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Int. J. Cancer: **119,** 2878–2884 (2006)
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SPAN-XB core promoter sequence is regulated in myeloma cells by specific CpG dinucleotides associated with the MeCP2 protein

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SPAN-Xb is a novel cancer-testis antigen in multiple myeloma. We recently demonstrated that SPAN-Xb expression in myeloma cells is regulated through promoter methylation and could be upregulated by IL-7 and GM-CSF. In this present study, we set out to investigate the mechanism of *SPAN-XB* expression and the promoter association with the methyl-CpG binding protein (MeCP2). Elucidation of these interactions is likely shed light on potential therapeutic strategies to upregulate antigen levels for SPAN-Xb-based tumor vaccines. Using a panel of truncated promoter constructs, we localize the core sequence of *SPAN-XB* promoter to the 73 bp at the 3′ end of the promoter, a region within the full length promoter that lacks CpG dinucleotides. Reporter gene expression assays showed that the core promoter function is significantly modulated by the adjacent CpG sequences. Chromatin immunoprecipitation assays revealed a specific association of MeCP2 with the promoter, and MeCP2 binding strongly correlated with repression of *SPAN-XB* gene. Reactivation of the *SPAN-XB* gene by 5-azacytidine treatment resulted in the loss of MeCP2 from this site. We, therefore, conclude that *SPAN-XB* core promoter function in myeloma cells is associated with MeCP2 protein binding and regulated by specific CpG dinucleotide sequences.
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Key words: SPAN-Xb; CT antigen; MeCP2 protein

SPAN-Xb is a spermatid-specific protein encoded by the *SPAN-XB* gene on chromosome Xq27.1. *SPAN-XB* has a genomic organization consisting of 2 exons with a small intron and belongs to the *SPAN-X* family of genes.[1] SPAN-Xb differs from its other family members (-C, -A1/2 and -D); it contains a 6 amino acid insertion in the middle of the sequence and also contains a potential *N*-linked glycosylation site. Also, unlike the other members of the SPAN-X protein that are predominantly nuclear in distribution, immunohistochemistry staining localized SPAN-Xb to the cytoplasm, suggesting that its function is likely to be distinct from those of its other family member proteins. There is no homology between the SPAN-X proteins and any other known proteins. SPAN-Xb was previously shown to be a novel cancer-testis antigen in hematologic malignancies, including multiple myeloma (MM).[2] This is a new class of antigens that consist of a group of normal testicular-specific protein aberrantly expressed in tumor cells. The aberrant expression of SPAN-Xb in tumor cells is associated *in vivo* with the generation of high titers IgG directed at SPAN-Xb protein, suggesting the immunogenicity of the protein in the cancer-bearing autologous host. The immunogenicity and highly restricted normal tissue expression pattern makes SPAN-Xb an excellent candidate protein to be used for the design of tumor vaccines.

One major problem preventing effective tumor immunotherapy is the heterogeneity of tumor antigen expression, even within individual specimens. Furthermore, these antigens are only detected in the tumor cells from approximately 30% of patients, although the expression frequency generally increases with disease progression.[3] Further study into the regulation of tumor antigen expression will, therefore, provide vital information that may be relevant to future design of tumor vaccines targeting that specific antigen.

On the basis of our previous study showing that DNA methylation regulates the expression of another CT gene, Sp17,[4] and the finding by other groups that CT antigen expression is associated with global DNA hypomethylation in the tumor genome,[5] we have recently investigated the role of promoter methylation in the transcriptional control of *SPAN-XB*.[6] Using a combination of bisulfite conversion with sequence analysis and *in vivo* methylation studies, we recently demonstrated that methylation at specific CpG dinucleotides within the promoter sequence provides the primary regulatory mechanisms for the expression of *SPAN-XB* gene. Pretreatment of the tumor cells with the hypomethylating agent, 5-azacytidine, resulted in the upregulating of SPAN-Xb expression that could further be enhanced with IL-7 and GM-CSF. However, the relationship between *SPAN-XB* promoter sequence and DNA methylation, and the interaction of these with the transcriptional repression machinery remains unclear. In the present study, we set out to determine the sequence of the minimal core promoter of *SPAN-XB* and the effect of the specific CpG dinucleotides within the promoter and association with methyl-CpG binding protein-2 (MeCp2) on the transcriptional control of the *SPAN-XB* gene. Elucidation of these interactions is likely shed light on the molecular mechanisms affecting SPAN-Xb expression and offers therapeutic strategies for upregulating the antigen levels for improving the efficacy of SPAN-Xb-based tumor vaccines.

