Transcription Factor BORIS (Brother of the Regulator of Imprinted Sites) Directly Induces Expression of a Cancer-Testis Antigen, TSP50, through Regulated Binding of BORIS to the Promoter

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Cancer-testis antigens (CTAs) are normally expressed in testis but are aberrantly expressed in a variety of cancers with varying frequency. More than 100 proteins have been identified as CTA including testes-specific protease 50 (TSP50) and the testis-specific parologue of CCCTC-binding factor, BORIS (brother of the regulator of imprinted sites). Because many CTAs are considered as excellent targets for tumor immunotherapy, understanding the regulatory mechanisms governing their expression is important. In this study we demonstrate that BORIS is directly responsible for the transcriptional activation of TSP50. We found two BORIS binding sites in the TSP50 promoter that are highly conserved between mouse and human. Mutations of the binding sites resulted in loss of BORIS binding and the ability of BORIS to activate the promoter. However, although expression of BORIS was essential, it was not sufficient for high expression of TSP50 in cancer cells. Further studies showed that binding of BORIS to the target sites was methylation-independent but was diminished by nucleosomal occupancy consistent with the findings that high expression of TSP50 was associated with increased DNase I sensitivity and high BORIS occupancy of the promoter. These findings indicate that BORIS-induced expression of TSP50 is governed by accessibility and binding of BORIS to the promoter. To our knowledge this is the first report of regulated expression of one CTA by another to be validated in a physiological context.

CTCF is a versatile chromatin factor originally identified as a repressor of Myc transcription (1–4). CTCF recognizes DNA target sequences through its 11 zinc finger (11ZF) domain that is highly conserved from Drosophila melanogaster to humans (5) and functions not only as a repressor but also as an activator to regulate expression of various genes including APP, RB1, TP53, TERT, and ARF (6–11). In addition to functioning as a conventional transcription factor, CTCF is known to regulate gene expression through organization of chromatin structure (12, 13). For instance, CTCF regulates expression of imprinted genes H19/lgf2 through binding to H19 ICR, which is methylated at the paternal allele and unmethylated at the maternal allele, in a CpG methylation-dependent manner (14, 15). The binding of CTCF to the H19 ICR regulates promoter activity of H19/lgf2 through formation of intrachromosomal loops, which results in allele-specific expression (16–18). Furthermore, recent reports identified additional unique functions of CTCF such as regulated stability of trinucleotide repeats (19) and latency of various viruses (20–22).

In mammals, there is a single CTCF paralogous gene named BORIS (brother of the regulator of imprinted sites), also known as CT27 (cancer-testis antigen 27; see CTDatabase) (23, 24). BORIS contains an 11ZF domain that is highly similar to the 11ZF domain of CTCF. We recently reported that BORIS plays important physiological functions in progression of spermatogenesis by regulating expression of a testis-specific splicing variant of cerebroside sulfotransferase (Cst), Cst form FTS (25).

CTAs such as BORIS are encoded by genes that show restricted expression in testis and tumors. For the restricted expression, CTAs are considered as candidate targets of cancer vaccines (26, 27). Immunization against BORIS was shown to be effective in treating mice injected with BORIS-expressing cancer cells (28, 29). Furthermore, it has been reported that BORIS regulates expression of genes encoding other CTAs including MAGE-A1, SPANX, and NY-ESO-1 (30, 31). Recent publications suggested BORIS was involved in DNA hypomethylation in cancers that is important for derepression of
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CTAs expression (32, 33). These studies suggest that BORIS is an important regulator of CTA expression in cancer cells.

PRSS50, also known as TSP50 (testes-specific protease 50) or CT20, encodes a protease originally found as a gene with a promoter that is hypomethylated in tumors (34). Like BORIS, TSP50 is known as a CTA. Although the physiologic function of TSP50 is not clarified, the characteristic of its expression makes it interesting as a potential target for cancer immunotherapy. Recent publications suggested that expression of TSP50 is regulated by CpG methylation of the promoter (35), similar to many other CTAs, and by various transcription factors including SP1, CCAAT/enhancer binding protein (C/EBP), and TP53 (36, 37). However, none of these factors was shown to regulate expression of TSP50 in a physiologic context, which make it unclear which factors are involved in vivo. Given the possible utility of TSP50 as a target for tumor immunotherapy, we felt that clarification of the regulatory mechanisms was an important issue. We recently reported that BORIS is involved in regulating Tsp50 expression in testis, although it was not clear whether reduced expression of Tsp50 in testis of Boris knock-out (KO) mice was a primary or secondary effect of the loss of BORIS (25).

