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Tissue-Specific Protein-DNA Interactions of the Mouse Protamine 2 Gene Promoter

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Abstract During spermiogenesis, the haploid phase of spermatogenesis, the genome is packaged into a highly compacted form and this process requires replacement of histones by protamines. In the mouse, protamines are encoded by two genes, which are transcriptionally regulated in testis. To understand the regulation of transcription of the mouse protamine 2 (mP2) gene, the tissue-distribution of sequence-specific interactions between nuclear proteins and promoter DNA sequences have been analyzed. Protein binding to the promoter region from −370 to +65 was studied using DNase I footprinting and gel shift assays. Five protein binding sites were identified, which are recognized by nuclear proteins from either testis or liver. Site 1 from −64 to −48, contains part of a cAMP responsive element (CRE), which in testis is recognized by CREMτ, an activator of post-meiotic transcription. Testicular protein(s) also binds to three other promoter domains: site 2, −87 to −67, a region containing a CAAT box, and sites 4 and 5, −239 to −210 and −328 to −311, sequences with similarity to consensus steroid hormone responsive elements (HRE). In contrast, interactions between the mP2 promoter and nuclear factors from liver, a tissue in which the mP2 gene is not transcribed, are observed at sites 1, 2, and 4, as well as at an additional region at site 3, −202 to −175. Because occupancy at site 3 appears to correlate with inactivation of the gene in non-testicular tissues, whereas testicular protein binding at site 5 appears to be associated with active transcription, we conclude that the mP2 promoter displays intricate tissue-specific patterns of protein/DNA interactions at key regulatory elements. J. Cell. Biochem. 64:94–105. © 1997 Wiley-Liss, Inc.

Key words: mouse protamine 2; gene promoter; protein-DNA interactions; spermatogenesis

Spermatogenesis consists of three distinct phases: mitotic proliferation of stem cells, the meiotic process, and spermiogenesis, the post-meiotic differentiation of spermatids [reviewed in Bellvé, 1979; Hecht, 1986]. Many genes for testis-specific proteins and testis-specific isoforms are expressed at defined stages during male germ cell development [Hecht, 1986, 1995; Propst et al., 1988; Willison and Ashworth, 1987; Wolgemuth and Watrin, 1991]. The ordered regulation of gene expression during differentiation makes the testis a desirable tissue to study interactions between cis-acting elements and protein binding factors. Since permanent cell lines of spermatogenic cells for transient expression are only beginning to be established [Cooker et al., 1993; Hofmann et al., 1992, 1994; Rassoulzadegan et al., 1993], most germ cell promoter studies have utilized in vitro transcription or transgenic mice to define essential cis-acting elements. DNA footprinting and gel shift assays are often used to define DNA-protein interactions [Howard et al., 1993; Tamura et al., 1992; van der Hoorn and Tarnasky, 1992].

Protamines are highly basic, small proteins present only in post-meiotic stages of male germ cells. Male germ cell DNA is packaged in a highly compacted form during spermiogenesis and this process requires in mammals a stepwise replacement of the nucleohistones by transition proteins and then by protamines. The mouse genome contains two genes for protamine, mP1 and mP2, that are transcribed in round spermatids and translated in elongating spermatids [Hecht, 1986]. The promoters of both mouse protamine genes have been analyzed in transgenic mice [Peschon et al., 1987; Stewart et al., 1988; Zambrowicz et al., 1993]. For protamine 2, a promoter sequence of 859 bp was shown to be sufficient for post-meiotic expression [Stewart et al., 1988], and in a more

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detailed analysis, 113 bp has been shown to be sufficient for the proper temporal expression of protamine 1 [Zambrowicz et al., 1993]. Promoter deletion assays using a testicular in vitro transcription system have identified a putative positive regulatory region in the mP2 promoter between −170 and −82 [Bunick et al., 1990b] and gel shift analyses have identified several regions of mP2 promoter that bind protein [Johnson et al., 1991].

Eukaryotic promoters bind numerous protein factors, and transcription is regulated by interactions among those factors. For the testis-specific protamine 1 promoter, several regulatory regions for tissue-specific and high-level transcription have been identified in transgenic mice [Zambrowicz et al., 1993; Zambrowicz and Palmiter, 1994]. Promoter analysis of the testis-specific variant of phosphoglycerate kinase, PGK2, has revealed several elements needed for tissue-specific expression and several elements for ubiquitous expression [Gebara and McCarrey, 1992]. To start to define the trans acting factors that regulate mP2 transcription, a region near the start of transcription of the mP2 promoter has been analyzed for protein binding sites using DNase I footprinting and gel shift assays with extracts from expressing and non-expressing tissues. Effort has been concentrated on the first 370 nucleotides of the protamine 2 promoter since 113 nucleotides of the highly homologous protamine 1 promoter are sufficient for round spermatid specific transcription [Zambrowicz et al., 1993]. Several known sequence elements that bind testicular protein "in vitro" have been identified: a cAMP responsive element (CRE) at site 1, a CAAT box at site 2, and two regions with similarity to steroid hormone responsive elements (HRE) at sites 4 and 5.

MATERIALS AND METHODS

Preparation of Nuclear Extracts

Nuclear protein extracts were prepared using a modification of the procedure of van der Hoorn and Tarnasky [1992]. Testes of 50 CD-1 mice, 4–6 months old, were decapsulated and homogenized in 20 ml of homogenization buffer (10 mM Hepes, pH 7.6, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 1 mM DTT, 2.2 M sucrose, 1% nonfat dry milk) and protease inhibitors [1 mM benzamidine, 0.5 mM phenylmethylsulfonylfluoride, 1% aprotinin, pepstatin (1 μg/ml), and leupeptin (1 μg/ml)] in a Teflon-glass homogenizer [Bunick et al., 1990a,b; van der Hoorn and Tarnasky, 1992]. After 10 strokes, the homogenate was diluted to 1.85 M sucrose and the nuclei were pelleted by centrifugation at 24,000 rpm for 1 h in a Beckman (Fullerton, CA) SW 28 rotor through a cushion of the same buffer containing 10% glycerol. Pelleted nuclei were resuspended in lysis buffer (10 mM Hepes, pH 7.6, 100 mM KCl, 0.1 mM EDTA, 3 mM $MgCl_2$, and 10% glycerol) and nuclear proteins were extracted with 0.36 M ammonium sulfate, precipitated with ammonium sulfate (0.3 g/ml of extract) and dissolved in dialysis buffer (25 mM Hepes, pH 7.6, 0.1 mM EDTA, 40 mM KCl, 10% glycerol, and 1 mM DTT). The concentration of recovered protein ranged from 5 to 15 mg/ml. Liver tissue was homogenized in a modified Waring (New Hartford, CT) Blender using the same buffers [Sierra, 1990] with the modification that liver nuclei were pelleted through 2.0 M sucrose.

Each preparation of nuclear extract was tested for transcriptional activity by an in vitro transcription assay [Gorski et al., 1986; van der Hoorn and Tarnasky, 1992]. Transcriptions with testis extracts were carried out using a construct consisting of the mP2 promoter DNA, −859 to +4, ligated upstream to a G-free cassette in p(C2AT)19. pAdMLP190, a plasmid containing the adenovirus major late promoter linked to a G-free cassette, was used as template to assay the liver extracts.

