Reciprocal Binding of CTCF and BORIS to the NY-ESO-1 Promoter Coincides with Derepression of this Cancer-Testis Gene in Lung Cancer Cells

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Abstract

Regulatory sequences recognized by the unique pair of paralogous factors, CTCF and BORIS, have been implicated in epigenetic regulation of imprinting and X chromosome inactivation. Lung cancers exhibit genome-wide demethylation associated with derepression of a specific class of genes encoding cancer-testis (CT) antigens such as NY-ESO-1. CT genes are normally expressed in BORIS-positive male germ cells deficient in CTCF and meCpG contents, but are strictly silenced in somatic cells. The present study was undertaken to ascertain if aberrant activation of BORIS contributes to derepression of NY-ESO-1 during pulmonary carcinogenesis. Preliminary experiments indicated that NY-ESO-1 expression coincided with derepression of BORIS in cultured lung cancer cells. Quantitative reverse transcription-PCR analysis revealed robust, coincident induction of BORIS and NY-ESO-1 expression in lung cancer cells, but not normal human bronchial epithelial cells following 5-aza-2'-deoxycytidine (5-azadC), Depsipeptide FK228 (DP), or sequential 5-azadC/DP exposure under clinically relevant conditions. Bisulfite sequencing, methylation-specific PCR, and chromatin immunoprecipitation (ChIP) experiments showed that induction of BORIS coincided with direct modulation of chromatin structure within a CpG island in the 5'-flanking noncoding region of this gene. Cotransfection experiments using promoter-reporter constructs confirmed that BORIS modulates NY-ESO-1 expression in lung cancer cells. Gel shift and ChIP experiments revealed a novel CTCF/BORIS-binding site in the NY-ESO-1 promoter, which unlike such sites in the H19-imprinting control region and X chromosome, is insensitive to CpG methylation in vitro. In vivo occupancy of this site by CTCF was associated with silencing of the NY-ESO-1 promoter, whereas switching from CTCF to BORIS occupancy coincided with derepression of NY-ESO-1. Collectively, these data indicate that reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter mediates epigenetic regulation of this CT gene in lung cancer cells, and suggest that induction of BORIS may be a novel strategy to augment immunogenicity of pulmonary carcinomas. (Cancer Res 2005; 65(17): 7763-74)

Introduction

It is increasingly recognized that many human diseases, including gene-imprinting disorders such as Beckwith-Wiedemann syndrome (1) and familial skewed X chromosome inactivation (2) as well as sporadic and inherited cancers (3, 4), involve common epigenetic derangements distributed nonrandomly throughout the genome. For example, during pulmonary carcinogenesis, genome-wide demethylation frequently results in loss of imprinting (LOI) of H19/IGF2 (5), as well as derepression of a growing class of germ cell–restricted genes such as NY-ESO-1 and MAGE-3, which encode immunogenic proteins, referred to as cancer-testis antigens (CTA), that are recognized by CTls from cancer patients (reviewed in ref. 6). Paradoxically, a variety of tumor suppressor genes including p16, p14/ARF, and RASSF1A are silenced by site-specific promoter methylation mechanisms in lung cancer cells (7).

The emerging relationships between epigenetics and malignant transformation provide impetus for the use of chromatin remodeling agents for lung cancer therapy (8, 9). Previously, we reported that the DNA-demethylating agent 5-aza-2'-deoxycytidine (5-azadC) and the HDAC inhibitor Depsipeptide FK228 (DP) synergistically induce apoptosis and markedly enhance NY-ESO-1 expression in lung cancer cells, facilitating their recognition by CTls specific for this CTA (10). Recently, several protocols have been conducted in the Thoracic Oncology Section, Surgery Branch, National Cancer Institute (NCI) in an attempt to recapitulate in clinical settings 5-azadC, DP, and sequential 5-azadC/DP exposure conditions that mediate apoptosis and CTA induction in cultured cancer cells. Nearly 40% of lung cancer patients receiving 5-azadC and/or DP infusions have exhibited induction of NY-ESO-1 expression in their tumors; several individuals in whom NY-ESO-1 was induced developed antibodies to this CTA following drug treatment.4

Presently, the mechanisms that control silencing of NY-ESO-1 expression in normal bronchial epithelia, and up-regulation of this CT gene in lung cancer cells remain unclear. However, recent studies regarding epigenetic regulation of the IGF2/H19 locus (11–13), the choice-imprinting center that regulates X chromosome inactivation (2, 14), and the few gene clusters that escape X chromosome inactivation (15), all of which involve methylation-sensitive binding of CTCF to a number of different CG-rich target sites, provide initial insight into putative mechanisms contributing to derepression of this CTA during pulmonary carcinogenesis.

Note: J. Hong and Y. Kang contributed equally to this work.

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4 D.S. Schrump et al., in preparation.
CTCF is a ubiquitous DNA binding protein that contributes to formation of all known chromatin insulators/boundaries, X chromosome inactivation, reading of gene-imprinting marks, and promoter-mediated regulation of a variety of genes involved in cell cycle progression and apoptosis (reviewed in refs. 16, 17). Regulation of gene expression by structurally distinct CTCF/DNA complexes results from combinatorial interactions of individual subsets of CTCF zinc fingers with highly diverse 50-bp-long DNA complexes results from combinatorial interactions of individual promoter-mediated regulation of a variety of genes involved in cell chromosome inactivation, reading of gene-imprinting marks, and CTCF is a ubiquitous DNA binding protein that contributes to Cancer Research (17, 22). During spermatogenesis, expression of imprinted sites (BORIS) encodes a protein with remarkably extensive homology to CTCF in the central 11 Zn-finger region but not in the NH2- and COOH-terminal regions that collectively account for nearly two thirds of the full-length amino acid sequences of these proteins (22). Thus, whereas BORIS and CTCF possess the same site-specific DNA-targeting domain, BORIS cannot mediate the same effects as CTCF upon interaction with DNA. BORIS lacks the motifs by which CTCF recruits functional “partner” cofactors, and does not contain modular substrates for specific post-translational modifications that are critical for CTCF function (reviewed in refs. 17, 20). For instance, BORIS lacks a highly conserved COOH-terminal phosphorylation motif required for CTCF-mediated growth suppression (22, 23).

Unlike CTCF, BORIS is not expressed in normal somatic cells (17, 22). During spermatogenesis, expression of BORIS is restricted to germ cells, and coincides with a marked decrease in CTCF expression, erasure of methylation patterns, and up-regulation of CT genes (22). Subsequently, CTCF expression is restored and BORIS is repressed, thereby reestablishing maternal methylation marks, including those within the IGF2/H19-imprinting control region (ICR) (22). Recent studies, including this report and an accompanying report by Vatolin et al. (24), indicate that BORIS is activated in a variety of human cancers, and that competition between BORIS and CTCF may contribute to epigenetic perturbations in these malignancies. Ulanel et al. (25) observed that aberrant activation of BORIS correlated with LOI by abnormal biallelic demethylation of CTCF sites in the H19 ICR in osteosarcomas. The present study was undertaken to ascertain whether BORIS contributes to derepression of NY-ESO-1 in lung cancer cells.