Here we report that BORIS is directly involved in activation of TSP50 expression by binding to the promoter region. We identified two BORIS target sites in the TSP50 promoter that are highly conserved among species. We found that BORIS-induced expression of TSP50 was dependent on regulated binding of BORIS to the promoter. The promoter of TSP50 was DNase I-hypersensitive in the cell line Delta47, which expresses TSP50 at high levels, but not in other cell lines with little or no expression. This suggested that accessibility to the promoter is the limiting factor for binding of BORIS and downstream expression of TSP50. To our knowledge, this is the first report demonstrating direct regulation of a CTA by BORIS in a physiologic context.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and penicillin-streptomycin. Normal human dermal fibroblasts (NHDF) and other human cancer cell lines, Delta47, EKXV, OVCAR8, K562, MM-S1, 8226, IGRov1, UO31, A498, and ARK were cultured in RPMI medium 1640 supplemented with 10% FBS and penicillin-streptomycin.

Mice—Boris knock-out mice were generated as described previously (25). All animal experiments were conducted in compliance with the Animal Care and Use Committee of the NIAID, National Institutes of Health.

Antibodies—The antibody against mouse BORIS used for chromatin immunoprecipitation (ChIP) analysis was described previously (25). Mouse monoclonal antibodies specific for human BORIS were produced using recombinant human BORIS protein prepared from larvae of Trichoplusia ni infected with baculovirus expressing full-length human BORIS. Six antibody-producing clones that produced antibodies active in mobility super-shift assay were chosen, and ChIP assays were carried out using a mixture of these antibodies.

Quantitative PCR (qPCR)—Total RNA was isolated from tissues and cells using RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was prepared with oligo-dT primers using SuperScript III First-strand Synthesis System (Invitrogen) according to the manufacturer’s protocol. qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the 7900HT sequence detection system (Applied Biosystems). The expression of TSP50 and BORIS in primary melanomas was analyzed using TissueScan Melanoma Tissue qPCR Panel I (Origene, Rockville, MD) and Mx3000p (Agilent Technologies, Santa Clara, CA). Fisher’s exact test was conducted to analyze statistical significance of correlation between expression of BORIS and TSP50 in melanomas. Primers for qPCR were designed using Primer Express software (Applied Biosystems). Primer sequences are listed in supplemental Table 1 or described in a previous publication (25) Expression levels were normalized against the housekeeping gene GAPDH except for the melanoma panel, which was pre-normalized against ACTB. Relative expression levels were determined by standard curve method or comparative Ct method according to the manufacturer’s protocol. Each sample was analyzed in triplicate. Student’s t test was performed to evaluate statistical significance. Data shown are the means ± S.D.

Purification of Spermatocytes and Round Spermatids—Spermatocytes and round spermatids were purified as previously described (25). Briefly, dissociated testicular cells were separated by centrifugal elutriation. Partially purified spermatocyte and round spermatid fractions were incubated with 10 μM Vybrant DyeCycle Green (Invitrogen) for 30 min at 32 °C followed by staining with 4,6-diamidino-2-phenylindole (DAPI). Spermatocytes and round spermatids were sorted according to DNA content to improve purity using FACS Aria (BD Biosciences). DAPI-positive dead cells were eliminated.