DNase I Footprinting

Footprinting analyses were performed using four DNA fragments: −170 to +8 (BspM I → Sph I), −170 to +65 (Sph I → Pst I), −269 to +65 (Nde I → Pst I), and −371 to +65 (Hind III → Pst I). One strand of each DNA fragment was labeled with an [α-^{32}P] dNTP at the 3′ end using Klenow enzyme. After labeling, the DNA was purified by polyacrylamide gel electrophoresis and the labeled band was eluted. DNase I footprinting assays were carried out using the procedure of Sierra [1990]. DNase I (0.5–1.5 U) was added to 20 μl reaction mixtures containing 1–3 × 10^4 cpm of DNA, 1 μg of poly(dI-dC)*(dI-dC), and 50–100 μg of nuclear proteins in footprinting buffer (final concentration: 40 mM Hepes, pH 7.6, 5 mM $MgCl_2$, 60 mM KCl, 6% glycerol, 0.06 mM EDTA, 0.6 mM DTT) and incubated for 2 min at room temperature. The digestions were terminated by adding 80 μl of

stop buffer (200 mM NaCl, 20 mM EDTA, pH 8.0, 1% SDS, 50 μg/ml yeast tRNA) and the DNAs were extracted, precipitated, and analyzed in 6% polyacrylamide DNA sequencing gels. Control DNAs were incubated with 0.02–0.1 U of DNase I under identical conditions in the absence of protein extract and processed as above.

Gel Shift Probes and Assay Conditions

Gel shift assays were performed as described below [Johnson et al., 1991] using the following double-stranded oligonucleotides: oligonucleotide 1, nucleotides −67 to −46 (5′-GGG CCG ACA GGT CAC AGT GGG-3′) and its complement (5′-CCC CAC TGT GAC CTG TCG GCC-3′); oligonucleotide 2, nucleotides −86 to −69 (5′-GGA ACA ATC AAT CAG GG-3′) and its complement (5′-CCC CTG ATT GAT TGT TCC-3′); oligonucleotide 3, nucleotides −202 to −174 (5′-GTG AGG CCA TCT CAC ATT CAA TAA GTC A-3′) and its complement (5′-CTG ACT TAT TGA ATG TGA GAT GGC CTC A-3′); oligonucleotide 4, nucleotides −226 to −210 (5′-AAG TTC AAG GTC ATT C-3′) and its complement (5′-ATA ATG ACC TTG AAC TT-3′); oligonucleotide 5, nucleotides −330 to −310 (5′-CAA CCA AGG CCA TGC ATG GG-3′) and its complement (5′-GCC CAT GCA TGG CCT TGG TT-3′); an NF1/CTF oligonucleotide (5′-ATT TTG GCT TGA AGC CAA TAT G-3′) and its complement; and a CREB oligonucleotide (5′-GAT TGG CTG ACG TCA GAG AGC T-3′).

Double-stranded oligonucleotides were made by incubating the two complementary single-stranded oligonucleotides at 80°C for 10 min and then slowly cooling the duplexes to room temperature. The recessed 3′ end of each oligonucleotide was labeled with α-^{32}P dNTP using Klenow enzyme, and then purified by polyacrylamide gel electrophoresis. Nuclear extracts (5–10 μg) were incubated with 1 μg of poly (dI-dC)*(dI-dC) in binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 3 mM $CaCl_2$, or 3 mM $MgCl_2$) at either 4°C or room temperature for 10 min. Competitor DNAs (a 10-fold molar excess of DNA for self-competitions or a 100-fold molar excess of DNA for non-self-competitions) were incubated for 15 min at 22°C before the radiolabeled DNA probes (2–3 × 10^4 cpm) were added and incubated 25 min at 22°C. The samples were analyzed in non-denaturing polyacrylamide gels in 0.25 × TBE buffer at either 4°C or at room temperature.

RESULTS

−370 to +4 Region of the mP2 Promoter Has Five Protein Binding Sites

Comparison of protein/DNA interactions at the mP2 promoter in expressing and non-expressing tissues provides a frame-work for understanding the tissue-specific regulation of the mP2 gene by its cognate factors. We performed DNase I footprinting analysis using a probe spanning nt −340 to +4 of the mP2 promoter using nuclear proteins from testis, where the gene is actively regulated at the transcriptional level, and a non-expressing tissue (liver), where the gene is physiologically inactive. Figure 1 shows the five protein/DNA interaction domains in the −370 to +4 region of the mP2 promoter. Nuclear factors from both liver and testis bind to sites 1, 2, and 4. However, proteins from liver but not testis nuclear extracts interact with site 3, whereas proteins from testis but not liver bind to site 5. Although testis and liver proteins bind to the same DNA sequences at sites 1, 2, and 4, the proteins bound from the liver and testis appear to be distinct, since the DNA footprints and the mobilities of the DNA-protein complexes in the gel shift assays differ between the two tissues (see below). Starting at the most upstream region, each binding site will be discussed in order.

Site 5, Protein Binding at −328 to −311

Of the five footprints detected in this region of the mP2 promoter, site 5 is an expressing promoter element, because occupancy of this domain is only observed with proteins from testicular extracts and not with non-testicular proteins (Fig. 2, compare lane 3 to lane 4) using equal amounts of protein activity based on in vitro transcription assays. This difference in site 5 binding activity between protein preparations was confirmed by gel shift assays with oligonucleotide 5, which spans nucleotides −330 to −310 and encompasses site 5. A prominent protein/DNA complex in gel shift assays was obtained with nuclear factors from testis (Fig. 3A, lane 1), but no equivalent DNA-protein complex was seen with the liver extract (Fig. 3A, lane 2). A faint band with a slower electrophoretic mobility could be detected, however,

-370
AGCTTTTGCT AGGAGTTGGT GAGTGCTCTC CTCGAATGCC

-330
CAACCAAGGC CATGCATGGG CTGCATCTCC AACACTGCAT
SITE 5

-290
GTCCAGGGCA TGGAAGCACA CATATGTGAT TCCAACCCCT

-250
GGGAGGTAAA GGGACCAGGG TTAGAAGTTC AAGGTCATTC
SITE 4

-210
TTCATTGTGT GAGGCCATCT CACATTCAAT AAGTCAGCAT
SITE 3

-170
GCTTCAAAGC AAGATGAGTA ACTTGGCCCC TAAGCCAGTC

-130
CTGCAAACCC TGTGCCGCCC TCACAGAGGG GACTGGGCAG

-90
GGTGGGAACA ATCAATCAGG GGTGGGCCGA CAGGTCACAG
SITE 2 SITE 1

-50
TGGGGTTTAC CTTTATATAT GAGCCCTCTG AGAGCCCCAA

-10 +1
ACACCAGACC ATCAT

Fig. 1. Summary of protein binding sites in the mP2 promoter region from −370 to +4. Binding sites identified by DNase I footprinting analyses are denoted as sites 1 to 5.

with the liver extract after a very long exposure (data not shown).