Materials and Methods

Drugs and chemicals. 5-azadC was purchased from Sigma, Inc. (St. Louis, MO), Desipreptide FK228 (DP) was obtained from the Developmental Therapeutics Program, NCI (Bethesda, MD). Cy3-dUTP and Cy5-dUTP were purchased from Amersham, Inc. (Buckinghamshire, England).

Cell lines and drug treatment regimens. All cancer lines were obtained from tissue culture banks at the NCI. Normal fibroblasts were purchased from American Type Culture Collection (Manassas, VA). Normal human bronchial epithelial (NHBE) cells were obtained from Cambrex (East Rutherford, NJ) and were cultured per vendor's recommendations. For reverse transcription-PCR (RT-PCR), quantitative RT-PCR, and immuno-histochemical analysis of BORIS and NY-ESO-1 expression following drug manipulation, cancer cells, NHBE cells, or normal dermal fibroblasts were exposed either to normal media × 96 hours; 5-azadC (0.1 μmol/L) × 72 hours followed by normal media × 24 hours; normal media × 72 hours followed by DP (25 μg/mL) × 6 hours then normal media × 18 hours; or 5-azadC (0.1 μmol/L) × 72 hours followed by DP (25 μg/mL) × 6 hours then normal media × 18 hours. RT-PCR, quantitative RT-PCR, and immunohistochemical analyses of BORIS or NY-ESO-1 expression were done at the 96-hour time point for each treatment condition unless indicated otherwise.

DNA and RNA isolation, reverse transcription-PCR, and quantitative reverse transcription-PCR analysis. Genomic DNA and total RNA were isolated from cultured cells or primary tumor specimens using DNeasy and RNeasy kits (Qiagen, Valencia, CA). mRNA was isolated from cancer cells using protocols and reagents contained in the Micro Fast Track 2.0 mRNA Isolation Kit (Invitrogen, Gaithersburg, MD). RT-PCR analysis of BORIS expression in untreated cells was done using the following primer sequences and thermal cycle conditions: BORIS forward 5′-CAGGCCCTTA-CAGTGTAAACGACTGCAA-3′ and BORIS reverse 5′-GCTATTGTAAGGGCTT-CTACCTGAGT-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5′-CCCTTTCATTGGACCTCAACTACATGG-3′ and GAPDH reverse 5′-CGTTGGTACACCCCTTCTGGATG-3′; IGF2/H19 expression was done using the following primer sequences and thermal cycle conditions: IGF2/H19 forward 5′-TGGGGTTTTTTAT-3′ and IGF2/H19 reverse 5′-CCCAAAACAACCTATACTCTTAA-3′.

Real-time quantitative RT-PCR analysis of BORIS and NY-ESO-1 expression was done using the following primer sequences and probes: BORIS forward 5′-CCCATTGTGGCACCACCTACA-3′ and BORIS reverse 5′-AGCATGCAA-GTTGGCATAT-3′; BORIS probe 6′AM-TGACAGGGAAACAGCCTAC-TGTGGTG-TAMRA; NY-ESO-1 forward 5′-GCTTCTTGACTCCTACCTG-3′ and NY-ESO-1 reverse 5′-GGGTCTGCGGGCAG-3′, and NY-ESO-1 probe 6′AM-TTGTGGACCAACAAGGAGGAGG-TAMRA; β-actin forward 5′-CGAGGAAGATGGACCCAGATCT-3′, β-actin reverse 5′-CAAGTGTAACGACTGCAA-3′, and β-actin probe 6′AM-CCAGGCGATGCTCGATCCAGGC-TAMRA; and techniques similar to those described previously (10). Tagman primers and probes were purchased from Applied Biosystems Oligonucleotide Factory (Foster City, CA).

Bisulfite sequencing of the BORIS and NY-ESO-1 promoters. BORIS and NY-ESO-1 promoter sequences were obtained from the University of California Santa Cruz Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat) and analyzed using the Cpg Software web site (http://www.ncbi.nlm.nih.gov). Genomic DNA from cultured cells was subjected to bisulfite modification as described by Herman et al. (26). A 335-bp sequence of the BORIS promoter containing a 279-bp region (27) was amplified by PCR techniques using the primer sequences: forward 5′-GTGTTTTTTTGTGGTTTTTTTAT-3′ and reverse 5′-CCCAAAACAACCCATCTTCAA-3′; Clontech Advantage GC 2 polymerase (Clontech, Palo Alto, CA), and the following thermal cycle variables: 95°C for 2 minutes followed by (denaturation at 95°C for 45 seconds, annealing at 56°C for 30 seconds, and elongation at 68°C for 1 minute) × 35 cycles then 68°C for 3 minutes. Two regions of the NY-ESO-1 gene (∼207 to −73, 135 bp; +168 to −299, 132 bp) were amplified using the following primer sequences: forward 5′-GGTTGTTTTTTTGGGTTTTTTTAT-3′ and reverse 5′-CCCAAAACAACCCATCTTCAA-3′; Clontech Advantage GC 2 polymerase (Clontech, Palo Alto, CA), and the following thermal cycle variables: 95°C for 1 minute, 72°C for 3 minutes then 68°C for 3 minutes. Before real-time quantitative PCR analyses, these PCR fragments were ligated into the TOPO TA cloning vector (Invitrogen, Carlsbad, CA). Following transformation, plasmids from individual bacterial colonies were isolated, and the BORIS and NY-ESO-1 promoter fragments were sequenced using an ABI 310 prism apparatus (Applied Biosystems, Carlsbad, CA), and protocols contained in the TOPO TA cloning kit.

Methylation-specific PCR. The Cpg Ware from Serologicals (Norriss, CA) was used to design primers to amplify 139-bp (unmethylated) and 135-bp (methylated) sequences within the Cpg island of the BORIS promoter, which would be susceptible to methylation changes. Methylation specific PCR (MSP) was done using bisulfite modified genomic DNA, Ampli-Taq Gold polymerase (Applied Biosystems), and the following thermal cycle variables: 94°C × 12 minutes, (95°C × 2 minutes, 65°C × 2 minutes, 72°C × 2 minutes) × 4 cycles, (95°C × 10 seconds, 62°C × 45 seconds, 72°C × 30 seconds) × 40 cycles followed by extension at 72°C for 30 minutes. PCR primers

K. Robertson and V. Lobanenkov, unpublished.

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included unmethylated forward 5'-GGTATGTTATATTTTTTTTGTGT- TAGGTT-3', unmethylated reverse 5'-ACCCCTCACCAAAAAATACACCA- CAA-3', methylated forward 5'-GTATGTTATATTTTTTTTGTAG- TTC-3', and methylated reverse 5'-CCTCCACCAGGAAAAAGCTACCGA-3'. PCR products were visualized by 3% NuSieve agarose ethidium bromide gel electrophoresis.

