

Author Proof

# Nectin-2 Expression in Testicular Cells Is Controlled via the Functional Cooperation Between Transcription Factors of the Sp1, CREB, and AP-1 Families

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Nectin-2, a major protein component of the adherens junctions (AJs), is found between Sertoli cells and germ cells in the seminiferous epithelium. Recent studies have shown that the expression of *nectin-2* gene in testis is crucial to maintain normal spermatogenesis since male knockout mice lacking *nectin-2* gene are sterile and possess morphologically abnormal spermatozoa. However, the molecular mechanisms governing its basal transcription remain poorly understood. By the use of Sertoli and germ cell-lines (TM4 and GC-2spd(ts) cells, respectively) in transient transfection studies, we showed that the minimal mouse nectin-2 promoter was located between nucleotides –316 and –211 (relative to the translation start site). Two putative Sp1 motifs and one each of the CRE, AP1, and AP2 motifs were identified within this region. Mutational studies showed that these two Sp1 motifs cooperated synergistically with the CRE motif, but not the AP1 and AP2 motifs, to regulate *nectin-2* gene transcription in both TM4 and GC-2spd(ts) cells. By EMSAs, we found that an AP-1 consensus sequence was able to inhibit DNA–protein complex formation with the CRE motif, suggesting an interaction between the AP-1 transcription factor (c-Jun) and CREB within the CRE motif. Overexpressions of CREB and c-Jun, but not c-Fos, also significantly increased the promoter activity, which suggests that CREB and c-Jun are the crucial transcription factors involved in regulating nectin-2 gene transcription. Chromatin immunoprecipitation assay has shown that, in vivo, CREB, c-Jun, and Sp1 family proteins are bound to the mouse nectin-2 promoter. Analysis of the staged tubules has confirmed that the cyclic expressions of CREB and nectin-2 coincide with the event of adherens junction restructuring between Sertoli cells and germ cells. The cross-talk between CREB, c-Jun, and Sp1 family protein is believed to be a major transcription machinery to drive nectin-2 expression in Sertoli cells. J. Cell. Physiol. 9999: 1–14, 2005. © 2005 Wiley-Liss, Inc.

Throughout spermatogenesis, germ cells must migrate from the basal to the adluminal compartment of the seminiferous epithelium, which associates with extensive restructuring of the actin-based cell–cell adherens junctions (AJs). Ectoplasmic specializations (ES) are specialized actin-based cell–cell AJs unique to the testis. They can be found between Sertoli cells at the basal region of the seminiferous epithelium (basal ES) or at the apical region of the seminiferous epithelium in which developing and mature spermatids attach onto Sertoli cells (apical ES) (for reviews, see Russell, 1977b, 1980). The turnover of basal ES allows the movement of spermatocytes across the seminiferous epithelium (Russell, 1977a), whereas the release of mature spermatids (spermatozoa) from the seminiferous epithelium at spermiation is accomplished by the disassembly of apical ES (Vogl et al., 2000). Nectins are found on both Sertoli cells and spermatids and function as interlocking proteins. They have been evident to involve in cell adhesion between Sertoli cells and spermatids at the apical ES (Ozaki-Kuroda et al., 2002).

Nectin is a Ca<sup>2+</sup>-independent cell adhesion molecule that belongs to the immunoglobulin-like superfamily (Morrison and Racaniello, 1992; Takahashi<sup>Q1</sup> et al., 1999). Unlike classic cadherins, nectins form not only homotypic AJs, but also heterotypic AJs (Kemler, 1992; Satoh-Horikawa et al., 2000). For instance, nectin-1 and nectin-3 heterotypic interactions are found at the pre- and post-synaptic regions of synapses (Satoh-Horikawa et al., 2000; Mizoguchi et al., 2002). Among the nectin family members, nectin-3 is most abundantly expressed and nectin-2 modestly expressed in the testis (Reymond et al., 2000; Satoh-Horikawa et al., 2000). Northern blot analysis revealed that all nectin-2 splicing variants were detected in Sertoli cells and germ cells at all ages, whilst all nectin-3 splicing variants were found mainly in spermatids of the germ cell fraction (Ozaki-Kuroda

et al., 2002). Studies from *nectin-2*<sup>-/-</sup> knockout mice showed that all male mice were infertile and produced morphological abnormal spermatozoa. For instance, the heads of spermatids showed irregular shapes with distorted nucleus. Mitochondria were present in the spermatid head and unable to pack tightly to form a helical sheath (Bouchard et al., 2000; Mueller et al., 2003).

Immunofluorescent analyses have showed that nectin-2 and nectin-3 colocalize with F-actin at Sertoli–spermatid junctions (apical ES). The appearance and disappearance of the nectin staining at apical ES were coincident to the assembly and disassembly of Sertoli–spermatid junctions, suggesting that actin-based heterotypic interaction between nectin-2 and nectin-3 might exist at apical ES in the seminiferous epithelium (Ozaki-Kuroda et al., 2002). Using transplantation techniques, *nectin-2*<sup>-/-</sup> spermatogonia when transplanted into *nectin-2*<sup>+/-</sup> testes were able to differentiate into normal spermatids, while *nectin-2*<sup>+/-</sup> spermatogonia transplanted into *nectin-2*<sup>-/-</sup> testes were not. These studies have indicated that nectin-2 in Sertoli cells contributes to the proper formation of heterotypic interaction

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between nectin-2 in Sertoli cells and nectin-3 in spermatids at Sertoli-spermatid junctions (Ozaki-Kuroda et al., 2002).

Not only proper formation of basal ES is crucial for germ cell differentiation, but also the timely disassembly of the apical ES is required to allow the release of mature spermatids. The disassembly and reassembly of ES appear to be controlled primarily by regulating the *nectin-2* and *nectin-3* genes at the transcriptional level. To address this issue, two nectin-2-expressing testicular cell lines, TM4 and GC-2spd(ts), were used to identify and characterize the transcriptional machinery important for the nectin-2 gene expression in Sertoli cells and germ cells. In the present study, we have demonstrated that the regulation of nectin-2 gene expression requires functional cooperation between multiple transcription factors.

## EXPERIMENTS

### Cells and cell culture

Mouse TM4 and GC-2spd(ts) were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM (Invitrogen, Calisbad, CA) containing 10% FBS. Cultures were maintained at 37°C in humidified atmosphere with 5% CO<sub>2</sub> in air.

### Preparation of nectin-2 promoter-luciferase constructs and site-directed mutagenesis

The 5'-flanking region of the *nectin-2* gene was generated using the mouse GenomeWalker Kit (Clontech, Palo Alto, CA) and primer #850 (Table 1). Various

5'-deleted regions generated by PCR were cloned into the promoterless pGL-3 Basic vector (Promega Corp., Madison, WI). Mutant plasmids were generated by a three-step PCR mutagenesis (Wong and Lee, 2002) using mutagenic primers (Table 1). All plasmids were prepared by Plasmid Midi Kits (Qiagen, Chatsworth, CA) and confirmed by sequencing analysis.

### Preparation of CREB-siRNA construct and its mutant

Oligonucleotides containing the CREB-siRNA or mutant CREB-siRNA sequences were annealed respectively. Annealed oligonucleotides and pSilencer 1.0-U6 siRNA expression vector (Ambion, Austin, TX) were digested by *Apa*I and *Eco*RI for subsequent cloning.

### Transient transfection and reporter gene assay

1 × 10<sup>5</sup> cells were seeded onto a 6-well culture plate a day before transfection. Luciferase constructs (1 μg) were co-transfected with pSV-β-gal (0.5 μg) using Lipofectamine reagent (Invitrogen) in serum-free media. Five hours after transfection, 20% FBS was added and cells were incubated overnight. Lipofectamine was replaced and cells were cultured for another 24 h before harvest. Luminescence was measured by a Lumat LB 9507 luminometer (EG&G, Berthold, Germany). β-Galactosidase activity was measured by a β-galactosidase enzyme assay system (Promega Corp.) and used to normalize transfection efficiency. Promoter activity was calculated as luciferase activity/β-galactosidase activity.

TABLE 1. Nucleotide sequence of primers used in plasmid construction, site-directed mutagenesis, and EMSA