Because protein binding sites 3, 4, and 5 share sequence-similarities, the possibility arises that similar proteins bind to these elements. We performed gel shift competition assays with a panel of oligonucleotides to examine specificity of binding to each region, and potential for cross-competition. The oligonucleotides spanning site 4 (Fig. 3B, lane 10), but not the site 3 oligonucleotide (Fig. 3B, lane 9) compete for the binding of testicular proteins to site 5. We note that site 3 is similar, but not identical to site 4. Consistent with competition between sites 4 and 5 for a shared factor, unlabeled oligonucleotide 5 successfully competes for protein binding with a radiolabeled site 4 probe (Fig. 3B, lane 5). However, oligonucleotide 2, a DNA probe with a totally different sequence (lanes 3 and 8), did not compete although self-competition gel shift assays demonstrated that the assays and the competitions

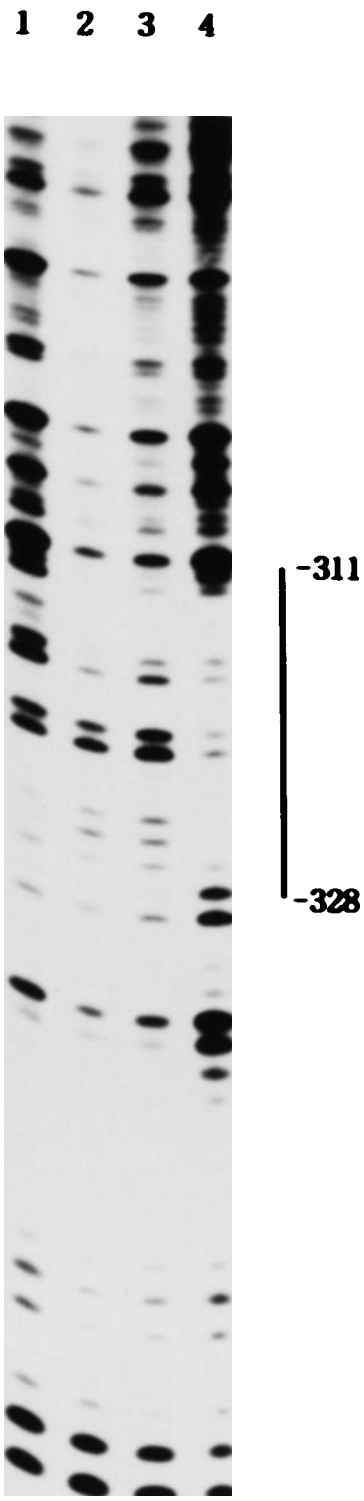

Fig. 2. DNase I footprinting of the mP2 promoter DNA fragment, −371 to +65. **Lanes 1** and **2,** control digestions in the absence of added proteins with 0.04 and 0.08 U of DNase I, respectively; **lane 3,** liver extract (66 μg); and **lane 4,** testis extract (78 μg). The latter two are digested with 0.75 U of DNase I.

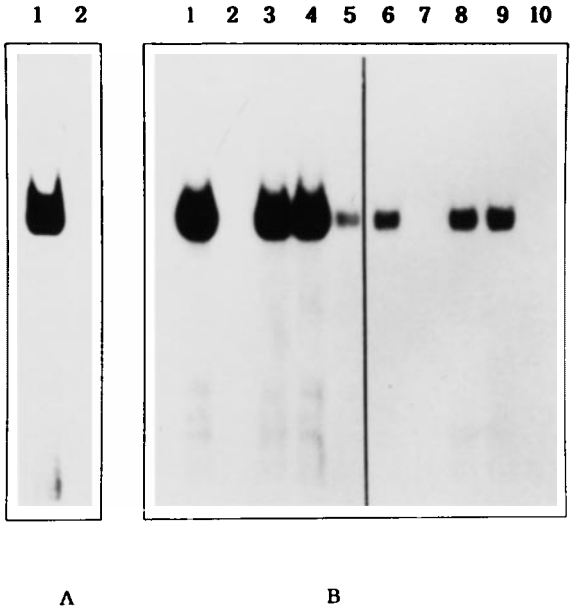

Fig. 3. Gel shift assays with oligonucleotides 4 and 5. **A:** Gel shifts with oligonucleotide 5. **Lane 1,** oligonucleotide 5 incubated with testis extract (5 μg); **lane 2,** oligonucleotide 5 incubated with liver extract (5 μg). **B:** Competition assays with oligonucleotides 4 and 5. Testis extract (6 μg) was incubated with radiolabeled oligonucleotide 4 **(lanes 1–5)** or radiolabeled oligonucleotide 5 **(lanes 6–10).** Lanes 1 and 6 are gel shifts without competition; lanes 2 and 7 are gel shifts with unlabeled oligonucleotide 4 and oligonucleotide 5 as competitors, respectively; lanes 3 and 8 are gel shifts with oligonucleotide 2 as competitor; lanes 4 and 9 are gel shifts with oligonucleotide 3 as competitor; lane 5 is a gel shift with unlabeled oligonucleotide 5 as competitor, and lane 10 is a gel shift with unlabeled oligonucleotide 4 as competitor. Tenfold molar excess of unlabeled oligonucleotides were used for self-competition assays (lanes 2 and 7) and a 100-fold molar excess of unlabeled oligonucleotides was used for the other competitions (lanes 3, 4, 5, 8, 9, and 10).

were specific (Fig. 3B, compare lane 1 to lanes 2 and 3, and lane 6 to lanes 7 and 8).

Site 4, Protein Binding at −239 to −210

Nuclear proteins from both testis and liver bind to mP2 promoter sequences at site 4, nucleotides −239 to −210 (Fig. 4A). When protein-DNA interactions of site 4 are analyzed by gel shift assays, complexes with different mobilities are observed with liver and testis extracts, with the liver complex migrating more slowly than that of the testis (Fig. 5A). Hence, although the testicular and non-testicular DNA binding activities recognize the same promoter domain, the proteins forming these somatic and testicular protein-DNA complexes are not identical.

A series of competition assays were performed with oligonucleotides 4 and 5 (Figs. 3 and 5). Oligonucleotide 5 competes efficiently for protein binding against oligonucleotide 4 (Fig. 3B, lane 5), but the amount of DNA-protein complex formed with oligonucleotide 4 is substantially greater than the DNA-protein complex that forms with oligonucleotide 5 (Fig. 3B). These results are consistent with the footprinting data which show that site 4 is better protected from DNase I digestion than site 5 (compare Figs. 2 and 4). This result could suggest that the amount of testis protein binding available for binding to site 4 is greater than that binding to site 5. However, as the similarities in electrophoretic mobilities of the DNA-protein complexes formed with oligonucleotides 4 and 5 suggest that the same protein binds to sites 4 and 5, we favor the interpretation that this putative factor interacts with a greater binding affinity to site 4 than site 5.

Although site 4 shares homology with the CREB binding site, ACGTCA, neither the liver nor the testis DNA-protein complexes were diminished by the addition of a CREB oligonucleotide at levels up to 100-fold (Fig. 5A, lanes 3 and 7). Moreover, oligonucleotide 5 competes only modestly for binding of liver proteins to oligonucleotide 4 (Fig. 5B, lane 5); the liver and testis proteins binding to this sequence may differ in affinity or identity.

Site 3, Protein Binding at −202 to −175

Based on our DNase footprinting results, liver extracts but not testis extracts contain DNA binding activities recognizing site 3 of the mP2 promoter (Fig. 4B). Gel shift analyses with oligonucleotide 3 confirms the sequence-specificity of the cognate factors (Fig. 6). Whereas testis extracts only show non-specific bands (Fig. 6A, lane 1; Fig. 6B, lane 2), nuclear proteins from liver mediate formation of two specific complexes (Fig. 6A, lane 2; the predominant band denoted by an arrowhead).