**Immunohistochemical analysis of BORIS and NY-ESO-1 expression.**

Drug treated CALU-6 and H1299 cells, as well as untreated NHBE cells were trypsinized, and pelleted into a thrombin-fibrin clot, fixed in formalin, and embedded in paraffin. BORIS antigen retrieval was carried out by heating sections in 0.01 mol/L citrate buffer (pH 6.6) in a high-pressure cooker for 4 minutes at full pressure. NY-ESO-1 antigen retrieval was done by incubating tissue sections in high pH target retrieval solution (DAKO, Carpinteria, CA) in a pressure cooker for 20 minutes at full pressure. Tissue sections were preblocked with dilute horse serum and incubated with affinity-purified chicken anti-BORIS ap-2-Ab antibodies (generously provided by A. Jungbluth, Ludwig Cancer Center, New York, NY) in 2% blocking solution overnight at 4°C in a humidified chamber. Immunoreactivity was detected by standard immunoperoxidase techniques using appropriate secondary antibodies, as well as reagents contained in the Vectastain avidin-biotin complex Elite kit (Vector Laboratories, Burlingame, CA).

**Plasmid constructs.** Full-length BORIS cDNA from the pCMV6-XL4/BORIS vector (22) was excised as an EcoRI and Smal fragment and inserted into the mammalian expression vector pIRE2-EGFP (BD Biosciences, Clontech, Palo Alto, CA) to generate the pIRE2-EGFP/BORIS plasmid expressing BORIS cDNA in sense orientation. The pBIG2/ (sense or antisense) BORIS plasmids, which express full-length BORIS cDNA in sense or antisense orientation under control of a tetracycline regulatable (tet-on) promoter were engineered as described by Vatolin et al. (24). A 761-bp (−207 to −968) fragment of the 5′-flanking region of NY-ESO-1 gene was PCR-amplified from human genome cDNA (BD Biosciences Clontech) and cloned into the MluXhoI sites of basic Luciferase reporter vector pGLO3 (Promega, Madison, WI) to generate pGLO3/ESO-762 for promoter-reporter experiments described below.

**Stable transfections.** SKLC-6 cells were transfected with pIRE2-EGFP/BORIS, and H1299 cells were transfected with pBIG2/antisense BORIS using LipofectAMINE 2000CD (Invitrogen) according to vendor’s instructions. To generate the pIRE2-EGFP/BORIS plasmid expressing BORIS cDNA in sense orientation, the pBIG2/ (sense or antisense) BORIS plasmids, which express full-length BORIS cDNA in sense or antisense orientation under control of a tetracycline regulatable (tet-on) promoter were engineered as described by Vatolin et al. (24). A 761-bp (−207 to −968) fragment of the 5′-flanking region of NY-ESO-1 gene was PCR-amplified from human genome cDNA (BD Biosciences Clontech) and cloned into the MluXhoI sites of basic Luciferase reporter vector pGLO3 (Promega, Madison, WI) to generate pGLO3/ESO-762 for promoter-reporter experiments described below.

**Luciferase reporter assays.** All transfactions were done using LipofectAMINE 2000CD (Invitrogen), and luciferase assays were carried out using Dual-Luc Reporter Assay System (Promega), according to vendor’s instructions. pGLO3/ESO-762 was transfected into SKLC-6/BORIS or H1299/ antiBORIS stable transfactants, and the cells were lysed 30 hours later. For transient transfection experiments, SKLC-6 cells were cotransfected with pIRE2-EGFP/BORIS and pGLO3/ESO-762 or control vectors; H1299 cells were cotransfected with pBIG2/antisense BORIS and pGLO3/ESO-762 or appropriate control plasmids. pGLO3/ESO-762 and pBIG2/antisense BORIS were cotransfected into SKLC/BORIS cells to examine the effects of reduced expression of BORIS on the activity of NY-ESO-1 promoter. Doxycycline (50 μg/mL) was added to culture media 14 hours after transfection to induce the expression of antisense BORIS in SKLC-6 and H1299 cells. Approximately 30 hours after transfections, cells were lysed, and luciferase activity of the NY-ESO-1 promoter-reporter plasmid or control luciferase construct were determined; all assays were done in triplicate, and luciferase values were normalized for transfection efficiency against Renilla luciferase activity as described previously (28).

**Nuclear protein extraction and electrophoretic mobility gel shift assays.** The luciferase as well as full-length CTFC and BORIS proteins were synthesized from the Luciferase T7 Control DNA, pCTFE-7.1, and pCITE4a-BORIS constructs, respectively (18, 29), with the TnT reticulocyte lysate coupled in vitro transcription-translation system (Promega). Six overlapping 250-bp fragments covering the NY-ESO-1 promoter were 32P-labeled, gel purified, and used as DNA probes for gel mobility shift assays with equal amounts of in vitro translated luciferase and CTFC proteins as described (18, 29). Supershifts used 1 μl of anti-CTFC antibodies as described by Pugacheva et al. (2), or 10 μl anti-peptide BORIS antibodies (see ref. 24 for details), or 10 μl anti-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as a negative control. Binding reactions were carried out in buffer containing standard PBS with 5 mM MgCl2, 0.1 mM ZnSO4, 1 mM DTT, 0.1% NP40, and 10% glycerol in the presence of poly(deoxyinosinic-deoxyCMP) and salmon sperm DNA. Reaction mixtures of 20 μl final volume were incubated for 30 minutes at room temperature and then analyzed on 5% nondenaturing PAGE run in 0.5× TBE buffer. For electrophoretic mobility gel-shift assay (EMSA) with in vitro methylated DNA probes, treatment with the Ssxl-methylase was done as previously described for CTFC-binding fragments DMD4 and DMD7 of the H19 ICR (11).

