Conditional Expression of the CTCF-Paralogous Transcriptional Factor BORIS in Normal Cells Results in Demethylation and Derepression of MAGE-A1 and Reactivation of Other Cancer-Testis Genes

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Abstract

Brother of the Regulator of Imprinted Sites (BORIS) is a mammalian CTCF paralog with the same central 11Zn fingers (11ZF) that mediate specific interactions with varying ~50-bp target sites. Regulated *in vivo* occupancy of such sites may yield structurally and functionally distinct CTCF/DNA complexes involved in various aspects of gene regulation, including epigenetic control of gene imprinting and X chromosome inactivation. The latter functions are mediated by meCpG-sensitive 11ZF binding. Because CTCF is normally present in all somatic cells, whereas *BORIS* is active only in CTCF- and 5-methylcytosine–deficient adult male germ cells, switching DNA occupancy from CTCF to BORIS was suggested to regulate site specificity and timing of epigenetic reprogramming. In addition to 11ZF-binding paternal imprinting control regions, cancer-testis gene promoters also undergo remethylation during CTCF/BORIS switching in germ cells. Only promoters of cancer testis genes are normally silenced in all somatic cells but activated during spermatogenesis when demethylated in BORIS-positive germ cells and are found aberrantly derepressed in various tumors. We show here that *BORIS* is also expressed in multiple cancers and is thus itself a cancer-testis gene and that conditional expression of *BORIS* in normal fibroblasts activates cancer-testis genes selectively. We tested if replacement of CTCF by BORIS on regulatory DNA occurs *in vivo* on activation of a prototype cancer-testis gene, *MAGE-A1*. Transition from a hypermethylated/silenced to a hypomethylated/activated status induced in normal cells by 5-aza-2′-deoxycytidine (5-azadC) was mimicked by conditional input of *BORIS* and is associated with complete switching from CTCF to BORIS occupancy at a single 11ZF target. This site manifested a novel type of CTCF/BORIS 11ZF binding insensitive to CpG methylation. Whereas 5-azadC induction of *BORIS* takes only few hours, derepression of *MAGE-A1* occurred 1 to 2 days later, suggesting that BORIS mediates cancer-testis gene activation by 5-azadC. Indeed, infection of normal fibroblasts with anti-*BORIS* short hairpin RNA retroviruses before treatment with 5-azadC blocked reactivation of *MAGE-A1*. We suggest that BORIS is likely tethering epigenetic machinery to a novel class of CTCF/BORIS 11ZF target sequences that mediate induction of cancer-testis genes. (Cancer Res 2005; 65(17): 7751-62)

Introduction

It is increasingly recognized that many human pathologies, including sporadic and inherited tumors, gene imprinting disorders, and familial skewed X chromosome inactivation (XCI) syndrome, may involve common types of epigenetic deregulation nonrandomly distributed throughout the genome. Specific targeting of epigenetic machinery cannot occur without factors capable of site-specific DNA recognition, but the very existence of such factors and their identity remained elusive until chromatin regions containing varying CTCF-binding sequences emerged as common places of epigenetic regulation and reprogramming (for reviews with a chronological survey, see refs. 1–6).

The first connection between region-specific epigenetic marking and formation of methylation-sensitive CTCF/DNA complexes was established in the *IGF2/H19* imprinting control region (ICR; refs. 7–10). It was later extended to include different nucleotide sequences of additional methylation-sensitive CTCF target sites that contribute to epigenetic regulation of XCI (11, 12) as well as the XCI escape mechanisms that exclude gene clusters from X-linked heterochromatinization (13). In mice, targeted mutation of the 11Zn fingers (11ZF)–contacting bases of a single CTCF target site within the *H19* ICR leads to loss of *IGF2* imprinting and complex patterns of *de novo* methylation on maternal inheritance (14). Remarkably, molecular studies of human pathologies revealed similarly critical roles of homologous 11ZF-binding sites in the human *H19* ICR. Recent reports indicate that several cases of Beckwith-Wiedemann syndrome (BWS) with and without Wilms' tumors in a familial setting are associated with precise microdeletions of two (15) or three (16) of a total of seven CTCF sites from the *H19* ICR in several different affected families.

Similarly, targeted mutagenesis of the other set of 11ZF sites in the *XIST/Tsix* locus (11) in mice gave insight into the complex control of choice/imprinting of XCI. These CTCF target sites are so functionally important that their targeted deletion in mice resulted in both skewing of Xi and lethality on maternal transmission (11). As with CTCF sites in BWS families, familial cases of point mutations in *XIST* revealed a correlation between CTCF binding and preemptive choices of XCI (12). Furthermore, identification of >200 novel CTCF-binding regions in mice, combined with analyses of their methylation and of *in vivo* occupancy by CTCF, MBD2, and

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MeCP2, suggested that many (but not all) of these regions maintain unmethylated loci throughout mammalian genomes (17). This study also showed that many of these sites seem to colocalize with a high density of meCpG clusters and with the heterochromatin marker, HP1, paradoxically suggesting that CTCF may contribute not only to maintaining demethylated regions but also to heterochromatin organization and/or gene silencing. Here, we asked if CTCF sites associated with silent heterochromatin may reflect a previously unrecognized ability of CTCF to bind DNA sequences in a methylation-insensitive manner and which functions such sites might be linked with.

The recent cloning of the *CTCF*-paralogous gene, designated brother of the regulator of imprinted sites (*BORIS*), helped us provide a possible solution to this question. *BORIS* exhibits extensive homology to *CTCF* in the central 11ZF region, encoded by the exact same duplicated exons, allowing for a similar DNA-binding spectrum. Because outside of the shared 11ZF domain these factors contain different NH_2 and COOH termini capable of recruiting different proteins and undergoing distinct types of post-translational modifications (see refs. 1, 2, 18, 19 and references therein), it was suggested that the regions of *BORIS* and *CTCF* flanking the 11ZF may mediate divergent effects at the overlapping spectrum of 11ZF target sites in chromatin (2, 20).

Unlike *CTCF, BORIS* is not expressed in normal somatic cells. During spermatogenesis, expressional switching from *CTCF* to *BORIS* coincides with the resetting of paternal methylation marks within the *H19* ICR as well as with transcriptional activation/demethylation of a unique class of gene promoter regions that are repressed in normal somatic cells but expressed only in testis and in various cancers and therefore named cancer testis genes. Thus, epigenetic regulation through a class of methylation-insensitive CTCF/BORIS–binding sequences may extend to cancer-testis genes in normal somatic cells in which methylation-associated heterochromatinization results in their silencing (reviewed in refs. 6, 21–25). One earlier study suggested that BORIS may play the role of a site-specific "demethylating factor" in osteosarcomas with biallelic demethylation of the *H19* ICR (26). Recent studies, including this and the accompanying report (27), indicate that *BORIS* is activated in a variety of human cancers and that competition between BORIS and CTCF may indeed contribute to epigenetic deregulation of cancer-testis genes.

In the present study, we have examined the possibility of cancer-testis gene reactivation by conditional ectopic expression of *BORIS* in cultured normal human dermal fibroblasts (NHDF), with emphasis on the mechanistic details of BORIS-induced derepression/demethylation of one of the best-studied examples of cancer-testis genes, *MAGE-A1*. We compared cancer-testis gene activation following exposure to 5-aza-2′-deoxycytidine (5-azadC) to that following ectopic *BORIS* expression. Bisulfite sequencing experiments confirmed that induction of *MAGE-A1* expression by BORIS coincided with demethylation of the *MAGE-A1* promoter in a pattern that was remarkably similar to the pattern seen following treatment with the DNA methylation inhibitor, 5-azadC. *In vitro* and *in vivo* binding analyzed by electrophoretic mobility shift assay (EMSA) and chromatin immunopurification (ChIP) revealed that CTCF and BORIS bind to one common region within the *MAGE-A1* promoter in a methylation-insensitive manner. In normal fibroblasts, CTCF occupies methylated and silenced *MAGE-A1* promoter, whereas activation/demethylation of this promoter induced in the same cells by BORIS (supplied ectopically by transfection of a tetracycline-inducible vector or induced endogenously by 5-azadC treatment) results in a complete exchange of CTCF for BORIS binding *in vivo*. To our knowledge, this is the first report describing that reciprocal binding of CTCF and BORIS to the *MAGE-A1* promoter coincides with derepression of this cancer-testis gene.

Moreover, because induction of *BORIS* by 5-azadC preceded up-regulation of the *MAGE-A1* cancer-testis gene by many hours, we suggested that activation by 5-azadC may be carried out through BORIS. To test this, a retroviral short hairpin RNA (shRNA) against *BORIS* was implemented to provide direct evidence that *BORIS* activation is a necessary step for reactivation of *MAGE-A1* by a chemical DNA methylation inhibitor, 5-azadC.

We also provide evidence that the novel type of CpG methylation-insensitive CTCF/BORIS binding [as shown here for *MAGE-A* and *NY-ESO-1* in the accompanying article by Hong et al. (27)] may be responsible for regional derepression of cancer-testis genes in cancers that are positive for BORIS. Therefore, aberrant activation of *MAGE-A1* in somatic cells cannot be a random consequence of genome-wide demethylation in cancer as thought previously (28) but rather a process of targeted epigenetic modifications directed in transformed cells by a novel class of CTCF/BORIS 11ZF-binding sequences.

Materials and Methods

Cells. NHDFs were purchased from Cambrex (Walkersville, MD). Various cancer cell lines were from our laboratory collection or obtained from American Type Culture Collection (Manassas, VA). A glioblastoma cell line, L299, was a generous gift of Dr. E.G. Van Meir (Emory University, Atlanta, GA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (Invitrogen, Carlsbad, CA). RNA from clinical samples were provided by our collaborators in National Cancer Institute (Bethesda, MD). Some breast cancer samples were kindly provided by Dr. A. Lindblom (Karolinska Hospital, Stockholm, Sweden).

Plasmids, transfection, and 5-aza-2′-deoxycytidine treatment. Plasmids expressing BORIS or the zinc finger domain of CTCF (pBIG2i-BORIS and pBIG2i-ZF-CTCF, respectively, with cDNA from refs. 20, 30, 31) were constructed on a template containing the tetracycline-responsive, auto-regulated, bidirectional expression vector pBIG2i that was a generous gift from Dr. Craig Strathdee (now at Immunex/Amgen Corp., Seattle, WA; ref. 29). The original plasmid was used as a control "empty" vector. Cells (1×10^7) were transfected with 5 μg DNA using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and doxycycline (2 μg/mL) was added immediately after transfection. Twenty-four hours after transfection, fresh medium with 2 μg/mL doxycycline was introduced and cells were allowed to grow for an additional 2 days before RNA/DNA/protein isolation. Cells were incubated with 1 or 10 μmol/L freshly prepared 5-azadC (Sigma-Aldrich, St. Louis, MO) in culture medium for 72 hours.