Material and methods

Primary myelomas and cell lines

Fresh myeloma cells were obtained, after informed consent and approval by the Institution Review Board, from the bone marrow of patients with a diagnosis of MM. Myeloma cells were enriched by CD138 positive selection (Miltenyi Biotec, Auburn, CA) and used for the experiments. Seven tumor cell lines were also used: ARK-B and ARP-1 (gifts from Joshua Epstein, PhD, University of Arkansas for Medical Sciences), RPMI 8226 (gifts from Raymond Comenzo, MD, Memorial Sloan Kettering Cancer Center) and IM 9 (gifts from Dharminder Chanhan, PhD, Dana Farber Cancer Center). All cell lines were maintained at liquid culture prior to being used for the experiments.

Generation of truncated and mutant SPAN-XB promoter sequences

Full length *SPAN-XB* promoter sequence was isolated from the genomic DNA derived from normal testis, amplified and cloned into the TA cloning system for sequence analysis. PCR primers for the amplification of *SPAN-XB* promoter were 5′-*GGT ACC ACC TGT AGG AGGGAA* ATG-3′ and 5′-*AGA TCT AAG GCA AAG CCA CAC* CCT-3′ and amplified a DNA segment of 445 bp (including the creation of restriction sites for cloning) spanning the nucleotides from position −536 to −104 from the translation

Grant sponsor: National Institute of Health/National Cancer Institute; Grant numbers: RO1 CA088434 and RO1 CA106283; Grant sponsor: Larry Wiley Memorial Fund.
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Received 10 May 2006; Accepted after revision 10 July 2006
DOI 10.1002/ijc.22259
Published online 11 October 2006 in Wiley InterScience (www.interscience.wiley.com).

