CTCFL/BORIS Is a Methylation-Independent DNA-Binding Protein That Preferentially Binds to the Paternal H19 Differentially Methylated Region

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Abstract

The CTCF paralog BORIS (brother of the regulator of imprinted sites) is an insulator DNA-binding protein thought to play a role in chromatin organization and gene expression. Under normal physiologic conditions, BORIS is predominantly expressed during embryonic male germ cell development; however, it is also expressed in tumors and tumor cell lines and, as such, has been classified as a cancer-germline or cancer-testis gene. It has been suggested that BORIS may be a pro-proliferative factor, whereas CTCF favors antiproliferation. BORIS and CTCF share similar zinc finger DNA-binding domains and seem to bind to identical target sequences. Thus, one critical question is the mechanism governing the DNA-binding specificity of these two proteins when both are present in tumor cells. Chromatin immunoprecipitation (ChIP) in HCT116 cells and their hypermethylated variant showed that BORIS binds to methylated DNA sequences, whereas CTCF binds to unmethylated DNA. Electromobility shift assays, using both whole-cell extracts and in vitro translated CTCF and BORIS protein, and methylation-specific ChIP PCR showed that BORIS is a methylation-independent DNA-binding protein. Finally, experiments in murine hybrid cells containing either the maternal or paternal human chromosome 11 showed that BORIS preferentially binds to the methylated paternal H19 differentially methylated region, suggesting a mechanism in which the affinity of CTCF for the unmethylated maternal allele directs the DNA binding of BORIS toward the paternal allele. [Cancer Res 2008;68(14):5546–51]

Introduction

Insulator DNA sequences are thought to partition the genome into functional chromosomal domains to regulate gene transcription (1). The compartmentalization of chromatin into distinct regulatory domains seems to alter interactions between target genes and nearby cis-acting enhancer elements (2). CTCF was the first human insulator DNA-binding protein identified that is a ubiquitously expressed, highly conserved, zinc finger protein that has multiple roles in gene regulation (3), including regulation of the imprinted maternal H19 allele (4). CTCF binds to the unmethylated DNA of the differentially methylated region (DMR), limiting access to an enhancer shared between H19 and IGF2 and causing silencing of the maternal IGF2 allele (5).

The CTCF paralog, CTCFL or BORIS (brother of the regulator of imprinted sites), is thought to be predominantly expressed in testes; it has also been detected in >100 cancer cell lines representing all major forms of human tumors (6). BORIS shares an 11 zinc finger domain with CTCF (6); however, CTCF and BORIS differ significantly in their NH2 and COOH termini, suggesting that these regions may interact with different binding partners, altering gene expression by different mechanisms. Indeed, in contrast to CTCF, BORIS seems to activate gene expression (7, 8). These results suggest that CTCF and BORIS use a similar DNA sequence to elicit very different changes in gene expression: one suppresses and the other induces gene expression at the same locus.

It has recently been shown that the human genome contains 13,804 CTCF DNA-binding sites in potential insulators of the human genome, and although these sequences are significantly different from transcriptional start sites, their distribution correlated with gene expression (9). Studies have also shown that CTCF is down-regulated in lobular carcinoma in situ of the breast (10), suggesting a potential role of the CTCF DNA-binding target sequences in carcinogenesis. Thus, a model could be proposed whereby the aberrant reexpression of BORIS, which is observed in tumor cells (6), might in turn activate a large number of genes resulting in a cellular phenotype permissive for transformation. However, both CTCF and BORIS seem to be expressed in tumor cells, suggesting a potential competition between these two proteins for CTCF DNA-binding sequences that may subsequently determine the expression of nearby target genes. Thus, one critical question about the activity of these related insulator DNA-binding proteins is the mechanism by which they specifically bind to CTCF target sequences.