Immunohistochemistry. Immunohistochemical analysis was done as described in ref. 17, 20. Briefly, cells were grown on glass eight-well chamber slides, washed twice in PBS, fixed in acetone for 15 minutes, washed 3×5 minutes in PBS, and processed according to the protocol for BORIS detection using chicken anti-BORIS antibodies.

Reverse transcription-PCR. RNA was isolated using a RNeasy kit (Qiagen, Valencia, CA). cDNAs were synthesized by using SuperScript III (Invitrogen, Carlsbad, CA) reverse transcriptase and oligo(dT) primers according to manufacturer's protocol: 2 units RNase OUT, 5× reverse transcription-PCR (RT-PCR) buffer, 50 mmol/L DTT, 0.5 mmol/L deoxynucleotide triphosphate, and 200 units SuperScript III reverse transcriptase. Reactions were run for 50 minutes at 57°C. The PCR reaction was done as follows: 2× PCR buffer B (Epicentre Technologies, Madison, WI), H_2O, 2 units Taq polymerase (Invitrogen, Carlsbad, CA), cDNA (1:20 of reverse transcription reaction mix), 40 to 45 cycles. The primers used to amplify each gene fragment and the correct fragment lengths are listed in Table S1. PCR products amplified from cell lines and primary tissues were sequence verified.

Bisulfite sequencing of the *MAGE-A1* promoter region. Genomic DNA was subjected to bisulfite modification using a CpGenome DNA modification kit (Serologicals Corp., Norcross, GA). A 335-bp sequence was amplified using PCR Platinum Taq polymerase and the following conditions: (*a*) 95°C for 2 minutes; (*b*) 95°C for 45 seconds, 60°C for 30 seconds, and 72°C for 1 minute × 37; (*c*) 68°C for 3 minutes; and (*d*) 4, ∞. PCR fragments were gel extracted using a Gel Extraction kit (Qiagen) and ligated into the TOPO TA cloning vector (Invitrogen, Carlsbad, CA). Following transformation, plasmids from individual bacterial colonies were isolated and sequenced.

Southern blot analysis. Genomic DNA was isolated from cells using a DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol. DNA was digested overnight with a mix of *Mse*I and *Aci*I restriction endonucleases (New England Biolabs, Beverly, MA) and loaded onto 1.5% agarose gel (1× Tris-borate EDTA). After electrophoresis, the gel was washed with water and incubated twice with 500 mL of 0.25 mol/L HCl followed by incubation twice with 500 mL alkaline denaturing solution and two rounds of 500 mL neutralization solution. Then, DNA was transferred overnight to Hybond-N (Amersham, Piscataway, NJ) nitrocellulose membranes with 20× SSC. The next day, the membrane was washed in 2× SSC, dried, and UV cross-linked. The DNA probe was prepared using a Prime-It RmT Random Primer Labeling kit (Stratagene, La Jolla, CA). The probe was hybridized to the membranes overnight in PerfectHyb Plus hybridization buffer (Sigma-Aldrich) followed by multistep washing with 2× SSC-0.1% SDS solution. Hybridization signals were then visualized on BioMax MR or MS films (Kodak, Rochester, NY).

Electrophoretic mobility shift assay. The luciferase and CTCF 11ZF DNA-binding domain proteins were synthesized from the luciferase T7 control DNA and pCITE-11ZF constructs, respectively (30, 31), with the TnT *in vitro* Transcription-Translation System (Promega, Madison, WI). Seven overlapping DNA fragments were ^{32}P-labeled, gel purified, and used as DNA probes for gel mobility shift assays with equal amounts of the *in vitro*–translated luciferase and CTCF proteins as described (12).

Chromatin immunopurification. NHDFs treated with 5-azadC (NDHF-5-azadC) for 3 days and NHDFs with BORIS expressed from the transiently transfected construct (NHDF-isB) were used for ChIP assays to show an association between CTCF and/or BORIS with the human *MAGE-A1* promoter. We used a ChIP Assay kit (Upstate, Charlottesville, VA) and followed the manufacturer's recommendation. One ChIP reaction used 10 μg anti-CTCF monoclonal antibodies as described in ref. 12 or the same amount of rabbit anti-BORIS peptide polyclonal antibody B3. Specificity of these antibodies for DNA-bound CTCF and for DNA-bound BORIS complexes was verified by gel supershifting experiments with various CTCF/BORIS–binding sites as described earlier (20). Immunopurified DNA was PCR amplified with primers corresponding to the S3 fragment of the *MAGE-A1* promoter: sense primer 5′-CAAATCACAAAGATGTCCGGCTTTCAATCTTCTAGGC-3′ and antisense primer 5′-AAGCTTCCAGCCCCGAGAGAGTAAGAAATATGGCTG-3′. For the positive controls for CTCF-ChIP analyses, we used the CTCF site N (see refs. 12, 32) in the human *MYC* 5′-insulator that was amplified using primers: sense primer 5′-GGCTCTGTGAGGAGGCAAGGTG-3′ and antisense primer 5′-GCTCTCTATTTGGAGTGGCGGG-3′. Quantitation of ChIPed DNA has been done by real-time PCR method suggested by Litt et al. (33) using the ABI Prism 7900 Sequence Detection System as described in detail by Pugacheva et al. (12).

Short hairpin RNA retroviral vector construction. Both sense and antisense oligonucleotide pairs were synthesized to BORIS target sequences, annealed, and then cloned into the puromycin and neomycin version of the retroviral vector, pSUPER (OligoEngine, Seattle, WA). *BORIS* target sequence 1 was 5′-CGGAGGAGAGCGAGAAGTACA-3′ and *BORIS* target sequence 4 was 5′-GTCCCCATTGTGCCACCATCA-3′. The retroviral plasmids were then transfected into the packaging line, PT67 (Clontech, Palo Alto, CA), and selected with either G418 (Invitrogen, Grand Island, NY) or puromycin (Clontech).

Infection of short hairpin RNA vectors. Untreated six-well plates were coated with retronectin (Cambrex) according to the manufacturer's directions. Retroviral supernatant was collected from packaging lines that were seeded at a density of 10^7 cells per 10 mL medium and incubated at 32°C overnight. The viral supernatant was filtered through a 0.45 μm filter (Millipore Corp., Bedford, MA) before addition to the retronectin-treated plates. The viral supernatant was incubated at 32°C for 7 hours. Supernatant was then removed, wells were washed with PBS, and the target cells were added. NHDF (p3) and normal human fibroblasts (NHF; p7) cells were added at a density of 10^5 cells per well to the pretreated wells. NHDFs were grown in FBM + FGM2 Singlequot supplement (Cambrex) and NHFs were grown in DMEM/F-12 supplemented with 10% FBS. Plates were incubated for 48 hours at 37°C, trypsinized, and then placed into a T-75 flask with selection. After 12 days of selection, cells were infected with the second virus using the same procedure as outlined above. After 48 hours, cells were selected using both selectable markers (puromycin and neomycin).

Quantitative reverse transcription-PCR. Total RNA was isolated from cultured cells using Trizol (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. RNA was then converted to cDNA using random primers and SuperScript II reverse transcriptase. *BORIS, MAGE-A1*, and *CTCF* expression was determined using the following primer sequences and probes: 5′-CCCATTGTGCCACCATCA-3′ (BORIS forward), 5′-AGCATGCAAGTTGCGCATAT-3′ (BORIS reverse), 6FAM-ACGGAAAAGCGACCTACMGB (BORIS probe), 5′-TGACACAGTCATAGCCCGAAAA-3′ (CTCF forward), 5′-TGCCTTGCTCAATATAGGAATGC-3′ (CTCF reverse), and 6FAM-TGATTTGGGTGTCCACTTGCGAAAGC-MGB (CTCF probe). *MAGE-A1* and human *β-actin* Taqman primers/probe sets were purchased as Predeveloped Assays (Applied Biosystems, Foster City, CA).

Results

Aberrant derepression of *BORIS* occurs often in cancer cell lines and primary tumors but not in primary cell cultures. We suggested previously that a combination of DNA demethylation and CTCF silencing in BORIS-positive germ cells that express other cancer-testis genes may indicate a common mechanism for transcriptional regulation of *BORIS* and other cancer-testis genes (20). Several established cancer cell lines were analyzed by Loukinov et al. (20) for *BORIS* activation, and high levels of *BORIS* mRNA and BORIS protein were observed in SV40-transformed mouse spermatogonia- and spermatocyte-derived cell lines, *a priori* expected to express most of the known 89 cancer-testis genes (see a summarizing table for cancer-testis gene expression patterns in ref. 24) as well as in the highly *BORIS*-positive erythroleukemia cancer cell line, K562, which by quantitative RT was also found to have a very high level of *MAGE-A1* (34). Next, we used RT-PCR analysis to further examine the extent of BORIS derepression in cancer cell lines. Ubiquitously expressed *CTCF* (35) served as an internal control of the quality of both RNA and first-strand cDNA. Because many of the cancer-testis genes were first cloned from melanoma cell lines (reviewed in ref. 36), we first examined a panel of well-established melanoma lines for *BORIS* expression. We readily detected *BORIS* expression in 9 of 10 lines (Fig. 1*A*). In addition, *BORIS* mRNA was detected in breast cancer (7 of 8), neuroblastoma (4 of 5), prostate (3 of 5), and colon cancer (3 of 4) cell lines. Importantly, similarly significant proportions of *BORIS*-positive samples were found among randomly selected primary tumors from breast (11 of 12), prostate (9 of 10), and colon (8 of 10) cancers (Fig. 1*B*). Cell lines shown to be *BORIS* negative on Fig. 1*A* turned out to be *BORIS* positive on treatment with 5-azadC, suggesting that *BORIS* can be up-regulated in a variety of cells by this agent (data not shown). To determine the fraction of cells that actually express BORIS in different tumors, we used immunohistochemical staining of cancer sections by the affinity-purified antibodies ap-2-ab described earlier (20). Representative examples of such staining experiments, shown in Fig. S3, had revealed mostly nuclear BORIS protein (in osteosarcoma, Wilms'

tumor, and pheochromocytoma samples) interspersed to various degrees with cytoplasmic fraction of the protein (for gliomas, breast tumors, and prostate tumors).

We conclude that strict silencing of *BORIS* observed in normal somatic tissues is frequently abrogated in various cancers, thereby confirming that *BORIS* is indeed a novel cancer-testis gene as we suggested (2, 20). Abnormal reactivation of *BORIS* does not occur during the establishment of primary tissue cultures of different adult mouse cells, even from *p53* knockout mice with a cancer-prone phenotype (Fig. 1*C*), suggesting that expression in tumor cells does not result from tissue culture stress conditions associated with the establishment of cancer cell lines.

