Research Paper

Nuclear factor-Y (NF-Y) regulates transcription of mouse *Dmrt7* gene by binding to tandem CCAAT boxes in its proximal promoter

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Abstract

Dmrt7, a member of the *Dmrt* family of genes, is required for spermatogenesis. However, promoter functions of the gene *Dmrt7* remain unknown. We have cloned and characterized the proximal promoter region of the mouse *Dmrt7* gene. Functional analysis of the 5' flanking region by sequential deletion mutations revealed crucial positive elements between -60 and +1, in which two highly conserved and tandem CCAAT boxes: the CCAAT box1 (-48/-44) and the CCAAT box2 (-7/-3) are located. Site-directed mutagenesis studies demonstrated that both CCAAT boxes are indispensable to the promoter activity. Electrophoretic mobility shift assays (EMSAs) and gel-supershift assays indicated that transcription factor NF-Y binds to the promoter. Chromatin immunoprecipitation (ChIP) analysis demonstrated that NF-Y interacts *in vivo* with the promoter of the *Dmrt7* gene in testis. Co-transfection and reporter analysis showed that over-expression of NF-Ys increased transcription of the *Dmrt7-luc* gene whereas expression of a dominant-negative NF-Ya decreased the transcription. This suggests that NF-Y can activate the *Dmrt7* promoter. These results provide evidence of a transcription regulatory mechanism that controls *Dmrt7* gene expression in mouse testis.

Key words: Endocrinology, Spermatogenesis, DM domain, Transcriptional regulation

Introduction

The genes *doublesex* in *Drosophila* and *mab-3* in *Caenorhabditie elegans* were first identified as transcription factors containing a conserved zinc finger-like DNA binding domain (DM domain), which plays a key role in sexual development (1-3). Many organisms have multiple *Dmrt* (doublesex and mab-3 related transcriptional factor) genes, the number of which varies between species. For example *Drosophila, Caenorhabditis elegans*, fish, mice and human have four, eleven, six, seven and eight copies respectively (4-9).

From arthropods to nematodes to vertebrates, most *Dmrt* genes are expressed in gonads and play roles in sex determination, which serves as a rare example of conserved mechanisms underlying sex

regulation during evolution. *Dmrt1* was the first DM domain gene identified in vertebrates (10). Previous research with *Dmrt1*-deficient mice strongly demonstrated that this gene was required for testis differentiation after determination, but dispensable for ovary development (11). In birds, *DMRT1* is sex-linked (12, 13) on chromosome Z and there is higher dosage of the gene in males (ZZ) as compared with females (ZW) (10, 13-15). The expression of *Dmrt1* in turtles and alligators was found to be related to sexual differentiation and was higher in developing male gonads than in female ones as well (16). Furthermore, the *Dmrt1y/DMY* gene, which is the homolog of

Dmrt1 in medaka, transposed to chromosome Y and became a master gene in male differentiation (17, 18).

However, evidence is being accumulated that some proteins in the DMRT family are involved in non-gonadal development. A null mutation for the gene *Dmrt1*, an ancient conserved gene common to sex-determining pathways, resulted in profound abnormalities in the development of anterior neural plate derivatives in Ciona (19). Both *Dmrt2a* and *Dmrt2b* are expressed in presomitic mesoderm and developing somites and contribute to somitogenesis and the creation of left-right asymmetry in the lateral-plate mesoderm (20-23). In chicken and mice, *Dmrt3* is expressed similarly in the forebrain, spinal cord, and nasal placode (24), while *Dmrt3* in zebrafish is expressed in the olfactory placode and the neural tube (25). *Xenopus Dmrt4* is expressed in the developing olfactory system and is required for neurogenesis (26). Both *Dmrt5* and *Dmrt6* are expressed primarily in the brain (9, 27).

In addition to *Dmrt1*, *Dmrt7* expression is restricted to embryonic gonads and adult testis (9). *Dmrt7* deficient mice show male infertility with spermatogenic arrest at the pachytene stage and defects in regulation of sex chromatin (28, 29). Kawamata M, et al. reported a possible post transcriptional role of poly-adenylation in *Dmrt7* expression (30). However, the transcriptional mechanisms regulating *Dmrt7* need to be studied further, which will contribute substantially to our understanding of the pathway of sex differentiation and spermatogenesis. Here we report on the nature of the mouse *Dmrt7* promoter. Deletion and site-directed mutagenesis were used to identify regions of the promoter that are required for transcriptional activity. EMSA and ChIP analysis was employed to determine the binding relationship between transcription factor NF-Y and the *Dmrt7* promoter. Finally, our study showed that NF-Y up-regulated the expression of *Dmrt7* by binding to two tandem CCAAT-boxes in the proximal promoter region of the gene.

Materials and Methods

In silico **sequence analysis.** Transcription factor binding sites were predicted using MatInspector (www.genomatrix.de) and TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html).