Materials and Methods

Cell lines, cell culture, and plasmids. HCT116 cells (human colon carcinoma) and the methyltransferase somatic knockouts DNMT1(−/−)/DNMT3B(−/−) double knockout (DKO) cells were cultured in McCoy's 5A medium containing 10% heat-inactivated fetal bovine serum (FBS). The murine hybrid A911P or A911M cells were grown in DMEM with 10% FBS. Media were supplemented with penicillin (100 units/mL) and streptomycin (100 μg/mL). Primers were used to PCR the DMR region (Supplementary Data) and this fragment was cloned into ptk-Luc (Clontech, Inc.) and transient assay was done as previously described (11).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were done using the Upstate Biotechnology, Inc. kit (12). ChIP was performed using CTCF (C-20; Santa Cruz Biotechnology, Inc.) or
BORIS (Supplementary Figs. S1 and S2) antibodies. Purified DNA samples were analyzed by real-time quantitative PCR (qPCR) using SYBR Green PCR Master Mix (Applied Biosystems) with primers to the human H19 DMR (Supplementary Materials and Methods). Data were collected and analyzed by comparative Ct methods.

**Bisulfite pyrosequencing and methylation-specific ChIP PCR.** DNA (10 ng) was treated with sodium bisulfite according to established methods (12). Treated DNA was resuspended in 40 μL of distilled water for PCR. All primers are listed in Supplementary Materials and Methods. PCR products were used directly for pyrosequencing according to the manufacturer’s instruction (Biotage). Methylation-specific ChIP PCR (MS-ChIP-PCR) was carried out in HCT116 cells. Following ChIP with an anti-BORIS or anti-CTCF antibody, the pulled-down chromatin was subjected to bisulfite conversion using the EZ DNA Methylation Gold kit (Zymo Research). Primer sets were designed using Methyl Primer Express software (Applied Biosystems). Each reaction contained 10 μL Fast Cycling Taq Master mix (Qiagen), 5 μL of converted DNA, and 1 μL each of 0.3 μmol/L forward and reverse primer. qPCR and PCR were done as described above.

**Preparation of nuclear extracts and electromobility shift assay.** Nuclear extracts were prepared as previously described method (11). In vitro BORIS and CTCF protein was prepared using Expressway In Vitro Protein Synthesis System (Invitrogen). For the electromobility shift assay (EMSA) methylation analysis of BORIS and CTCF DNA binding, 1 μg of PCR product was used. Each reaction consisted of 30 μL total volume containing 1 μg DNA, 4 units of SsoI methylase enzyme, and buffer, and reactions were carried out at 37°C for 1 h. The same procedure was performed for unmethylated DNA. The DNA reactions were gel purified, and 50 ng of DNA were used for labeling with [32P]ATP. For each EMSA, 10,000 cpm of labeled DNA were used with in vitro transcribed CTCF or BORIS protein.

**Results**

**BORIS binds to the H19 DMR in cultured cells.** We initially determined whether BORIS, like CTCF (3), binds to the DMR of the H19 locus (Fig. 1A). ChIP analysis was done using either an anti-CTCF (Santa Cruz Biotechnology) or an anti-BORIS (Supplementary Fig. S2) antibody. Primers were used that overlap the primary H19 CTCF DNA-binding site that has been shown to regulate IGF2/H19 gene expression (2). ChIP of Tera-1 testicular tumor or HCT116 colon cancer cells followed by PCR or qPCR yielded a significant enrichment of the H19 DMR with both CTCF and BORIS antibodies (Fig. 1B–D). As a negative control, an H19 genomic region without a CTCF consensus site was amplified (data not shown) using primers that have been previously described (14). These results show that, like CTCF, BORIS binds to the H19 DMR (15).

**BORIS preferentially binds to the methylated H19 DMR.** Although BORIS and CTCF both seem to bind to the H19 DMR, the mechanism governing DNA binding is unknown, and it seemed reasonable to suggest that methylation might determine the specificity of binding. To address this idea, two cell lines were used, (a) HCT116 cells and (b) HCT116 somatic DKO cells, which have both DNMT1 and DNMT3B deleted. These cells have been previously characterized; the methylation is decreased ~95% in the DKO cells (13) compared with the parental HCT116 cells.

