly(dI·dC)·(dI·dC) were supplemented to suppress unspecific protein interactions. For immunological assays antibodies and normal serum were applied to the mixtures and incubated for 30 min on ice before the labeled probes were added. The following antibodies were used: anti-YY1 polyclonal rabbit antibody (C-20, Santa Cruz Biotechnology), anti-CREM (cAMP response element modulator) antibody [Delmas et al., 1993], rabbit anti-FRGY1 (frog Y-box protein 1) antibody [Tafuri and Wolffe, 1992; Tafuri et al., 1993], and anti-FRGY2 antibody [Matsumoto et al., 1996].

TABLE I. Oligonucleotides Used as Probes in EMSA

Cis-acting element	Oligonucleotide sequence[a]
Y-box	5′-GTCACCC**TGCTGATTGGCCA-G**AAGGCT-3′ 3′-GTGGG**ACGACTAACCGGTC**TTCC-GACG-5′
CRE site 2	5′-ATAAAGTG**AGACGTCA-**GAAGGCTCGCCAAG-3′ 3′-TTTCAC**TCTGCAGT**CTTCC-GAGCGGTTCGG-5′
CRE-like site	5′-GCCTTC**TGACATTG**TTACTGGGAA-3′ 3′-GAAG**ACTGTAAC**AATGACCCTTTG-5′
YY1 element	5′-CTAGAACTTCA**AAATGG**CTCC-3′ 3′-TTGAAGT**TTTACC**GAGGAGCT-5′

[a]Protein binding motifs are shown in bold.

RESULTS

Localization and Sequences of Binding Motifs

In the present study we analyzed putative *cis*-acting elements localized in 0.9 kb of the rat proacrosin promoter which is sufficient to direct a temporal and stage-specific correct expression of a CAT reporter gene in transgenic mice [Nayernia et al., 1994b]. We used the TF search program [Heinemeyer et al., 1998] and online browser services to recognize *cis*-acting elements for which a possible implication in the germ cell-specific transactivation of the proacrosin gene was indicated. In addition, we restricted our analysis to the motifs conserved between the mouse and rat proacrosin gene [Kremling et al., 1991a,b].

Figure 1 illustrates localization and sequences of the respective binding motifs. A Y-box motif (5′-TGCTGATTGGCCAG-3′) is present in the proximal promoter region (55–66 bp upstream of ATG) that matches the consensus sequence in 12 of the 14 bp, deduced from conserved Y-box motifs (5′-T$^{T}/_{G}$CTGATTGG$^{T}/_{C}$-T$^{A}/_{C}$$^{A}/_{C}$-3′) [Dorn et al., 1987]. Y-box proteins exert various functions on gene regulation at the trancriptional and translational level [reviewed in Matsumoto and Wollfe, 1998]. The proximal promoter region of the proacrosin gene contains also two cAMP response elements (CREs) located from 30 to 37 bp (CRE site 1, 5′-TGAGGTCA-3′) and 252 to 259 bp (CRE site 2, 5′-AGACTGCA-3′) upstream of ATG showing either a 7/8 bp homology to the palindromic CRE consensus motif 5′-TGACGTCA-3′. An element designated here as the CRE-like site (5′-TGACATTG-3′) shares a 5/8 bp homology to the CRE