**Chromatin immunoprecipitation assays.** CALU-6 cells were plated at a density of 2.6 × 106 cells per 10-cm dish and incubated overnight. The following day, cells were exposed to normal media with or without 25 ng/mL DP for 6 or 24 hours. Acetylation status of core histones associated with the BORIS promoter was assessed by chromatin immunoprecipitation (ChIP) techniques described by Steiner et al. (30), using rabbit polyclonal antibodies recognizing acetylated histone H3 and acetylated histone H4 (Upstate Biotechnology, Inc., Lake Placid, NY). The following primers were used for PCR analysis of immunoprecipitated DNA as well as input samples: forward 5′-CTCTTTTCGCCCTGGCCG-3′ and reverse 5′-GCCCAGCCCGACGACGC-3′. These primers amplified a 218-bp region within the Cpg island of the BORIS promoter. The variables for ChIP-PCR were 94°C × 3 minutes followed by (94°C × 30 seconds, 61°C × 60 seconds, 68°C × 60 seconds) × 32 cycles and extension at 68°C for 3 minutes. PCR products were analyzed by 1.2% agarose ethidium bromide gel electrophoresis. For ChIP analysis of the NY-ESO-1 promoter, 1 × 106 untreated NHBE cells or H1299 cells were prepared using a ChIP Assay Kit from Upstate Biotechnology according to the manufacturer’s recommendations. Briefly, sheared chromatin were precleared with Protein A beads and incubated overnight with either a mixture of nine anti-CTFC mouse monoclonal antibodies (10 μL), or 10 μL of rabbit polyclonal BORIS antisera “B3” produced at the NCI. Immunopurified rabbit polyclonal BORIS was amplified with primers corresponding to the E3 fragment of the NY-ESO-1 promoter: forward 5′-ACCCCGAACCCACCCCACAC′- and reverse 5′-GGGGCGACGCCCTTTAAGTGCGG′-3. As a positive control, a well-known CTFC site in the human c-myc promoter (myc-NS) was amplified by using primers forward 5′-GCTCTTGTACGGCACGAGTGT-3′ and reverse 5′-GCTCTTGTATTTGAGTGCGG′-3. Quantitation of ChIP qDNA was done by real-time PCR using the ABI Prism 7900 Sequence Detection System according Applied Biosystem’s SYBR Green PCR Master Mix Protocol. Real-time PCR was carried out in triplicate using 1 μL of immunopurified chromatin, control, and input DNA, and the following thermal cycling variables: 95°C for 10 minutes followed by (95°C for 15 seconds and 60°C for 1 minute) × 40 cycles. Data were collected at 60°C and analyzed by Comparative Ct methods (31).

**Results**

**Analysis of BORIS and NY-ESO-1 expression in cultured cells.** Preliminary experiments were initiated to evaluate the NY-ESO-1 promoter in an attempt to develop MSP methods for analyzing NY-ESO-1 expression in biopsies from patients on our gene induction trials. Specifically, a region pertaining to the NY-ESO-1 coding sequence, as well as 3 kb proximal to it was identified using CpG software provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/cpgplot/index.html). Interestingly, no sequence was identified that fulfilled current criteria for CpG island as defined by Takai and Jones (ref. 27; length > 500 bp, G + C content > 0.55, observed CpG/expected CpG > 0.65). Using less stringent criteria, two smaller regions of CG dinucleotides were identified in the first exon and 5′-flanking sequence
of the NY-ESO-1 gene. Bisulfite sequencing analysis indicated that the methylation patterns in these regions did not coincide with NY-ESO-1 expression in cultured cells (data not shown). These preliminary results suggested that NY-ESO-1 expression might be mediated to a significant extent by more subtle epigenetic mechanisms targeted to the promoter region of this CT gene.

RT-PCR experiments were done to examine BORIS and NY-ESO-1 expression in cultured lung cancer cells. As shown in Fig. 1, BORIS transcripts were detected in 9 of 15 lung cancer lines. NY-ESO-1 transcripts were clearly seen in five of the cancer lines that expressed BORIS. NY-ESO-1 expression seemed much lower in two additional lung cancer lines that were deficient for BORIS under these experimental conditions. NHBE cells as well as normal human dermal fibroblast (NHDF) cells had no detectable BORIS or NY-ESO-1 expression. In general, these preliminary results suggested that NY-ESO-1 expression coincided with derepression of BORIS in lung cancer cells.

Quantitative RT-PCR experiments were done to examine the relationship between BORIS and NY-ESO-1 expression in lung cancer cells exposed to chromatin remodeling agents under treatment conditions achieved in our clinical trials. Results of representative experiments are summarized in Table 1. 5-azadC (0.1 \( \mu \)M \times 72 hours) mediated robust induction of BORIS in cultured lung cancer cells. DP (25 ng/mL \times 6 hours) had variable effects in cells exhibiting low basal levels of BORIS expression but markedly enhanced 5-azadC-mediated induction of this gene in most of the cancer lines; DP also augmented expression of BORIS in H1299 cells that normally exhibit extremely high BORIS mRNA levels. In contrast, CTCF mRNA levels remained relatively constant, or diminished somewhat following drug exposure. Interestingly, the drug treatment regimens were insufficient to induce BORIS or NY-ESO-1 expression in NHBE cells. Collectively, these data suggested that the extent of NY-ESO-1 derepression in lung cancer cells was proportional to the induction of BORIS expression following 5-azadC, DP, or sequential 5-azadC/DP exposure.

Immunohistochemistry experiments were done to further evaluate BORIS and NY-ESO-1 expression in lung cancer cells exposed to chromatin remodeling agents under treatment conditions achieved in our clinical trials. Results of representative experiments are summarized in Table 1. 5-azadC (0.1 \( \mu \)M \times 72 hours) mediated robust induction of BORIS in cultured lung cancer cells. DP (25 ng/mL \times 6 hours) had variable effects in cells exhibiting low basal levels of BORIS expression but markedly enhanced 5-azadC-mediated induction of this gene in most of the cancer lines; DP also augmented expression of BORIS in H1299 cells that normally exhibit extremely high BORIS mRNA levels. In contrast, CTCF mRNA levels remained relatively constant, or diminished somewhat following drug exposure. Interestingly, the drug treatment regimens were insufficient to induce BORIS or NY-ESO-1 expression in NHBE cells. Collectively, these data suggested that the extent of NY-ESO-1 derepression in lung cancer cells was proportional to the induction of BORIS expression following 5-azadC, DP, or sequential 5-azadC/DP exposure.