Electrophoretic Mobility Shift Assay (EMSA) and Methylation Interference Assay—EMSA and methylation interference assays were performed as described previously (25). Mouse and human TSP50 promoters were analyzed with BORIS protein of mouse and human origin, respectively. Because the amino acid sequences of human and mouse CTCF are highly conserved, we used mouse CTCF for analyses of both the mouse and human TSP50 promoters. Partially purified recombinant human BORIS protein prepared from larva of T. ni infected with baculovirus expressing full-length human BORIS was used for EMSA. Briefly, larvae were homogenized in buffer containing 40 mM HEPES (pH 7.6), 100 mM NaCl, 2 mM MgSO4, 10 μM ZnSO4, and 4× Complete protease inhibitor mixture without EDTA (Roche Applied Science). Homogenate was centrifuged at 10,000 × g for 10 min. The bulk of the BORIS protein was detected in the pellet using antibodies against the V5 tag. The pellet was washed sequentially with homogenization buffer containing 1, 2, and 4 M urea. After final washing, BORIS was extracted using homogenization buffer containing 6 M urea and 500 mM NaCl. The DNA binding ability and specificity of the recombinant protein was confirmed in EMSA tests using a variety of known BORIS binding DNA sequences as well as negative controls. The binding specificity was further confirmed by

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mobility supershift assay using antibodies against human BORIS (Abcam, Cambridge, MA). Partially purified recombinant mouse BORIS and CTCD proteins were prepared from *Escherichia coli* transformed with pET16b-mouse Boris and mouse Ctf, respectively, following the procedure described above. Bacteria were disrupted by sonication. Samples prepared from *E. coli* transformed with pET16b empty vector were used as a negative control. For EMSA with nucleosomal DNA fragments, nucleosomes were reconstituted using a Chromatin Assembly kit (Active Motif, Carlsbad, CA) according to the manufacturer’s protocol. Luciferase and control lysate from larva of *T. ni* were used as negative controls in EMSA for *in vitro* translated protein and recombinant protein prepared with baculovirus, respectively. Primers to prepare DNA fragments of human TSP50 promoter for EMSA are listed in [supplemental Table 1](#).  

**Luciferase Assay**—Fragments containing the entire 5’-untranslated region (5’-UTR) sequences of Tsp50 and its promoter sequences starting from position −150, −300, or −600 were cloned into pGL3-basic vector. NIH3T3 cells were cotransfected using FuGENE 6 (Roche Applied Science) with luciferase constructs together with pRL-CMV vector as an internal control and expression vectors for BORIS or CTCD in pCIneo according to the manufacturer’s protocol. Cells were cultured for 48 h at 37 °C with 5% CO₂. Luciferase assays were carried out with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s protocol. The luciferase activities were normalized to the empty vector-transfected samples for each luciferase construct. Student’s *t* test was performed to evaluate statistical significance. Data shown are the means ± S.D.  

**ChIP assay**—ChIP assays were carried out as previously described (25) with slight modifications. Briefly, fixed cells or tissue lysates corresponding to 1 × 10⁶ cells or 20 mg of tissue in 100 μl of SDS lysis buffer (20 mM Tris-HCl (pH 8), 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100) were incubated with Dynabeads M-280 (Invitrogen) preconjugated with antibodies for 1.5 h at 4 °C with rotation. DNA fragments binding to beads were purified and subjected for qPCR. Primers used for qPCR are listed in [supplemental Table 1](#). Student’s *t* test was performed to evaluate statistical significance. Data shown are the means ± S.D.  

**Bisulfite Sequencing**—Bisulfite modification of genomic DNA was carried out using the Imprint DNA Modification kit (Sigma) according to the manufacturer’s protocol. The bisulfite-modified TSP50 promoter region from −198 to −32 was amplified using 5’-GGTTLAAGGAGGGAATGGTATG-3’ and 5’-CTAATAAATCAAAAAATCTGTTAATA-3’ as primers with PCR Platinum Taq Polymerase (Invitrogen) under the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min. A CpG island from −65 to +159 was amplified using 5’-GGG- GTATTAGGATTTTTTTTATGTTATAT-3’ and 5’-ACCTCAACAAAAACACACACACAA-3’ as primers under the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 5 min. Amplicons were purified and cloned into pGEM-T vector (Promega). After transformation, plasmids from individual bacterial colonies were extracted and subjected to sequencing (Genomics Research Facility, Rocky Mountain Laboratories, NIAID, National Institutes of Health).  