Plasmid constructions. Six deletion mutants of the mouse *Dmrt7* promoter were constructed using PCR cloning from mouse genomic DNA and primers: forward primer, 5'TATACGCGTCTAGAGGTCACACACAAAATCAGAAG3' for construct -948/+116, 5'TGCACGCGTAAAGGCAGGCTAAAAAGCCTGCC3' for construct -407/+116, 5'GGGACGCGTCA

GCCTCGCTCTGGCTGAGGT3' for construct -245/+116, 5'TTGACGCGTCGGAGAAGCGGGTAGGCAAGAAA3' for construct -104/+116, 5'CGGACGCGTATTGCAAACCCTATTGGCTGCGC3' for construct -60/+116, 5'CTCACGCGTCCTTGTGTGAAGGAGCGAGCGG3' for construct +1/+116, and a common reverse primer, 5'CACAAGCTTCACAGCCTCGAGCCGAATCACAG3'. PCR products were double-digested with *Mlu*I and *Hind*III and cloned into the *Mlu*I - *Hind*III site of the pGL3-basic vector (Promega, Madison,WI).

Site-directed mutagenesis for two CCAAT boxes was performed using the following primers as follows: Mut1 for CCAAT box1 mutant: 5'TGCAAACCCTCTGATCTGCGCGGCGCCG3' and 5'CGGCGCCGCGCAGATCAGAGGGTTTGCA3'; mut2 for CCAAT box2 mutant, 5'GTGCTTGGAGCTCCTGATTCCTTGTGTG3' and 5'CACACAAGGAATCAGGAGCTCCAAGCAC3'; mut1/2 for both CCAAT box 1 and 2 mutants using mut1 as a template: 5'GTGCTTGGAGCTCCTGATTCCTTGTGTG3' and 5'CACACAAGGAATCAGGAGCTCCAAGCAC3'. All constructs were sequenced.

For the construction of expression plasmids, the full-length cDNAs of mouse wild-type NF-Ya, NF-Yb and NF-Yc were PCR-amplified from mouse testis cDNAs and cloned into *Eco*RI site of the pCX-EGFP vector by replacing the EGFP fragment to generate expression plasmids (pCX-NFYa, pCX-NFYb and pCX-NFYc) using primers:, 5'CGTGAATTCGCCATGGAGCAGTATA3' and 5'CTCGAATTCTTAGGAAACTCGGATGA3' for NF-Ya; 5'ATAGAATTCATCATGACAATGGACGG3' and 5' TCCGAATTCTCATGAAAACTGAATTTG3' for NF-Yb; 5'GCCGAATTCAAAATGTCCACAGAAG3' and 5'CTCGAATTCTCAGTCTCCAGTCACCT3' for NF-Yc. The mouse dominant negative plasmid for NF-Ya (NF-YAm29) was a generous gift from Dr. Roberto Mantovani (University of Milano). All constructs were confirmed by DNA sequence analyses.

Cell culture, transient transfection and dual-luciferase reporter assay. GC-1 (a cell line from type B spermatogonia of mouse and show characteristics of a stage between type B spermatogonia and primary spermatocytes) and COS 7 cells (a cell line was obtained by immortalizing a CV-1 cell line derived from kidney cells of the African green monkey) were maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, plated in 48-well plates and transfected using Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA). The cells were transfected with 0.4 µg of each deletion construct (-948/+116, -407/+116, -245/+116,

-104/+116, -60/+116, or +1/+116) along with 10 ng/well pRT-TK (an internal control). For luciferase assays, 0.2 µg of construct -60/+116 or mutated constructs, and 0.2 µg NF-Y expression vectors (NF-Ya, NF-Yb and NF-Yc) or the dominant negative NF-Y plasmid (NF-YAm29) were co-transfected into the cells. Empty vector pGL3-basic or pCX (pCX-EGFP without the EGFP coding sequence between two *Eco*RI sites) was added to equalize the final DNA content in each well. Luciferase activity was measured 24 hours after transfection using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) and a Modulus Single Tube Multimode Reader (Turner Biosystems, Sunnyvale, CA, USA) following the manufacturer's protocol. Assays were performed in triplicate and expressed as means ± S.D.

Electrophoretic mobility shift assays (EMSA). Oligonucleotides corresponding to the CCAAT box1 and box2 of the *Dmrt7* promoter were synthesized and annealed into double strands. Their sequences are as follows: 5'TGCAAACCCTATTGGCTGCGCG GCGCCG3' and 5'CGGCGCCGCGCAGCCAATAG GGTTTGCA3' for oligo1; 5'TGCAAACCCTCTG ATCTGCGCGGCGCCG3' and 5'CGGCGCCGCG CAGATCAGAGGGTTTGCA3' for oligo1-ccaat mut; 5'GTGCTTGGAGCTCATTGGTCCTTGTGTG3' and, 5'CACACAAGGACCAATGAGCTCCAAGCAC3' for oligo2; 5'GTGCTTGGAGCTCCTGATTCCTTGT GTG3' and 5'CACACAAGGAATCAGGAGCTCCAA GCAC3' for oligo2-ccaat mut. Radiolabeled probes were generated by incubation of 250 ng annealed oligonucleotides with 20 µCi [γ-32P] dATP in the presence of T4 Polynucleotide Kinase (Promega, Madison, WI, USA) for 1 h at 37°C, and were subsequently separated from free nucleotides for purification using a G-50 column (Amersham Biosciences, Uppsala, Sweden). Mouse testis nuclear extract used for Electrophoretic mobility shift assays was prepared as described previously (31). Then incubated at room temperature for 30 min with a 100,000 dpm radiolabeled probe and 1 µg poly (dI-dC) in a buffer of 10 mmol/l Tris-HCl, pH 7.5, 50 mmol/l NaCl, 1 mmol/l dithiothreitol, 1 mmol/l EDTA, and 5% glycerol. For supershift experiments, binding reactions were subsequently incubated with anti-NF-Ya (Abcam Inc., Cambridge, CA, USA) for 30 min at room temperature. For competition experiments, the unlabeled competitor oligos (in 50-fold molar excess) were added together with probe at the start of the incubation. Samples were resolved in 5% polyacrylamide gels in 0.5% TBE running buffer at 10 V/cm for 2 h. The dried gel was exposed to a phosphorimager cassette and scanned with typhoon 9200 instrument (GE-Healthcare, Amersham bioscience, Uppsala, Sweden).