Immunohistochemistry experiments were done to further evaluate BORIS and NY-ESO-1 expression in lung cancer cells

**Table 1.** BORIS, CTCF, and NY-ESO-1 expression in cultured cells following 5-azadC, DP, or 5-azadC/DP exposure (copy number/β-actin × e4)

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<td>3</td>
<td>726</td>
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<td>0</td>
<td>1</td>
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**Note:** Representative results of quantitative RT-PCR analysis of BORIS, CTCF, and NY-ESO-1 expression in cultured lung cancer cells and NHBE cells following exposure to normal media, 5-azadC, DP, or sequential 5-azadC/DP. Whereas CTCF mRNA levels remained relatively constant, or diminished somewhat following drug treatment, BORIS and NY-ESO-1 mRNA copy numbers increased substantially in all of the lung cancer lines following 5-azadC, DP, or sequential 5-azadC/DP exposure. BORIS and NY-ESO-1 mRNA copy numbers were very high in normal testes, as well as untreated H1299 lung cancer cells, yet exceedingly low in NHBE cells. Overall, induction of BORIS coincided with derepression of NY-ESO-1 in lung cancer cells following exposure to chromatin remodeling agents. Drug treatment had no significant effect on BORIS or NY-ESO-1 mRNA copy numbers in NHBE cells.
using antibodies previously shown to recognize these proteins (22, 32). Consistent with RT-PCR and quantitative RT-PCR data, immunohistochemical analysis showed derepression of BORIS in H1299 cells but not in NHBE cells, or in untreated CALU-6 cells (Fig. 2). BORIS immunoreactivity was markedly enhanced in CALU-6 cells following sequential 5-azadC/DP exposure. Consistent with previously published data pertaining to BORIS expression in HeLa cells (22), cytoplasmic as well as nuclear staining was observed in untreated H1299 as well as drug-exposed CALU-6 cells. Additional experiments indicated that NY-ESO-1 expression coincided with induction of BORIS in lung cancer cells. The pattern of NY-ESO-1 immunoreactivity in drug-treated CALU-6 (Fig. 2H) cells seemed somewhat more focal than that observed for BORIS (Fig. 2F), possibly reflecting differences regarding NY-ESO-1 and BORIS protein levels, relative efficiencies of antigen retrieval, and affinities of the antibodies used to detect these proteins in cancer cells. Collectively, these data confirmed that derepression of BORIS following 5-azadC, DP, or sequential 5-azadC/DP treatment coincided with induction of NY-ESO-1 expression in cultured lung cancer cells.

**Figure 2.** Immunohistochemical analysis of BORIS (A) and NY-ESO-1 (C) expression in untreated NHBE cells: untreated H1299 lung cancer cells (B and D, respectively), CALU-6 cells exposed to normal media (E and G, respectively), and sequential 5-azadC/DP (F and H, respectively). Overall, induction of BORIS expression coincided with derepression of NY-ESO-1 in these cancer cells. These immunohistochemical results were consistent with data pertaining to quantitative RT-PCR analysis of BORIS and NY-ESO-1 expression in lung cancer cells and NHBE cells.
Bisulfite sequencing, methylation-specific PCR, and chromatin immunoprecipitation analysis of histone modifications in the 5′-flanking noncoding region of BORIS gene. Induction of BORIS expression by 5-azadC, DP, and sequential 5-azadC/DP strongly suggested that this gene is regulated, at least in part, by localized alterations of chromatin structure. A 279-bp CpG island was identified in the 5′-flanking noncoding region of the BORIS gene; bisulfite sequencing experiments were done to examine methylation status of this CpG island (Fig. 3A). Overall, in NHBE cells that do not express BORIS (Fig. 1; Table 1), only five CpG-pairs are demethylated, whereas 18 of 23 CpGs are completely methylated. Similarly, in CALU-6 cells grown in normal media, extremely low level of BORIS expression (Table 1) correlates with methylation of the majority of CpG pairs. Notably, the methylation profile of this region from untreated CALU-6 cells was considerably different from that observed in NHBE cells. H1299 cells with high level BORIS expression (Table 1) exhibited extensive demethylation of this CpG island. Partial demethylation of this CpG island coincided with markedly enhanced BORIS expression in CALU-6 cells following low-dose 5-azadC exposure (Table 1). B: MSP analysis of the CpG island in the 5′-flanking noncoding region of BORIS gene in 5-azadC- and/or DP-treated CALU-6 or NHBE cells, untreated H1299 lung cancer cells, normal testes, and representative samples of normal lung (NL), primary tumor tissues (TU), or lymph node metastases (LN) from 6 of 12 lung cancer patients (Pt 1-6). H1299 and testis showed the expected presence of both “unmethylated” and “methylated” templates. Treatment with either 5-azadC alone, or sequential 5-azadC/DP resulted in demethylation of this CpG island evidenced by the appearance of a PCR product corresponding to unmethylated template in bisulfite-treated genomic DNA from CALU-6 lung cancer cells. This phenomenon was not observed following drug treatment of NHBE cells. MSP analysis revealed the presence of “demethylated” template in crude lysates from lung cancers but not adjacent normal tissues from patients 2, 3, 4, and 6; this phenomenon was not observed in tissue samples from patient 5. Interestingly, demethylation of the BORIS-regulatory region was observed in tumor as well as adjacent normal lung tissue from two patients (one of whom is patient 1). These findings suggest an epigenetic field effect associated with these cancers. C: histone acetylation–specific ChIP analysis of the CpG island within the 5′-flanking noncoding region of BORIS gene in CALU-6 cells exposed to normal media, or to DP (25 ng/ml) for 6 or 24 hours. Time-dependent increases in PCR products pertaining to DNA associated with acetylated histone H3 and H4 confirm that DP enhances acetylation of core histones within this CpG island.
was observed in H1299 cells exhibiting high-level BORIS expression relative to this region from NHBE cells deficient in BORIS (a three-log fold difference in BORIS expression, as shown in Table 1). Interestingly, the methylation profile of this region in untreated CALU-6 cells with extremely low level BORIS expression was considerably different from that observed in NHBE cells or H1299 cells; low level 5-azadC exposure mediated partial demethylation of this CpG island, coincident with an ~40-fold increase in BORIS expression in CALU-6 cells (Table 1).

MSP experiments were done to further evaluate methylation status of the 5’-flanking noncoding region of BORIS in cultured cancer cells and patient tissues. As shown in Fig. 3B, MSP revealed demethylation within the BORIS CpG island in H1299 cells and testes, both of which exhibit high level BORIS expression. Additional cell lines, including A549 and H2227, which expressed BORIS in the basal state, also exhibited demethylation of this CpG island (data not shown). Consistent with results of the quantitative RT-PCR and bisulfite sequencing experiments, demethylation was observed in this CpG island in CALU-6 cells (but not NHBE cells) following 5-azadC, or sequential 5-azadC/DP exposure. Similar analysis of patient samples revealed a relative demethylation in this putative BORIS-regulatory region in 6 of 12 primary lung cancers but not in adjacent normal lung tissues (representative data pertaining to six patients depicted in Fig. 3B). Demethylation of the BORIS-regulatory region was noted in the primary tumor as well as adjacent, histologically normal lung tissue from two additional patients (including patient 1, Fig. 3B). These preliminary data suggest that BORIS is activated in primary lung cancers, and raise the possibility that derepression of BORIS is an early event during multistep pulmonary carcinogenesis.