**Methylation-specific PCR**—Methylation status was analyzed by PCR using bisulfite-treated DNA as templates with primers specific for methylated or unmethylated sequences with designs based on the promoter region or the CpG island inside the transcribed region of TSP50. Primers used for these experiments are shown in [supplemental Table 1](#). PCR was performed under the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, annealing temperature of 57 °C (unmethylated promoter)/57 °C (methylated promoter)/53 °C (unmethylated transcriptional region)/60 °C (methylated transcriptional region) for 30 s, 72 °C for 30 s; 72 °C for 5 min. PCR products were electrophoresed on 3% agarose gels and visualized by ethidium bromide staining.  

**DNase I Hypersensitive Assay**—DNase I hypersensitive assays were carried out as previously described with slight modifications (38). Briefly, 2 × 10⁷ cells were homogenized with a Dounce homogenizer were split into four samples and treated with 0, 80, 160, or 320 units/ml of DNase I for 3 min at 25 °C. Samples were then treated with RNase A followed by treatment with proteinase K. 70 μg of purified DNA digested with Nhel and Kpnl was separated on 0.8% agarose gels. The probe for hybridization was generated by PCR using 5’-CA-GTCTTCTGTCCACGG-3’ and 5’-CCCTGGGATCTGTATGTTGGC-3’ as primers.  

**Nucleosome Phasing**—Nucleosome positioning was analyzed as described previously with modifications (39, 40). 1.5 × 10⁷ cells were harvested and washed with cold PBS. Cells were suspended in solution A (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 0.4% Nonidet P-40, protease inhibitor mixture) and incubated for 10 min on ice followed by treatment with solution B (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 0.4% Nonidet P-40, 1 mM CaCl₂, protease inhibitor mixture). Samples were treated with 120 units/ml micrococcal nuclease for 10 min at 37 °C, and the reaction was terminated by adding solution C (100 mM EDTA, 4% SDS). After treatment with 0.3 mg/ml RNase A for 30 min at 37 °C, samples were treated overnight at 50 °C with 20 mg/ml proteinase K. Purified DNA was separated on 1.5% agarose gels, and the band corresponding to mononucleosomes was excised from the gel followed by purification with QIAquick Gel Extraction kit (Qiagen). Nucleosome positioning was identified by tiling qPCR using mononucleosomal DNA as a template. Primers were designed by Primer3 from the Whitehead Institute to produce overlapping amplimers covering the promoter region of TSP50. Primers used for qPCR are shown in [supplemental Table 1](#). Data were normalized to the signal of the highest peak.  

**RESULTS**  

**Characterization of Tsp50 Expression in Wild Type (WT) and Boris KO Mice**—As a first step in evaluating the role played by BORIS in expression of Tsp50, we quantified levels of Tsp50 transcripts in WT and Boris KO mice by qPCR (Fig. 1A). As reported previously (34), Tsp50 transcripts were present at high levels only in testis and were significantly lower in testis of Boris KO mice (25). To determine if Tsp50 was differentially ex-
pressed among subsets of spermatogenic cells, we compared the levels of transcripts in purified spermatocytes and round spermatids. We found that transcript levels in round spermatids were 16-fold higher than in SC (Fig. 1B, left panel). The differential distribution of Tsp50 expression between these subsets of spermatogenic cells was quite similar to that described previously for Csf1r, a reporter target of BORIS, and Boris as illustrated in Fig. 1B (middle and right panels, respectively) (25).

The Mouse Tsp50 Promoter Contains Two BORIS Binding Sites—The above findings prompted us to determine if BORIS was directly involved in governing Tsp50 expression by binding to the promoter region. To examine this issue, we generated five DNA fragments (Fr), designated Fr1 to Fr5, that span the promoter region (Fig. 2A) and tested them for BORIS binding. NIH3T3 cells were cotransfected with empty, BORIS, or CTCF expression vector. Luciferase activities were normalized against the empty vector-transfected sample. Asterisks indicate statistical significance versus SC (p < 0.005).