Chromatin immunoprecipitation (ChIP) and quantitative real-time PCR. Samples of mouse testis and liver were chopped into small pieces with a scalpel in cold phosphate-buffered saline (PBS) and cross-linked in 1% formaldehyde-PBS for 15 min with constant shaking. The tissues were rinsed in cold PBS and homogenized with a Dounce homogenizer in 1 ml cold cell lysis buffer (10 mM Tris-Cl, pH 8.0, 10 mM NaCl, 3 mM MgCl2, 0.5% NP-40) supplemented with protease inhibitors (Roche Diagnostics Ltd, Mannheim, Germany). The cells were incubated at 4°C for 5 min to allow the release of the nuclei. The nuclei were collected by centrifugation at 13,000 × g for 5 min. After lysis in the buffer (1% sodium dodecyl sulfate [SDS], 5 mM EDTA, 50 mM Tris-Cl, pH 8.1), sonication was performed with a Sonic Dismembrator model 100 sonicator (Fisher Scientific, Inc., Pittsburgh, PA, USA). The supernatant chromatin after centrifugation was immunoprecipitated with no antibody (beads only), preimmuno IgG (IgG) and anti-NF-Ya (Abcam Inc., Cambridge, CA, USA), together with Protein G PLUS-Agarose (Santa Cruz, Biotech, CA, USA) respectively. DNA isolated from the immunoprecipitated complex was amplified by PCR with primers flanking both CCAAT boxes. The primer sequences are: 5'AGAAGCGGGTAGGCAAGA3' and 5'CAGTGGCGAAGAGGAACG3'. The amplified PCR fragments were analyzed on a 2% agarose gel. A region of 257 bp around 10 kb downstream of the first exon of the *Dmrt7* was amplified as a control with primers: 5'AATAAGTTTCCAACCGTGAAGT3' and 5'TATCCAAGGAGATGGGTAAGTG3'. The PCR products were cloned into T-easy vector (Promega, Madison, WI, USA) and sequenced for confirmation. Precipitated DNA in the ChIP assay was amplified by quantitative real-time PCR using the multichannel RotorGene 3000 (Corbett Research, Australia). The cycling conditions were: 5 min at 94°C; 45 cycles of 94°C, 20 s; 60°C, 20 s and 72°C, 15 s in a 25 µL reaction mix containing Sybr Green I. The P1 region primers were 5'AGAAGCGGGTAGGCAAGA3'; and 5'CAGTGGCGAAGAGGAACG3'. The P2 region primers were 5'AATAAGTTTCCAACCGTGAAGT3'; and 5'TATCCAAGGAGATGGGTAAGTG3'. Each sample was performed in triplicate at least. Data were analyzed by the software Rotor-gene version 4.6 and then plotted in Microsoft Excel.

Western blot analysis

Western blots were performed according to routine protocols. Aliquots of tissue extracts (50 mg) were separated by electrophoresis in 10%

SDS-polyacrylamide gel (PAGE) and transferred onto a 0.45 µm membrane (Hybond-P; Amersham Pharmacia Biotech). Dmrt7 was detected by Dmrt7 antibody (1:500, GenWay Biotech, Inc., San Diego, CA) and β-Actin was used as an internal control (1:1000, Santa Cruz Biotech, CA, USA). Membranes were incubated with primary antibody for 1 h at room temperature, washed in TBST and probed with HRP conjugated secondary antibody (1:10000, Santa Cruz Biotech, CA, USA). After several washes in TBST, detection of the secondary antibody was performed using the SuperSignal Chemiluminescent Substrate system (Pierce, Rockford, USA). The chemical illumination signals were exposed onto Fuji medical X-ray film.

Results

Identification of regulatory elements in the mouse *Dmrt7* promoter region

To characterize functional elements in the *Dmrt7* promoter, the sequence from -407 to +116 of *Dmrt7* was run in MatInspector and TFSEARCH to find putative transcriptional elements. Several possible transcription-factor binding sites, which included AP-1, Sp1, GATA1, E2F and CCAAT box, were identified (Figure 1a). BLAST alignment of the promoter region of mouse, rat, and human *Dmrt7* genes revealed two highly conserved CCAAT boxes: the CCAAT box1 (-48/-44) and the CCAAT box2 (-7/-3) (Figure 1b). To determine the functional elements required for promoter activity, six 5'-deletion mutants (-948/+116, -407/+116, -245/+116, -104/+116, -60/+116 and +1/+116) were constructed and analyzed for promoter activity using luciferase reporter in GC-1 and COS 7 cells (Figure 1c). The maximum luciferase activity was observed in the region of the -407/+116. The deletion of the region between -245 and -104 appears to increase promoter activity in GC-1 cells, whereas the opposite result was obtained in COS 7 cells. To explain this discrepancy, we speculate that there could be other GC-1 specific transcription factors that could bind to this region and play a negative role in *Dmrt7* promoter activity. Deletion of the -60 to +1 sequence markedly decreased the activity, indicating the region of the -60 to +1 is essential for promoter function (Figure 1c). Two highly conserved CCAAT boxes were located in this region. To confirm the role of both CCAAT boxes, point mu-