| Primer name              | Location  | Orientation | Sequence (5' → 3')  | Purpose                    |
|--------------------------|-----------|-------------|---|----------------------------|
| 847                      | 28/47     | S           | 5' TCC AGA TTG TCA CCG ACG CT 3'  | Amplification of cDNA      |
| 848                      | 549/569   | AS          | 5' GAT GAG ATC CAG GTG ATT CGG 3'   | Amplification of cDNA      |
| 850 GSP                  | 33/58     | AS          | 5' GCA ACA ACG GCA GCG TCG GTG ACA AT 3'  | GenomeWalk                 |
| 861                      | -90/-106  | S           | 5' ACG CGT GGA GCC GGA CAC TTC A 3'   | Deletion                   |
| 862                      | -196/-211 | S           | 5' ACG CGT CTG GAG CTA AGC GAG G 3'   | Deletion                   |
| 895                      | -299/-316 | S           | 5' GGA CGC GTG ATG GGC GGG 3'   | Deletion                   |
| 863                      | -381/-399 | S           | 5' ACG CGT GGC TCC ATG TCG AGT G 3'   | Deletion                   |
| 864                      | -510/-528 | S           | 5' ACG CGT GGT CTG GGC GAG AAG 3'   | Deletion                   |
| 865                      | -646/-662 | S           | 5' ACG CGT GAC CCC GAC CTA CCA 3'   | Deletion                   |
| 879                      | -813/-830 | S           | 5' ACG CGT GAC ATA GGC ACA TGG ACA 3'   | Deletion                   |
| 880                      | -928/-946 | S           | 5' ACG CGT CTG GGC TGG TAT TAA GAG T 3'   | Deletion                   |
| 894                      | 20/34     | AS          | 5' GAA GAT CTG GAC GGC GGG A 3'   | ChIP assay                 |
| 1099                     | 1322/1346 | S           | 5' AGA TGC CTC GGT ATC ACG AGC TGC C 3'   | ChIP assay                 |
| 1100                     | 1566/1592 | AS          | 5' CAC ACA TAC ATG GCC CGT GAC ACA AA 3'  | ChIP assay                 |
| MPB                      |           | S           | 5' GGA GTA CTA ACC CTG GCC TAG CAA AAT AGG CTG TCC C 3'   | Mutagenic universal primer |
| MPC                      |           | AS          | 5' CTT TAT GTT TTT GGC GTC TTC CA 3'  | Mutagenic universal primer |
| MPD                      |           | S           | 5' GGA GTA CTA ACC CTG GC 3'  | Mutagenic universal primer |
| 901 CRE-BP               | -216/-241 | S           | 5' CCG GAC TCA <i>aGt</i> CGT <i>gAC</i> AGG CCC CG 3'  | Site-directed mutagenesis  |
| 902 pSP-1                | -227/-254 | AS          | 5' ACG TCA CAG GCC <i>CtG</i> <i>aaC</i> CTC TTC GCC A 3'   | Site-directed mutagenesis  |
| 920 dSP1                 | -288/-315 | AS          | 5' AGG GCT AAG ACC <i>CtG</i> <i>aaC</i> ATC ACG CGT C 3'   | Site-directed mutagenesis  |
| 931 CREB-dSP1            | -217/-251 | AS          | 5' CGG ACT <i>CAa</i> <i>GtC</i> <i>GTg</i> ACA GGC <i>CCT</i> <i>Gaa</i> CCT CTT CG 3'                   | Site-directed mutagenesis  |
| 904 GS-CREB-S            | -217/-237 | AS          | 5' GCC TGT GAC GTC ATG AGT CCG 3'   | Gel-shift                  |
| 906 GS-CREB*-S           | -231/-252 | S           | 5' GCC TGT <i>cAC</i> <i>GaC</i> <i>tTG</i> AGT CCG 3'  | Gel-shift                  |
| 908 GS-pSP1-S            | -231/-252 | S           | 5' GCG AAG AGG GGC GGG GCC TGT G 3'   | Gel-shift                  |
| 910 GS-pSP1*-S           | -231/-252 | S           | 5' GCG AAG AGG <i>tC</i> <i>aGG</i> GCC TGT G 3'  | Gel-shift                  |
| 912 GS-dSP1-S            | -291/-313 | S           | 5' CGC GGG ATG GGC GGG GTC TTA GC 3'  | Gel-shift                  |
| 914 GS-dSP1*-S           | -291/-313 | S           | 5' CGC GGG ATG <i>tC</i> <i>aGG</i> GTC TTA GC 3'   | Gel-shift                  |
| 1037 GS-CREBmAP1-S       | -217/-237 | S           | 5' GCC TGT GAC GTC <i>ATt</i> <i>cGT</i> <i>tCG</i> 3'  | Gel-shift                  |
| 1059 specific CREB-siRNA |           | S           | 5' ACG AAG GGA AAT CCT TTC ATT CAA GAG ATG AAA GGA TTT CCC TTC GTT TTT TT                                 |                            |
| 1060 specific CREB-siRNA |           | AS          | 5' AAT TAA AAA AAC GAA GGG AAA TCC TTT CAT CTC TTG AAT GAA AGG ATT TCC CTT CGT GGC C                      |                            |
| 1061 mCREB-siRNA         |           | S           | 5' ACG AAG <i>Get</i> AAT CCT TTC ATT CAA GAG ATG AAA GGA <i>TtA</i> <i>gCC</i> TTC GTT TTT TT            |                            |
| 1062 mCREB-siRNA         |           | AS          | 5' AAT TAA AAA AAC GAA <i>GGe</i> <i>tAA</i> TCC TTT CAT CTC TTG AAT GAA AGG ATT <i>agC</i> CTT CGT GGC C |                            |

S, sense; AS, anti-sense; italic bases indicate nucleotide mutation.

\*Mutated probe.

### Electrophoretic mobility shift assay (EMSA)

Oligonucleotides containing the putative Sp1 [d(distal) Sp1, p(proximal)Sp1] and CRE and the corresponding mutated oligonucleotides were annealed to form double-stranded DNA (Table 1). Probes were end-labeled with [ $\alpha$ - $^{32}$ P]-ATP and separated from unincorporated nucleotides via the Microspin G-25 columns (Amersham Biosciences, Piscataway, NJ). Nuclear extracts were prepared as described (Wong and Lee, 2002). EMSA was performed in a 20  $\mu$ l reaction mixture containing 20 mM HEPES (pH 7.5), 50 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM EDTA, 10% glycerol, 1  $\mu$ g poly (dI:dC), 50 fmol radiolabeled probe (50,000 cpm), and nuclear extract. For competitive assay, competitor oligonucleotides were added simultaneously with the radiolabeled probes. For supershift assay, nuclear extracts were preincubated with the antibodies (1–2.5  $\mu$ g) for 30 min. Anti-Sp1, anti-Sp3, and anti-CREB antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The binding reaction was carried out at room temperature for 15 min and the reaction products were separated on a 6% polyacrylamide gel. The gels were dried and then exposed to X-ray film (Eastman Kodak Co., Rochester, NY) at  $-70^{\circ}\text{C}$  overnight.

### Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as described (Baek et al., 2001). Macromolecules in exponentially growing cells were cross-linked with 1% formaldehyde. Cells were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, 1 mM PMSF, and protease inhibitors) and sonicated by Sonifier 450 (Branson, Danbury, CT). Lysates were cleared by centrifugation and diluted in IP buffer (0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 140 mM NaCl, 1 mM PMSF, and protease inhibitors). Antibody (10  $\mu$ g) and 20  $\mu$ l protein A/G agarose were added to the precleared chromatin solution for incubation overnight at  $4^{\circ}\text{C}$ . Beads were washed with washing buffer (0.1% Triton X-200, 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA) and eluted in the elution buffer (1% SDS and 0.1 M NaHCO<sub>3</sub>). The solution was heated at  $65^{\circ}\text{C}$  for 4 h followed by proteinase K digestion and phenol/chloroform extraction. The extracted DNA was used for PCR using the primer pairs #895/#894 and #1099/#1100, respectively.

### Microdissection of staged tubules by transillumination microscopy

Separation of staged tubules was performed under a stereomicroscope. The wave of the seminiferous tubules was determined as earlier described (Parvinen and Vanha-Perttula, 1972) and tubules were dissected into two groups as follows: Group 1 containing dark zone and dark spot zone (stages II–VIII) and Group 2 containing pale zone and weak spot zone (stages IX–I). Isolated staged tubules were used for RNA extraction.

### RT-PCR

Total RNA was isolated from cells by TRIZOL reagent (Invitrogen). RT-PCR was performed essentially as previously described (Lui and Lee, 2005). RT product (2  $\mu$ l) was used as template for RT-PCR with a pair of nectin-2 and S16 primers (Table 1). Co-amplifications of nectin-2 and S16 were in their linear phases. The authenticity of the PCR product was confirmed by nucleotide sequencing.

### Data analysis

For all transfection assays, data were shown as mean  $\pm$  SD of duplicate assays in three independent experiments. For EMSAs, all studies were repeated three times and consistent results were obtained. Data from mutation study were analyzed by one-way ANOVA, followed by Tukey's multiple comparison tests using the computer software PRISM (GraphPad Software, Inc., San Diego, CA).

## RESULTS

### Expression of mouse nectin-2 in testicular cell lines

RT-PCR was performed to study the expression of nectin-2 at the mRNA levels in two testicular cell lines, TM4 (Sertoli cell line) and GC-2spd(ts) (germ cell line). A 542-bp PCR fragment was obtained in both cell lines with a higher mRNA level in GC-2spd(ts) cells (Fig. 1A). The authenticity of the PCR products was confirmed by DNA base sequencing.

### Mapping of the mouse nectin-2 promoter in TM4 and GC-2spd(ts) cells

Although the transplantation study demonstrated that *nectin-2* gene in germ cells is not responsible for the Sertoli-germ cell adhesion, it is possible that nectin-2 exerts a yet-to-identified function other than cell adhesion in the seminiferous epithelium since germ cells express a relatively high level of nectin-2 in the testis (Ozaki-Kuroda et al., 2002).

To locate the active promoter regions of the *nectin-2* gene, progressive 5'-deletion mutants were constructed and analyzed in TM4 and GC-2spd(ts) cells (Fig. 1B). Results of transient transfection study revealed that both TM4 and GC-2spd(ts) cells show similar promoter activity profiles. Deletion of the nectin-2 5'-flanking sequence from nt  $-946$  to  $-662$  had no apparent effect on the promoter activity in both cell lines. Further deletion of 134-bp from p( $-662/+34$ )Luc increased the promoter activities in both cell lines. Also, 5'-deletion of a 84-bp fragment from p( $-399/+34$ )Luc increased the promoter activity by 59.9% and 53.3% in TM4 and GC-2spd(ts) cells, respectively (Fig. 1B), suggesting that negative regulatory elements might locate between nt  $-399$  and  $-316$ .