Because CTCF is known to share target sequences in the Tsp50 promoter region, we conducted methylation interference assays using the Fr2 sequence as a probe (Figs. 3, A–C). Because CTCF is known to share target sequences with BORIS in vitro for the highly conserved DNA recognition domain, we first probed the Fr2 sequence using the 11ZF domain of CTCF (Fig. 3C). This result clearly shows that BORIS and CTCF recognize the same target sequences in the Tsp50 promoter. Furthermore, we conducted EMSA using either WT Fr2 (Fr2-wt) or Fr2 carrying mutations at contact nucleotides within B1 and/or B2 (Fig. 3C). These results indicated that the activation of Tsp50 promoter was directly dependent on binding of BORIS to sequences downstream of −150 of the Tsp50 promoter. To more precisely define the target sites for BORIS on the Tsp50 promoter, we conducted methylation interference assays using the Fr2 sequence as a probe (Figs. 3, A–C). Because CTCF is known to share target sequences with BORIS in vitro for the highly conserved DNA recognition domain, we first probed the Fr2 sequence using the 11ZF domain of CTCF that efficiently binds to target sequences (Fig. 3C). This result clearly shows that BORIS and CTCF recognize the same target sequences in the Tsp50 promoter. Furthermore, we conducted EMSA using either WT Fr2 (Fr2-wt) or Fr2 carrying mutations at contact nucleotides within B1 and/or B2 (Fig. 3, A and D). BORIS was bound to Fr2-wt as well as Fr2 with mutations.
within either B1 (Fr2-B1mut) or B2 (Fr2-B2mut). In contrast, almost no band-shift was seen with Fr2-carrying mutations in both B1 and B2 (Fr2-B1/B2mut). We then tested the Fr2-wt and mutated Fr2 sequences in EMSA for binding of either full-length CTCF or the 11ZF domain (Fig. 3E). The results of these studies showed that, like BORIS, both CTCF and the 11ZF domain bound to Fr2-wt, B1mut, and B2mut but that binding to Fr2-B1/B2mut was markedly reduced or undetectable. It is noteworthy that EMSA with BORIS, CTCF, and 11ZF domain showed the slower migrating shifted bands due to double occupancy of Fr2-wt (Fig. 3D, arrow 1; Fig. 3E, arrow 1; Fig. 3E, arrow 3, respectively) and the more rapidly migrating shifted band due to single occupancy of the single mutants, Fr2-B1mut and Fr2-B2mut (Fig. 3D, arrow 2; Fig. 3E, arrow 2; Fig. 3E, arrow 4, respectively).

To assess the functional relevance of these findings, we performed luciferase assays in NIH3T3 cells using Tsp50 promoter constructs starting from -600 that were WT or mutated at the B1, B2, or both the B1 and B2 sites. BORIS-induced activation of the promoter was reduced by mutations in either site and lost
almost completely with a construct bearing mutations in both
sites (Fig. 3F). Taken together, these observations indicated that
recognition of the Tsp50 promoter by BORIS, involving binding
to both the B1 and B2 sites, was responsible for the promoter
activation.

These findings prompted us to determine if BORIS bound to
Tsp50 promoter sequences in vivo. To do this we performed
ChIP-qPCR analyses using BORIS antibodies with testis tissue
from WT and Boris KO mice (Fig. 3G). The results showed
marked binding of BORIS to sequences in the Tsp50 promoter
region in WT but not in Boris KO testis, whereas enrichment
was not found in the 5' negative control region of either sample.
From this, we conclude that BORIS binds directly to specific
sequences within the Tsp50 promoter and is responsible for
transcriptional activation of the gene in testis.

BORIS Binding Sites in the TSP50 Promoter Are Conserved
between Mice and Humans—Because some CTAs are recog-
nized to be important targets for immunotherapy of cancer and
others, such as BORIS, to have potential for preventative vac-
cines (28, 41–43), we felt it is important to characterize mech-
nanisms governing expression of TSP50 in human cells. A com-
parison of the TSP50 promoter regions of various mammalian
species revealed areas of strong conservation around the B1 and
B2 BORIS target sites defined for mice (Fig. 4A), suggesting that
similar regulatory mechanisms might be operative across spe-
cies lines. This suggestion was strengthened by the results of
methylation interference assays of the human TSP50 promoter
region (Fig. 4B). These studies identified two BORIS binding
regions with contact guanines lying in the regions of homology
to the mouse B1 and B2 binding sites. We validated the impor-
tance of these two sites for binding of BORIS by EMSA using
probes with WT sequences and sequences mutated at contact
residues in B1, B2, or both sites (Fig. 4C). Studies performed
with a WT probe revealed two shifted bands with the slower
migrating band (Fig. 4C, arrowhead), corresponding to the
occupancy of both target sites and the more rapidly migrating
band (Fig. 4C, arrow), corresponding to the occupancy of single
sites as seen with probes mutated at only B1 or B2. No band
shifts were observed with a probe mutated at both B1 and B2,
paralleling similar studies of the mouse TSP50 promoter. Addi-