tants were created by site-directed mutagenesis (Figure 1d). The mutation of CCAAT box1 in -60/+116 construct resulted in a decrease of approx.60-70% compared to the promoter activity of -60/+116 construct, the CCAAT box2 mutation in -60/+116 construct approx.70-80%. Nearly no activity was observed in the double mutations of both boxes in -60/+116 construct. However, the double mutations of both CCAAT boxes in the -948/+116 region resulted in only a decrease of approx.40-50% compared to the promoter activity of -60/+116 construct. Although there may be other important cis-regulatory sites upstream of the -60/+116, which may compensate the loss of these CCAAT boxes (Figure 1d), the region of the -60/+116 including two highly conserved CCAAT boxes is important for the promoter function. These results showed that both CCAAT box1 and CCAAT box2 are important for transcriptional activation of the mouse *Dmrt7* gene.

Transcription factor NF-Y binds to *Dmrt7* promoter *in vitro* and *in vivo*

Transcription factor prediction using the MatInspector and TFSEARCH showed that potential transcription factor NF-Y may bind to the CCAAT boxes. To determine if NF-Y binds to the CCAAT boxes, electrophoretic mobility shift assays were carried out using nuclear extracts prepared from mouse testis and double stranded oligonucleotides. Incubation of probe1 spanning from -58 to -31 (containing CCAAT box1) with the extract gave rise to the formation of a DNA–protein complex (Figure 2 lane1), which could be competed by the addition of an excessive amount of unlabelled oligo1 DNA (Figure 2 lane5), but not by the oligo1 DNA with mutated CCAAT box (Figure 2 lane7). Furthermore, addition of NFYa antibody to the binding reaction caused the disappearance of the specific DNA/protein complex and the appearance of the super-shift band (Figure 2 lane3). These results indicate that the CCAAT box1 within the promoter region is capable of binding with transcription factor NF-Ya *in vitro*. A similar result was also observed in incubation of probe2 spanning from -20 to +8 (containing CCAAT box2) with mouse testis nuclear extract (Figure 2). These results indicate that transcription factor NF-Ya can bind to *Dmrt7* promoter *in vitro*.
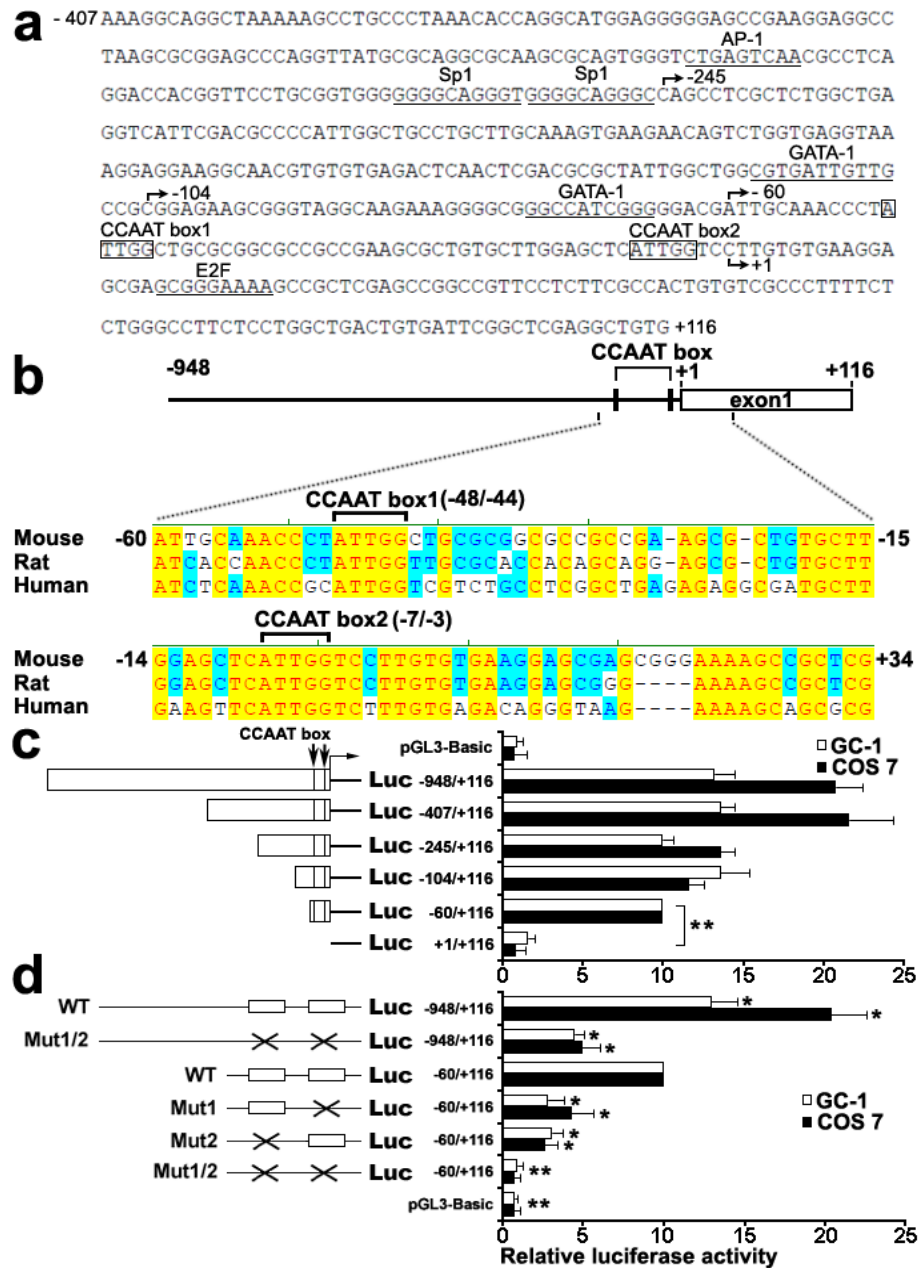