ChIP experiments were next done to ascertain if DP modulated acetylation status of core histones within the CpG island of the BORIS 5’-flanking region previously analyzed for alterations of DNA methylation. As shown in Fig. 3C, time-dependent increases in PCR products pertaining to DNA associated with acetylated histone H3 and acetylated histone H4 were observed in CALU-6 cells following DP exposure. These results confirmed that DP enhances 5-azadC-mediated induction of BORIS, at least in part, via modulation of chromatin structure within the CpG island involved in regulating BORIS expression in lung cancer cells.

Analysis of BORIS-mediated regulation of NY-ESO-1. A series of transfection experiments using a NY-ESO-1 promoter luciferase-reporter construct were done to examine regulation of NY-ESO-1 by BORIS in lung cancer cells. In the first series of experiments, SKLC-6 cells that normally exhibit very low level BORIS expression (Table 1), were stably transfected with pIRE2-EGFP/BORIS or “empty” pIRES-vector; subsequently, stable transfectants were transiently transfected with the pGL3/ESO-762 NY-ESO-1 promoter-reporter construct or pGL3 basic control vector. As shown in Fig. 4A, NY-ESO-1 promoter activity was significantly higher in BORIS-transfected SKLC-6 cells relative to pIRES-vector control-transfected cells. Promoterless control plasmids generated

![Figure 4](https://example.com/figure4.png)

Figure 4. Cotransfection experiments indicating that activity of the NY-ESO-1 promoter is positively regulated by BORIS in SKLC-6 or H1299 lung cancer cells. A, representative results of luciferase-reporter assays in SKLC-6 cells stably transfected with either the pIRE2-EGFP/BORIS construct (SKLC-BO), or pIRE2-EGFP vector control (SKLC-V), following transient transfection with the pGL3/ESO-762 NY-ESO-1 promoter-reporter construct or the pGL3 basic control vector. B, transient cotransfection assays with either the NY-ESO-1 promoter-reporter construct or luciferase-control without promoter, with or without BORIS-expressing (pIRE2-EGFP/BORIS) and nonexpressing (pIRES) vectors. Once again, NY-ESO-1 promoter activity was markedly enhanced in SKLC-6 cells following transfection with BORIS cDNA. C, luciferase activity in H1299 lung cancer cells stably expressing either pBIG2i/antisense-BORIS (H1299-Bo/AS), or pBIG2i control vector (H1299-V) following transient transfection of pGL3/ESO-762 or control luciferase plasmid and doxycycline exposure. D, NY-ESO-1 promoter activity in SKLC-6 cells stably transfected with pIRE2-EGFP/BORIS (SKLC-6-Bo) and transiently cotransfected with pBIG2i/antisense-BORIS or pBIG2i vector control and the pGL3/ESO-762 construct followed by doxycycline treatment.
background luciferase values in both types of stably transfected SKLC-6 cells (Fig. 4A). Transient cotransfection experiments with pIRES2-GFP/BORIS and pGL3/ESO-762 showed similar dependence of NY-ESO-1 reporter activity on the presence of BORIS in SKLC-6 cells (Fig. 4B).

In the second series of experiments shown in Fig. 4C, H1299 cells with very high level BORIS expression (Table 1) were stably transfected with pBIG2i/antisenseBORIS, or the pBIG vector control plasmid; stable transfectants were subsequently transiently transfected with the pGL3/ESO-762 NY-ESO-1 promoter-reporter construct, or pGL3 vector control. Following doxycycline exposure, NY-ESO-1 promoter-reporter activity was markedly reduced in H1299 cells that expressed the BORIS antisense compared with that observed in pBIG vector control-transfected cells. Additional...
experiments, depicted in Fig. 4D, indicated that NY-ESO-1 promoter activity was obviously diminished in pRES2-EFGP/ BORIS-transfected SKLC-6 cells following transient cotransfection of pBIG2i/antisense BORIS plasmid with pGL3/ESO-762 reporter and subsequent doxycycline treatment. Additional experiments using Northern blot techniques confirmed that induction or repression of BORIS message resulted in concordant changes in NY-ESO-1 expression in lung cancer cells (data not shown). Together with data from the quantitative RT-PCR and immunohistochemistry experiments, results of these transfection experiments strongly suggested that BORIS mediates NY-ESO-1 expression in lung cancer cells.

To provide evidence that regulation of the NY-ESO-1 promoter is a direct effect of BORIS competition with CTCF for a target site recognized by the 11 ZF DBD shared by these two paralogous factors, electrophoretic mobility shift assays (EMSA) were done to examine if CTCF and BORIS bind to the NY-ESO-1 promoter in vitro. As shown in Fig. 5A and B, overlapping fragments of the NY-ESO-1 promoter region were radiolabeled and incubated with either recombinant in vitro translated full-length CTCF and BORIS proteins. Recombinant luciferase protein prepared by the same in vitro translation reaction was used as a negative control for site-specific DNA-binding experiments. As shown in Fig. 5C, EMSAs showed specific interaction between CTCF and the E3 fragment of the NY-ESO-1 promoter, which was verified by the supershifted band generated by incubation of the complex formed between CTCF and the E3 probe with the anti-CTCF monoclonal antibodies but not following incubation with the α-actin control antibody (Fig. 5D, left). Moreover, binding of BORIS to the same E3 DNA fragment was detected and verified by specific supershifting of the E3/BORIS complex by anti-BORIS but not by irrelevant antibodies (Fig. 5D, right). Collectively, these data indicated that both CTCF and BORIS directly interact with the E3 region of the NY-ESO-1 promoter in vitro.

Previous studies have shown that CpG methylation regulates binding of CTCF to a majority of the 11 ZF DBD recognition sequences mapped in the context of epigenetically regulated loci, including the IGF2/H19 ICR (11, 13, 33, 34), XIST/Tsix choice-imprinting center for X chromosome inactivation (14), and certain genes that can escape X chromosome inactivation (15). To examine if CpG methylation affects interaction of the 11 Zn finger DBD common to CTCF/BORIS, additional EMSAs were done using unmethylated and in vitro methylated DNA probes corresponding to the E3 region of the NY-ESO-1 promoter; results of these experiments are depicted in Fig. 5E. Interestingly, this analysis revealed no apparent difference in binding of CTCF to methylated versus unmethylated E3 probes, indicating that interaction of CTCF with the NY-ESO-1 promoter in vitro is resistant to CpG methylation. This unexpected finding allowed us to speculate that in vivo occupancy of the CTCF site in the NY-ESO-1 promoter can be maintained within silenced heterochromatin in normal cells. To examine this issue, ChIP experiments were done to evaluate in vivo occupancy of the E3 region by CTCF in BORIS-negative NHBE cells. A ~40-fold enrichment for the E3 DNA sequence was observed in dispersed, sheared chromatin immunoprecipitated with a mixture of nine anti-CTCF mouse monoclonal antibodies (2) compared with a background fold enrichment obtained with a nonspecific control antibody. The human c-myc oncogene 5′-flanking insulator site (N site), which is known to be constitutively occupied by CTCF in normal somatic cells (2, 35), was used as a positive control for in vivo CTCF binding for these experiments. ChIP analysis showed an ~100- and 20-fold enrichment of CTCF binding to the myc-N insulator in NHBE and H1299 cells, respectively (Fig. 5F). In contrast to what was observed in NHBE cells, the E3 region manifested no CTCF-specific enrichment in H1299 lung cancer cells expressing very high levels of BORIS and NY-ISO-1.