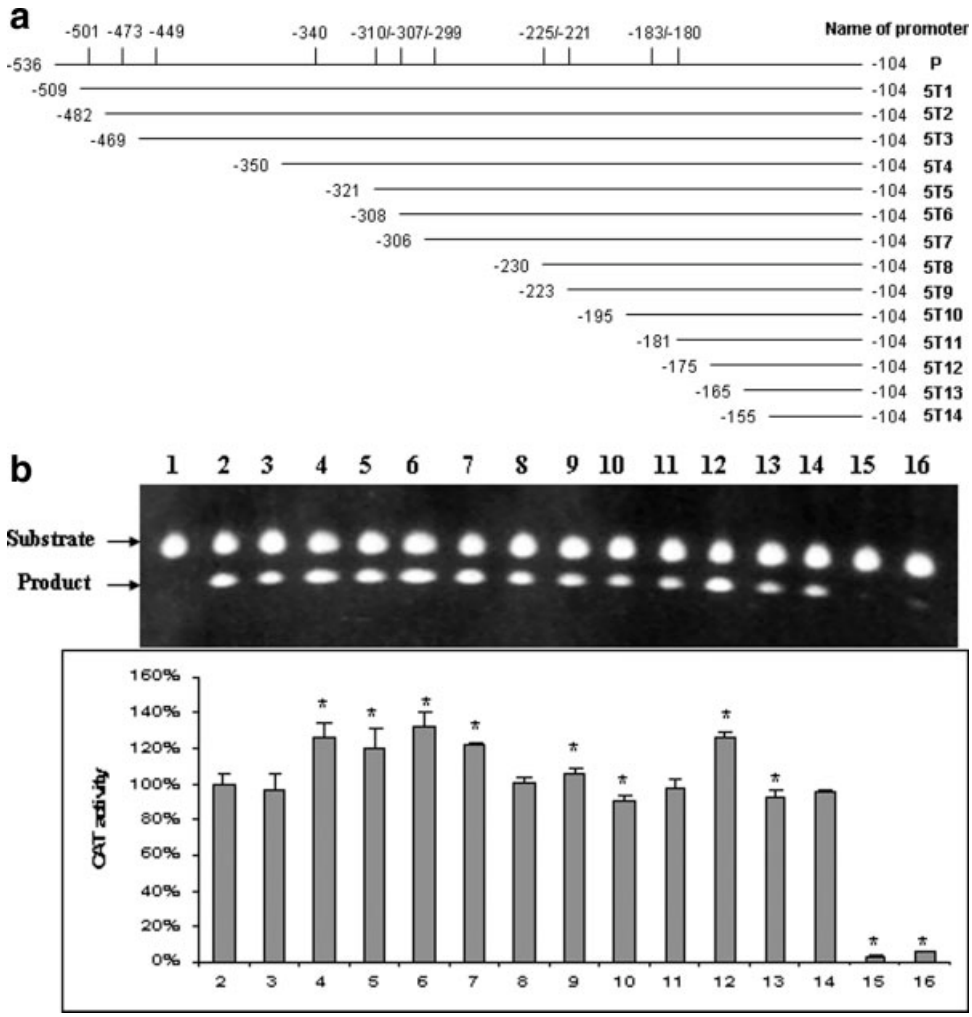

FIGURE 1 – (*a*) Panel of 5′ truncated *SPAN-XB* promoters, including the positions of the CpG dinucleotides. (*b*) Functions of 5′ truncated *SPAN-XB* promoters, as measured by CAT activities. The promoter function was essentially intact until the sequence was truncated to less than 73 bp at the 3′ end of the full length sequence when suddenly the promoter function was abrogated in 5T13 and 5T14 (Lane 1 = No *SPAN-XB* promoter; Lane 2 = Full length promoter; Lane 3 = 5T1; Lane 4 = 5T2; Lane 5 = 5T3; Lane 6 = 5T4; Lane 7 = 5T5; Lane 8 = 5T6; Lane 9 = 5T7; Lane 10 = 5T8; Lane 11 = 5T9; Lane 12 = 5T10; Lane 13 = 5T11; Lane 14 = 5T12; Lane 15 = 5T13; Lane 16 = 5T14) (All results were expressed as mean + SD of 3 readings) (Results are from 1 of 2 similar experiments).

start site. This full length gene was subsequently used for the generation of truncated and mutant *SPAN-XB* promoter sequences.

A panel of truncated *SPAN-XB* promoters was generated by polymerase chain reactions from the 5′ end and then from the 3′ end of the full length genes. These truncated promoters were generated by using oligonucleotide primers specific for the 5′ end or 3′ end of the full length promoter with a panel of oligonucleotide primers spanning various regions along the full length *SPAN-XB* promoter. Successfully amplified DNA fragments were isolated and cloned into the TA cloning system for sequence confirmation prior to being used for subcloning into the pCAT*3-Enhancer vector (Molecular Probes Company, Eugene, OR) between *Kpn I* and *Bgl II*.

A panel of mutant *SPAN-XB* promoters was also generated from the full length wild-type *SPAN-XB* promoter sequence by oligonucleotide-directed mutagenesis, mutating individually the cytosine or guanine of each of the CpG dinucleotides within the promoter sequence using GeneEditor (Promega), according to the manufacturer's instruction. Briefly, the full length wide-type *SPAN-XB* promoter template was denatured by 0.2 N NaOH solution for 2 min and then neutralization by 0.2 N NH_4Ac (pH 4.5). The denatured DNA template was then precipitated and recovered by ethanol precipitation followed by resuspension in water. The single-stranded DNA was annealed to 2 oligonucleotides, 1 containing the mutated CpG nucleotides and another to the ampicillin resistant gene. Following polymerization, the plasmid was transformed into BMH 71-18 mutS competent cells and grown on Ampicillin + Selection Mix LB plate. Recombinant colonies were picked and grown in LB. The plasmid was extracted and then transformed into JM109 for selection of mutants. The mutants were confirmed by DNA sequencing.