Figure 1. Identification and function characterization of the mouse *Dmrt7* promoter. a), Nucleotide sequence of the promoter region (from -407 to +116) of mouse *Dmrt7*. The putative binding sites for transcriptional factors are underlined. The CCAAT boxes are boxed. The numbering of the nucleotides starts at the first nucleotide of the cDNA (+1). **b),** Sequence alignment of 1 kb upstream of the first exon of *Dmrt7* from mouse, rat and human. Two highly conserved CCAAT boxes are indicated. Conserved nucleotides among all three species are denoted by red letters and are shaded in yellow. The positions of the nucleotides in the mouse *Dmrt7* gene are indicated. +1 represents the first nucleotide of the cDNA. **c),** Deletion analysis of the mouse *Dmrt7* promoter using the luciferase assay. GC-1 or COS 7 cells were transiently transfected with 0.4 µg of the deletion constructs together with 10 ng *Renilla* luciferase plasmid, pRT-TK, which served as an inner control for transfection efficiency. The relative activities to the -60/+116 construct of a series of deletion mutants was determined by the luciferase assay. The results are the mean ± S.D. of three independent experiments. *, P < 0.05; **, P < 0.001. **d),** Mutation analysis in the CCAAT boxes of the *Dmrt7* promoter by the luciferase assay. The -948/+116 and -60/+116 constructs were used as a template to generate point mutants. GC-1 or COS 7 cells were transiently transfected with 0.4 µg of these constructs together with 10 ng *Renilla* luciferase plasmid, pRT-TK. The relative activities of the -60/+116 construct of these mutants was determined by the luciferase assay. The results are the mean ± S.D. of three independent experiments. *, P < 0.05; **, P < 0.001 with compared to the -60/+116 wild-type construct. The box indicates the intact CCAAT box. The fork indicates the corresponding mutations.
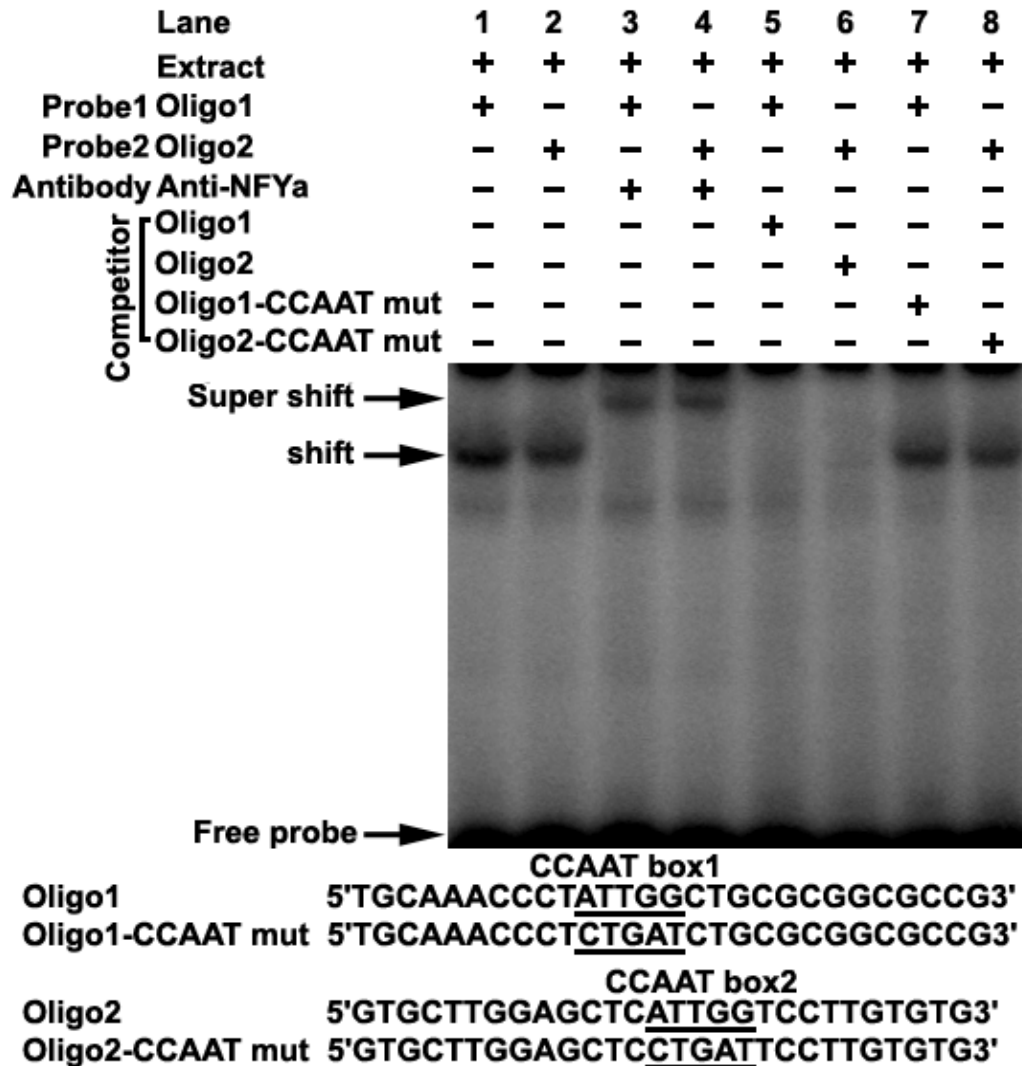
Figure 2. Electrophoretic mobility shift assay of NF-Y binding to *Dmrt7* promoter. The oligo1 corresponding to -58/-31 and oligo2 corresponding to -20/+8 were γ-32P-ATP labelled and incubated with 5μg of nuclear extract of mouse testis in the absence or presence of 50-fold excess various competitor DNA (mutant or non-labeled oligo) or anti-NFYa antibody, as indicated on the top of the gel image. Samples were resolved on 5% polyacrylamide gels in 0.5% TBE running buffer at 10 V/cm for 2 h. The specific DNA/protein complex and the super-shift bands were indicated by arrows. The shift bands cannot be competed with mutated DNA competitor (lane 7, 8). The sequences of oligo1, oligo2 and corresponding mutants are shown in the lower panel.