DNA-protein binding specificity was confirmed with competition assays using oligonucleotides 2, 3, 4, and 5. The most prominent liver DNA-protein complex (indicated by an arrowhead in Fig. 6B) is not detected with the testis extract (Fig. 6B, compare lanes 1 and 2). Interestingly, the amount of DNA-protein complex is reduced by the presence of oligonucleotide 2, suggesting that one protein(s) recognizes similar sequences in sites 2 and 3 (Fig. 6B, lane

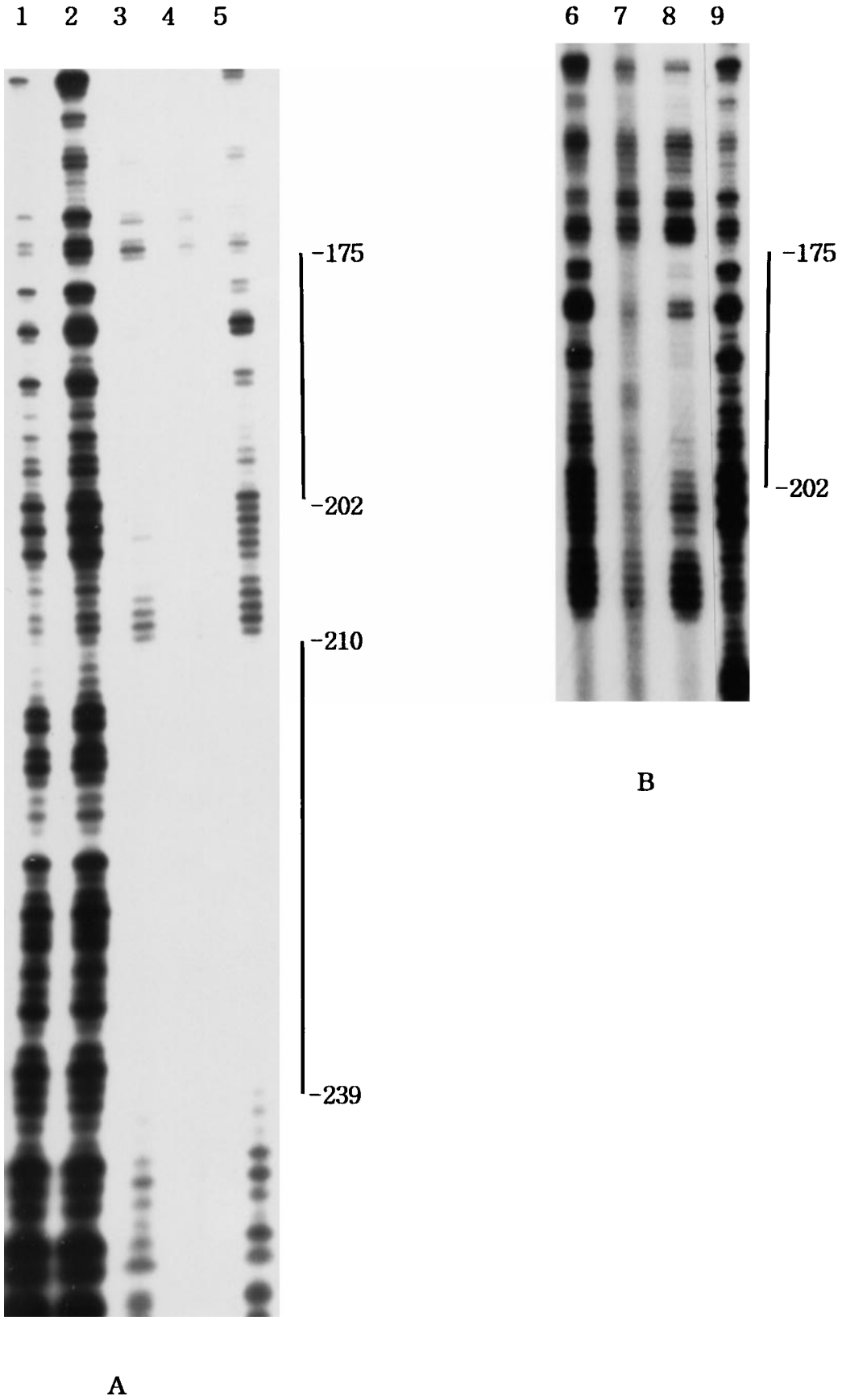

Fig. 4. DNase I footprinting of the mP2 promoter fragment −269 to +65. DNA fragment −269 to +65 was end labeled at nucleotide −269. **Lanes 1–5** show protein binding to sites 3 and 4 **(A), lanes 6–9** show protein binding to site 3 **(B).** Lanes 3, 4, 7, 8, the radiolabeled DNAs were incubated with liver extract (100 μg) and digested with DNase I (lanes 3 and 8 with 0.2 U of DNase I, and lanes 4 and 7 with 0.4 U of DNase I). Lanes 5 and 6, radiolabeled DNAs were incubated with testis extract (90 μg) and 0.4 U of DNase I; and lanes 1, 2, and 9, radiolabeled DNAs and no added extracts were incubated with DNase I (0.04 U, lanes 1 and 9; 0.02 U, lane 2).