Cell transfection and analysis of chloramphenicol acetyl transferase expression

Once generated, the pCAT*3 Enhancer-promoter vectors were used to transfect SPAN-Xb-positive RPMI 8226 cells. Transfection was carried out using the FuGENE 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's recommendation. Briefly, the cells were seeded into a 6-well cluster plate and grown to 50% confluence. The cultures were transfected with 2 μg of the recombinant plasmids and assayed for chloramphenicol acetyl transferase (CAT) activities after 72 hr. FAST CAT Green (deoxyl) Chloramphenicol Acetyltransferase Assay Kit (Molecular Probes) was used to detect CAT activity. The transfectants were first lysed and a cytoplasmic extract prepared. The extract was

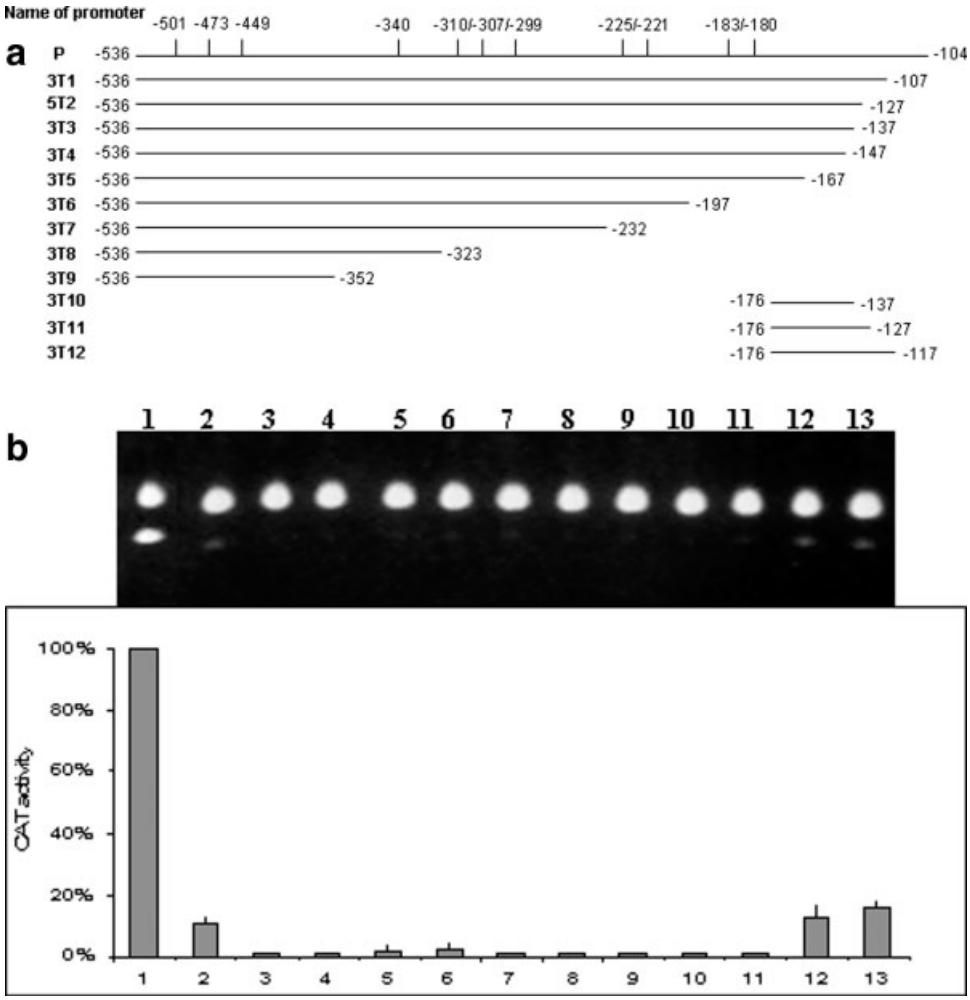

FIGURE 2 – (*a*) Panel of 3′ truncated SPAN-XB promoters, including the positions of the CpG dinucleotides. (*b*) Functions of 3′ truncated *SPAN-XB* promoters, as measured by CAT activities. Shortening of the sequence, even by only 3 bp (promoter 3T1), resulted in a marked drop in the promoter function of the sequence. All the truncated promoters generated from the 3′ end produced sequences that did not exhibit any promoter function. (Lane 1 = Full length promoter; Lane 2 = 3T1; Lane 3 = 3T2; Lane 4 = 3T3; Lane 5 = 3T4; Lane 6 = 3T5; Lane 7 = 3T6; Lane 8 = 3T7; Lane 9 = 3T8; Lane 10 = 3T9; Lane 11 = 3T10; Lane 12 = 3T11; Lane 13 = 3T12) (All results were expressed as mean + SD of 3 readings) (Results are from 1 of 2 similar experiments).