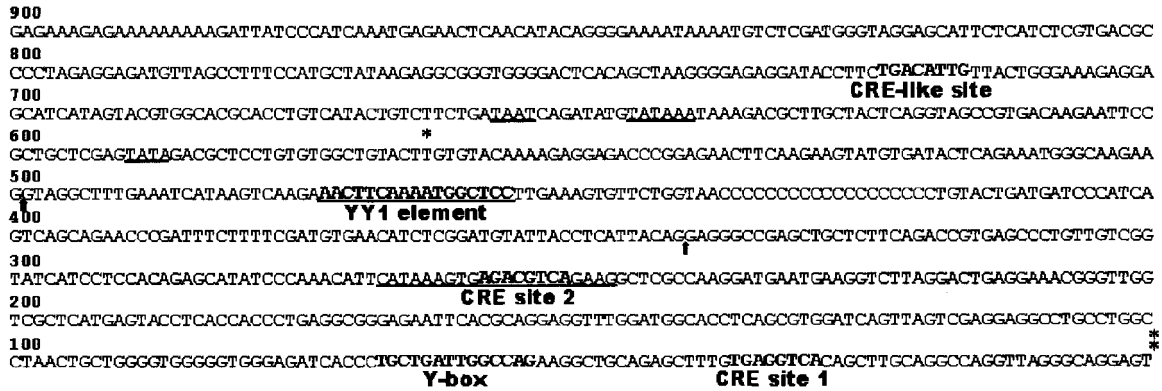

Fig. 1. Nucleotide sequence of 0.9 kb of the rat proacrosin promoter including *cis*-acting elements analyzed in this study for specific DNA-protein interactions. The positions of these *cis*-acting elements (in bold) upstream of ATG (indicated by two asterisks) are: Y-box, 55–66 bp; CRE site 1, 30–37 bp; CRE site 2, 252–259 bp; CRE-like site, 717–724 bp; YY1 element, 457–473 bp. The promoter fragment F45 containing the proximal 45 bp upstream of ATG was used to analyze the CRE site 1 in EMSA. The Y-box, the two CRE sites, the CRE-like site, and the YY1 element were assayed in EMSA using specific oligonucleotides (Table I). Two putative TATA boxes, a modified CAAT (TAAT) motif, and two DNase I footprint sequences [Kremling et al., 1995] comprising the YY1 element and the CRE site 2 are underlined. The major transcriptional start site (indicated by an asterisk) is positioned at 564 bp upstream of ATG. Intron 1 is located between 322 and 499 bp upstream of ATG (intron-exon boundaries indicated by arrows).

consensus sequence. This site is present in the distal promoter from 717 to 724 bp upstream of ATG. The CRE mediates transactivation via the cAMP response pathway and is involved in the germ cell-specific expression of genes [reviewed in Foulkes and Sassone-Corsi, 1996]. A conserved binding motif for the zinc finger protein YY1 is located in the central promoter region from 457 to 473 bp upstream of ATG (5′-AACTTCAAAATGGCTCC-3′). This motif contains the core consensus sequence 5′-CCATNT-3′ in minus strand orientation [Schulten et al., 1999].

Binding of Nuclear Factors to Motifs

Oligonucleotides (Table I) and the F45 element including specific protein binding motifs were used with nuclear extracts and specific antibodies in EMSA to confirm binding of nuclear factors. Using a FRGY2 antibody with a proven reactivity against a mouse Y-box protein a testis-specific shift could be suppressed in EMSA (Fig. 2A, lane 4). In control reaction with normal serum in lane 6 and in brain nuclear extracts in lane 7, no suppression was observed, which indicates the testis specificity of the Y-box interaction with a protein immunologically related to FRGY2. In contrast, the FRGY1 antibody specifically inhibited a brain-specific shift of high abundance (lane 8) as compared to the testicular shift suppressed by the FRGY2 antibody. Binding of CREM to the CRE site 1 was confirmed by using a DNA fragment of the proximal 45 bp promoter region containing this CRE site. Figure 2B illustrates inhibition of shifts by the CREM antibody in testis extracts (lane 3) and a supershift effect in brain extracts (lane 6) whereas normal serum had no inhibitory effects (lanes 4 and 7). Inhibition of shifts was also observed in testis and brain extracts (Fig. 2C, lanes 4 and 8) when an oligonucleotide containing the CRE site 2 and the CREM antibody were used in EMSA. When an oligonucleotide comprising the CRE-like site was applied to rat nuclear extracts several shifts were detected (Fig. 2D). Binding of a complex that migrated with low mobility was specifically suppressed by the CREM antibody (lane 4). Similar to the homologous YY1 element present in the mouse proacrosin promoter, the YY1 antibody inhibited binding of YY1 to the YY1 binding site in EMSA in rat and brain nuclear extracts (Fig. 2E, lanes 4 and 8). The complexes that migrated with high mobility in testis extracts represent YY1 truncates (lanes 3 and 4).

To examine whether these YY1 truncates are germ cell-specific or not, an EMSA was performed including the YY1 oligonucleotide and nuclear extracts from W/W^v mice that harbor only somatic testicular cells (Fig. 3). Testis extracts from adult mice were used for control reactions. The W/W^v extracts contained only small amounts of the truncates (lanes 2 and 3)