To further determine whether NF-Y binds to the mouse *Dmrt7* promoter *in vivo*, we performed chromatin immunoprecipitation analysis. Because Dmrt7 protein was only detected in testis (Figure 3a), we chose the testis for ChIP analysis. As shown in Figure 3b, a 160 bp of DNA fragment was amplified from the precipitate by NF-Ya antibody from testis, but not from control tissue liver or negative control immunoprecipitations using no antibody (beads only) or normal rabbit IgG. The amplified fragment was confirmed by sequencing. To rule out the possibility of non-specific binding of the NF-Ya antibody, an additional PCR amplification of a distinct genomic region was performed on all of the precipitated chromatin DNAs, which showed no amplification out of the NF-Ya-binding region (Figure 3b,d). A quantitative ChIP PCR was also performed to quantify the binding extent of NF-Ya on the *Dmrt7* promoter (Figure 3c). These results indicate that NF-Y specifically binds to the *Dmrt7* promoter *in vivo*.
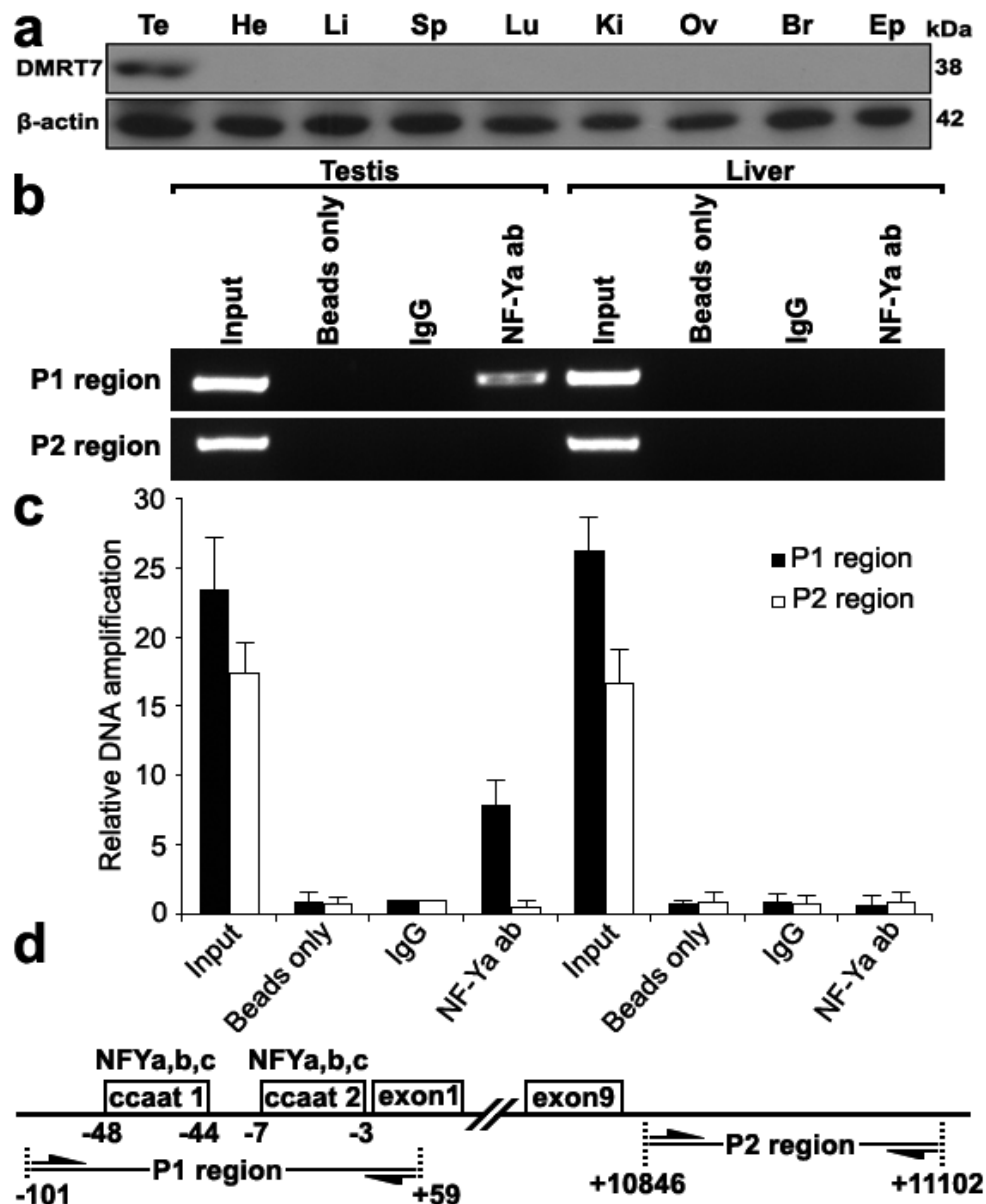
Figure 3. ChIP assay of NF-Y binding to the *Dmrt7* promoter in testis. a), Western blot analysis of the Dmrt7 protein in the adult testis, heart, liver, spleen, lung, kidney, ovary, brain and epididymis. The Dmrt7 expression was only detected in the testis. β-actin was used as an internal control. **b),** Interaction of NF-Y with the *Dmrt7* promoter *in vivo* was determined by chromatin immunoprecipitation analysis. Samples of mouse testis and liver were chopped into small pieces and cross-linked in 1% formaldehyde to cross-link endogenous proteins and DNA. Samples of sonicated chromatin were immunoprecipitated with anti-NFYA, no antibody (beads only) and preimmuno IgG (control) respectively. DNA isolated from immunoprecipitated material was amplified by PCR with primers to amplify the 160-bp sequence of mouse *Dmrt7* promoter corresponding to the −101 to +59 region. Primers for an unrelated region were used as negative control. The amplified PCR fragments were analyzed on 2% agarose gel. **c),** A q-ChIP PCR was performed to quantify the binding extent of NF-Ya on the *Dmrt7* promoter. All values were initially expressed relative to relevant IgG DNA content. **d),** The relative positions of the primers are shown.