then incubated with the fluorescent deoxylchloramphenicol substrate and acetyl CoA at 37°C. The reaction was terminated by the addition of ice-cold ethyl acetate. After drying and dissolution in ethyl acetate, the reaction substrate and products were resolved by thin-layer chromatography on silica gel plates and eluted with a chloroform:methanol mixture (85:15 v/v). Quantitation of the products was performed using a Bio-Rad imaging system. The product amount in the CAT reaction was calculated as percentage of activity of wild-type promoter. Experiments were carried out in triplicates in 2 independent settings, and Student's *t* test was applied for statistical validation.

Chromatin immunoprecipitation

This was carried out using the ChIP assay kit and an anti-MeCP2 rabbit polyclonal antibody purchased from Upstate Biotechnologies (Charlotteville, VA). Briefly, tumor cells (2×10^6) grown in 100 mm dishes were harvested, and the proteins were crosslinked to DNA *in situ* with formaldehyde (1%) for 10 min. The cells were then allowed to settle in a 1% SDS buffer followed by sonication in the presence of a protease cocktail. The soluble chromatin fraction was mixed with 5 µl of MeCP2 antibodies, and incubated overnight with rotation. Immune complexes were collected with salmon sperm DNA-protein agarose, washed and eluted with buffer (1% SDS, 0.1 M $NaHCO_3$). The protein–DNA crosslinks were treated with proteinase K, recovered by phenol–chloroform extraction and ethanol precipitated. The DNA samples were dissolved in water and subjected to PCR using *SPAN-XB* promoter sequence-specific primers for detecting the sequences that interacted with the MeCP2 protein. PCR products were run on 1% agarose ethidium bromide gel and the products visualized on an ultraviolet light transilluminator.

Results

Identification of the core promoter sequence of SPAN-XB

The putative *SPAN-XB* promoter resides within exon 1 and contains 433 bp, starting from position −546 to position −104 upstream of the translational start site.[6] To determine the core promoter sequence, a panel of truncated *SPAN-XB* promoters was generated, by PCR, from the 5′ end (Fig. 1*a*). The truncated promoter sequences were subcloned into the pCAT vector and transfected into RPMI 8226 cells. Cell lysates from transfectants were used to measure the CAT activities. Compared to full length promoter sequence, the function was altered in truncated *SPAN-XB* promoters (Fig. 1*b*). Truncation generally resulted in an increase in the CAT activity, except for truncated promoters 5T8 and 5T11