Immunohistochemical and Western blot analysis of BORIS and MAGE-A1 expression in normal human dermal fibroblasts treated with 5-aza-2′-deoxycytidine or transfected with a BORIS-expression vector. To determine if *BORIS* transcripts induced by 5-azadC were associated with expression of protein, we used anti-BORIS antibodies in immunohistochemical (Fig. 2*A*) and Western blot analyses of proteins extracted from treated cells (Fig. 2*B*). As a control, we examined cells carrying a BORIS expression vector. We also tested cells transfected with empty vector or a vector expressing the 11ZF of CTCF as negative controls. BORIS was not expressed in NHDFs transfected with either empty vector or the zinc finger domain of CTCF (Fig. 2*A*). In contrast, cells transfected with full-length *BORIS* cDNA or treated with 5-azadC expressed BORIS at high levels. BORIS expressed either from a plasmid or induced by 5-azadC showed some cytoplasmic but a predominantly nuclear localization. As shown in the magnified insets in boxes B and Aza in Fig. 2*A*, in cells treated with BORIS vector or with 5-azadC, BORIS expression within the nucleus was speckled, suggesting association with specific subnuclear structures. In addition, BORIS vector-transfected and 5-azadC-treated fibroblasts exhibited a dramatic decrease of MeCP2-containing granules (see Fig. S1), representing high-density HP1-positive heterochromatin (data not shown; see also figure in ref. 17 showing HP1 + CTCF staining). These observations indicate that transient expression of BORIS from our tetracycline-regulated vectors was not toxic to the cells and that ectopic BORIS caused noticeable alteration of nuclear chromatin packaging similar to that observed by 5-azadC treatment. Protein extracts prepared from normal testis and from cells in these experimental groups were examined by Western blot analysis for expression of BORIS and MAGE-A1 (Fig. 2*B*). As expected, both proteins were present in extracts from testis and, consistent with the RT-PCR data described above, in extracts from cells treated with 5-azadC. We found that MAGE-A1 protein was readily detected in extracts from NHDFs expressing BORIS from the vector. In contrast, neither MAGE-A1 nor BORIS was detected in extracts from cells transfected with empty vector or vector expressing the 11ZF region of CTCF.

Brother of the regulator of imprinted sites reactivates expression of many cancer-testis genes. To determine if aberrant expression of BORIS in normal somatic cells might influence the expression of other cancer-testis genes, RT-PCR was done on the same set of samples, namely NHDF with male karyotype (XY) transiently transfected with empty vector, with a full-length *BORIS* cDNA, with a cDNA encoding only the 11ZF region of *CTCF* cDNA, or NHDFs treated with 5-azadC (at 1 μmol/L final concentration for 72 hours). The presence of a single X chromosome ensured the analysis of only one allele of cancer-testis genes, most of which map to this chromosome (36, 37). As shown in Fig. 3, transient expression of BORIS results in a

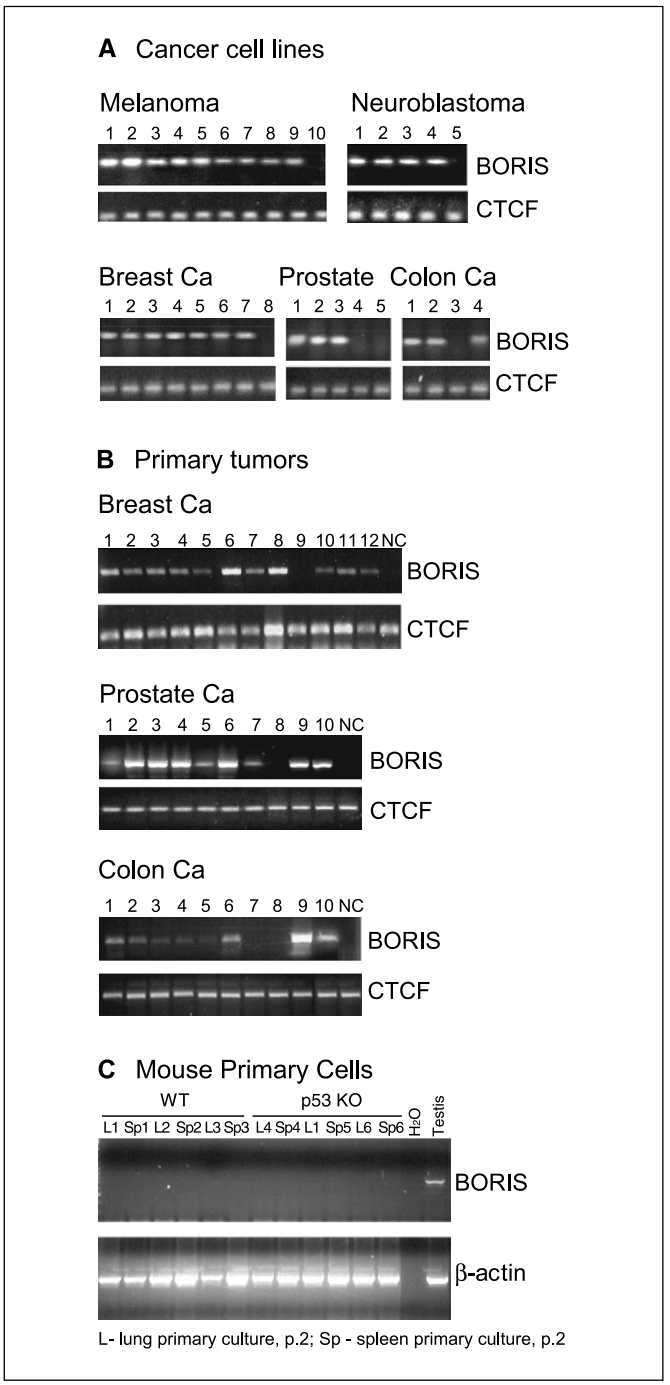


Figure 1. Aberrant reactivation of *BORIS* in various cancer cell lines and primary tumors. *A,* multiple cancer cell lines show expression of *BORIS* mRNA by RT-PCR. Lanes are marked according the type of cancer: melanoma (*lanes 1-10*): SK-MEL-5, MALME-3M, SK-MEL-2, SK-MEL-28, Godowns-MEL, 624.28-MEL, 928-MEL, 1359-MEL, A375, and 1123-MEL; neuroblastoma (*lanes 1-5*): SK-N-D2, GoTo, SK-N-SH, SK-N-DZ, and NBL-W; breast cancer cell lines (*lanes 1-8*): Du4475, CAMA-1, T47D, MDA231, MDA435, MDA453, ZR75-1, and MCF7; prostate cancer (*lanes 1-5*): Vcap, DuCap, TP2, Du145, and PC3; colon cancer (*lanes 1-4*): Colo320 HSR, DLD1, SW48, and Colo205. *B,* representative examples of abnormal *BORIS* activation in random samples of primary breast, prostate, and colon tumors and lack of activation in corresponding normal control tissues (*lane NC*). *C,* activation of *BORIS* does not occur as an adaptation to tissue culture conditions. Representative lung and spleen tissue samples from three healthy adult wild-type and p53 knockout mice have been used for preparing primary cell cultures grown for 1 week under standard conditions.

marked induction of a series of cancer-testis genes, including *MAGE-A1, MAGE-A2, MAGE-B1, MAGE-B4, GAGE-3-8, RAGE-2, Oct-3/4* (*POU5F1*), *NY-ESO-1* (*CTAG1B*), and *LAGE-1* (*CTAG2*), in cells either transiently transfected with the BORIS expression vector or treated with 5-azadC. Virtually identical results were observed after either treatment (Fig. 3*A*). All cancer-testis genes analyzed were strongly induced by 5-azadC and readily detected by RT-PCR (Fig. 3). For most cancer-testis genes, comparable levels of transcripts were seen in cells expressing BORIS from a vector. In contrast, the expression of genes, which do not encode cancer-testis antigens and are not activated in concert with CTCF-BORIS switching during spermatogenesis, such as *IL-2, FGF4, SOX1*, and *REX1*, was unaffected in NHDF transfected with BORIS vector or treated with 5-azadC. An important control for selectivity of the activation of cancer-testis genes by BORIS in NHDF was the *IL-2* gene, which can be actively demethylated and activated in T cells in ~30 minutes (ref. 38; see a summary in Fig. 3). Similar results of cancer-testis gene activation studies have been obtained not only with NHDF and primary cultures of adult mouse normal cells but also with other normal fibroblasts, including those derived from neonatal foreskin (NHF) that, in addition to NHDF, were used in the anti-*BORIS* shRNA experiments described below. In addition, cancer-testis gene expression was not observed in cells transfected with either empty vector or a plasmid encoding the zinc finger region of *CTCF*. The observation that cancer-testis genes are expressed after introduction of *BORIS* cDNA into normal cells suggests that BORIS may contribute to disruption of the normally tight repression of cancer-testis genes in somatic cells.

As *BORIS* itself is a cancer-testis gene, it seemed possible that BORIS might regulate its own expression. To examine this issue, we studied induction of the endogenous *BORIS* gene in adult murine fibroblasts in response to transient expression of human *BORIS*. Because cDNAs for mouse and human *BORIS* differ markedly in the 5′ end of their respective coding sequences, RT-PCR primers were designed to specifically recognize murine *BORIS* cDNA. As shown in Fig. 3, transient expression of human *BORIS* resulted in a marked induction of endogenous *BORIS* expression in adult mouse fibroblasts. It is also worth noting that transient transfection with antisense *CTCF* can induce *BORIS* (Fig. 3), further supporting the idea of a possible mutual regulation loop between the two paralogs (for details, see refs. 2, 20). Activation of *BORIS* by down-regulation of *CTCF* is in line with the recent findings that loss of one allele of *CTCF* in normal rat RIE cells resulted in both soft-agar growth (39) and *BORIS* activation in *CTCF*+/− cells.[4] Importantly, expression of endogenous *BORIS* in mouse adult primary cells, initially induced by human *BORIS* from the tetracycline vector, continued after removal of doxycycline from the medium in these cells. Finally, Fig. 3 also shows that gene induction by *BORIS* was not only limited to cancer-testis genes but also included other male germ cell–specific genes like *Oct-3/4* (*POU5F1*), a marker of murine and human pluripotent stem cells that is coexpressed with BORIS in testis and embryonic stem cells.[5]

Expression of *BORIS* is associated with changing methylation patterns of the *MAGE-A1* promoter. To further examine the role of *BORIS* in mediating cancer-testis gene expression, bisulfite sequencing experiments were done to evaluate the methylation status of the *MAGE-A1* promoter in NHDFs transfected with a plasmid containing *BORIS* cDNA as well as in cells treated with 5-azadC (Fig. 4*A-D*). Our bisulfite sequencing analyses covered 23 CpG dinucleotides contained in a fragment sequence from −399 to