The maximal promoter activities [13.6-fold for TM4 cells and 12.8-fold for GC-2spd(ts) cells] were obtained when 5'-sequence was deleted to nt  $-316$ . However, further removal of sequence to nt  $-211$  completely abolished the promoter activity. These results indicated that the core promoter of *nectin-2* gene is located within the region between nt  $-316$  and  $-211$  in both cell lines (Fig. 1B).

### Mutational analysis of putative CRE, Sp1, AP-1, and AP-2 motifs within the core promoter region

Two putative Sp1 binding sites, namely distal Sp1 (dSp1) (5'-TGGGCGGGGT-3', located from nt  $-306$  to  $-297$ , with 94.5% homology to the Sp1 consensus motif) and proximal Sp1 (pSp1) (5'-GGGGCGGGGC-3', located from nt  $-245$  to  $-235$ , with 94.5% homology to Sp1 consensus motif), and one each of the motifs including CRE, AP-1, and AP-2 (5'-TGACGTCA-3', located from nt  $-232$  to  $-225$ , with 100% homology to CRE consensus motif; 5'-TGAGTCC-3', located from nt  $-224$  to  $-218$ , with 85.3% homology to AP-1 consensus motif; 5'-GGCCCG-3', located from nt  $-217$  to  $-212$ , with 89% homology to AP-2 consensus motif) were identified within the core promoter region (Fig. 2A). To examine

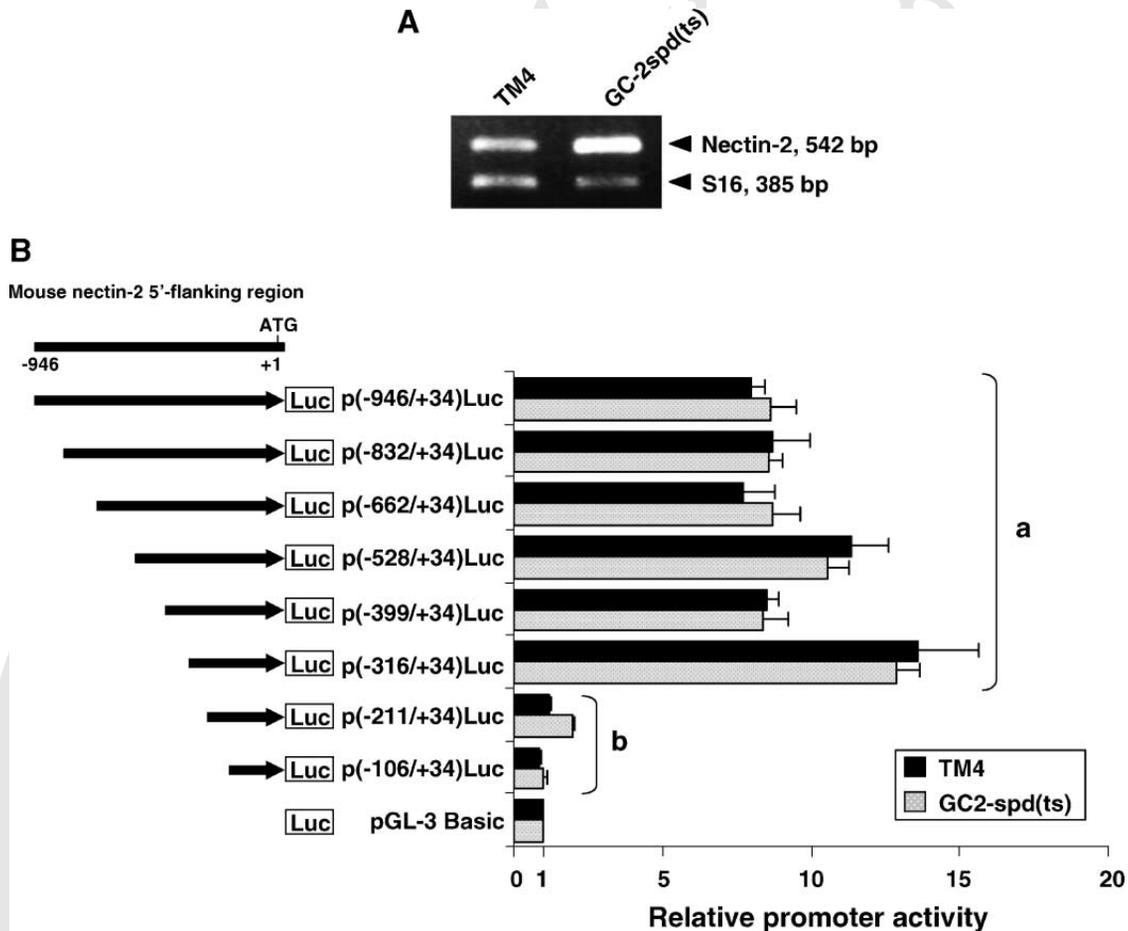


Fig. 1. **A–B:** Expression of mouse nectin-2 mRNA in TM4 and GC-2spd(ts) cells and progressive 5'-deletion analysis of nectin-2 promoter. **A:** Co-amplification of nectin-2 and S16 cDNAs from TM4 and GC-2spd(ts) cells using a pair of primers specific to *nectin-2* and *S16* genes. The authenticity of the PCR product was confirmed by sequencing analysis. **B:** Progressive 5'-deletion analysis of the mouse nectin-2 5'-flanking region was performed between nt -946 and +34 by direct PCR amplification of the corresponding regions, followed by subsequent cloning of the amplified fragments into the promoterless pGL-3

Basic vector. Various nectin-2 promoter-luciferase constructs were co-transfected with the pSV- $\beta$ -gal vector. The promoter activity of each construct was normalized by  $\beta$ -galactosidase activity from pSV- $\beta$ -gal plasmid. The relative promoter activity was represented as the fold induction when compared to the promoterless pGL-3 Basic vector. Values represent the mean  $\pm$  SD of three independent experiments each performed in duplicate. a,  $P < 0.01$  versus pGL-3 Basic; b, not significant versus pGL-3 Basic.

the functional significance of these motifs in regulating nectin-2 gene transcription in the testicular cells, site-directed mutants were constructed and transiently transfected into TM4 and GC-2spd(ts) cells, respectively (Fig. 2B). Single mutation of either dSp1, AP-1, and AP-2 motifs had no significant effect on the promoter activities of TM4 and GC-2spd(ts) cells (mutants A, D, and E vs. wild type), whilst mutation of the pSp1 motif caused a 24.8% and a 32.5% reduction of promoter activities in TM4 and GC-2spd(ts) cells (mutant B vs. wild type), respectively. Mutation of the CRE motif also significantly reduced the promoter activities in the TM4 (50.6% reduction) and GC-2spd(ts) cells (44.8% reduction) (mutant C vs. wild type). These results showed that mutation of either one of these motifs (pSp1 and CRE) could only partially abolish the promoter activity.

To examine whether there is any functional cooperation among these *cis*-acting elements, constructs containing double or triple mutation were analyzed in TM4 and GC-2spd(ts) cells. As depicted in Figure 2B, no significant further change was observed in TM4 and GC-2spd(ts) cells when two of the elements (dSp1 + pSp1, dSp1 + CRE, and AP-1 + CRE) were mutated concurrently (mutants F, G, and J vs. mutants B and C). However, a significant increase was observed in GC-

2spd(ts) cells, but not in TM4 cells, when double mutations of pSp1 and CRE motifs were performed compared with the corresponding pSp1 or CRE single mutation (mutant H vs. mutants B, C). A significant reduction (almost 70% reduction in both cell lines) was observed when triple mutation (dSp1, pSp1, and CRE motifs) was performed (mutant I vs. mutants F–H and wild type), indicating that these three regulatory motifs functionally co-operate with one another to stimulate the basal nectin-2 gene transcription.

#### Analysis of DNA–protein interactions of the dSp1 and pSp1 motifs by EMSAs

Results from site-directed mutagenesis suggest that the three motifs (dSp1, pSp1, and CRE) within the region between nt -316 and -212 are required for the basal promoter activity of *nectin-2* gene, we sought to examine and identify the transcription factors from these two cell lines that bound to these three motifs. EMSAs showed that DNA–protein complexes were formed in a dose-dependent manner with synthetic oligonucleotides containing either dSp1 or pSp1 motifs when nuclear extracts from TM4 and GC-2spd(ts) cells were used (Fig. 3A,C and 4A,C). As depicted in Figure 3A,C, three DNA–protein complexes (complexes