The methylation status of the H19 DMR was determined by bisulfite pyrosequencing (12) and clearly showed hypomethylation of the DMR in the DKO compared with HCT116 cells (Fig. 2A). ChIP analysis with an anti-BORIS antibody showed that BORIS binding was highest in the HCT116 cells and lowest in the unmethylated DKO cells (Fig. 2B, left). In contrast, CTCF DNA binding is highest in the unmethylated DKO cells and lowest in the HCT116 cells (Fig. 2B, right). Western blot analysis showed similar BORIS and CTCF protein levels in all HCT116 cell lines, ruling out that DNA binding is a function of total BORIS or CTCF levels (Supplementary Fig. S3). These experiments suggest that methylation may be at least one mechanism governing the DNA binding of CTCF or BORIS.

Although these experiments suggest that methylation may regulate CTCF and BORIS DNA binding, they do not a priori reveal the binding characteristics in a tissue culture model system.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** BORIS is an H19 DMR DNA-binding protein. **A.** diagram of the H19 region and the location of the primers used for ChIP analysis. **B.** BORIS binds to the H19 DMR in testicular tumor cells. Tera-1 testicular tumor cells were fixed with 1% formaldehyde to cross-link protein-DNA interactions and then sonicated, and fixed cells were immunoprecipitated with either an anti-CTCF or anti-BORIS (Supplementary Fig. S2) antibody. DNA was eluted and purified before analysis by real-time QPCR with primers that cover the CTCF-binding site in the H19 DMR region. BORIS binds to the H19 DMR in HCT116 colon tumor cells as measured by PCR (C) and QPCR (D). HCT116 cells were harvested and CTCF and BORIS DNA binding was measured as described above. All ChIP experiments were done in triplicate. **Columns, mean; bars, SD.**
To confirm the role of methylation in DNA binding, MS-ChIP-PCR was done in HCT116 cells. In these experiments, ChIP was performed with either an anti-BORIS or an anti-CTCF antibody and the pulled-down chromatin was then subjected to bisulfite conversion. These samples were subsequently used for methylation-specific PCR with primers (Methyl Primer Express software) designed to detect unmethylated versus methylated DNA. These experiments showed that BORIS preferentially binds to methylated DNA (Fig. 2C, left). In contrast, CTCF seems to preferentially bind to unmethylated chromatin (Fig. 2C, right).

**H19 gene expression is decreased in HCT-derived DKO cells.**

The results above show that BORIS preferentially binds to the H19 DMR in HCT116 cells, whereas CTCF preferentially binds to the H19 DMR in DKO cells (Fig. 2B). Thus, it seemed logical to suggest that the expression of H19 would correlate with the levels of BORIS or CTCF binding to the DMR. As such, HCT116 and DKO cells...
were harvested and H19 RNA levels were determined by RT-PCR. These experiments showed a roughly 70% decrease in H19 RNA levels in DKO compared with HCT116 cells (Fig. 2D). The H19 DMR was subsequently cloned upstream of ptk-Luc and this plasmid (DMR-tk-Luc) was transfected into both HCT116 and DKO cells. A roughly 5-fold increase in luciferase activity was observed in HCT116 cells transfected with DMR-tk-Luc compared with DKO cells (Fig. 2E).

BORIS binding is methylation independent. EMSAs and supershift experiments were done using HCT116 nuclear lysates mixed with a 32P-labeled Ssxl-methylated oligonucleotide containing the H19 DMR CTCF sequence. Supershift experiments with either an anti-BORIS (Fig. 3A, lanes 3 and 4) or an anti-CTCF (lanes 7 and 8) antibody showed that BORIS, but not CTCF, binds to methylated DNA. In contrast, supershift experiments using an unmethylated 32P-labeled oligonucleotide showed preferential CTCF binding (Supplementary Fig. S4A) with some minimal binding of BORIS (Supplementary Fig. S4B, lane 2 versus lane 4). This was our first indication that BORIS might also bind to unmethylated DNA.

Follow-up experiments were done using in vitro translated BORIS and CTCF with either an Ssxl-methylated or an unmethylated oligonucleotide containing a CTCF DNA-binding consensus sequence. EMSA analysis showed that the DNA binding of BORIS was unaltered by the methylation status of the oligo (Fig. 3B, left, lane 2 versus lane 4). In contrast, CTCF bound to the 32P-labeled unmethylated oligo (Fig. 3B, right, lane 6) but not to the SssI-methylated oligo (lane 8). The results of these experiments imply that, at least in vitro, BORIS seems to bind equally well to both methylated and unmethylated DNA, suggesting that BORIS, in contrast to CTCF, is a methylation-independent DNA-binding protein.