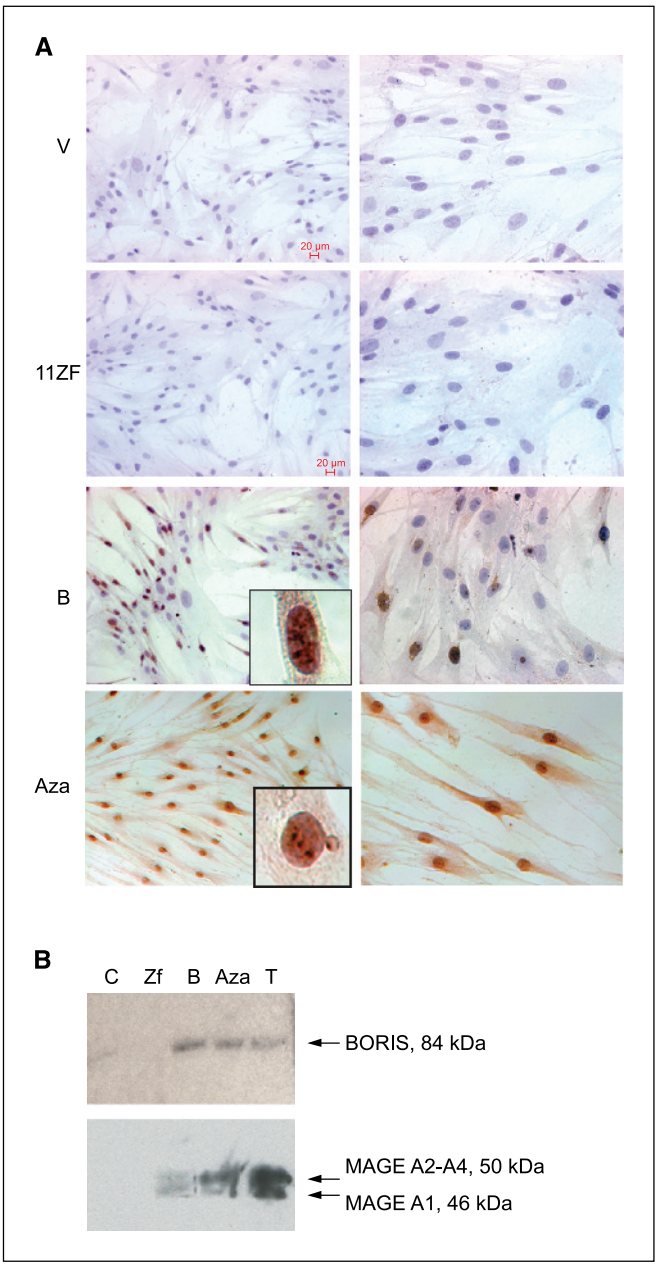
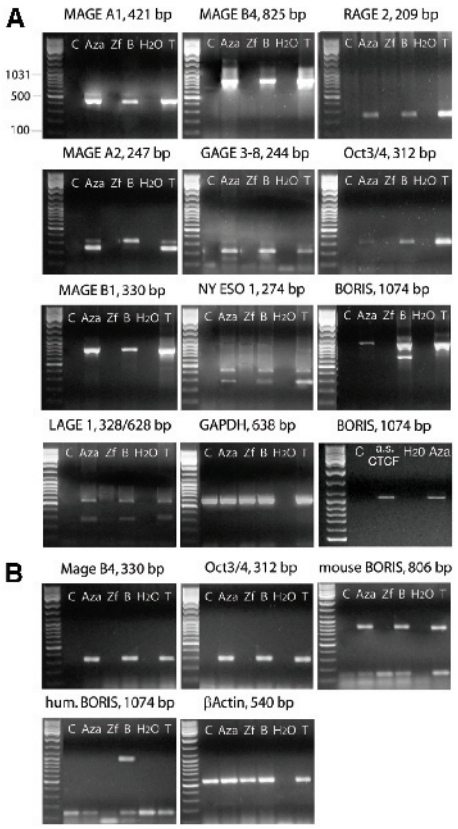


Figure 2. Immunohistochemical analysis of *BORIS* expression in normal primary human fibroblasts treated with *BORIS* expression vector or 5-azadC. *A,* cells were fixed with methanol, permeabilized with Triton X-100, and incubated with ap-2-ab chicken antibodies specific for BORIS protein and then secondary horseradish peroxidase conjugates. Original magnification, ×10 (*left*) and ×40 (*right*). *V,* control fibroblasts transfected with empty vector; *11ZF,* fibroblasts transfected with plasmid expressing BORIS/CTCF zinc finger domain only; *B,* fibroblasts transfected with *BORIS* expression plasmid; *Aza,* cells treated with 1 μmol/L 5-azadC for 3 days. *B,* Western blot analyses of BORIS and several MAGE family proteins in control fibroblasts transfected with empty vector (*lane C*), in the same fibroblasts transfected with the vector for the 11ZF domain of CTCF/BORIS (*lane Zf*) or with the full-length BORIS expression plasmid (*lane B*), and in cells treated with 1 μmol/L 5-azadC (*lane Aza*). Total protein lysate from normal adult human testis was also included as a positive control (*lane T*). Human BORIS protein is represented by a 84-kDa band (*top*). Expression of MAGE-A proteins was detected by monoclonal antibody 6C1 (these antibodies detect both MAGE-A1 and MAGE-A2).

[4] S. Pack et al., unpublished data.
[5] S. Vatolin et al., unpublished data.



C

Gene	Induction with: 5azadC	11ZF	BORIS	Normal Coexpression with BORIS
MAGE-A1*	+	-	+	Yes
MAGE-A2*	+	-	+	Yes
MAGE-A3*	+	-	-	Yes
MAGE-A4*	+	-	+	Yes
MAGE B1*	+	-	+	Yes
MAGE B2*	+	-	-	Yes
MAGE B4*	+	-	+	Yes
MAGE C4*	-	-	-	Yes
GAGE 1/2*	+	-	-	Yes
GAGE 3 8*	+	-	+	Yes
LAGE 1*	+	-	+	Yes
NY-ESO-1*	+	-	+	Yes
PAGE 1*	+	-	-	Yes
SSX-2*	+	-	-	Yes
SCP-1	+	-	-	Yes
SPANX s*	+	-	+	N/D
RAGE	+	-	+	N/D
RAGE 2	+	-	+	N/D
RAGE 3	+	-	-	N/D
POU5F1 [Oct3/4]	+	-	+	**Yes**
BORIS	+	-	+	**Yes**

D

Gene	Induction with: 5azadC	11ZF	BORIS	Normal Coexpression with BORIS
MageB4*	+	-	+	**Yes**
Pou5F1 [Oct3/4]	+	-	+	**Yes**
Boris	+	-	+	**Yes**
Rex1	-	-	-	**No**
Sox1	-	-	-	**No**
α-fetoprotein	-	-	-	**No**
IL-2	-	-	-	**No**

*** CTA-genes on X-chromosome are marked by an * asterisk.**

+169 relative to the transcription start site, which extend across the distal promoter region, first exon, and a portion of the first intron of *MAGE-A1*. In Fig. 4*A*, CpG dinucleotides 1 to 17 correspond to the nomenclature of De Smet et al. (40). We also included 6 CpGs upstream of the 17 already mapped, which we have designated −1 to −6. Figure 4*A* also shows that this region of the *MAGE-A1* gene was densely methylated in parental cells that do not normally express this gene. Previous studies showed that multiple CpGs located in the regulatory region of a gene must be substantially demethylated to make the promoter accessible for transcriptional machinery (28, 40–43). We expected to see similar pattern induced by *BORIS* and/or 5-azadC on activation in NHDF. However, demethylation of a very small number of CpGs (predominantly −1 and/or −2 and 17; see Figs. 4*A* and 5*A*) was sufficient to trigger reexpression of MAGE-A1 after cells had received an input of *BORIS*. Statistical analysis of alterations in the demethylation pattern leading to *MAGE-A1* expression shows change at CpG sites −1 and −2 from 20% demethylated (in the repressed promoter) to >80% demethylated (in the activated promoter). A group of CpGs from 13 to 17 also changes their demethylation status on activation from 17% unmethylated (in inactive promoter) to >40% unmethylated (in the activated chromatin conformation). It is worth noting that ectopic *BORIS* expression leads predominantly to demethylation of CpGs −1 and 17, whereas treatment of NHDF with 5-azadC yields a somewhat different pattern: demethylation of −2 and −1 CpGs plus scattered changes in the promoter region around CpGs 13 to 17. Although some CpGs were demethylated after treatment with 5-azadC at 1 μmol/L, the methylation status of other CpGs was completely unaffected in the face of increased concentrations as high as 10 μmol/L.

In contrast to normal cells, cancer cells showed a more scattered pattern of methylation in the *MAGE-A1* promoter depending on their origin and status of *MAGE-A1* expression (see Fig. S2). This suggests that in cancer cell lines the primary demethylation event associated with promoter derepression can be masked by later acquisition/selection (in culture or in tumor progression) of additional changes possibly leading to an altered binding potential for different tissue-specific transcription factors, such as Ets-1 (44), which apparently do not directly participate in the initial, precisely localized demethylation events seen in normal cells only.

To verify our bisulfite data, additional evidence for BORIS-induced altered methylation of the *MAGE-A1* promoter was obtained by Southern blot analysis using a meCpG-sensitive restriction endonuclease for digesting DNA prepared from NHDF

Figure 3. Activation of cancer-testis gene expression in human and mouse normal cells by treatment with 5-azadC or by transient transfection of BORIS (each gene analyzed is indicated on *top* of each RT-PCR gel). *A,* RT-PCR analysis of cancer-testis gene expression in NHDF. *C,* control fibroblasts transfected with empty vector; *AZA,* cells treated with 1 μmol/L 5-azadC; *ZF,* fibroblasts transfected with plasmid expressing CTCF zinc fingers only; *B,* fibroblasts transfected with BORIS expression plasmid; H_2O, negative control for RT-PCR; *T,* normal testis; *a.s. CTCF,* for reverse transcription outcomes from NHDF transfected with an expression vector for the antisense CTCF. *B,* RT-PCR analysis of cancer-testis gene expression in normal mouse adult primary fibroblasts. Lanes are the same as in (*A*). *C,* comparison of the normal coexpression (in human fibroblasts) with human BORIS (*Yes*) versus patterns for each cancer-testis gene analyzed by RT-PCR for activation by treatment with 1 μmol/L 5-azadC for 72 hours or transfected with the either CTCF 11ZF expression plasmid or with BORIS expression plasmid as described in Materials and Methods section. *D,* same as (*C*), with mouse fibroblasts.