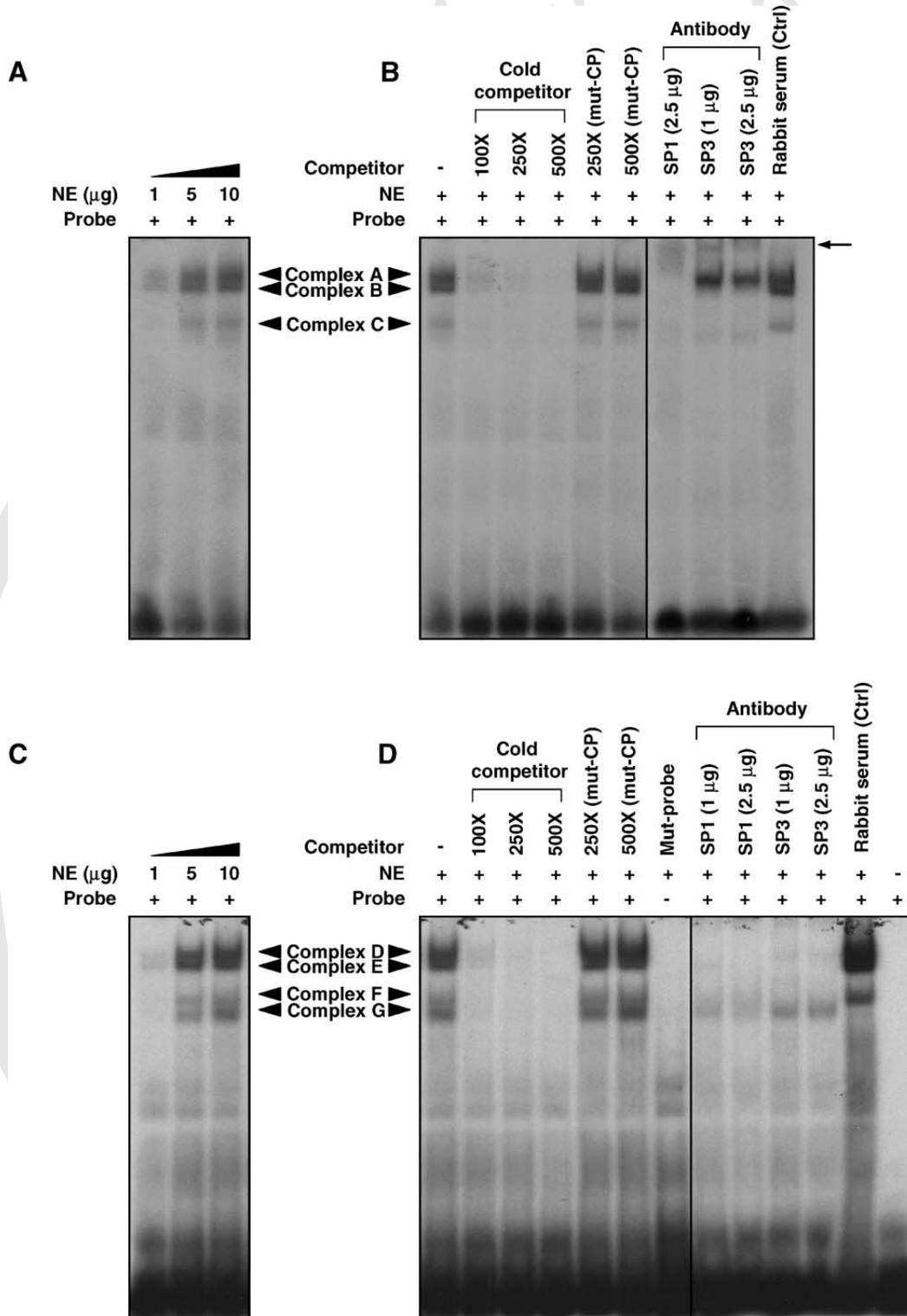


Fig. 3. **A–D**: EMSAs of the dSp1 motif using nuclear extracts from mouse TM4 and GC-2spd(ts) cells. Synthetic oligonucleotides containing the dSP1 sequence were annealed to form double strand DNA, end-labeled with  $^{32}\text{P}$  and incubated with nuclear extracts from TM4 and GC-2spd(ts) cells. Formation of DNA–protein complexes (complexes A–G, arrowheads) with increasing amount (1–10  $\mu\text{g}$ ) of TM4 (A) and GC-2spd(ts) (C) nuclear extracts. Nuclear extracts from TM4 (B) and GC-2spd(ts) (D) cells (10  $\mu\text{g}$ ) were incubated with the radiolabeled probe ( $\sim 50$  fmol) in the presence of an increasing amount

of a cold competitor (100- to 500-fold excess, lanes 2–4) or competitor containing the corresponding mutated sequence (250- and 500-fold excess, lanes 5 and 6). Mutated labeled probe was also incubated with the nuclear extracts as a control (D, lane 7). Nuclear extracts (10  $\mu\text{g}$ ) from TM4 (B, lanes 7–10) and GC-2spd(ts) (D, lanes 8–12) cells were pre-incubated with anti-Sp1, anti-Sp3 antibody, or rabbit serum before addition of the radiolabeled probes. The arrow represents the supershifted bands.

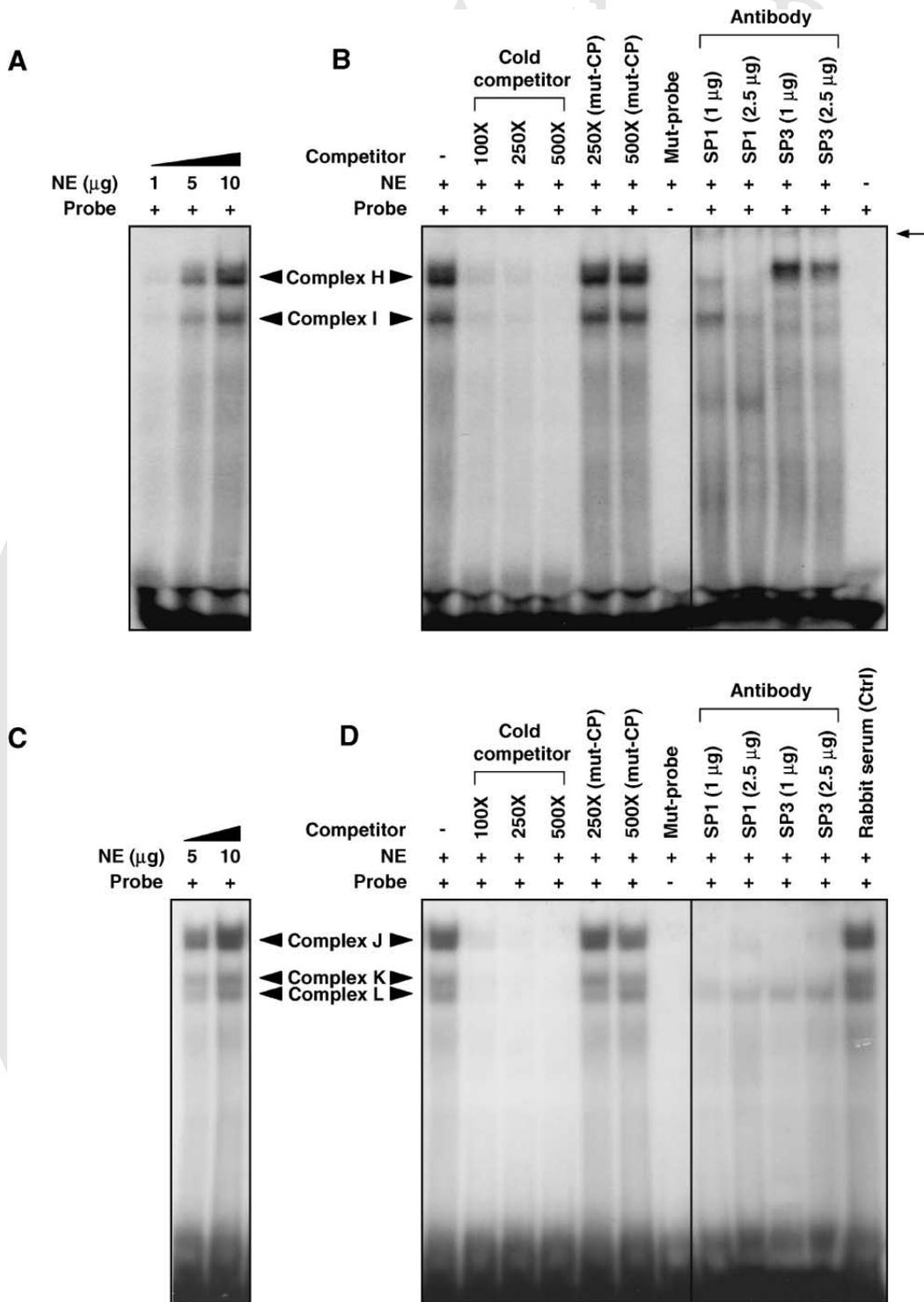


Fig. 4. **A–D**: EMSAs of the pSp1 motif using nuclear extracts from mouse TM4 and GC-2spd(ts) cells. Synthetic oligonucleotides containing the pSP1 sequence were annealed to form double strand DNA and end-labeled with <sup>32</sup>P and incubated with nuclear extracts from TM4 and GC-2spd(ts) cells. Formation of DNA–protein complexes (complexes H–L, arrowheads) with increasing amount (1–10 μg) of TM4 (A) and GC-2spd(ts) (C) nuclear extracts. Nuclear extracts from TM4 (B) and GC-2spd(ts) (D) cells (10 μg) were incubated with the radiolabeled probe (~50 fmol) in the presence of an increasing amount

of a cold competitor (100- to 500-fold excess, lanes 2–4) or competitor containing the corresponding mutated sequence (250- and 500-fold excess, lanes 5 and 6). Mutated labeled probe was also incubated with the nuclear extracts as a control (lane 7). Nuclear extracts (10 μg) from TM4 (B, lanes 8–11) and GC-2spd(ts) cells (D, lanes 8–12) cells were pre-incubated with either anti-Sp1, anti-Sp3 antibody, or rabbit serum before addition of the radiolabeled probes. The arrow represents the supershifted bands.