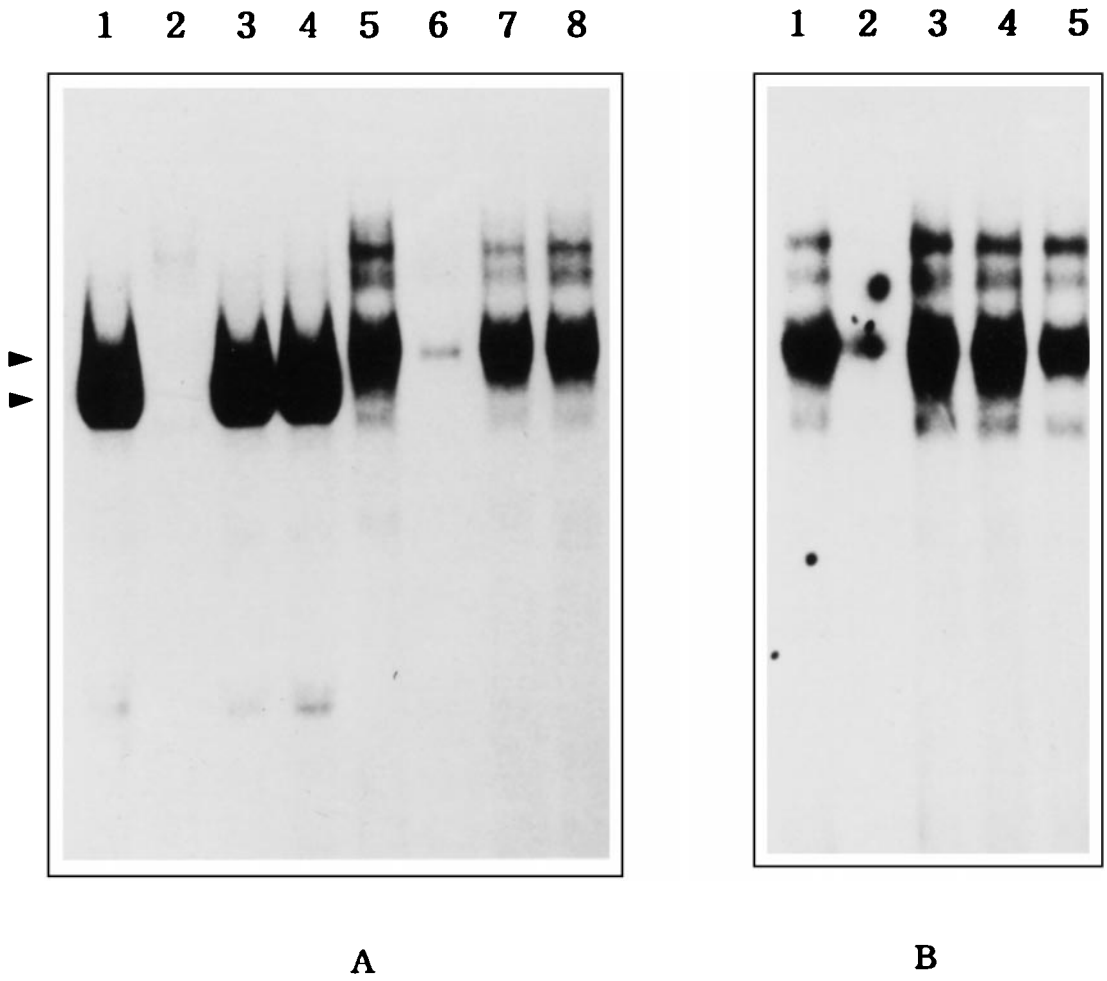

Fig. 5. Gel shift assay with oligonucleotide 4. **A:** Competition assay for oligonucleotide 4. **Lanes 1** to **4,** incubations with oligonucleotide 4 and testis extract (10 μg); **lanes 5** to **8,** incubations with oligonucleotide 4 and liver extract (10 μg). Lanes 1 and 5, no added competitors; lanes 2 and 6 were self-competitions (10-fold molar excess of unlabeled oligonucleotide 4). Lanes 3 and 7 were competed with 100-fold molar excess of CREB binding oligonucleotide; lanes 4 and 8 were competed with 100-fold molar excess of oligonucleotide 2. **B:** Liver extracts (10 μg) were incubated with oligonucleotide 4. **Lane 1,** no added competitors; **lane 2** was self-competition (10-fold molar excess unlabeled oligonucleotide); **lanes 3** and **4** were competed with a 100-fold molar excess of oligonucleotides 2 or 3, respectively; and **lane 5** was competed with a 100-fold molar excess of oligonucleotide 5.

3). Even though site 3 shares some of the same sequence as site 5, AGGCCA, no competition is seen with up to 100-fold amounts of oligonucleotides 4 and 5 (Fig. 6B, lanes 4 and 5).

Site 2, Protein Binding at −87 to −67

Both testis and liver proteins bind to site 2 in the mP2 promoter (Fig. 7). Similar protein binding patterns are seen with each of the strands of DNA (Fig. 7A and B). In gel shift assays, three specific DNA-protein complexes (indicated by a, b, and c in Fig. 8) are seen with the testis or liver extracts. The major DNA-protein complex, complex b, has a similar mobility with both testis and liver extracts. In addition, a slower migrating liver DNA-protein complex, complex a, and a more rapidly migrating testis DNA-protein complex, complex c, are seen. These latter two complexes can be successfully competed by oligonucleotide 3 (Fig. 8, lanes 4 and 9). Addition of an unlabeled oligonucleotide for NF1/CTF diminishes complex a (lane 10) but not complex c (lane 5).

Site 1, Protein Binding at −64 to −48

This sequence element contains the sequence GTCA, a half site of the cAMP response element (CRE) of CREMτ, a CREM activator protein that serves as a regulatory transcription factor for several post-meiotic testicular genes

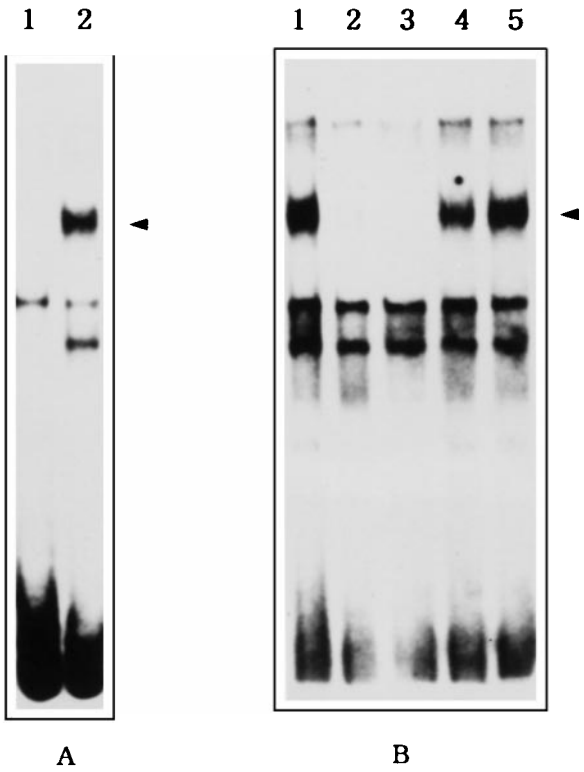

Fig. 6. Gel shift assay with oligonucleotide 3. **A: Lane 1,** radiolabeled oligonucleotide 3 incubated with testis extract (5 µg); **lane 2,** radiolabeled oligonucleotide 3 incubated with liver extract (5 µg). The arrowhead points to the predominant specific complex. **B:** Competition assay for oligonucleotide 3 binding. **Lane 1,** no competition; **lane 2,** oligonucleotide 3 competed with 10-fold molar excess of unlabeled oligonucleotide 3; **lanes 3, 4,** and **5,** oligonucleotide 3 competed with 100-fold molar excess of oligonucleotides 2, 4, and 5, respectively.

[Delmas et al., 1993]. Although we detect DNA footprints from both liver and testis extracts in this region (Fig. 7), the differences we detect when gel shift assays are performed with a CREMτ oligonucleotide (Fig. 9) indicate different proteins from liver and testis are recognizing this sequence element.

DISCUSSION

To start to define the trans-acting factors that recognize cis-acting elements in the mP2 promoter, DNase I footprinting and gel shift assays have been employed. Within a 370 nucleotide promoter region for mP2, five protein binding sites have been identified (Fig. 1). Each site shows differences between testis and liver binding proteins in either amount or mobility of the DNA-protein complexes formed. This comparison of extracts from testis and liver underestimates differences in trans-acting factors present in expressing and non-expressing cells since protamine is only transcribed in round spermatids and the testicular extracts contain proteins from all testicular cells. We have found testicular protein binding to four regions (sites 1, 2, 4, and 5) and liver protein binding to four regions (sites 1, 2, 3, and 4). Although protein from both extracts binds to similar sites, gel shift analyses suggest the protein-DNA complexes differ. Previous studies have analyzed protein binding to the mP2 promoter, from −419 to −23, by gel shift assays using large DNA fragments as probes. One DNA-protein complex was detected using a 118 nt fragment from −141 to −23 (see below), and in non-expressing tissues another complex was seen with a DNA fragment from −419 to −141. These data serve as the background for the studies present here where smaller probes, i.e., oligonucleotides of 17 to 26 nucleotides, have allowed resolution of multiple complexes within the fragments analyzed earlier [Johnson et al., 1991].