Additional ChIP experiments were done to examine whether BORIS is recruited to the NY-ESO-1 promoter in vivo in H1299 cells that express BORIS relative to NHBE cells deficient for BORIS expression. As shown in Fig. 5F (BORIS-IP), the E3 region from DNA isolated from H1299 cells but not NHBE cells was enriched in a chromatin fraction pulled down with the same affinity-purified anti-BORIS-peptide antibodies, which were shown to interact specifically with the E3 DNA/BORIS complex in EMSA supershifting experiments (Fig. 5D). Of note was the finding of no enrichment in BORIS-specific ChIP for the site N CTCF target from the c-myc insulator in H1299 or NHBE cells (which showed the ChIP fold difference values characteristic for a nonspecific background). Whereas the magnitude of reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter may have been underestimated due to technical limitations related to the BORIS antisera that was used for the ChIP experiments, the data strongly suggested that induction of NY-ESO-1 expression in lung cancer cells coincides with a CTCF-to-BORIS switch of occupancy within the E3 region of this CT gene promoter.

Figure 5. Characterization of the in vitro binding and in vivo occupancy by CTCF and BORIS within the NY-ESO-1 promoter. A, the NY-ESO-1 promoter sequence with indicated positions of primers used to generate consecutively overlapping radiolabeled probes for screening CTCF-binding by EMSA. A transcriptional +1 start site corresponds to nucleotide 906 of the sequence from Genbank (accession no. AJ275977) shown here by an arrow at a capital T, B, a schematic map of the overlapping restriction fragments (E1 to E6) used as EMSA probes for in vitro detection of CTCF-binding sites. C, results of EMSA analyses showing binding of recombinant full-length CTCF (CTCF) only to the E3 fragment. Recombinant luciferase protein generated by the same in vitro methods (Luc) served as negative controls. CTCF-bound (B) versus unbound free (F) DNA probes (arrowheads). D, results of supershifting EMSA experiments with binding reactions containing the E3 radiolabeled probe, recombinant CTCF, and anti-CTCF monoclonal antibodies, or recombinant BORIS and anti-BORIS B3 antibodies (for more detail, see refs. 2 and 24), as well as an unrelated control antibodies (EMSA-gel lanes CTCF + α-Actin or BORIS + α-Actin). In addition to CTCF-binding (B) and unbound free (F) DNA probes, supershifted bands (S) generated by interactions of CTCF-E3 or BORIS-E3 complexes with corresponding antibodies are also indicated. E, results of the EMSA analyses of CpG methylation effects on binding of CTCF/BORIS 11 ZF DBD to the control (left) and to the SssI-treated methylated E3 DNA probe (right). Completion of the CpG methylation generated in the E3 probe by SssI methylase treatment was verified by digestion of control and of SssI-treated probes by methylation-sensitive restriction enzymes as described in detail previously (11). In addition, the DmD4 probe of the H19 ICR served as control for methylation-sensitive 11 ZF DBD binding (data not shown here but included in the similar EMSA experiments with the SssI-methylated CTCF/BORIS target site of the MAGE-A1 promoter; ref. 24). Note that unbound and 11 ZF-bound bands (F and B) are indicated as in (C) and (D), whereas an additional minor band (B′) may be generated by the relative excess of the 11 ZF-protein for reasons not yet clear. F, results of real-time PCR analysis of the fold difference for the presence of DNA with the CTCF/BORIS-binding sites in the input chromatin versus in the CTCF-containing or the BORIS-containing chromatin fraction obtained by ChIP techniques with NHBE cells and H1299 lung cancer cells. Note a much stronger in vivo binding of CTCF to the E3 region of the NY-ESO-1 promoter in NHBE cells that do not express NY-ESO-1 relative to H1299 cells in which expression of this CTA is very high. In contrast, BORIS interaction with this region was more pronounced in H1299 cells relative to NHBE cells, suggesting that binding of CTCF to CTCF and BORIS coincides with induction of NY-ESO-1 in lung cancer cells. The constitutive chromatin insulator myc-N site analyzed for CTCF occupancy previously (2, 36) served as a control target site that does not manifest any significant switching to the occupancy by BORIS in H1299 lung cancer cells with a very high level of BORIS expression.
Discussion

NY-ESO-1 is the most immunogenic CTA identified to date; nearly 50% of patients whose tumors express NY-ESO-1 exhibit serum antibodies to this CTA, which fluctuate with extent of disease (reviewed in ref. 8). Vaccines using either CD4- or CD8-restricted peptide epitopes, or full-length recombinant NY-ESO-1 protein have enhanced anti-NY-ESO-1 reactivity in cancer patients, some of whom have exhibited disease regression following immunization (36, 37). Whereas nearly 30% of non–small cell lung cancer and 75% of small cell lung cancer (SCLC) express NY-ESO-1 (38, 39), immune response to this CTA seems limited in lung cancer patients (40). Although this apparent lack of immune response to NY-ESO-1 may be attributable, at least in part, to deficiencies regarding antigen processing/presentation, particularly in SCLC (41, 42), and the immunosuppressive effects of regulatory T cells, which are abundant within the primary tumors as well as the systemic circulation of lung cancer patients (43, 44), in many instances, levels of NY-ESO-1 expression in primary lung carcinomas may simply be below the threshold for immune recognition. Indeed, our experience concerning induction of NY-ESO-1 in tumor tissues from lung cancer patients and detection of NY-ESO-1 antibodies in several of these individuals following exposure to chromatin-remodeling agents\(^6\) clearly indicate the potential use of gene induction regimens for enhancing the immunogenicity of lung cancer cells in vivo.