K was abolished in the presence of anti-Sp1 antibody (Fig. 4B,D, lanes 8 and 9), whilst the antibody against Sp3 inhibited the formation of complexes I, J, and K (Fig. 4B,D, lanes 10 and 11). Supershifted bands were

observed in TM4 cells when nuclear extracts were incubated with either anti-Sp1 or anti-Sp3 antibodies (Fig. 4B, lanes 8–11, arrow). Rabbit serum was used to determine the specificity of protein–protein interaction

(Fig. 4D, lane 12). The identity of complex L in GC-2spd(ts) cells remains to be determined since antibody targeted against the Sp1 family transcription factors did not affect the formation of complex L.

Taken together, these results confirmed that interactions of Sp1 and Sp3 transcription factors over the dSp1 and pSp1 motifs were responsible for the formation of those complexes in both cell lines except complexes G (dSp1 motif) and L (pSp1 motif) found in GC-2spd(ts) cells. Presence of unidentified transcription factors in complexes G and L suggests that the transcriptional machinery of *nectin-2* gene in TM4 and GC-2spd(ts) cells might not be exactly the same.

### Effects of overexpression of Sp1 and Sp3 on mouse *nectin-2* promoter activity

To ascertain the functional significance of Sp1 and Sp3 in regulating *nectin-2* gene transcription, expression plasmids encoding wild type Sp1 and Sp3 were co-transfected with p(-316/+34)Luc in TM4 and GC-2spd(ts) cells. As shown in Figure 5A, overexpression of Sp1 upregulated the *nectin-2* promoter activities in TM4 with an 1.5-fold induction when 1.5  $\mu$ g of Sp1 expression plasmids was used, although this induction was apparently not so significant in the case of GC-2spd(ts) cells. When the p(-316/+34)Luc construct was co-transfected

with Sp3 expression vector, a dose-dependent increase in promoter activity was observed in TM4 cells (Fig. 5B). However, no regulatory effect from forced expression of Sp3 was detected in GC-2spd(ts) cells (Fig. 5B), indicating that although Sp1 and Sp3 are capable to bind dSp1 and pSp1 motifs in both cell lines, Sp1 and Sp3 might exert their regulatory effect on *nectin-2* promoter in a cell type-specific manner.

### Analysis of DNA-protein interactions of the CRE motif by EMSAs

One DNA-protein complex (complexes M and N) was formed in TM4 and GC-2spd(ts) cells, respectively with the synthetic oligonucleotide containing the CRE motif (Fig. 6A,C). Formation of the complex was abolished dose-dependently in the presence of increasing fold-excess of unlabeled oligonucleotide (Fig. 6B,D, lanes 2–4), whereas addition of mutated CRE sequence failed to inhibit the formation of the complex (Fig. 6B,D, lanes 5 and 6). No DNA-protein complex was observed when mutated labeled probe or no nuclear extract was used (Fig. 6B, lanes 7 and 11; Fig. 6D, lane 7). To get insights into the nature of nuclear protein bound to the CRE motif, antibody supershift assay was performed. A slight supershifted band was observed in TM4 and GC-2spd(ts) cells when nuclear extracts were incubated with anti-CREB antibody (2.5  $\mu$ g) (Fig. 6B, lane 9; Fig. 6D, lane 10, arrow), but not with rabbit serum (Fig. 6B, lane 10; Fig. 6D, lane 11). Addition of anti-CREB antibody influenced partially the formation of DNA-protein complex, suggesting that unidentified transcription factors other than CREB bind to the CRE motif.

### CREB and mCREB (mutated at Ser133), but not KCREB and PKA, activate *nectin-2* promoter activity in both cell lines

To confirm the involvement of CREB in controlling the mouse *nectin-2* promoter activity, the pCMV-CREB expression vector was co-transfected with the p(-316/+34)Luc construct. As shown in Figure 7A, there were 34- and 13-fold increases in promoter activities in TM4 and GC-2spd(ts) cells, respectively, which confirms that CREB is one of transcription factors involved in the *nectin-2* gene transcription. It is well-documented that the phosphorylation of Ser133 in CREB is crucial for protein kinase A (PKA)-mediated gene activation (Lalli and Sassone-Corsi, 1994). It is of interest to investigate whether the *nectin-2* gene transcription requires the activation of PKA signaling pathway. Construct having CREB mutated at serine 133 (mCREB) was co-transfected with the p(-316/+34)Luc construct. To our surprise, significant increases (28- and 13-fold, respectively) of transcriptional activity persisted in TM4 and GC-2spd(ts) cells when the phosphorylation of CREB at serine 133 was blocked (Fig. 7A). These results suggest that the serine positioned at 133 in CREB is not the crucial phosphorylation site in the activation of *nectin-2* gene transcription and the activation of *nectin-2* transcription is PKA-independent. A dominant-negative mutant (KCREB) having mutations in its DNA-binding domain was co-transfected with the p(-316/+34)Luc construct, promoter activity was similar to the control (pCMV vector alone) observed in both cell lines when KCREB formed inactive dimers with endogenous CREB. These results suggest that transcription factor(s), other than CREB, might be able to bind CRE motif and exert its effect without the presence of CREB homodimer.

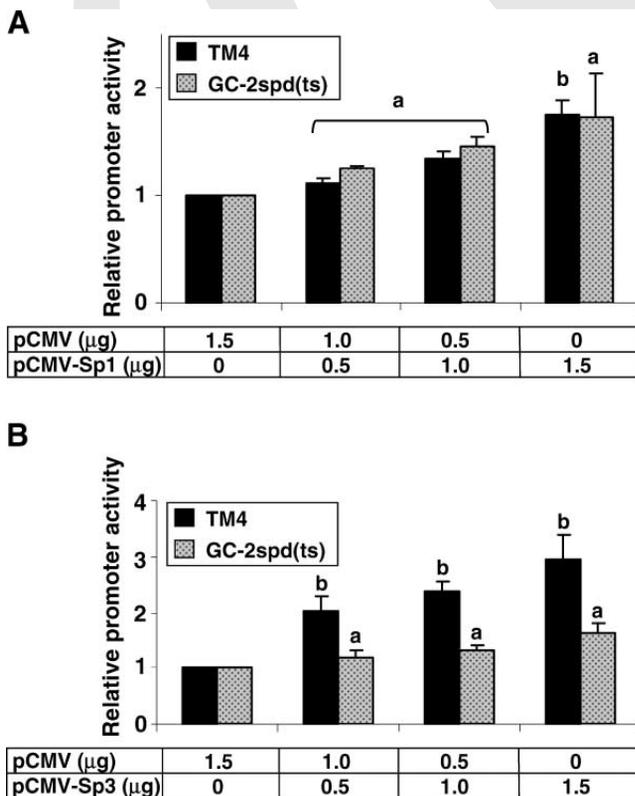


Fig. 5. **A–B.** Effects of overexpression of Sp1 and Sp3 on regulating the *nectin-2* promoter activity in TM4 and GC-2spd(ts) cells. The construct p(-316/+34)Luc was co-transfected with an increasing amount (0–1.5  $\mu$ g) of pCMV-Sp1 expression vector (A) or pCMV-Sp3 expression vector (B) into TM4 (solid) and GC-2spd(ts) (hatched) cells. The pCMV vectors were added to ensure that equal amounts of plasmids were used for transfection. The relative promoter activity was represented as the fold induction when compared to the control (pCMV vector) after normalized by  $\beta$ -galactosidase activity. Values represent the mean  $\pm$  SD of three independent experiments each performed in duplicate. a, not significant versus pCMV alone; b,  $P < 0.01$  versus pCMV alone.