NF-Y activates the mouse *Dmrt7* promoter

To investigate the role of transcription factor NF-Y in the activation of the *Dmrt7* promoter, both GC-1 and COS 7 cells were co-transfected with luciferase reporter driving by wild-type *Dmrt7* promoter (-60/+116 construct) or its mutants for CCAAT sites, and NF-Ya/b/c expression plasmids. The luciferase activity increased obviously when a wild-type promoter construct (-60/+116) was co-transfected with NF-Ya/b/c (Figure 4a). However, the luciferase ac-

tivity was not upregulated obviously when co-transfected with mutants for CCAAT box1 or box2 and was not upregulated absolutely when co-transfected with double mutants for CCAAT box1 and box2 (Figure 4a). This result indicated tandem arrangement of the CCAAT box1 and box2 may ensure maximum promoter activity of the *Dmrt7*. In addition, replacing the NF-Ya/b/c expression plasmid with the dominant-negative construct

(NF-YAm29) resulted in a marked reduction of luciferase activity in a dosage-dependent manner (Figure 4b). NF-YAm29 has three amino acid substitutions at the C-terminal region of the NF-Ya, which impairs its DNA binding and acts as a dominant-negative mutant by sequestering the NF-Yb/Yc subunits into a defective complex (32). These results indicated that NF-Y can activate the *Dmrt7* promoter.
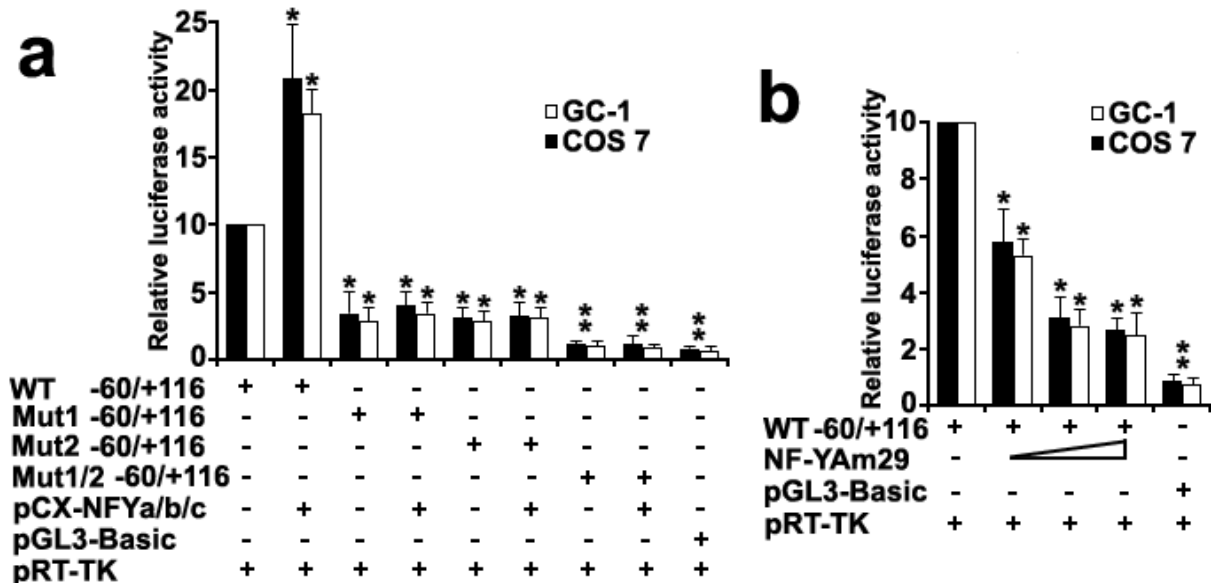