Based upon in vitro transcription assays [Bunick et al., 1990a,b], a sequence element from −170 to −82 is predicted to serve as a positive regulatory element. From the previous DNA deletion analyses and the protein binding we find in this study, site 2, from −87 to −67, could contain one of the positive elements responsible for the mP2 transcription. Although oligonucleotide 2, derived from site 2, forms complexes with both liver and testis extracts, one complex appears to be specific to the expressing tissue while the second appears common to both liver and testis. The testicular complex shares sequence homology with oligonucleotide 3 (Fig. 6B, lane 3; Fig. 8, lane 4) and sequences in oligonucleotides 2 and 3 have homology with the CCAAT box. We know that the binding protein for this element is not NF1/CTF since this sequence does not effectively reduce DNA-protein complex formation (Fig. 8, lane 5). Perhaps this element interacts with H1TF2/HiNF-B, another type of CCAAT box protein, which recognizes the CCAAT box of the histone H1 gene promoter and is distinct from NF1/CTF [Gallinari et al., 1989; van Wijnen et al., 1988]. The major complexes formed with site 2 DNA have similar electrophoretic mobilities for both the testis and liver extracts. Other than the CCAAT box sequence, the sequences of site 2 show no apparent consensus homology with the cis-acting elements of known transcription factors.

Transcription of eukaryotic genes is complex, requiring multiple proteins, tissue-specific fac-

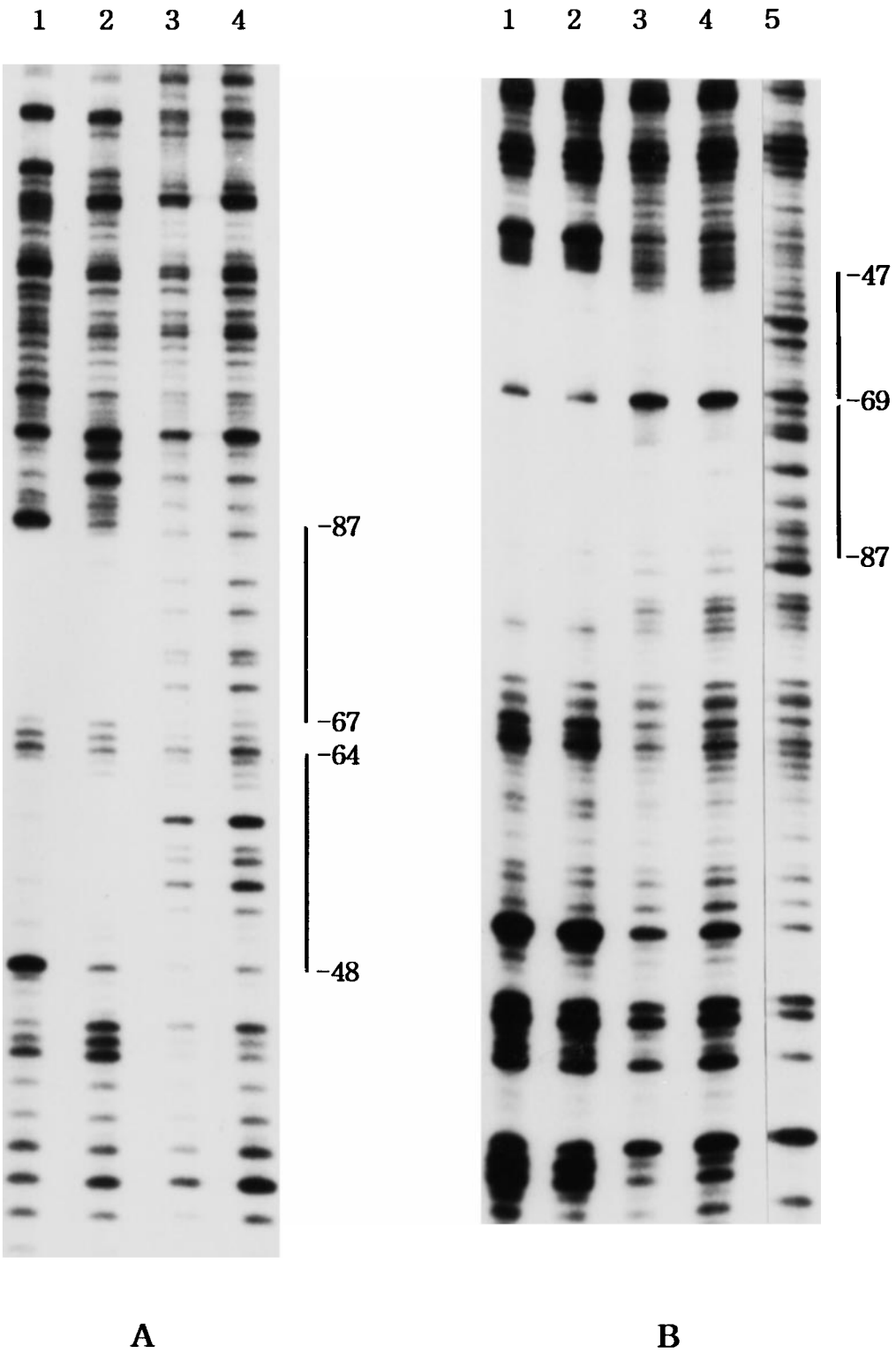

Fig. 7. DNase I footprinting of the mP2 promoter fragment, −170 to +8. **A:** DNase I footprinting of coding stand. **B:** DNase I footprinting of non-coding strand. A: DNA fragment −170 to +8 was labeled at nucleotide +8. **Lane 1,** incubation with liver extract (100 µg) and digestion with DNase I (0.4 U); **lane 2,** incubation with testis extract (100 µg) and digestion with DNase I (0.4 U); **lanes 3** and **4,** no protein added. B: DNA fragment −170 to +65 was labeled at nucleotide −170. **Lanes 1** and **2,** incubation with liver extract (100 µg) and digestion with DNase I (0.5 U for lane 1 and 0.3 U for lane 2); **lanes 3** and **4** incubation with testis extract (100 µg) and digestion with DNase I (0.4 U for lane 3 and 0.75 U for lane 4); **lane 5,** DNA without protein.