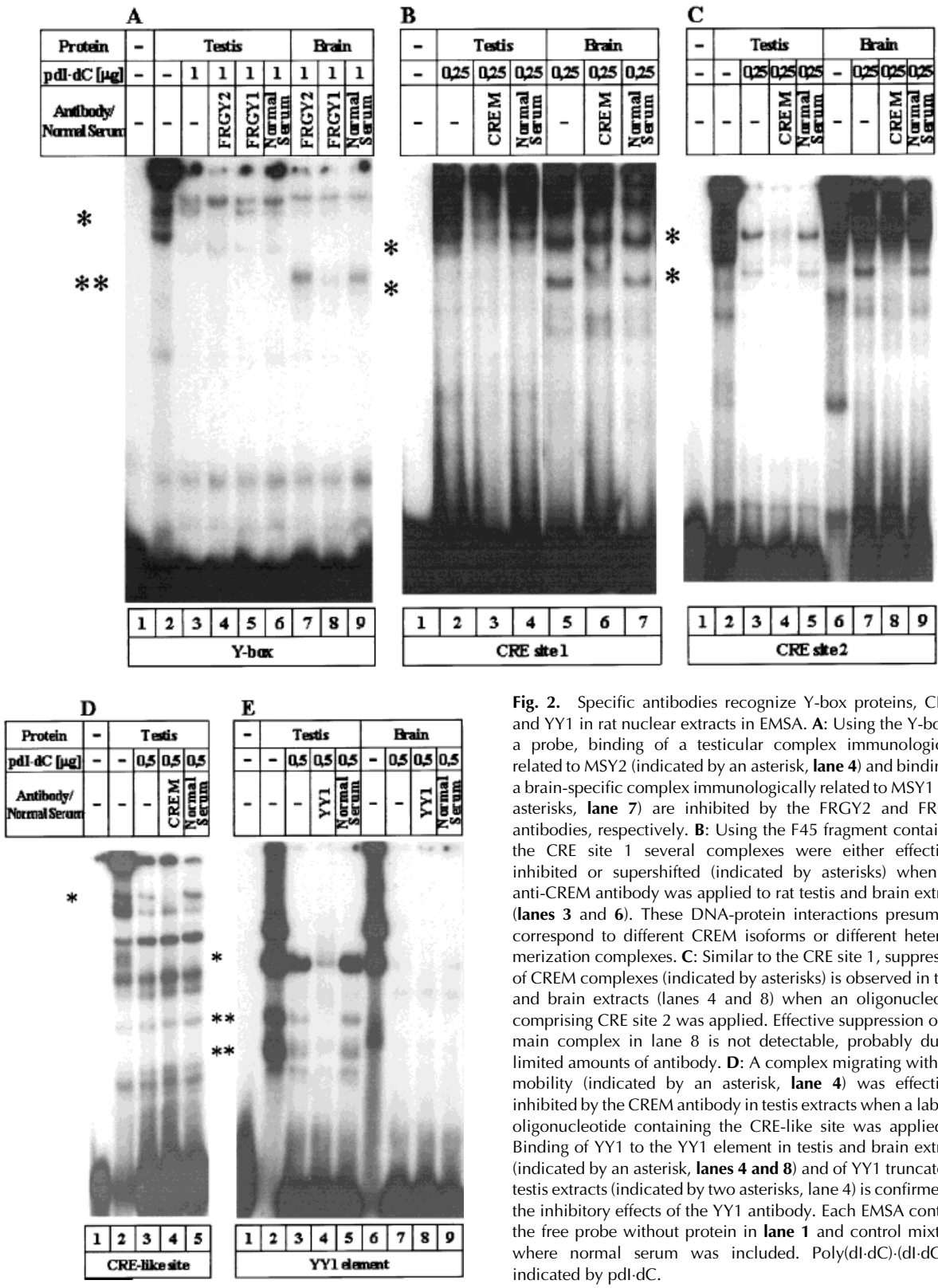

Fig. 2. Specific antibodies recognize Y-box proteins, CREM and YY1 in rat nuclear extracts in EMSA. **A**: Using the Y-box as a probe, binding of a testicular complex immunologically related to MSY2 (indicated by an asterisk, **lane 4**) and binding of a brain-specific complex immunologically related to MSY1 (two asterisks, **lane 7**) are inhibited by the FRGY2 and FRGY1 antibodies, respectively. **B**: Using the F45 fragment containing the CRE site 1 several complexes were either effectively inhibited or supershifted (indicated by asterisks) when the anti-CREM antibody was applied to rat testis and brain extracts (**lanes 3** and **6**). These DNA-protein interactions presumably correspond to different CREM isoforms or different heterodimerization complexes. **C**: Similar to the CRE site 1, suppression of CREM complexes (indicated by asterisks) is observed in testis and brain extracts (lanes 4 and 8) when an oligonucleotide comprising CRE site 2 was applied. Effective suppression of the main complex in lane 8 is not detectable, probably due to limited amounts of antibody. **D**: A complex migrating with low mobility (indicated by an asterisk, **lane 4**) was effectively inhibited by the CREM antibody in testis extracts when a labeled oligonucleotide containing the CRE-like site was applied. **E**: Binding of YY1 to the YY1 element in testis and brain extracts (indicated by an asterisk, **lanes 4 and 8**) and of YY1 truncates in testis extracts (indicated by two asterisks, lane 4) is confirmed by the inhibitory effects of the YY1 antibody. Each EMSA contains the free probe without protein in **lane 1** and control mixtures where normal serum was included. Poly(dI·dC)·(dI·dC) is indicated by pdI·dC.