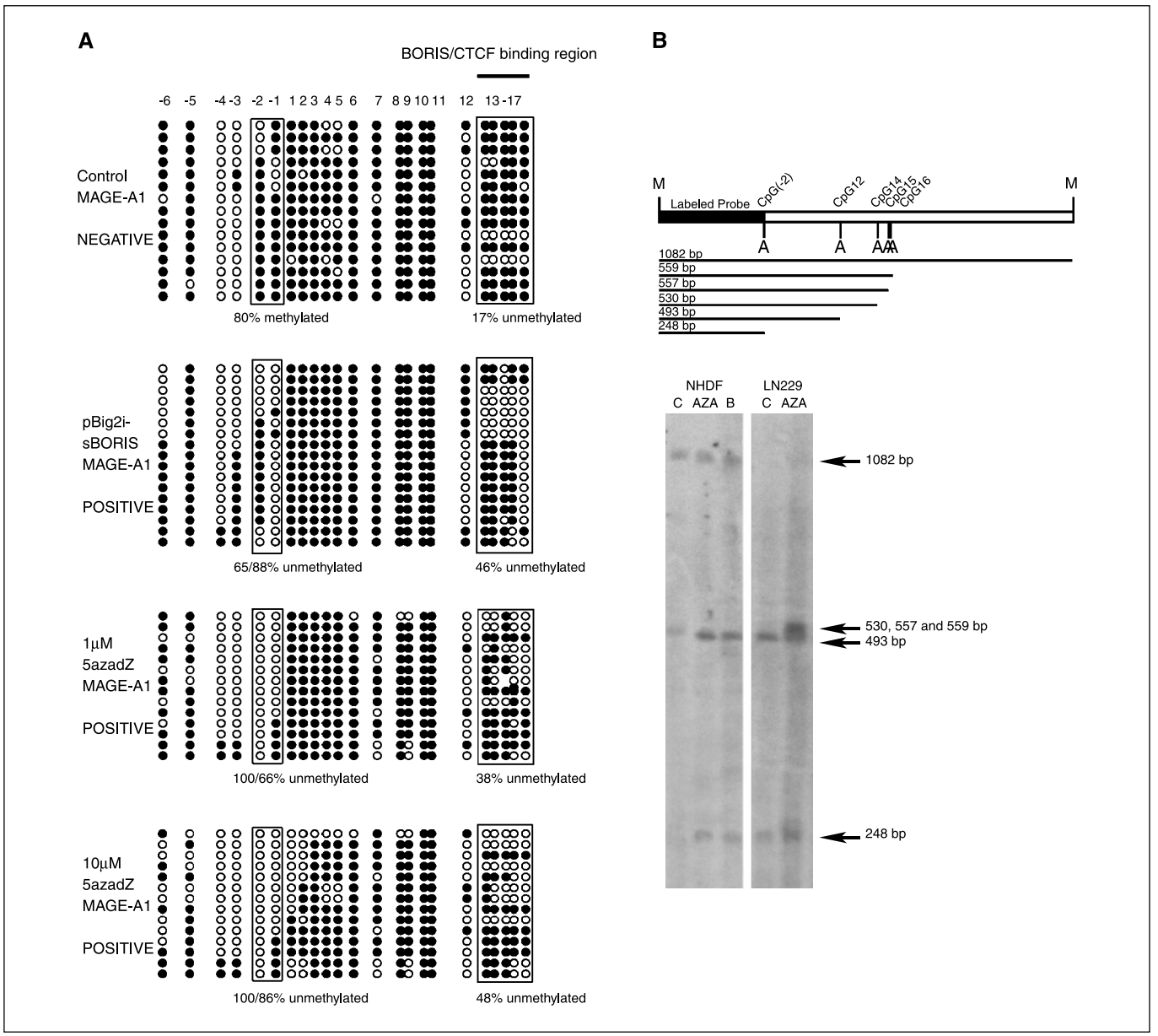
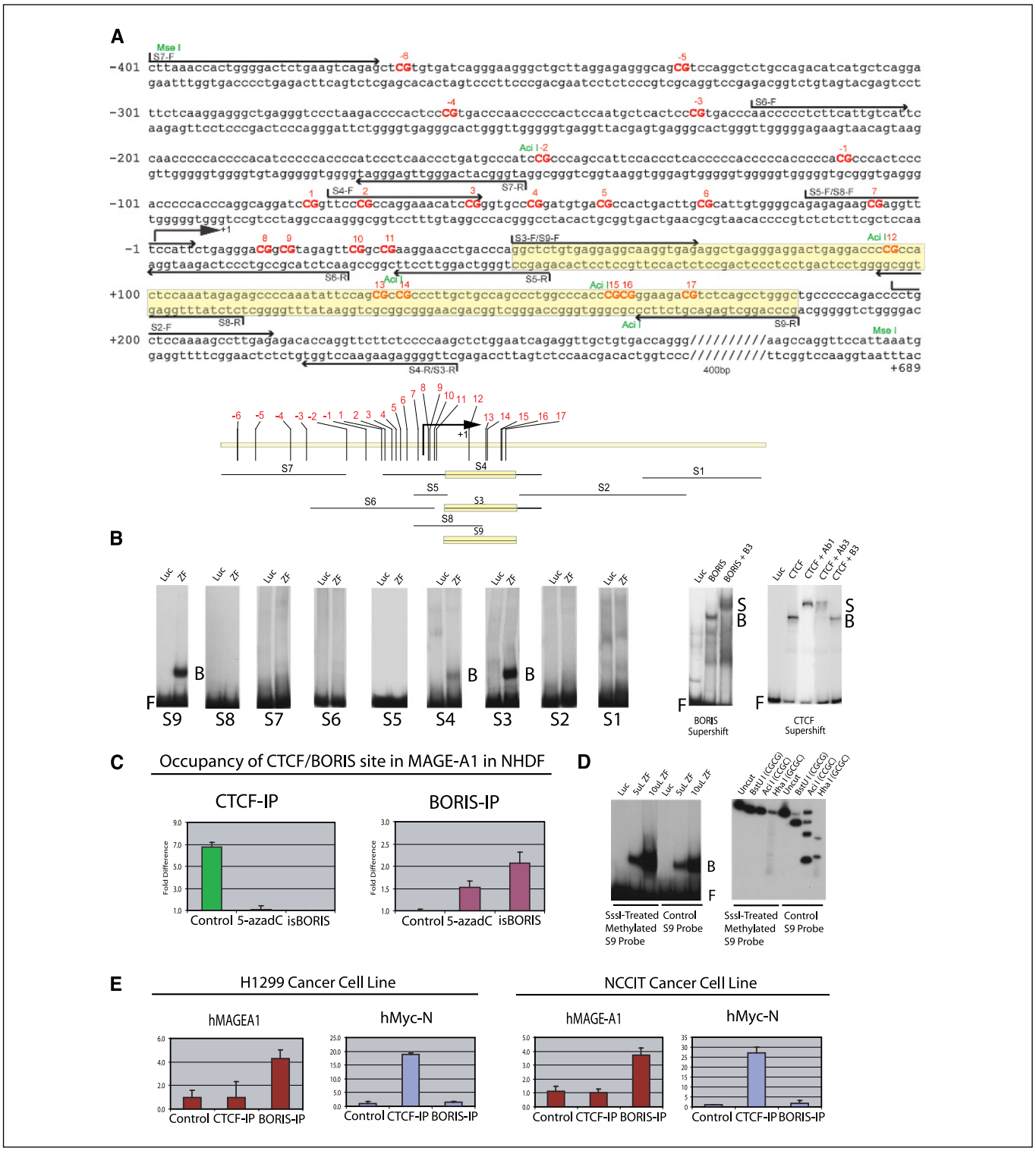


Figure 4. *A,* methylation status analyses of the 5′-flank of the *MAGE-A1* gene, including the promoter region, first exon, and a part of first intron (for a detailed map and the distribution of CpGs in this sequence, see Fig. 5*A* and *B*). Each circle represents a single CpG dinucleotide. *Black circles,* methylated cytosines; *open circles,* unmethylated cytosines. Each row of circles corresponds to a single DNA molecule analyzed by bisulfite sequencing. CpG dinucleotides 1 to 17 were numbered according to De Smet et al. (40). Six additional CpG dinucleotides from the 5′-end were numbered in the reverse direction −1 to −6. *First subpanel,* methylation profile of DNA in control NHDF (which are *MAGE-A1* negative); *second subpanel,* NHDF transfected with a BORIS expression plasmid (*MAGE-A1* positive); *third* and *fourth subpanels,* positive controls for *MAGE-A1* expression, NHDF treated with two different concentrations of 5-azadC. *B,* methylation-sensitive enzyme mapping of the *MAGE-A1* promoter region in NHDF and LN229 cancer cell lines by Southern blot analysis. *Top,* schematic presentation of expected restriction fragments after treatment of genomic DNA with two endonucleases: *Aci*I (*A*) and *Mse*I (*M*). *Horizontal bar, Mse*I fragment of the *MAGE-A1* promoter region that contains the entire DNA sequence analyzed. *Aci*I is a methylation-sensitive endonuclease that is able to cut DNA at a CCGC site only if the second cytosine is unmethylated. *Black bar,* sequence used as a labeled probe for detection of DNA fragments (−400 to −152 bp on Fig. 5). *Vertical bars,* restriction enzyme cutting sites. CpGs −2, 12, 14, 15, and 16 contain the *Aci*I restriction site. Only one 1,082-bp length fragment could be detected in the fully methylated sequence. A combination of 1,082- and 248-, 493-, 530-, 557-, and 559-bp fragments (all schematically shown as *black lines* labeled respectively) could be detected in the cases of demethylated CpGs −2, 12, 14, 15, and 16, respectively. *Bottom,* a methylation profile of the *MAGE-A1* promoter region as detected by Southern blot in normal cells: *C,* control fibroblasts transfected with empty vector show two bands corresponding to fully methylated DNA and demethylation at CpG 12 (493 bp). Cells treated with 1 μmol/L 5-azadC and fibroblasts transfected with BORIS expression plasmid (*B*) show similar patterns of methylation corresponding to demethylation at CpGs −2 (248 bp) and additional demethylation at positions 14, 15, and 16 with 530-, 557-, and 559-bp length fragments, respectively. Cancer cell line L299 under normal culture conditions (*C*) and after treatment with 2 μmol/L 5-azadC for 3 days shows patterns characteristic of demethylation at all sites analyzed and also the presence of a fully methylated promoter.

transfected with empty vector, cells transfected with a plasmid containing *BORIS* cDNA, and cells treated with 5-azadC (Fig. 4*B*). DNA prepared from L299 cells served as a positive control with a previously defined methylation (Fig. S2). DNA samples were first digested with *Mse*I enzyme to generate fragment containing all 23 CpGs from −6 to 17. The samples were then digested with *Aci*I, which cuts at CpGs −2, 12, 14, 15, and 16 only when they are unmethylated. DNA from control normal cells shows two bands,

one corresponding to a fully methylated promoter (band size, 1,082 bp) and another reflecting the partial demethylation of CpG 12 (band size, 493 bp). Fibroblasts treated with 5-azadC or transfected with a BORIS expression vector displayed the appearance of an additional band (248 bp) that corresponds to demethylation of CpG −2. DNA from glioblastoma cells revealed primarily bands corresponding to demethylation of CpG −2, 12, 14, 15, and 16. These data are in close agreement with the bisulfite sequencing results and reinforce the concept that aberrant expression of *BORIS* is associated with demethylation of the *MAGE-A1* promoter.