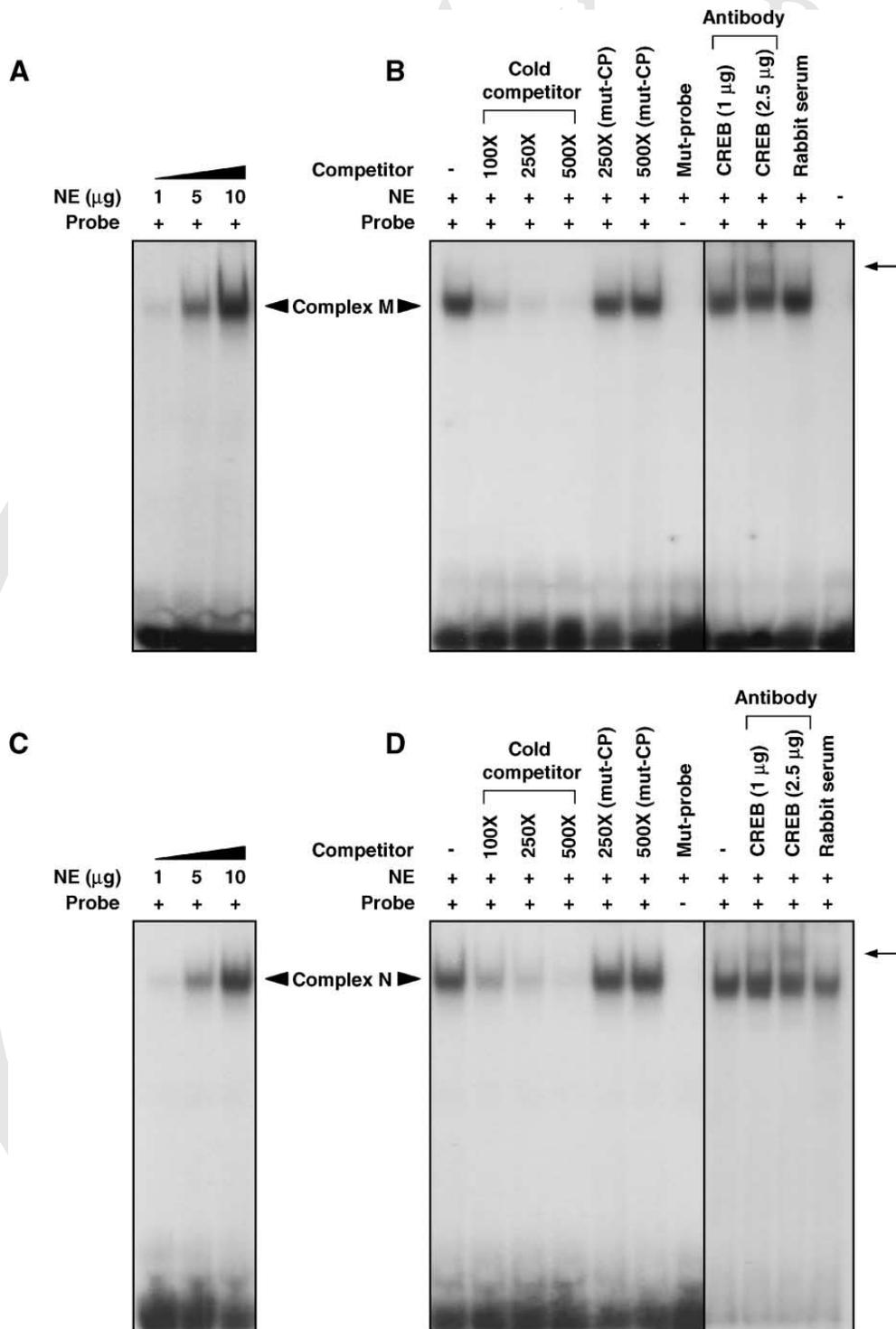


Fig. 6. **A–D**: EMSAs to characterize the CRE motif using nuclear extracts from TM4 and GC-2spd(ts) cells. Synthetic oligonucleotides containing the CRE motif were annealed to form double-stranded DNA and end-labeled with  $^{32}\text{P}$ . The labeled probe were incubated with an increasing amount of nuclear extracts (1–10  $\mu\text{g}$ ) derived from TM4 (A) and GC-2spd(ts) (C) cells to allow the formation of DNA–protein complex. Nuclear extracts (10  $\mu\text{g}$ ) from TM4 (B) and GC-2spd(ts) (D) cells were incubated with the radiolabeled CRE probe in the presence of an increasing amount of cold competitor (100- to 500-fold excess,

lanes 2–4) or mutated cold competitor (250- and 500-fold excess, lanes 5–6). No DNA–protein complex was formed when mutated labeled probe (B and D, lane 7) was used. Supershift analyses were performed by pre-incubation of anti-CREB antibody or rabbit serum with TM4 (B, lanes 8–10) and GC-2spd(ts) (D, lanes 9–11) nuclear extracts (10  $\mu\text{g}$ ) before the addition of the labeled CRE probe. Specific DNA–protein complexes are indicated with black arrowheads (complexes M and N) and the arrows represents the supershifted bands.

Overexpression of mCREB was unable to inhibit the nectin-2 promoter activity, suggesting that the activation of nectin-2 gene transcription is in a PKA-independent manner. To investigate whether nectin-2 gene transcription requires the activation of PKA signaling

pathway, we tested the effect of PKA overexpression, forskolin (adenylate cyclase activator), and H-89 (protein kinase A inhibitor) on nectin-2 promoter activities of two cell lines. Cells co-transfected with p(-316/+34)Luc construct and pCMV-PKA vector showed no

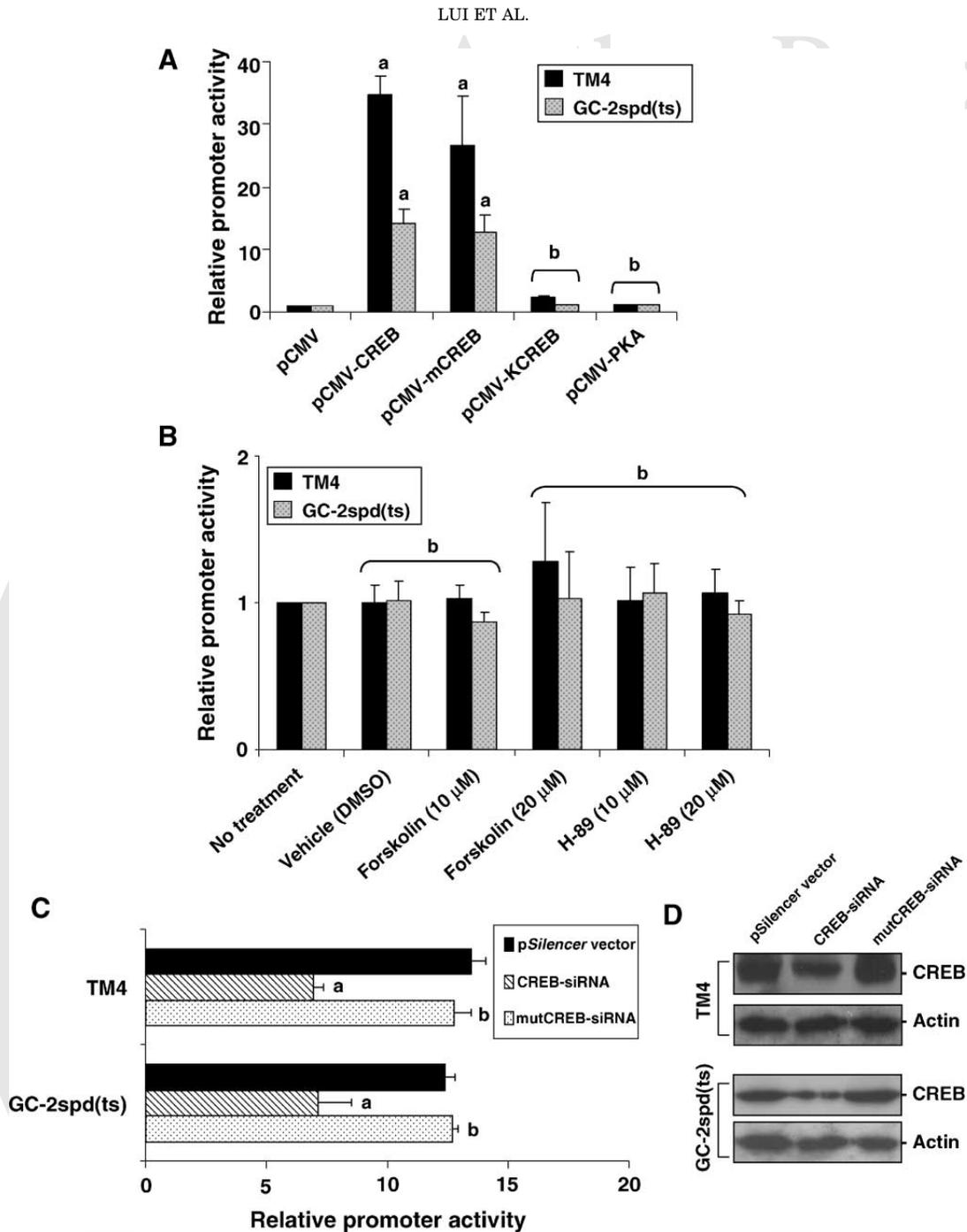


Fig. 7. **A–D**: Effects of CREB, mCREB, KCREB, PKA, forskolin, H-89, and CREB-siRNA on nectin-2 promoter activity in TM4 and GC-2spd(ts) cells. **A**: The p(-316/+34)Luc construct was co-transfected with the expression vectors (pCMV-CREB, pCMV-mCREB, pCMV-KCREB, and pCMV-PKA) into TM4 and GC-2spd(ts) cells. **B**: Cells transfected with p(-316/+34)-Luc construct were treated with different concentrations of forskolin (10  $\mu$ M or 20  $\mu$ M) or H-89 (10  $\mu$ M or 20  $\mu$ M) for 3 h before harvest. **C**: p(-316/+34)-Luc construct was cotransfected with pSilencer vector alone, specific CREB-siRNA, or mutant CREB-siRNA (mutCREB-siRNA) in TM4 and GC-2spd(ts).

The promoter activity of each test group was normalized by  $\beta$ -galactosidase activity. The relative promoter activity is represented as the fold induction when compared to pCMV alone in (A), control group (no treatment) in (B) or pGL-3 basic vector in (C). **D**: Proteins were extracted from cells and probed with anti-CREB and anti-actin antibodies. Values represent the mean  $\pm$  SD of three independent experiments each performed in duplicate. a,  $P < 0.01$  versus control (pCMV alone); b, not significant versus control (pCMV alone, no treatment or pSilencer alone).

significant change in promoter activities (Fig. 7A). Cells transfected with p(-316/+34)Luc construct were incubated with either forskolin or H-89 for 3 h before harvest for luciferase assay. It was found that neither forskolin nor H-89 alter the promoter activities compared to the control (no treatment) in both cell lines (Fig. 7B). These data strengthen the notion that the basal transcription of *nectin-2* gene in TM4 and GC-2spd(ts) cells do not require the activation of PKA pathway.