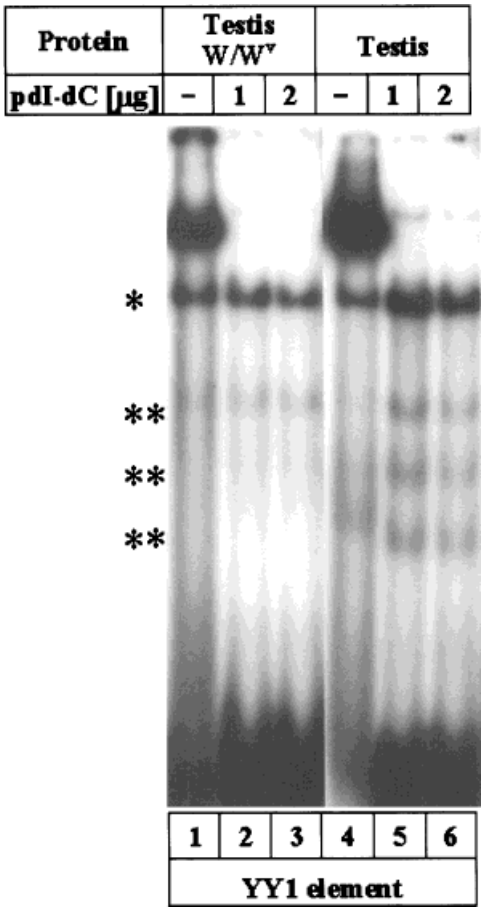

Fig. 3. YY1 truncates are almost germ cell-specific. In all nuclear extracts binding of untruncated YY1 protein (indicated by an asterisk) to a probe containing the YY1 element was assayed in EMSA. However, binding of YY1 truncates (indicated by two asterisks) is reduced in testis nuclear extracts of W/W^v mutant mice (**lanes 2** and **3**) compared to testis extracts of adult mice (**lanes 5** and **6**). Poly(dI·dC)·(dI·dC) is indicated by pdI·dC.

as compared to the corresponding shifts in testis extracts (lanes 5 and 6).

DISCUSSION

The present study assesses putative promoter elements of the rat proacrosin gene for their involvment in the temporal and spatial-specific expression of the gene. Binding to promoter elements was shown for two Y-box proteins, and for CREM and YY1.

Y-box-like sequence motifs are present in the 5′-flanking regions of several genes specifically expressed in male germ cells, e.g., phosphoglycerate kinase 2, protamine 1, protamine 2, transition protein 2, and the alternatively transcribed cytochrome C [discussed in Nikolajczyk et al., 1995]. The functional relevance of the Y-box sequence in the protamine 2 promoter was proven by in vitro transcription assays using testicular nuclear extracts [Yiu and Hecht, 1997]. Our in vitro studies of the proacrosin promoter revealed the presence of a Y-box element 55 bp upstream of ATG. The identity of the testicular Y-box protein that bound to the Y-box was demonstrated by using an antibody against *Xenopus* Y-box protein FRGY2 which is known to immunoreact with Y-box proteins of *Xenopus* [Matsumoto et al., 1996]. Recently, a mouse cDNA was isolated which encodes the FRGY2 homologue MSY2 (mouse Y-box protein 2) [Gu et al., 1998]. The expression profile of MSY2 is restricted to female and male germ cells with a high level of transcripts detected in round spermatids. On the basis of both the expression profile of MSY2 in testicular germ cells and our result in EMSA, it can be concluded that the homologous rat Y-box protein is involved in the transcriptional regulation of the proacrosin gene. The immunological relationship of the protein that bound to the Y-box in brain nuclear extracts was shown with the anti-FRGY1 antibody. This antibody has been successfully used to identify Y-box proteins in mouse [Tafuri and Wolffe, 1992; Tafuri et al., 1993]. The mouse homologous protein for FRGY1 is MSY1 [Tafuri et al., 1993] which is part of the testicular ribonucleoprotein fraction that serves as the storage form for testicular mRNAs. An abundant transcript was found in pachytene spermatocytes and round spermatids. Overexpression of MSY1 in transient transfection experiments repressed transcription of the growth hormone receptor gene [Schwartzbauer et al., 1998] suggesting a similar role of this Y-box protein in proacrosin gene regulation.