Small interfering RNA knockdown of CTCF increases BORIS binding to the H19 DMR. Because the results above suggested that BORIS can bind to unoccupied unmethylated CTCF DNA-binding sites in a cell-free system, we hypothesized that BORIS may be excluded from binding to unmethylated CTCF DNA-binding sites simply due to the competitive occupancy of CTCF. To investigate this idea, HCT116 cells were treated with either control or CTCF small interfering RNA (siRNA; Supplementary Fig. S5) followed by ChIP with either an anti-CTCF or an anti-BORIS antibody. These results clearly showed a decrease in CTCF (Fig. 3C, left) and an increase in BORIS (right) binding to the H19 DMR in the absence of normal CTCF protein (Supplementary Fig. S5). MS-ChIP-PCR (described

**Figure 3.** BORIS, but not CTCF, DNA binding is methylation independent. A, BORIS, but not CTCF, binds to a methylated H19 DMR CTCF DNA-binding sequence. HCT116 cells were harvested and nuclear cell extracts were used for EMSA with a 32P-labeled Ssxl-methylated oligonucleotide containing the H19 CTCF-binding sequence. Lanes 1 and 5, probe alone; lanes 2 to 4 and 6 to 8, incubated with nuclear extract. Supershift assays were done by preincubating extracts with increasing concentrations (+ or +++) of either an anti-BORIS (lanes 3 and 4) or an anti-CTCF antibody (lanes 7 and 8). Arrows, position of the protein-DNA complex and free probe; small arrows, shifted bands. B, BORIS, but not CTCF, DNA binding in vitro is independent on methylation. EMSAs were done with in vitro translated BORIS (left) or CTCF (right) with either an unmethylated oligonucleotide containing a CTCF-binding site (lanes 1, 2, 5, and 6) or an identical Ssxl-methylated oligonucleotide (lanes 3, 4, 7, and 8). All gel and supershift experiments were done in triplicate. Sections of fluorograms from native gels using a Typhoon phosphorimager are shown. Arrows, the supershift as well as the protein-CTCF-DNA complex and free unbound oligonucleotide probe. C and D, siRNA knockdown of CTCF decreases CTCF and increases BORIS binding to the H19 DMR. HCT116 cells were transfected with either control (Cont) or CTCF siRNA (see Supplementary Fig. S5 for decreased CTCF levels). ChIP was done followed by either (C) qPCR with primers that cover the H19 DMR or (D) MS-ChIP-qPCR via bisulfite treatment and qPCR with either methylated or unmethylated primer sets to the H19 DMR. All ChIP experiments were done in triplicate. Columns, mean; bars, SD. Statistical significance was established by Student’s t test. *, P < 0.05.
confirmed that siRNA knockdown of CTCF significantly increased BORIS binding to unmethylated DNA (Fig. 3D, 3rd versus 4th column) with little change to methylated DNA (1st versus 2nd column). siRNA knockdown of CTCF proportionally decreased CTCF DNA binding (Supplementary Fig. S6). These results suggest that CTCF may preferentially bind to unmethylated DNA, and if these sites become unoccupied, then BORIS can subsequently bind.

**BORIS binding is limited to the paternal H19 DMR.** It is well established that the H19 DMR from the maternal allele is hypomethylated, whereas the paternal allele is methylated and that this plays a key role in the regulation of IGF2 imprinting (14, 15). To address whether BORIS binds to the H19 DMR of either the maternal or paternal origin, two murine hybrid cell lines that contain human chromosome 11 of either maternal (A911M) or paternal (A911P) origin were used (16). ChIP analysis with an anti-BORIS antibody showed preferential binding to the paternal allele (Fig. 4A, left) with minimal binding to the maternal allele (right). In contrast, ChIP analysis with an anti-CTCF antibody showed preferential binding of CTCF to the maternal allele (right) as has been previously shown (16) with very little binding to the paternal allele (left). Methylation-specific PCR (Fig. 4B) and bisulfite pyrosequencing (Fig. 4C) of these two murine hybrid cell lines clearly showed that the maternal H19 DMR is hypomethylated, whereas the paternal H19 DMR is methylated.