To further confirm a functional role of CREB on nectin-2 transcription, the siRNA approach was used. Co-transfection of a specific CREB-siRNA with p(-316/+34)Luc blunted the nectin-2 promoter activity by approximately 50% in both TM4 and GC-2spd(ts) cells, whereas a mutated version of the sequence and the pSilencer 1.0-U6 vector alone had no effect (Fig. 7C). The CREB-siRNA reduced of about 50% the protein level, in agreement with the reduced activity of the reporter

construct. The mutated siRNA and vector alone showed no effect (Fig. 7D). These data strongly support the notion that CREB is a major transcription regulator of nectin-2 promoter.

**The basic helix-loop-helix (bHLH) transcription factor (c-Jun) binds to the putative CRE motif**

Both EMSA analyses and overexpression studies demonstrated that an unknown transcription factor bound to the CRE motif was playing a crucial role in

regulating nectin-2 gene transcription. To identify the proteins that participated in the above interaction, EMSA was performed in the presence of several consensus oligonucleotides including AP-1, Egr, and Smad3/4 (Fig. 8A). The formation of complexes M and N was partially abolished in the presence of AP-1 consensus oligonucleotide (250- to 500-fold excess) in both TM4 and GC-2spd(ts) cells when compared to the control (Fig. 8B,C, lanes 2 and 3 vs. lane 1), but not inhibited by other sequences such as the Egr consensus or Smad3/4

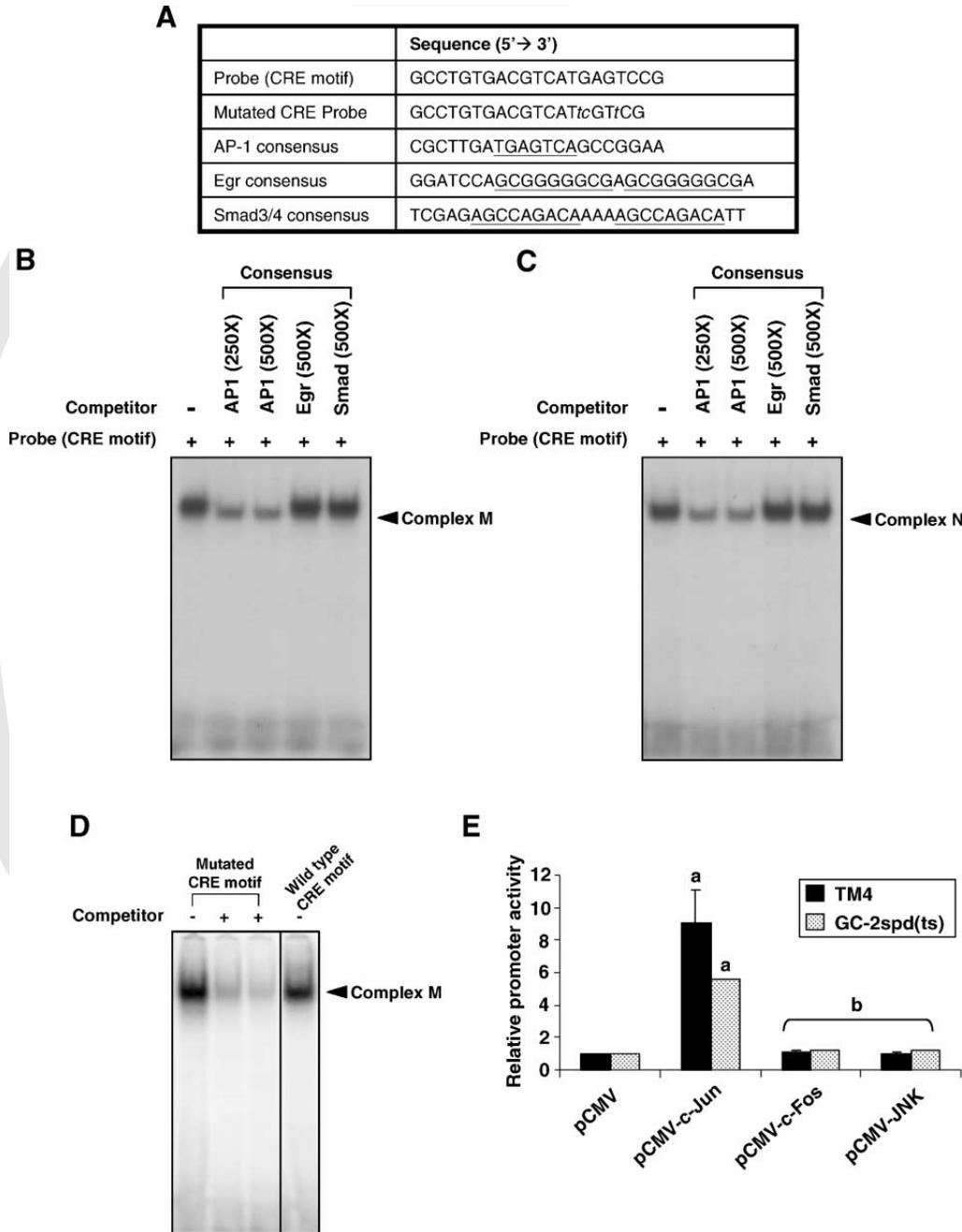


Fig. 8. **A–E**: Binding of AP-1 transcription factor c-Jun, but not c-Fos, to the CRE/AP-1 motif and effects of AP-1 transcription factors on nectin-2 promoter activity. **A**: Nucleotide sequences of CRE motif (probe) and consensus AP-1, Egr, and Smad3/4 oligonucleotides used in EMSAs are shown. Nuclear extracts (10  $\mu$ g) from TM4 (**B**) and GC-2spd(ts) (**C**) cells were incubated with the radiolabeled probe (CRE/AP-1 motif) in the presence of consensus AP-1 (250- and 500-fold excess), Egr (500-fold excess) or Smad3/4 (500-fold excess) oligonucleotides. A CRE motif having the intact CRE site and mutated AP-1

site (**D**, lanes 1–3 vs. wild type probe, lane 4) was used in EMSA to assess the complex formation using nuclear extracts from TM4 cells. **E**: The p(-364/+34) construct were co-transfected with either empty expression vector (pCMV) or expression vector encoding c-Jun, c-Fos, and JNK cDNAs. The relative promoter activity was represented as the fold induction when compare to the control (pCMV alone) after being normalized by  $\beta$ -galactosidase activity. Values represent the mean  $\pm$  SD of three independent experiments each performed in duplicate. a,  $P < 0.01$  versus control; b, not significant versus control.

binding motif (500-fold excess) (Fig. 8B,C, lanes 4 and 5), suggesting specific interactions between the putative CRE motifs and AP-1 transcription factors.

To ensure that the AP-1 protein binds to the CRE motif rather than the nearby AP-1 motif, a mutated labeled probe having the intact CRE motif along with mutated AP-1 site was used in EMSA analysis. As shown in Figure 8D, the mutated labeled probe was capable to form the complex M using TM4 nuclear extract (lane 1 vs. lane 4, wild type probe). When cold competitors were added, the complex formation was inhibited dose-dependently (lanes 2 and 3). Similar result was obtained when germ cell nuclear extract was used (data not shown). These results indicate that complexes M and N were bound to the CRE motif instead of AP-1 motif.

AP-1 transcription factor consists of either Jun homodimers or Fos/Jun heterodimeric complexes. As shown in Figure 8E, co-transfection of the pCMV-c-Jun expression vector increased the promoter activity of p(-316/+34)Luc construct more than eightfold and fivefold over the controls in TM4 and GC-2spd(ts) cells, respectively. No such increase was detected when pCMV-c-Fos or pCMV-JNK overexpression vector was co-transfected with p(-316/+34)Luc into TM4 and GC-2spd(ts) cells. These results demonstrated that c-Jun, an AP-1 transcription factor family member, is capable to bind to the CRE motif to drive the nectin-2 transcription machinery.

#### In vivo binding of CREB, Sp1, and c-Jun to the mouse nectin-2 promoter

To assess the in vivo association of CREB, Sp1, and c-Jun with the mouse nectin-2 promoter, the ChIP assay was performed in TM4 and GC-2spd(ts) cells (Fig. 9B, upper part). Positive PCR signals were detected in both TM4 and GC-2spd(ts) using primer pair #895/#894 when immunoprecipitation was performed using anti-CREB (lane 2), anti-Sp1 (lane 3), anti-Sp3 (lane 4), anti-c-Jun (lane 5) antibody, but not anti-p53 antibody (lane 6), supporting the notion that these proteins including CREB, Sp1, and c-Jun interact with the nectin-2 promoter in in vivo situation. No PCR signal was observed from the negative controls including: rabbit serum (lane 7) and no template (lane 9). Negative PCR signal were obtained in both cell lines using primer pair #1099/#1100, which amplifies a non-specific sequence of the *nectin-2* gene about one kilobase downstream from the promoter region (Fig. 9B, lower part).