CTCF and BORIS bind directly to the *MAGE-A1* promoter region. The experiments described above show that normal cells treated with 5-azadC or induced to express BORIS exhibited



alterations in the methylation status of the *MAGE-A1* promoter associated with expression of the gene at the protein level. To determine if there might be a direct relationship between BORIS expression and changes in the *MAGE-A1* promoter, we used EMSA to determine if the 11ZF region shared by CTCF and BORIS binds to the *MAGE-A1* promoter *in vitro*. As diagrammed in Fig. 5*A* and *B*, overlapping radiolabeled probes were created to cover ~1.1 kb surrounding the transcription start site and covering the same region shown in Fig. 4. Recombinant luciferase as a negative control or recombinant 11ZF domain of CTCF and BORIS was incubated with these probes and analyzed by EMSA. Figure 5*B* shows that the 11ZF protein bound strongly to probes S3 and S9 and less well to probe S4 but did not bind to S8, S5, or S2. These results suggested that CTCF and BORIS can bind *in vitro* within the region downstream of the transcription start site, highlighted in yellow, and covering CpGs 12 to 17. To investigate whether CTCF or BORIS is recruited to the MAGE-A1 promoter *in vivo*, we used chromatin immunoprecipitation (ChIP) analyses with cross-linked DNA prepared from NHDF, cells transiently transfected with BORIS, and cells treated with 5-azadC. For immunoprecipitations, we used a mixture of nine mouse monoclonal antibodies to CTCF that produced supershifts and displayed one specific band of correct size on Western blots (12) and anti-BORIS rabbit polyclonal antibodies to BORIS peptide and a nonspecific IgG as a negative control. Using fragment S3 we observed strong *in vivo* binding of CTCF to the MAGE-A1 promoter in normal cells (Fig. 5*C, left box*). Treatment with 5-azadC or with inducible BORIS led to a dramatic decrease in CTCF occupancy on the promoter-proximal region (Fig. 5*C, left box*). Conversely, although the same region was not occupied by BORIS in control cells that do not express it, BORIS/DNA complexes were detected in chromatin of cells treated with 5-azadC or induced to express BORIS by transfection (Fig. 5*C, right box*). The specificity of each antibody used in the ChIP experiments was shown in EMSA experiments by their ability to supershift DNA-bound CTCF and BORIS bands (Fig. 5*B, two right boxes*). To further analyze the specificity of CTCF and BORIS binding recognized by these antibodies, we did ChIP assays on the NCCIT and H1299 cell lines and analyzed promoter regions of both *MAGE-A1* and *c-MYC,* which contains the well-characterized CTCF-binding site N of the *MYC* chromatin insulator (see ref. 1 for DNA sequence of this site, and refs. 12, 32 for the similar ChIP assay with the same site N in various cells). These two cell lines, derived from testicular (NCCIT) and lung (H1299) tumors, were chosen for these experiments because both express high levels of *BORIS* and *MAGE-A1* mRNAs as shown in ref. 45 and in the accompanying article (27), respectively, whereas expression of *CTCF* and *BORIS* in the NCCIT cells has been verified by us[4] (data not shown). This experiment showed that the insulator site N of the *MYC* gene exhibited permanent occupancy by CTCF but not by BORIS (Fig. 5*E*). Conversely, this same experiment showed binding of BORIS to the *MAGE-A1* promoter with concurrent lack of CTCF binding (Fig. 5*E*). Collectively, the results in Fig. 5 show that antibodies used in ChIP experiments specifically select CTCF- and BORIS-containing chromatin fragments and that CTCF and BORIS bind to the same site in the promoter of the *MAGE-A1* gene in a mutually exclusive manner. Replacement of CTCF binding for BORIS binding on a target gene promoter *in vivo* also provides a first direct support to the idea that two "cancer hits" affecting various functions of CTCF targets may occur at two different chromosomes; that is, that an aberrant activation of *BORIS* at 20q13 gain/amplification region can act as "interfering mutation" for tumor suppressor function of *CTCF* mapped on 16q22 to the smallest region of overlap for loss of heterozygosity in multiple cancers (see ref. 2 for more details).

Methylation of *MAGE-A1* promoter does not alter CTCF binding. Fragment S9 from the *MAGE-A1* promoter positive for CTCF binding was methylated *in vitro* using *Sss*I DNA methylase. EMSA analysis of methylated and unmethylated probes with the CTCF 11ZF DNA-binding domain shows no difference in the binding of probes (Fig. 5*D, left box*). The degree of probe methylation was tested by methylation-sensitive restriction digestion using three different enzymes cutting in different positions within the probe. None of the enzymes tested produced even faint band of the expected size compared with unmethylated control, suggesting that methylation was close to 100% at least at the restriction sites (Fig. 5*D, right box*). In addition, the DMD4 probe (10) of the *H19* ICR treated by *Sss*I in parallel with the MAGE S9 DNA-probe did not bind the 11ZF (data not shown) as expected for this widely characterized meCpG-sensitive site (8–12). Therefore, the CTCF/BORIS 11ZF-binding site in the S9 *MAGE-A1* fragment is the first example thus far that has shown methylation-insensitive binding.

Treatment of primary human fibroblasts with 5-aza-2′-deoxycytidine induces expression of *BORIS* that is followed by a much later reactivation of *MAGE-A1*. It is well known that 5-azadC can induce expression of most cancer-testis genes in a wide range of normal and cancerous cell types by inducing demethylation of CpG islands in the promoter regions of these

Figure 5. *In vitro* and *in vivo* analyses of CTCF and BORIS binding to the *MAGE-A1* 5′-flanking regulatory region. *A, top,* sequence of the region analyzed by EMSA, with CpGs *capitalized in bold* and *numbered in red*. Numbering is shown relative to the transcription start site, which is marked with a *bold arrow* and +1. Primers for generating labeled EMSA probes are marked with *arrows. Bottom,* a schematic of EMSA probes S1 to S9 used in the initial screening of *MAGE-A1*. Positions of CpGs, *numbered in red* above this map, are indicated together with the transcription start site. *B,* screening of promoter fragments S1 to S9 by EMSA for the presence of a binding site recognized by the 11ZF domain of the BORIS and CTCF factors. Labeled DNA fragments shown in (*A*) were used in EMSA-binding reactions either with *in vitro*–translated 11ZF-domain of CTCF (*ZF*) or with an equal amount of luciferase protein (*Luc*) as a negative control. Protein was synthesized from pETchZF and luciferase T7 control DNA (Promega) for zinc finger and luciferase, respectively. *Two far right subpanels,* assessment of the CTCF and BORIS ChIP antibodies to interact specifically with CTCF/DNA and BORIS/DNA complexes by verifying their specificity in supershift EMSA experiments. We preincubated *in vitro*–translated CTCF and BORIS proteins with anti-CTCF and/or anti-BORIS antibodies. *Left, Luc,* control sample with *in vitro*–translated luciferase; *BORIS, in vitro*–translated BORIS protein; *BORIS + B3, in vitro*–translated BORIS protein supershifted with polyclonal rabbit anti-BORIS antibodies (*clone B3*). *Right, Luc,* control sample with *in vitro*–translated luciferase; *CTCF, in vitro*–translated CTCF protein; *CTCF + Ab1, in vitro*–translated CTCF protein supershifted with monoclonal mouse anti-CTCF antibodies (*clone 1*); *CTCF + Ab3, in vitro*–translated CTCF protein supershifted with monoclonal mouse anti-CTCF antibodies (*clone 3*); *CTCF + B3, in vitro*–translated CTCF protein was not supershifted with polyclonal rabbit anti-BORIS antibodies (*B3*). *C,* CTCF and BORIS *in vivo* occupancy of the *MAGE-A1* promoter via ChIP of male NHDF. For each ChIP, sonicated chromatin was immunoprecipitated with either anti-CTCF or anti-BORIS antibodies and then analyzed by real-time PCR with pairs of primers spanning fragment S3 corresponding to (*A* and *B*). Each panel (in *C*) shows representative results of three independent PCR analyses of ChIP; *bars,* SD. Fold enrichment is calculated as described in Materials and Methods. *D,* binding of CTCF 11ZF DNA-binding domain to the S9 fragment of the MAGE-A1 promoter is *not* sensitive to meCpG methylation. EMSA shows no decrease in binding of BORIS/CTCF 11ZF domain on methylation of its target DNA site in the S9 DNA sequence (*left*). The completion of *in vitro* methylation was assayed by three different methylation-sensitive restriction enzymes of the same probe used in EMSA (*right*), and the overall degree of methylation is estimated close to 100%. *E,* CTCF and BORIS *in vivo* occupancy of the *MAGE-A1* promoter and the MYC insulator site N in the NCCIT cell line. CTCF and BORIS *in vivo* occupancy of the *MAGE-A1* promoter and the MYC insulator site N in H1299 cell line.

genes (28, 40, 43, 46–48). The pattern of BORIS expression in normal tissues is similar to that of cancer-testis genes, as it is expressed only in testes (20). This suggested that epigenetic alterations similar to those responsible for activation of other cancer-testis genes may support the expression of endogenous *BORIS*. With these facts in mind, we decided to determine whether 5-azadC can induce *BORIS* expression following treatment of NHDF. To examine this possibility, RNA prepared from NHDFs treated with 1 μmol/L 5-azadC for 1 to 72 hours was tested by RT-PCR for expression of *BORIS* and *MAGE-A1* mRNAs. A total RNA prepared from normal testis served as a positive control for normal expression of *BORIS* (20). Figure 6*A* shows that *BORIS* transcription was detected as early as 6 to 8 hours after treatment with 5-azadC, whereas transcripts for *MAGE-A1* were not detected until a much later time (48 hours) of treating same cells with this demethylating agent. After transcription of *MAGE-A1* was induced at 48 hours, it remained for a prolonged period (Fig. 6*A*; data not shown). This kinetics of the two gene's activation by 5-azadC suggested that BORIS may be the necessary mediator of the capability of 5-azadC to activate at least one of the 11ZF target cancer-testis genes, *MAGE-A1*.