FIGURE 4. Binding of BORIS to human TSP50 promoter. A, alignment of sequences around B1 and B2 sites of TSP50 promoter from various species is shown. Gray boxes indicate conserved residues among species. The TSS is marked by an arrow. Asterisks denote contacting guanine residues of human or mouse. Residues marked with squares were converted to adenine in mutant constructs. B, shown is a methylation interference assay using DNA fragment containing B1 and B2 sites of human TSP50 promoter. Partial sequences of the fragment around B1 and B2 sites are shown. Only the top strand is shown, as the bottom strand did not show any differences. Left lane, unbound fragments (Free); right lane, bound fragments (Bound). Asterisks denote contacting guanine residues. C, binding of BORIS to the mutated fragments of human TSP50 promoter was analyzed by EMSA as Fig. 3D. Arrowhead, shifted band for simultaneous binding of BORIS to both B1 and B2 site; arrow, shifted band for binding of BORIS to one site. An asterisk indicates a shifted band most likely for binding of degraded BORIS protein. D, binding of CTCF and 11ZF to the mutated fragments of human TSP50 promoter was analyzed by EMSA as Fig. 3E. Arrow 1, shifted band for simultaneous binding of CTCF to both B1 and B2 site; arrow 2, shifted band for binding of CTCF to one site; arrow 3, shifted band for simultaneous binding of 11ZF to both B1 and B2 site; arrow 4, shifted band for binding of 11ZF to one site. N, negative control; ZF, 11ZF; C, CTCF.
tional studies of the WT and mutant probes were performed in EMSA using CTCF and 11ZF domain (Fig. 4D). In keeping with the observations made with the WT probe and BORIS, we observed two shifted bands in the presence of CTCF. Consistently, the patterns of band shifts seen with 11ZF protein resembled those seen with CTCF. Taken together, these results indicated that the mechanisms governing regulation of TSP50 expression by BORIS are conserved between mice and humans.

Expression of BORIS Is Essential but Not Sufficient for Expression of TSP50 in Human Cancer Cell Lines—To analyze the regulation of TSP50 expression by BORIS in human cells, we quantified expression of both BORIS and TSP50 in five BORIS-positive cell lines, five BORIS-negative cell lines, and NHDF, which are supposed to be negative for both BORIS and TSP50 (Fig. 5A). The results showed that TSP50 transcripts were expressed by three BORIS-positive cell lines of different origins (Delta47, a multiple myeloma cell line; EKVVX, a non-small cell lung carcinoma line; OVCA8, an ovarian tumor cell line) but were almost completely negative in two other BORIS-positive lines (K562, a myelogenous leukemia cell line; MM-S1, a multiple myeloma cell line). It is noteworthy that the levels of TSP50 transcripts in Delta47 cells were more than 17-fold higher than in the other expressing lines and that there was no direct correlation between the levels of transcripts for TSP50 and BORIS in the cell lines expressing both genes. None of the BORIS-negative cancer cell lines including NHDF expressed TSP50. To investigate the relationship between the expression of BORIS and TSP50 in primary cancers, we analyzed their expression in melanomas, which are known to express various CTAs (Fig. 5B). Among 40 melanoma samples analyzed, nine showed higher expression of TSP50 than the background detected in normal skin samples (Fig. 5B, 1–9). Of these nine cases, five melanomas, including samples showing high expression of TSP50 (Fig. 5B, 1–3 and 5) expressed BORIS. There was a statistically significant correlation between the expression of BORIS and TSP50 ($p = 0.016$), consistent with the idea that BORIS has a crucial function in activation of the TSP50 expression. On the other hand, BORIS expression was not sufficient to induce expression of TSP50 in melanomas as four cases were BORIS-positive but TSP50-negative (Fig. 5B, 13, 17, 36, and 37), similar to the observations made with cancer cell lines. These results suggest that the mechanisms involved in regulation of TSP50 expression by BORIS in cancer cell lines as well as primary melanomas are not completely congruent with those operative in normal testis.