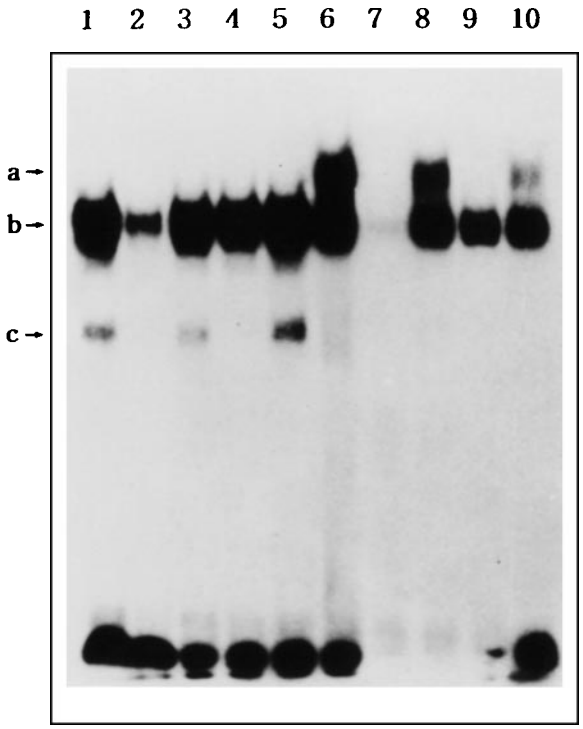

Fig. 8. Gel shift assay with oligonucleotide 2. **Lanes 1–5** were incubated with testis extract (8 µg); **lanes 6–10** were incubated with liver extract (8 µg). Lanes 1 and 6, no competition; lanes 2 and 7, 10-fold molar excess of oligonucleotide 2; lanes 3 and 8, 100-fold molar excess of oligonucleotide 1; lanes 4 and 9, 100-fold molar excess of oligonucleotide 3; and lanes 5 and 10, 100-fold excess of the NF1/CTF binding oligonucleotide. The three DNA-protein complexes detected are designated a, b, and c.

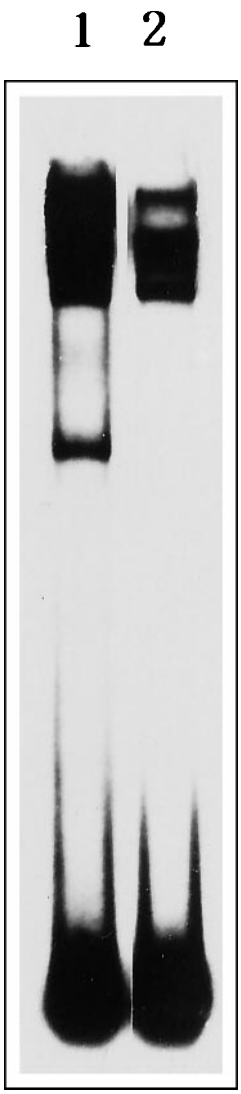

Fig. 9. CREMτ binding at site 1. **Lane 1,** oligonucleotide 1 incubated with testis extract (10 µg); **lane 2,** oligonucleotide 1 incubated with liver extract (12 µg).

tors, and ubiquitous transcription factors. Analysis of transcription of the mouse protamine 1 gene in transgenic mice has defined elements apparently needed for spermatid-specific transcription and elements required for high-level transcription that are not essential for testis-specific transcription [Zambrowicz et al., 1993; Zambrowicz and Palmiter, 1994]. Although site 1, situated downstream of site 2, has not been characterized by in vitro transcription or deletion analysis, sequence analysis identifies a half element CRE binding sequence, CREMτ, produced from a CRE modulator (CREM) gene by alternative splicing that may function in protamine transcription [Delmas et al., 1993; Foulkes et al., 1992]. Unlike other CREM spliced forms such as CREM α, -β, or -γ, which act as repressors, CREMτ retains its transactivation domain and functions as an activator. During spermatogenesis, CREM α, -β, and -γ transcripts are present at low levels in pre-meiotic germ cells. As meiosis proceeds, splicing produces high levels of CREMτ transcripts in post-meiotic round spermatids. Several genes expressed in post-meiotic cells, including protamines 1 and 2 [Delmas et al., 1993; Tamura et al., 1992], ACE [Howard et al., 1993], RT7 [van der Hoorn and Tarnasky, 1992], and TP1 [Kistler et al., 1994] contain a CRE-binding protein sequence and deletion of these sequences decreases transcription [Howard et al., 1993; Tamura et al., 1992; van der Hoorn and Tarnasky, 1992]. CREMτ has been implicated as an activator of transcription [Howard et al., 1993; Kistler et al., 1994; Tamura et al., 1992; van der Hoorn and Tarnasky, 1992] and CREMτ protein has been shown to bind to the promoters of post-meiotically expressed genes including mP2 [Delmas et al., 1993]. The recent

gene targeting of the CREMτ gene has led to male sterility due to a cessation of spermatogenesis [Nantel et al., 1996; Blendy et al., 1996]. The sequences of these binding sites contain at least a conserved half-site CRE, GTCA, in either strand, although the neighboring sequences can diverge greatly [Delmas et al., 1993]. The affinities for binding vary among the different CREs, but the wide range of binding suggests that CREMτ recognizes a number of different promoter targets in spermatids. Of special note is the proximity of site 2 and the CREMτ site in the mP2 promoter. From footprinting data it appears that the sites are separated by only one hypersensitive base (Fig. 7B). There are numerous examples of adjacent regulatory elements in promoters, such as the adjacent CRE and CCAAT motifs in the human fibronectin promoter [Muro et al., 1992], the adjacent NFY and C/EBP binding sites in the mouse serum albumin promoter [Milos and Zaret, 1992], and a CREB-associated protein site adjacent to a CRE element in the promoter of the rat glucagon gene [Miller et al., 1993]. The abilities of protein to bind DNA and/or function as transcription factors are often modulated by the presence of another protein at an adjacent site.

We have detected protein binding from testis extracts at sites 4 and 5. From the DNA footprinting and gel shift assays, one or more similar proteins may bind to sites 4 and 5 although site 4 appears to have a stronger binding affinity than site 5. When the sequences of site 4 are compared to similar sequences that do not bind this protein (by comparing the sequences of oligonucleotides 4 and 5 to non-binding sequences such as oligonucleotide 3 and the CREB-binding oligonucleotide), we conclude that CAAGGT/cCAT may be an essential shared sequence needed for binding. In fact, AGGTCA is the consensus sequence for a hormone response element (HRE) to which a group of steroid hormone receptors bind [Beato, 1989]. The estrogen response element (ERE) is AGGTCAnnnTGACCT [Beato, 1989; Kumar and Chambon, 1988] and the consensus sequences for thyroid hormone responsive element (TRE) and retinoic acid responsive element (RRE) contain half palindromes (AGGTCA) similar to those of the ERE but with different spacing [Beato, 1989; Glass et al., 1988]. Although the HREs in sites 4 and 5 are not arranged as palindromes, there is a half palindromic sequence in site 5, AGGCCA, and two in tandem in site 4, AGTTCAAGGTCA. Although site 3 contains a HRE with a sequence the same as site 5, the HRE in site 3 does not appear to bind the same protein that binds to site 4, perhaps because of adjacent elements. The promoter of mP1 also has a similar sequence in box C, ATGCCA [Zambrowicz and Palmiter, 1994]. Deletion of box C decreases the amount of the mP1 transcript to 3–5% of endogenous levels in transgenic mice, indicating this region of mP1 is needed for high levels of transcription [Zambrowicz et al., 1993]. Comparison of the promoter sequences of mouse and rat protamine 2 (rP2) further suggest that HRE regions facilitate transcription since the rP2 transcript is present at approximately 5% of that of the mP2 mRNA, and the rP2 promoter is transcribed poorly in an in vitro system [Bunick et al., 1990]. Sequence comparisons between mP2 and rP2 reveal differences in sites 3, 4, and 5 while sites 1 and 2 are the same [Johnson et al., 1991]. Moreover, similar protein binding was detected in the region of the rP2 promoter containing sites 1 to 4 (up to −280 bp) by DNase I footprint analysis (data not shown). Homologous regions from sites 1 to 4 in the rP2 promoter bind similar proteins and no new additional binding sites were found. These data suggest that the HRE binding protein may be needed for maximal mP2 transcription.