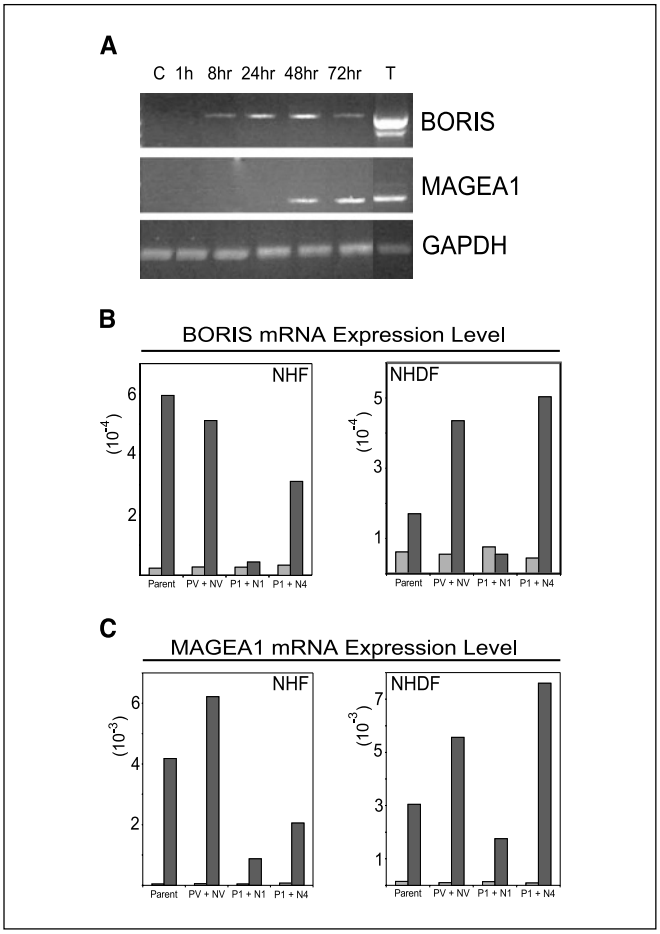


Figure 6. *A,* time course of *BORIS* and *MAGE-A1* expression in NHDF after treatment with 1 μmol/L 5-azadC. Total RNA preps for RT-PCR were made at several time points (in hours) as indicated. RT-PCR results for *GAPDH* at each time point shown as controls. *Lanes C* and *T,* negative and positive (adult human testis sample) control, respectively. *B* and *C,* "preventive" inhibition of *BORIS* before derepression by 5-azadC in normal fibroblasts results in a noticeable decrease in 5-azadC induction of BORIS-targeted *MAGE-A1* gene. Levels of BORIS and MAGE-A1 expression in BORIS-shRNA-infected NHF and NHDFs with and without 5-azadC treatment for 48 hours. Cells were infected with a combination of either puromycin vector + neomycin vector (*PV + NV*), puromycin vector containing oligonucleotide 1 + neomycin vector containing oligonucleotide 1 (*P1 + N1*), or puromycin vector containing oligonucleotide 1 + neomycin vector containing oligonucleotide 4 (*P1 + N4*). Expression levels were calculated relative to testis using the comparative C_T method.

***BORIS*–specific short hairpin RNAs introduced by retroviruses into normal fibroblasts before 5-aza-2′-deoxycytidine treatment prevents *MAGE-A1* activation.** To further show that expression of BORIS in NHFs selectively derepresses *MAGE-A1*, we used RNA silencing technology to silence *BORIS* expression in target cells. Both NHFs (derived from neonatal foreskin) and NHDFs were infected with *BORIS* shRNA retroviral constructs. Two different *BORIS* target sequences were cloned into a neomycin and a puromycin selectable version of retroviral plasmid, pSUPER, to allow for dual infection/selection. Empty vectors were used as controls. Cells were selected using both antibiotics and then treated with 5-azadC for 48 hours. Real-time quantitative PCR analysis of *BORIS* and *MAGE-A1* expression levels was determined and is shown in Fig. 6. Both NHDF and NHF cells infected with both *BORIS* shRNA 1 vectors blocked induction of *BORIS* and *MAGE-A1* expression in 5-azadC-treated cells compared with either uninfected cells or cells infected with empty vector. This experiment further shows that BORIS expression specifically regulates derepression of *MAGE-A1* in normal cells by one of most common chemical methylation-inhibitors, 5-azadC.

Discussion

In mammals, epigenetic regulation of differential gene expression involves a sophisticated structure-function interplay of DNA methylation, various histone modifications, and chromatin remodeling that often goes along with moving nascent sense and antisense noncoding transcripts (21, 23). Disruption of one or more of these interacting systems leads to inappropriate expression or silencing of genes, thereby contributing to chromosomal instability, mental retardation, and cancer. The correct targeting of these processes to specific loci in the genome ensures that normal patterns of silenced versus activated states are maintained and reestablished in a fashion properly coordinated in both time and space (21, 23).

In this study, we asked if such targeting may involve the 11ZF-binding sites of CTCF and BORIS and showed that unprogrammed expression of BORIS in normal cells resulted in replacement of CTCF by BORIS on sequences present in the *MAGE-A1* promoter, which was associated with site-specific demethylation of the promoter and expression of MAGE-A1 protein. In addition to *MAGE-A1*, several other cancer-testis and germ cell specific genes were found induced in normal cells forced to express BORIS. These data are significant because, although there is a large body of evidence describing induction of cancer-testis genes in primary tumors or cancer cell lines (28, 36, 37, 40, 42, 47, 49, 50), there are just a few examples of cancer-testis gene expression in normal tissues or normal cultured cells (40, 43, 48). In tumor cells already positive for both BORIS and its target cancer-testis genes like *MAGE-A1*, regulation of both by maintaining a certain ratio between BORIS and CTCF seems to play a critical role, as changing this balance regulates targets that contain binding sites for their shared 11ZF DNA-binding domain. For example, using a model system with tetracycline-inducible CTCF in the osteosarcoma cell line UTA6/

U2OS, we observed that increasing the level of CTCF results in down-regulation of *MAGE-A1* expression,[5] and, moreover, we have also shown that forced expression of antisense to *CTCF* in NHDF led to activation of *BORIS*.

Bisulfite sequencing and Southern blot analysis with methylation-sensitive restriction enzymes identified the exact targets within the *MAGE-A1* promoter that had undergone demethylation following expression of BORIS or treatment with 5-azadC. As expected, these sequences contained CTCF-binding sites. The exact location of these targets within the promoter was determined by detailed EMSA analysis and ChIP assays. Both techniques showed that CTCF and BORIS can bind to the *MAGE-A1* promoter and that BORIS replaced CTCF at these sites in cancer cell lines. Because BORIS and CTCF have been shown to compete for binding to common targets and thus cannot simultaneously occupy the same sequences, we suggest that the same regulatory region of DNA that can bind either CTCF or BORIS has different functions *in vivo* depending on which of the two paralogous transcription factors is bound to the site. The exact function will likely depend on the availability of cofactors that reflect the differentiation and activation state of the cell.

Taken together, these results support a model suggesting that BORIS is directly involved in changing the epigenetic status of the *MAGE-A1* promoter and possibly other germ cell–specific regulatory regions but only of those genes that are normally coexpressed with BORIS in CTCF- and meCpG-depleted testicular germ cells. In addition, our results imply that only a minor but spatially defined demethylation is necessary to overcome the suppressed state of a methylated promoter. Finally, these data indicate that the ratio of two paralogous regulatory factors with the same DNA-binding specificity, CTCF and BORIS, are capable of such epigenetic regulation of chromatin that allows a cancer-testis gene in the off position to be turned on and vice versa.

In addition, our data show that BORIS-treated normal cells have an open and active *MAGE-A1* promoter that has a slightly altered DNA methylation pattern. The observation that the promoter is only slightly altered suggests that the promoter of *MAGE-A1* in some expressing cells is ready to be shutdown at any moment, which may reflect the natural ability of normal cells to constrain and control inappropriately expressed genes. Possibly, this important feature may gradually degrade during *in vitro* immortalization/transformation of normal cells (when *BORIS* was noticed to be derepressed in various models tested, including DNMT1-deficient cells)[5] or in BORIS-positive tumors *in vivo*. Most importantly, however, we showed that changing the identity of one of the *MAGE-A1* promoter-occupying transcription factors from CTCF to BORIS (Fig. 5*C*) in freshly cultured normal fibroblasts resulted in recruitment of new functions to CTCF sites, exemplified here by selective demethylation and reactivation of target promoters normally regulated in accord with the CTCF-BORIS switching in male germ cells (2, 20). These targets also include promoters of other X-linked cancer-testis genes, such as *NY-ESO-1* (27). Thus, two cancer-testis gene promoters were found to contain novel meCpG-insensitive binding sites for the 11ZF DNA-binding domain shared by CTCF and BORIS, which were mapped by direct, serial gel-shift screening with recombinant CTCF and BORIS protein. This screening was followed by ChIP analyses of BORIS- or 5-azadC-treated cells, which resulted in the *in vivo* changing of promoter occupancy from CTCF to BORIS.

Furthermore, we have observed that normally *BORIS* is activated/expressed within just several hours after 5-azadC treatment, which is 2 days before derepression of *MAGE-A1* by 5-azadC. Although ChIP experiments directly showed that both ectopic input of BORIS and 5-azadC-induced BORIS in normal cells result in the same switching of the occupancy on the 11ZF target site in the *MAGE-A1*-regulatory region from CTCF to BORIS in association with its activation and demethylation, these experiments nevertheless did not provide direct evidence for the necessary contribution of BORIS into the *MAGE-A1*-activating effect of 5-azadC. However, we blocked this process using shRNA constructs targeted to BORIS to confirm the direct role of BORIS in *MAGE-A1* derepression by 5-azadC, and infection of two types of NHFs before 5-azadC treatment with retroviruses that expressed anti-*BORIS* shRNA markedly blocked reactivation of both genes.

Thus, taken together, our ChIP and shRNA experiments provided, for the first time, direct evidence that a particular transcriptional factor, BORIS, is required for the promoter-specific demethylation and derepression of a cancer-testis gene by 5-azadC, one of the most widely used and best-studied inhibitors of DNA methylation (21–23, 51).