#### Changes in the steady-state mRNA levels of CREB and nectin-2 in staged seminiferous tubules

Tubules were isolated by transillumination stereomicroscopy and divided into two groups. These included the dark zone combined with dark spot zone (stages II–VIII) and weak spot zone combined with pale zone (stages IX–I). The steady-state CREB mRNA was detected in all stages of the seminiferous tubules, yet their levels became lower in stages IX–I (Fig. 10, upper part). This pattern of CREB expression was consistent with previous studies that there is a cyclic expression of CREB in the seminiferous epithelium where high level of CREB mRNA is present in stages I–VIII of the spermatogenic cycle (Waerber et al., 1991). The steady-state nectin-2 mRNA level exhibits a similar expression pattern with a higher mRNA level at stages II–VIII (middle part). This coordinated relationship between CREB and nectin-2 supports the hypothesis that cyclic expression of CREB plays a role in controlling the timely

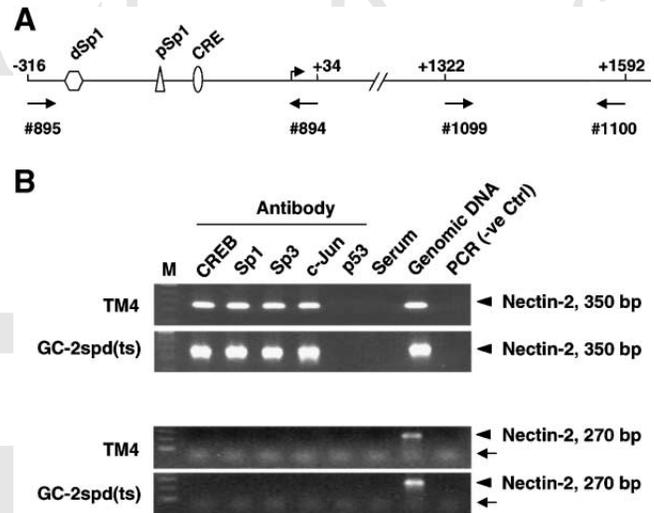


Fig. 9. A–B: Chromatin immunoprecipitation analysis for CREB, Sp1, and c-Jun association with mouse nectin-2 promoter. A: A diagram of the mouse nectin-2 promoter showing the two Sp1 binding sites and the CRE motif. Arrows indicate the primers used for PCR analysis and their relative location. B: Nuclear proteins from TM4 and GC-2spd(ts) cells and genomic DNA were cross-linked by formaldehyde and isolated genomic DNA was immunoprecipitated with anti-CREB, anti-Sp1, anti-Sp3, anti-c-Jun, or anti-p53 antibody (lanes 2–6, respectively). After immunoprecipitation, the mouse nectin-2 promoter (-316/+34) was amplified by PCR using two sets of primer pairs: #895/#894 (upper part) and #1099/#1100 (lower part). PCR products were analyzed by an agarose gel. Immunoprecipitation using anti-p53 antibody or rabbit serum were carried out as negative control. Black arrows represent the primer dimers.

expression of *nectin-2* gene in the seminiferous epithelium, which in turn modulates the dynamic of cell junctions between Sertoli cells and germ cells.

#### DISCUSSION

In the present study, we have characterized the regulatory elements involved in the expression of mouse *nectin-2* gene in two testicular cell lines, TM4 (Sertoli) and GC-2spd(ts) (germ) cells. The nectin-2 core promoter is relatively short (~100 bp in length) and lacks canonical TATA and CAAT boxes. It contains two putative Sp1 binding sites and one each of the CRE, AP-1, and AP-2 motifs next preceding to each other.

Mutational analysis showed that single mutation of either pSp1 or CRE motifs partially abolished the promoter activity in TM4 and GC-2spd(ts) cells. Interestingly, a significant rebound in promoter activity was observed in GC-2spd(ts) cells, but not in TM4 cells when

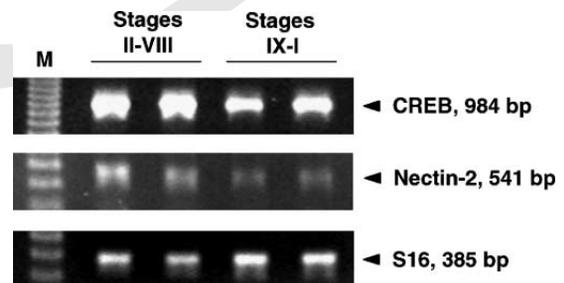


Fig. 10. Changes in the steady-state mRNA levels of CREB and nectin-2 in staged seminiferous tubules. Staged tubules were isolated by transillumination stereomicroscopy. Total RNA was extracted and RT-PCR was performed using gene-specific primers. The authenticity of the PCR product was confirmed by sequencing analysis.

both pSp1 and CRE motifs were mutated. Since in the EMSA analysis, there was an apparent difference in the pattern of DNA–protein complex formation the pSp1 motif when nuclear extracts from TM4 and GC-2spd(ts) cells were used, an unknown trans-acting factors of the pSp1 motif in GC-2spd(ts) cells might be able to interact with the pSp1, CRE, or dSp1 motif, but with the preference of interacting with the pSp1 and CRE motifs to form a core complex. When both pSp1 and CRE motifs were mutated, this unknown nuclear factor element still exerted its positive effect via interaction with the dSp1 motif. Therefore, the promoter activity could be maintained in GC-2spd(ts) cells. When both dSp1, pSp1 and CRE motifs are concurrently mutated, this unknown factor failed to interact with either one of the motifs, which results in a significant drop in promoter activity. In addition, co-transfection of either Sp1 or Sp3 overexpression plasmids significantly increased nectin-2 promoter activity in TM4 cells, whereas overexpression of Sp1 and Sp3, exerted no significant regulatory effect on nectin-2 promoter activity in GC-2spd(ts) cells. These results from EMSAs and overexpression analyses unequivocally demonstrated that the regulatory machinery of nectin-2 gene transcription in TM4 and GC-2spd(ts) cells might not be exactly the same.

Another intriguing finding in this study is the involvement of CREB and AP-1 family proteins in the regulation of nectin-2 gene transcription. Overexpression of CREB in TM4 as well as in GC-2Spd(ts) cells exert 34- and 14-fold increase in promoter activity respectively, suggesting that CREB acts as an activator in nectin-2 gene transcription. The involvement of CREB family members in transcriptional regulation in the testis is not limited to *nectin-2* gene, several genes involved in spermatogenesis such as murine spermatogenesis-associated protein-2 gene and murine sperm adhesion molecule-1 gene have been found to be regulated by CREB via the CRE motif (Zheng and Martin-Deleon, 1999; Slongo et al., 2003). Previous studies using in situ hybridization analysis has showed that Sertoli CREB mRNA is present in stages I–VIII of the spermatogenic cycle and the amount decreases to an undetectable level during stages IX–XIV (Waeber et al., 1991). The time frame of high CREB expression level at stages I–VIII coincides with the formation of nectin-2-based Sertoli–spermatid AJs (for reviews, see Don and Stelzer, 2002; Ozaki-Kuroda et al., 2002). Our analysis of the staged tubules provides an additional line of evidence to show cyclic expressions of CREB and nectin-2 on junction dynamics in the seminiferous epithelium. It is logical to speculate that the presence of high level of CREB at stages II–VIII might upregulate the nectin-2 gene transcription and allow the formation of nectin-2-based Sertoli–spermatid AJs. At stage IX–I, spermiation takes place to allow the release of mature spermatids, which is concomitant with low level of CREB expression. It is believed that the basal nectin-2 gene transcription is greatly inhibited without the presence of CREB at these stages, resulting in the disassembly of AJs between Sertoli cells and spermatids.

Other than CREB, it has also shown that the formation of DNA–protein complexes M and N on CRE labeled probe (CRE motif + AP motif) were partially abolished in the presence of the nectin-2 AP-1 consensus oligonucleotides. These observations suggest that the member(s) of AP-1 transcription factor family is capable to interact with the CRE/AP-1 motif. The formation of complexes M and N was not affected by the use of the mutated labeled CRE probe (intact CRE motif +

mutated AP motif). These results clearly demonstrate the CRE motif is the *cis*-acting element involved in nectin-2 gene transcription, but not the AP-1 motif. To ascertain the functional importance of the AP-1 protein to the CRE motif, overexpression of two members of AP-1 protein including c-Jun and c-Fos were performed. To our surprise, a significant increase in the promoter activity in TM4 and GC-2spd(ts) cells [eightfold in TM4 and sixfold GC-2spd(ts) cells] was observed when c-Jun, but not c-Fos, was overexpressed. Studies have revealed that AP-1 proteins can form either Jun homodimers or Fos/Jun heterodimers and interact with the CRE motif to drive the promoter activity, as in the case of human neurotensin/neuromedin N promoter (Hai et al., 1989; Hadman et al., 1993; Chatton et al., 1995; Evers et al., 1995). Our data clearly demonstrated that induced expression of c-Fos has no apparent regulatory effect on nectin-2 promoter as heterodimers formed by exogenous c-Fos protein with endogenous Jun proteins, like c-Jun and Jun D, were unable to activate nectin-2 promoter in both cell lines. Since c-Jun is capable to form either homodimers or heterodimers with CREB (c-Jun/CREB) and exert its regulatory effect via the interaction with the CRE motif on certain promoters such as human cyclin D1 promoter (Sabbah et al., 1999), it is possible that the positive regulatory effect on nectin-2 promoter is mediated by either c-Jun homodimer or c-Jun/CREB heterodimer.

In conclusion, the mouse nectin-2 gene transcription in TM4 and GC-2spd(ts) cells required functional co-operation of multiple transcription factors including Sp1, Sp3, CREB, c-Jun, and an unknown transcription factor. Given the fact that the heterotypic intercellular junctions with Sertoli cells are downregulated when spermatids are released (Takai and Nakanishi, 2003), it is possible that the cyclic expression of CREB in seminiferous epithelium might be a crucial mechanism to regulate the assembly and disassembly of Sertoli–spermatid AJs via the control of nectin-2 gene transcription.

#### ACKNOWLEDGMENTS

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