**Discussion**

BORIS and CTCF share similar zinc finger DNA-binding domains and bind to the same cis-acting DNA-binding element; however, their NH2-terminal and carboxy amino acid sequences are distinct, suggesting that these two insulator DNA-binding proteins may have divergent cellular functions (6, 17). Whereas CTCF plays a central role in IGF2/H19 imprinting (4), the intracellular function of BORIS is unclear. However, it is well established that BORIS is expressed in malignancies, and in this setting, both CTCF and BORIS are present in tumor cells (6). Thus, the mechanism governing either CTCF or BORIS DNA binding to CTCF DNA-binding target sequences is very likely to provide insight into the cellular role of these two insulator DNA-binding proteins and the subsequent expression of downstream genes, some of which may be prosurvival or pro-proliferative.

In this work, we found that BORIS DNA-binding activity is largely independent of the methylation status of its target sequence. In contrast, CTCF DNA binding is limited to unmethylated DNA. We also showed that BORIS preferentially binds to the paternal H19 DMR using matched murine hybrid cells that contain human chromosome 11 of either maternal or paternal origin. In contrast, CTCF seems to preferentially bind to the maternal H19 DMR. These results are surprising because these two proteins

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**Figure 4.** BORIS preferentially binds to the paternal H19 DMR. A, BORIS binds preferentially to the paternal allele. A911M and A911P mouse hybrid cells that contain human chromosome 11 of either the paternal (left) or maternal (right) origin, respectively, were analyzed via ChIP using either an anti-CTCF or an anti-BORIS antibody with primers to the H19 DMR. DNA was eluted and analyzed by QPCR with primers to the human H19 DMR (Fig. 1A). All ChIP experiments were done in triplicate. Columns, mean; bars, SD. B and C, methylation-specific analysis of the paternal and maternal H19 DMR in the murine hybrid cell lines. A911M and A911P mouse hybrid cells were harvested, bisulfite treated, and subjected to QPCR with either methylated or unmethylated primer sets to the human H19 DMR (B) or analyzed by bisulfite pyrosequencing (C).
BORIS Is a Methylation-Independent Binding Protein

...contain a significant degree of homology in their zinc finger DNA-binding domains and both bind to identical cis-acting DNA regulator elements (6). siRNA knockdown of CTCF decreased CTCF binding to the DMR with a reciprocal increase in binding by BORIS, an effect that seems to involve binding of BORIS to unmethylated DNA.

Thus, these results suggest a regulatory mechanism whereby BORIS preferentially binds to methylated cis-acting CTCF DNA-binding sites because it is prevented from binding to unmethylated CTCF DNA-binding sites by the physical occupancy of CTCF. In isolation, BORIS preferentially binds to methylated DNA-binding sites but also binds readily to unmethylated DNA-binding sites. In contrast, when both CTCF and BORIS are present within the cell, which is the case in tumor (HCT116) and immortalized (A911P/A911M) cell lines, BORIS can bind to unmethylated CTCF DNA-binding sites only when CTCF is displaced. This explains why BORIS preferentially binds to the methylated paternal H19 DMR in the hybrid mouse cells.

In conclusion, these results strongly suggest that DNA methylation may be at least one mechanism governing the specificity of insulator DNA-binding proteins in the regulation of target gene expression. Because CTCF seems to be ubiquitously expressed in all cells, we would expect that the main targets of BORIS in tumor cells would be the induction of genes that contain methylated promoter regions. However, the empirical fact that CTCF only binds to the unmethylated DMR suggests that the DNA binding of BORIS and CTCF in tumor cells is quite complicated, and although the DNA-binding domains are highly similar, there might be key differences that lead to different binding properties. In addition, different NH$_2$ and COOH termini of these two proteins might interact with different protein complexes leading to dissimilar DNA-binding behavior. The answers to these questions should help to better understand the binding dynamic under different physiologic conditions leading to better understanding of the role of BORIS and CTCF in normal and malignant physiology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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