Whereas the number of newly discovered CT genes continues to increase (6), it remains unclear if activation of particular subsets of CTA(s) coincides with tumor histology. To date, no specific CT gene expression profiles have been consistently associated with lung cancer (reviewed in ref. 9). Indeed, using the "INCLUSive" (integrated clustering, upstream sequence retrieval and motif sampling; ref. 45), we have observed that relatively few of the 89 individual CT genes described to date have been analyzed extensively in human malignancies. Furthermore, no common features have been identified in CT gene promoters that can account for the strict silencing of these genes in all normal somatic cells and their concordant activation during male germ cell development (46). Although initial studies on transcriptional regulation of MAGE-A1 have been done (47, 48), little has been done to elucidate the mechanisms regulating other CT genes such as NY-ESO-1, particularly in a clinically relevant manner.

In the present study, we sought to examine the epigenetic mechanisms by which chromatin-remodeling agents enhance expression of NY-ESO-1 in lung cancer cells relative to NHBE cells. Our experiments indicated that 5-azadC, DP, or 5-azadC/DP-mediated induction of NY-ESO-1 coincided with derepression of BORIS in lung cancer cells. Additional experiments suggested that BORIS is activated in primary lung cancer cells, and that 5-azadC- and DP-mediated induction of BORIS expression coincides with DNA demethylation and enhanced acetylation of core histones within a CpG island of the BORIS promoter. Subsequent studies revealed that BORIS physically interacts with the NY-ESO-1 promoter to enhance expression of this CT gene. Experiments are presently under way to extend these observations by systematically evaluating BORIS expression relative to NY-ESO-1 and other CTAs in primary lung cancers and adjacent normal bronchial epithelia by RT-PCR, quantitative RT-PCR, MSP, and immunohistochemistry techniques.

Although our data clearly indicate that derepression of BORIS coincides with induction of NY-ESO-1 in lung cancer cells, the precise mechanisms by which BORIS enhances NY-ESO-1 transcription remain unknown; indeed, our data suggest that BORIS is necessary but not always sufficient for induction of NY-ESO-1 in lung cancer cells. Whereas the contact nucleotides for BORIS and CTCF binding within the NY-ESO-1 promoter have not been determined, results of the ChIP experiments strongly suggest that BORIS competes with CTCF for binding to the NY-ESO-1 promoter, presumably recruiting additional proteins that facilitate derepression of this gene. In related studies, we have observed that ectopic expression of BORIS (but not the 11 Zn finger DBD alone) induces a variety of CTAs (including NY-ESO-1 and MAGE-A1) in NHDF; reciprocal binding of CTCF and BORIS to the MAGE-A1 promoter coincides with demethylation and induction of MAGE-A1 in a manner remarkably similar to that observed following 5-azadC exposure (24).

It is intriguing that whereas interaction of CTCF with insulator/ boundaries is methylation specific (2, 11, 13–15, 25, 49), binding of CTCF to the NY-ESO-1 (this report) or to the MAGE-A1 (24) promoters seems unaffected by methylation status of the recognition region for the 11 ZF DBD. Furthermore, it is interesting that BORIS-mediated derepression of MAGE-A1 in NHDF seems contingent on demethylation mechanisms, whereas induction of NY-ESO-1 expression in lung cancer cells does not correlate in an obvious manner with demethylation of the NY-ESO-1 promoter, which seems to lack CpG islands typically associated with CT genes (47). Indeed, the fact that the NY-ESO-1 promoter region does not seem densely methylated, could account for our observations that NY-ESO-1 is more robustly induced in lung cancer cells compared with other CTAs such as MAGE-A1, MAGE-3, or MAGE-12 following exposure to chromatin remodeling agents (9, 10). Nevertheless, our data do not exclude the possibility that BORIS facilitates demethylation of individual CG dinucleotides, thereby enhancing binding of other transcription factors to the NY-ESO-1 promoter. Furthermore, the data do not exclude the possibility that alterations in chromatin structure within extended “looped” regions of the X chromosome which contain interacting CTCF-binding sites, as recently suggested for the IGF2/H19 (13, 50) and XIST (2) loci, as well as for several gene clusters that escape X chromosome inactivation (15), could indirectly enhance BORIS-mediated induction of NY-ESO-1 in lung cancer cells. Conceivably, differential regulation of CT genes in lung cancer cells is dependent on the stoichiometry of BORIS and CTCF, combinatorial interaction of the 11 Zn finger DBD of these transcription factors with target sequences, methylation status of promoters, and recruitment of accessory chromatin-remodeling proteins.

It is intriguing that to date, we have not been able to express BORIS in NHBE cells using gene transfer techniques, and we have not been able to induce BORIS expression in these cells by 5-azadC, DP, or sequential 5-azadC/DP treatment regimens that potently induce this gene in lung cancer cells; the lack of BORIS induction coincides with negligible NY-ESO-1 derepression in NHBE cells. Previously, we reported that 5-azadC, DP, or sequential 5-azadC/DP treatment regimens identical to those used in our current experiments induced MAGE-3 expression in lung cancer cells but were insufficient to mediate up-regulation of this CT gene in NHBE cells (51). In contrast, 5-azadC (at a log-fold higher concentration than used for our lung cancer studies) induces BORIS expression, with subsequent derepression of CTAs in NHDF (24). Whereas relative proliferation rates and global methylation status could account, in

\(^6\) D.S. Schrump et al., in preparation.
part, for the magnitude of BORIS induction in cultured cancer cells compared with NHBE cells, these observations suggest that the BORIS promoter in lung cancer cells is more susceptible to the chromatin remodeling effects of DNA-demethylating agents and HDAC inhibitors. Experiments are in progress to define the mechanisms that contribute to preferential induction of BORIS in lung cancer cells following exposure to chromatin remodeling agents.

Data presented in this manuscript as well as those described in an accompanying article (24) suggest that BORIS contributes to derepression of a wide variety of CTAs. As such, induction of BORIS expression by sequential 5-azaC/DP infusion may be a novel strategy to augment antitumor immunity via up-regulation of numerous, potentially relevant CTAs, particularly in the context of vaccine or cell transfer protocols (52). Furthermore, it is conceivable that BORIS itself may be a novel target for lung cancer immunotherapy. Studies are currently under way to ascertain if BORIS is immunogenic in cancer patients, and to define peptide epitopes that may be used for future vaccine trials.

The fact that a CTCF-to-BORIS switch in occupancy within the NY-ESO-1 promoter seems to modulate expression of this CT gene in lung cancer cells, prompts one to ponder the events, and mechanisms that mediate derepression of BORIS during pulmonary carcinogenesis. Further investigation of this issue will undoubtedly provide considerable insight regarding the epigenetics of lung cancer and possibly reveal novel targets for the treatment and prevention of this disease.

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References


Reciprocal Binding of CTCF and BORIS to the NY-ESO-1 Promoter Coincides with Derepression of this Cancer-Testis Gene in Lung Cancer Cells

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