In conclusion, our results reveal an intricate pattern of expressing and non-expressing tissue protein/DNA interactions at the mouse protamine P2 gene promoter, which are mediated by several distinct classes of DNA binding activities. The possibility arises that these complex binding events at the mP2 promoter may facilitate the tight regulation of the protamine 2 gene in testicular tissue, where the gene is physiologically expressed in a discrete germ cell type and developmental stage, and inactivation of the gene in other testicular cells and non-testicular tissues.

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REFERENCES

Beato M (1989): Gene regulation by steroid hormones. Cell 56:335–344.

Bellvé AR (1979): The molecular biology of mammalian spermatogenesis. In Finn CA (ed): "Reviews of Reproductive Biology." New York: Oxford University Press, pp 159–261.

Blendy J, Kaestner K, Weinbauer G, Nieschlag E, Schütz G (1996): Severe impairment of spermatogenesis in mice lacking the CREM gene. Nature 380:162–165.

Bunick D, Balhorn R, Stanker LH, Hecht NB (1990a): Expression of the rat protamine 2 gene is suppressed at the level of transcription and translation. Exp Cell Res 188:147–152.

Bunick D, Johnson PA, Johnson TR, Hecht NB (1990b): Transcription of testis-specific mouse protamine 2 gene in a homologous in vitro transcription system. Proc Natl Acad Sci USA 87:891–895.

Cooker LA, Brooke CD, Kumari M, Hofmann MC, Millán JL, Goldberg E (1993): Genomic structure and promoter activity of the human testis lactate dehydrogenase gene. Biol Reprod 48:1309–1319.

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 implication for haploid germ cell differentiation. Mol Endocrinol 7:1502–1517.

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

Gallinari P, La Bella F, Heintz N (1989): Characterization and purification of H1TF2, a novel CCAAT-binding protein that interacts with a histone 1 subtype-specific consensus element. Mol Cell Biol 9:1566–1575.

Gebara MM, McCarrey JR (1992): Protein-DNA interaction associated with the onset of testis-specific expression of the mammalian PGK-2 gene. Mol Cell Biol 12:1422–1431.

Glass CK, Holloway IM, Devary OV, Rosenfeld MG (1988): The thyroid hormone receptor binds with opposite transcriptional effect to a common sequence motif in thyroid hormone and estrogen response elements. Cell 54:313–323.

Gorski KM, Carneiro M, Schibler U (1986): Tissue-specific in vitro transcription from the albumin promoter. Cell 47:767–776.

Hecht NB (1986): Regulation of gene expression during mammalian spermatogenesis. In Rossant J, Pedersen RA (eds): "Experimental Approaches to Mammalian Embryonic Development." New York; Cambridge University Press, pp 151–193.

Hecht NB (1995): The making of a spermatozoon: A molecular perspective. Dev Genet 16:95–103.

Hofmann MC, Narisawa S, Hess RA, Millán JL (1992): Immortalization of germ cells and somatic testicular cells using the SV40 large T antigen. Exp Cell Res 201:417–435.

Hofmann MC, Hess RA, Goldberg E, Millán JL (1995): Immortalized germ cells undergo meiosis in vitro. Proc Natl Acad Sci USA 91:5533–5537.

Howard T, Balogh R, Overbeek P, Bernstein K (1993): Sperm-specific expression of angiotensin-converting enzyme (ACE) is mediated by a 91 base pair promoter containing a CRE-like element. Mol Cell Biol 13:18–27.

Johnson PA, Peschon J, Yelick PC, Palmiter RD, Hecht NB (1988): Sequence homologies in the mouse protamine 1 and 2 genes. Biochim Biophys Acta 950:45–53.

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

Kistler MK, Sassone-Corsi P, Kistler WS (1994): Identification of a functional cyclic adenosine 3′,5′-monophosphate response element in the 5′-flanking region of the gene for transition protein 1(TP1), a basic chromosomal protein of mammalian spermatids. Biol Reprod 51:1322–1329.

Kumar V, Chambon P (1988): The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145–156.

Miller CP, Lin JC, Habener JF (1993): Transcription of the rat glucagon gene by the cyclic AMP response element-binding protein CREB is modulated by adjacent CREB-associated proteins. Mol Cell Biol 13:7080–7090.

Milos PM, Zaret KS (1992): A ubiquitous factor is required for C/EBP-related proteins to form stable transcription complexes on an albumin promoter segment in vitro. Genes Dev 6:991–1004.

Muro AF, Bernath VA, Kornbihtt AR (1992): Interaction of the −170 cyclic AMP response element with the adjacent CCAAT box in the human fibronectin gene promoter. J Biol Chem 267:12767–12774.

Nantel F, Monaco L, Foulkes S, Masquilier D, LeMeur M, Henriksén K, Dierich A, Parvinen M, Sassone-Corsi P (1996): Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. Nature 380:159–162.

Peschon J, Behringer RR, Brinster RI, Palmiter RD (1987): Spermatid-specific expression of protamine 1 in transgenic mice. Proc Natl Acad Sci USA 84:5316–5319.

Propst F, Rosenberg MP, Vande Woude GF (1988): Proto-oncogene expression in germ cell development. Trends Genet 4:183–187.

Rassoulzadegan M, Paquis-Fluckinger V, Bertino B, Sage J, Jasin M, Miyagawa K, van Heyningen V, Besmer P, Cuzin F (1993): Transmeiotic differentiation of male germ cells in culture. Cell 75:997–1006.

Sierra F (1990): "A Laboratory Guide to In Vitro Transcription." Basel, Boston, Berlin: Birkhauser Verlag.

Stewart TA, Hecht NB, Hollingshead PG, Johnson PA, Leong JAC, Pitts SL (1988): Haploid-specific transcription of protamine-*myc* and protamine-T-antigen fusion genes in transgenic mice. Mol Cell Biol 8:1748–1755.

Tamura T, Makino Y, Mikoshiba K, Muramatsu M (1992): Demonstration of a testis-specific trans-acting tet-1 in vitro that binds to the promoter of the mouse protamine 1 gene. J Biol Chem 267:4327–4332.

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 USA 89:703–707.

van Wijnen AJ, Wright KL, Massung RM, Gerretsen M, Stein JL, Stein GS (1988): Two target sites for protein binding in the promoter region of a cell cycle regulated human H1 histone gene. Nucleic Acids Res 16:571–592.

Willison K, Ashworth A (1987): Mammalian spermatogenic gene expression. Trends Genet 3:351–355.

Wolgemuth DJ, Watrin F (1991): List of cloned mouse genes with unique expression patterns during spermatogenesis. Mammal Genome 1:283–288.

Zambrowicz BP, Palmiter RD (1994): Testis-specific and ubiquitous proteins bind to functionally important regions of the mouse protamine 1 promoter. Biol Reprod 50:65–72.

Zambrowicz BP, Christopher J, Harendza JW, Zimmerman RL, Brinster RD, Palmiter RD (1993): Analysis of the mouse protamine 1 promoter in transgenic mice. Proc Natl Acad Sci USA 90:5071–5075.