Two CRE sites and one CRE-like site are present in the rat proacrosin promoter for which binding of the transcription factor CREM was confirmed by using the CREM antibody which abolished or supershifted complexes in EMSA. Several genes expressed during spermatogenesis have been found to contain CRE sites which interact with the postmeiotically transactivator form of CREM (CREMτ) [Delmas et al., 1993]. The CREM antibody used in our study recognizes all CREM isoforms with equal efficiency [Delmas et al., 1993]. Therefore, the suppressive effects observed in EMSA were not restricted to testicular extracts. The functional

relevance of CRE sites and CREM protein has been shown in both cell transfection experiments and in knock-out mice. Cotransfection of JEG-3 cells with a construct containing the promoter of the gene for the testis angiotensin-converting enzyme and an expression vector generating CREMτ resulted in a specific transcriptional promoter activation [Zhou et al., 1996]. CREMτ-induced promoter activation was also demonstrated for the testis-specific RT 7 gene and transition protein 1 gene [Delmas et al., 1993; Kistler et al., 1994]. Mice homozygous for a targeted disruption of the CREM gene were infertile due to an arrest of spermatogenesis in early round spermatids [Blendy et al., 1996; Nantal et al., 1996]. In these mice the haploid-expressed genes for protamine 1, RT 7, mitochondrial capsule selenoprotein, and transition protein 1 were not expressed at all, whereas the transcription level of the diploid-expressed gene for proacrosin was slightly reduced. Since CRE sites are situated in the promoters of all these genes it can be suggested that CREMτ is involved in transcriptional activation of these genes. Alignment analysis of the mouse proacrosin promoter revealed three putative CRE sites (451–458, 170–177, and 30–37 bp upstream of ATG), and one CRE-like site (734–741 bp upstream of ATG).

Several shifts for the CRE-like site were observed in EMSA which were not suppressed by the CREM antibody. Further studies will be necessary to recognize these binding factors. Of particular importance in this context is the 5′-AACAAT-3′ motif which overlaps in minus strand orientation (in bold) with the CRE-like motif (in italics): 5′-*TGAC**ATTG**T*T-3′. The 5′-AACAAT-3′ motif is known as the conserved core binding motif for Sox (Sry-related HMG box) proteins [Mertin et al., 1999]. Sox proteins are particularly involved in the regulation of developmental processes. Binding of Sox factors (Sox 5 and 6) to a testis-specific promoter element of the gene for hormone-sensitive lipase was recently shown [Blaise et al., 1999]; these Sox factors may also bind to the CRE-like site.

Alignment analysis revealed the presence of putative YY1 binding sites in the promoters of various germ cell-specific genes of the mouse, including the proacrosin gene [Schulten et al., 1999]. In this study, binding of YY1 to the YY1 motif in the rat proacrosin promoter was observed, similiar to the result obtained with the mouse homologous YY1 element. YY1 truncates were detected with nuclear extracts of adult testes which were visible only as faint bands in nuclear extracts of testes of W/W^v mice and which were not detectable in brain extracts. The truncation of YY1 protein, in our analysis almost restricted to germ cells, could indicate a functional relevance for the germ cell-specific transactivation of the proacrosin gene. This hypothesis is supported by the results of Walowitz et al. [1998] who demonstrated that YY1 loses its repressor function on the α-actin promoter during muscle cell differentiation following proteolytic cleavage caused by proteases. Thus, YY1 protein might act as a repressor for the proacrosin gene and is cleaved by germ cell-specific proteases which results in proacrosin gene expression.

Besides the CRE-like site, all other elements investigated in this study are located downstream of the major transcriptional start site either in intron 1 (YY1 element) or in the 5′UTR (both CRE sites and the Y-box). In recent years it has been well established that transcriptional regulation is mediated by elements located not only 5′ but also 3′ of the transcriptional start site in introns or exons [reviewed in Ogbourne and Antalis, 1998]. In particular, YY1 is known to bind in several promoters to downstream intronic or 5′UTR elements [Hariharan et al., 1991; Shi et al., 1997; Yan et al., 2001].

In summary, we have shown that factors either known for an ubiquitous expression (a rat FRGY1/MSY1 homologue and YY1) or known for their germ cell-specific functions (a rat FRGY2/MSY2 homologue and CREM) bind to elements present in the rat proacrosin promoter. It can be suggested that these DNA-protein interactions are involved in the testis-specific regulation of the proacrosin gene.

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