There is the possibility that as for CTCF (reviewed in refs. 1, 2) formation of BORIS-DNA complexes have a distinct three-dimensional structure that depends on the zinc finger–contacting sequence of various 11ZF-binding sites and on the correspondingly different usage of zinc finger involved in the recognition of each unique DNA-binding site. This three-dimensional structure may allosterically determine the type of post-translational modifications and interacting partners for DNA-bound BORIS, which in turn ultimately define the functional outcome of CTCF-to-BORIS switching in a given chromatin zone. Because full-length BORIS expression activates cancer-testis genes (in contrast to the 11ZF domain alone), we suggest that the COOH and/or NH_2 terminus of BORIS is likely tethering epigenetic machinery to a novel class of 11ZF target sequences that mediate induction of cancer-testis genes. Therefore, based on the data presented here, one can envision previously unattainable experiments to determine which DNMTs, HMTases, MBDs, and/or dMTase may possibly be recruited by BORIS on replacing CTCF *in vivo*, thereby resulting in gene activation through demethylation of CpGs at and around different BORIS/CTCF target sites. We hope that molecular studies of the roles of CTCF-to-BORIS switching may help us resolve a long-standing "methylation paradox" in cancer cells (51); that is, to not only address BORIS-induced hypomethylation processes associated with activation of cancer-testis and other male germ cell–specific genes but also decipher CTCF site-specific targeting and recruiting mechanisms that generate tumor-specific hypermethylation patterns (21–23). This hope is based on our preliminary data indicating that sustained expression of ectopic BORIS (rather than short-term expression of BORIS analyzed here) results in aberrant hypermethylation at DNA regions different from 5′-flanks of cancer-testis genes, including several CTCF/BORIS–binding sequences mapped in promoters of tumor suppressor genes that have been often reported as aberrantly silenced in various cancers (for review, see ref. 51).

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References

1. Ohlsson R, Renkawitz R, Lobanenkov V. CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends Genet 2001;17:520–7.
2. Klenova EM, Morse HC, Ohlsson R, Lobanenkov VV. The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer. Semin Cancer Biol 2002;12:399–14.
3. Lee JT. Molecular links between X-inactivation and autosomal imprinting: X-inactivation as a driving force for the evolution of imprinting? Curr Biol 2003;13: R242–54.
4. Ohlsson R, Kanduri C, Whitehead J, Pfeifer S, Lobanenkov V, Feinberg AP. Epigenetic variability and the evolution of human cancer. Adv Cancer Res 2003; 88:145–68.
5. Feinberg AP, Tycko B. The history of cancer epigenetics. Nat Rev Cancer 2004;4:143–53.
6. Rousseaux S, Caron C, Govin J, Lestrat C, Faure AK, Khochbin S. Establishment of male-specific epigenetic information. Gene 2005;345:139–53.
7. Szabo P, Tang SH, Rentsendorj A, Pfeifer GP, Mann JR. Maternal-specific footprints at putative CTCF sites in the H19 imprinting control region give evidence for insulator function. Curr Biol 2000;10:607–10.
8. Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature 2000;405:486–9.
9. Bell AC, Felsenfeld G. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature 2000;405:482–5.
10. Kanduri C, Pant V, Loukinov D, et al. Functional association of CTCF with the insulator upstream of the H19 gene is parent of origin-specific and methylation-sensitive. Curr Biol 2000;10:853–6.
11. Chao W, Huynh KD, Spencer RJ, Davidow LS, Lee JT. CTCF, a candidate trans-acting factor for X-inactivation choice. Science 2002;295:345–7.
12. Pugacheva E, Tiwari VK, Abdullayev Z, et al. Familial cases of point mutations in the XIST promoter reveal a correlation between CTCF binding and pre-emptive choices of X chromosome inactivation. Hum Mol Genet 2005;14:953–65.
13. Fillipova GN, Cheng MK, Moore JM, et al. Boundaries between chromosomal domains of X inactivation and escape bind CTCF and lack CpG methylation during early development. Dev Cell 2005;8:31–42.
14. Pant V, Kurukuti S, Pugacheva E, et al. Mutation of a single CTCF target site within the H19 imprinting control region leads to loss of Igf2 imprinting and complex patterns of *de novo* methylation upon maternal inheritance. Mol Cell Biol 2004;24:3497–504.
15. Sparago A, Cerrato F, Vernucci M, Ferrero GB, Silengo MC, Riccio A. Microdeletions in the human H19 DMR result in loss of IGF2 imprinting and Beckwith-Wiedemann syndrome. Nat Genet 2004;36:958–60.
16. Prawitt D, Enklaar T, Gartner-Rupprecht B, et al. Microdeletion of target sites for insulator protein CTCF in a chromosome 11p15 imprinting center in Beckwith-Wiedemann syndrome and Wilms' tumor. Proc Natl Acad Sci U S A 2005;102:4085–90.
17. Mukhopadhyay R, Yu W, Whitehead J, et al. The binding sites for the chromatin insulator protein CTCF map to DNA methylation-free domains genome-wide. Genome Res 2004;14:1594–602.
18. Dunn KL, Davie JR. The many roles of the transcriptional regulator CTCF. Biochem Cell Biol 2003;81:161–7.
19. Klenova E, Ohlsson R. Poly(ADP-ribosyl)ation and epigenetics: is CTCF PARt of the Plot? Cell Cycle 2005; 4:96–101.
20. Loukinov DI, Pugacheva E, Vatolin S, et al. BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. Proc Natl Acad Sci U S A 2002;99:6806–11.
21. Bird A. DNA methylation patterns and epigenetic memory. Genes Dev 2002;16:6–21.
22. Jones PL, Wolffe AP. Relationships between chromatin organization and DNA methylation in determining gene expression. Semin Cancer Biol 1999;9:339–47.
23. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003;33 Suppl: 245–54.
24. Kalejs M, Erenpreisa J. Cancer/testis antigens and gametogenesis: a review and "brain-storming" session. Cancer Cell Int 2005;5:4–15.
25. Koslowski M, Bell C, Seitz G, et al. Frequent nonrandom activation of germ-line genes in human cancer. Cancer Res 2004;64:5988–93.
26. Ulaner GA, Vu TH, Li T, et al. Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site. Hum Mol Genet 2003;12:535–49.
27. Hong JA, Kang Y, Abdullaev Z, et al. Reciprocal binding of CTFC and BORIS to the NY-ESO-1 promoter coincides with depression of this cancer-testis gene in lung cancer cells. Cancer Res 2005;65:7751–62.
28. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T. The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci U S A 1996; 93:7149–53.
29. Strathdee CA, McLeod MR, Hall JR. Efficient control of tetracycline-responsive gene expression from an autoregulated bi-directional expression vector. Gene 1999;229:21–9.
30. Awad TA, Bigler J, Ulmer JE, et al. Negative transcriptional regulation mediated by thyroid hormone response element 144 requires binding of the multivalent factor CTCF to a novel target DNA sequence. J Biol Chem 1999;274:27092–8.
31. Filippova GN, Fagerlie S, Klenova EM, et al. An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-*myc* oncogenes. Mol Cell Biol 1996;16: 2802–13.
32. Garrett FE, Emelyanov AV, Sepulveda MA, et al. Chromatin architecture near a potential 3′ end of the Igh locus involves modular regulation of histone modifications during B-cell development and *in vivo* occupancy at CTCF sites. Mol Cell Biol 2005;25: 1511–25.
33. Litt MD, Simpson M, Recillas-Targa F, Prioleau MN, Felsenfeld G. Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci. EMBO J 2001;20:2224–35.
34. Suyama T, Ohashi H, Nagai H, et al. The MAGE-A1 gene expression is not determined solely by methylation status of the promoter region in hematological malignancies. Leuk Res 2002;26:1113–8.
35. Filippova GN, Lindblom A, Meincke LJ, et al. A widely expressed transcription factor with multiple DNA sequence specificity, CTCF, is localized at chromosome segment 16q22.1 within one of the smallest regions of overlap for common deletions in breast and prostate cancers. Genes Chromosomes Cancer 1998;22:6–36.
36. Zendman AJ, Ruiter DJ, Van Muijen GN. Cancer/testis-associated genes: identification, expression profile, and putative function. J Cell Physiol 2003; 194:272–88.
37. Chen YT, Scanlan MJ, Venditti CA, et al. Identification of cancer/testis-antigen genes by massively parallel signature sequencing. Proc Natl Acad Sci U S A 2005;102:7940–5.
38. Bruniquel D, Schwartz RH. Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. Nat Immunol 2003;4:235–40.
39. Sheng J, Organ EL, Hao C, Wells KS, Ruley HE, Rubin DH. Mutations in the IGF-II pathway that confer resistance to lytic reovirus infection. BMC Cell Biol 2004;5:32–45.
40. De Smet C, Lurquin C, Lethe B, Martelange V, Boon T. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol Cell Biol 1999; 19:7327–35.
41. Janssen BL, van de Locht LT, Fourkour A, et al. Transcription of the MAGE-1 gene and the methylation status of its Ets binding promoter elements: a quantitative analysis in melanoma cell lines using a real-time polymerase chain reaction technique. Melanoma Res 1999;9:213–22.
42. Honda T, Tamura G, Waki T, et al. Demethylation of MAGE promoters during gastric cancer progression. Br J Cancer 2004;90:838–43.
43. De Smet C, Loriot A, Boon T. Promoter-dependent mechanism leading to selective hypomethylation within the 5′ region of gene MAGE-A1 in tumor cells. Mol Cell Biol 2004;24:4781–90.
44. De Smet C, Courtois SJ, Faraoni I, et al. Involvement of two Ets binding sites in the transcriptional activation of the MAGE1 gene. Immunogenetics 1995; 42:282–90.
45. Zhang C, Kawakami T, Okada Y, Okamoto K. Distinctive epigenetic phenotype of cancer testis antigen genes among seminomatous and nonseminomatous testicular germ-cell tumors. Genes Chromosomes Cancer 2005;43:104–12.
46. Zakut R, Topalian SL, Kawakami Y, Mancini M, Eliyahu S, Rosenberg SA. Differential expression of MAGE-1, -2, and -3 messenger RNA in transformed and normal human cell lines. Cancer Res 1993;53:5–8.
47. Jungbluth AA, Busam KJ, Kolb D, et al. Expression of MAGE-antigens in normal tissues and cancer. Int J Cancer 2000;85:460–5.
48. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA. Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine. Mol Pharmacol 2004;65:18–27.
49. Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T, Lucas S. An overview of the MAGE gene family with the identification of all human members of the family. Cancer Res 2001;61:5544–51.
50. Aubry F, Satie AP, Rioux-Leclercq N, et al. MAGE-A4, a germ cell specific marker, is expressed differentially in testicular tumors. Cancer 2001;92:2778–85.
51. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3: 415–8.

Conditional Expression of the CTCF-Paralogous Transcriptional Factor BORIS in Normal Cells Results in Demethylation and Derepression of MAGE-A1 and Reactivation of Other Cancer-Testis Genes

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