Figure 4. Co-transfection analysis of the mouse *Dmrt7* promoter. a), Effects of expression of NF-Y on the transcriptional activity of the *Dmrt7* 5'-flanking sequence (-60/+116). Cells were transfected with 0.2μg -60/+116 construct or mutants and 0.2μg NF-Y expression plasmids (pCX-NFYa, pCX-NFYb, pCX-NFYc). **b),** Effects of expression of the dominant negative mutant of NF-Y on the transcriptional activity of the *Dmrt7* 5'-flanking sequence (-60/+116). The dominant negative NF-Ya expression plasmid NF-YAm29 (0.1μg, 0.2μg, 0.3μg) was cotransfected with 0.2μg -60/+116 construct, which inhibits *Dmrt7*-luciferase activity. pGL3-basic was employed as a negative control. pRT-TK served as an inner control for transfection efficiency. The relative luciferase activities to the -60/+116 construct were determined by luciferase assay. Cells were harvested 24 h post-transfection, and luciferase activity was measured and normalized to Renilla luciferase activity. All transfection experiments were repeated at least three times and the activities are showed relative to that of the -60/+116 construct, which was set to 10. The results are the mean ± S.D. of three independent experiments. *, P < 0.05; **, P < 0.001 with compared to the -60/+116 wild-type construct.

Discussion

Dmrt7 is a member of the DM domain family of genes, which encodes proteins with a conserved DNA binding DM domain. In addition to Dmrt1, some DM domain proteins such as Dmrt3, Dmrt4 and Dmrt7 may be involved in sexual development. *Dmrt7* mRNA could be detected in testis and fetal ovary but not in other tissues (9). The expression of Dmrt7 protein was detected in testis (29), but not in the fetal ovary. *Dmrt7* mutants were infertile with spermato-

genic arrest at the pachytene stage, while the mutant females showed normal fertility (28, 29). Possible post transcriptional role of poly-adenylation in *Dmrt7* expression regulation was previously proposed (30). Because transcriptional regulation of the gene *Dmrt7* during spermatogenesis is an important process, in this study, we have explored promoter function of the gene and found that nuclear factor-Y (NF-Y) regulates transcription of mouse *Dmrt7* gene through binding to two conserved CCAAT boxes in its proximal promoter region. Deletion analysis of the 5' flanking region of the mouse *Dmrt7* revealed crucial positive elements

between -60 and +1, in which two evolutionarily conserved CCAAT boxes (the CCAAT box1 and box2) were identified. The conservation of transcription factor binding sites between orthologue gene promoters may indicate their highly significant roles in maintaining transcriptional regulation of *Dmrt7* gene. Further site-directed mutagenesis and functional analysis were performed to confirm their role in maintenance of promoter function of *Dmrt7*. The results suggest that binding by NF-Y to these two tandem CCAAT boxes is important for regulation of the basal transcription of the *Dmrt7* gene in the mouse testis.

NF-Y protein (also known as CBF) binds with high specificity to the CCAAT sequence and is a ubiquitous heteromeric transcription factor (33). NF-Y is a complex composed of three subunits: NF-Ya (CBF-B, HAP2 in yeast), NF-Yb (CBF-A, HAP3) and NF-Yc (CBF-C, HAP5), which are all required for DNA-binding (34-36). Both NF-Yb and NF-Yc have conserved histone folding motifs resembling H2B-H2A and their heterodimerization is essential for NF-Ya association (33, 37). NF-Ya is a regulatory subunit of the trimeric complex, whose levels vary in different cell types and/or growth conditions (38). It has been suggested that NF-Y can gain access to its genomic locations even in the absence of methyl histone marks and then leads the positioning of methyl histone marks typical of active chromatin independently (39). NF-Y appears to be essential for the activation of transcription (40). A number of mammalian promoters have been shown to contain CCAAT boxes and have been regulated by NF-Y alone or together with other transcriptional factors (41-44). In accordance with these results, the transcription factor NF-Y may activate the mouse *Dmrt7* gene and play an important role in the regulation of *Dmrt7* expression in testis. This, therefore, suggests that NF-Y regulation of *Dmrt7* plays a functional role in spermatogenesis.

In summary, we have found two important CCAAT boxes in the 5' flanking region of the mouse *Dmrt7* promoter. NF-Y is a major transcriptional activator of the *Dmrt7* gene through binding to these two CCAAT boxes. These results provide evidence of the regulatory mechanisms that control the expression in testis of the mouse *Dmrt7* gene and will form the basis for understanding mammalian spermatogenesis.

Acknowledgements

Conflict of Interest

There are no conflicts of interest with the financing of this project and its subject matter.

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