Our studies about mouse Tsp50 promoter indicated that BORIS-induced expression of Tsp50 was controlled by binding of BORIS to the promoter. To determine if this mechanism was also operative in cancer cells, we performed ChIP-qPCR assays of the TSP50 promoter and an irrelevant site 3’ to the promoter with antibodies against human BORIS and against BORIS of the mouse Tsp50 promoter region (Pr) and 10 kb downstream from TSS (3’) in each of the cell lines was examined by ChIP-qPCR using anti-human BORIS antibodies ($n = 3$). An asterisk denotes statistical significance against enrichment in other cell lines ($p < 0.005$).

FIGURE 5. Correlation between expression of TSP50 and binding of BORIS to TSP50 promoter. A, expression of TSP50 and BORIS in various human cell lines was analyzed by qPCR ($n = 3$). Expression levels are shown as the ratio to NHDF. B, expression of TSP50 and BORIS in melanoma samples was analyzed by qPCR. Expression levels are shown as the ratio to testis. Asterisks shown on the top panel indicate BORIS-positive samples. C, binding of BORIS to TSP50 promoter region (Pr) and 10 kb downstream from TSS (3’) in each of the cell lines was examined by ChIP-qPCR using anti-human BORIS antibodies ($n = 3$). An asterisk denotes statistical significance against enrichment in other cell lines ($p < 0.005$).
These results suggested that demethylation of the promoter was not sufficient to recruit BORIS to the promoter and induce expression of TSP50. Because the CpG island of the TSP50 promoter extends into the body of the gene, we also analyzed the methylation status of CpGs downstream of the TSS. Interestingly, CpGs inside the transcribed region of TSP50 were completely unmethylated in Delta47 cells, whereas they were fully methylated in other cell lines (Fig. 6A, right column). Parallel studies of the same CpGs in NHDF also showed relatively high methylation of the sequences. To extend our understandings of the relations between the methylation status of CpGs downstream of the TSS, we performed DNase I hypersensitive assays of the TSP50 promoter region to analyze the status of chromatin in relation to binding of BORIS using cells that expressed both TSP50 and BORIS (Delta47), cells that are BORIS-positive but TSP50-negative (K562), and cells that were negative for the expression of both genes (8226) (Figs. 7, A and B). The results of these studies showed that Delta47 alone had a DNase I hypersensitive site in the TSP50 promoter region. To precisely determine the DNase I hypersensitive site in the promoter, we stud-
**DISCUSSION**

Previous studies from our laboratory demonstrated that Tsp50, a gene encoding CTA, was differentially expressed in testis from WT and Boris KO mice, suggesting that BORIS may be directly or indirectly involved in governing its expression (25). The results of the current study provide definitive evidence that TSP50 is a direct transcriptional target of BORIS in both mice and humans. It was recently reported that TSP50 is expressed at higher levels in conjunction with BORIS in spermatocytic seminomas than in seminoma/dysgerminomas (45). Our findings suggest that the high expression of TSP50 in spermatocytic seminoma can be ascribed to expression of BORIS.

Transcriptional activation of TSP50 by BORIS was shown to be dependent on occupancy of two target sites in the promoter region that were conserved between mice and humans. Both binding sites share sequence similarity with the BORIS binding sequences we found in the promoter of Cst form FTS (25) as well as...
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Finally, it was reported that down-regulation of BORIS (49) or TSP50 (50) expression results in apoptosis of cancer cell lines, suggesting that induction of TSP50 expression by BORIS may contribute to the survival of cancer cells. Although the potential clinical relevance of these observations is at present only conjectural, there is increasing evidence that BORIS and other CTAs may be important targets for immunotherapy (28, 41). Further studies are clearly required to explore these possibilities.

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