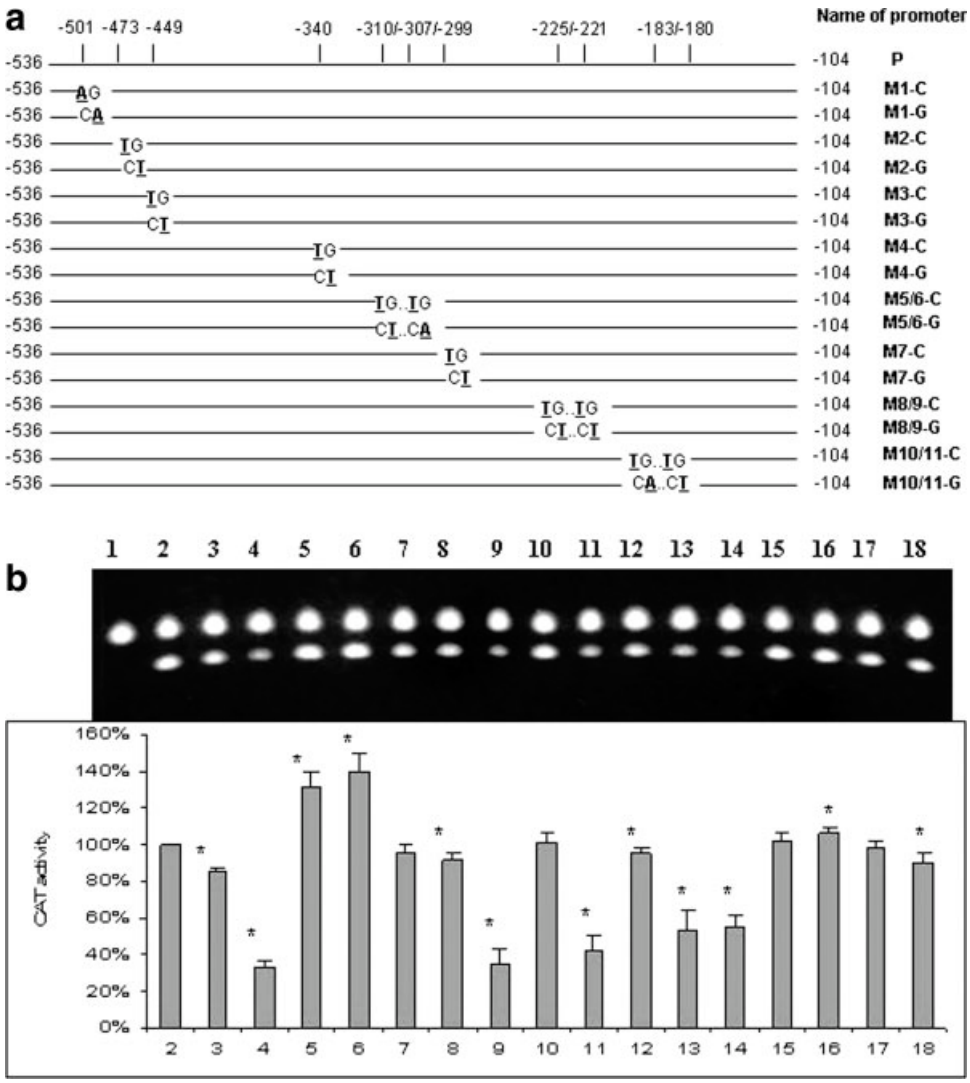

FIGURE 3 – (*a*) Panel of mutated (in bold) full length SPAN-XB promoters, including the positions of the CpG dinucleotides. (*b*) Functions of mutated full length *SPAN-XB* promoters, as measured by CAT activities. Mutation of the CpG nucleotides resulted in changes in the promoter function (Lane 1 = No promoter; Lane 2 = wild-type promoter; Lane 3 = M1-C; Lane 4 = M1-G; Lane 5 = M2-C; Lane 6 = M2-G; Lane 7 = M3-C; Lane 8 = M3-G; Lane 9 = M4-C; Lane 10 = M4-G; Lane 11 = M5/6-C; Lane 12 = M5/6-G; Lane 13 = M7-C; Lane 14 = M7-G; Lane 15 = M8/9-C; Lane 16 = M8/9-G; Lane 17 = M10/11-C; Lane 18 = M10/11-G) (All results were expressed as mean + SD of 3 readings) (Results are from 1 of 2 similar experiments).

in which the promoter function was slightly reduced. The promoter function was essentially intact until the sequence was truncated to less than 72 bp at the 3′ end of the full length sequence when suddenly the promoter function was abrogated. The results, therefore, suggest that the core promoter sequence is contained within the 73 bp at the 3′ end of the full length sequence.

To confirm this, a panel of truncated promoters was generated from the 3′ end of the full length sequence (Fig. 2*a*). Shortening of the sequence, even by only 3 bp (promoter 3T1), resulted in a marked drop in the promoter function of the sequence (Fig. 2*b*). All the truncated promoters generated from the 3′ end produced sequences that did not exhibit any promoter function. Taken together with the results obtained from truncated promoter 5T12, we conclude that the core promoter sequence of *SPAN-XB* is contained within the 72 nucleotides at the 3′ end of the full length sequence. We next studied the promoter activity of sequences in this region, from position −176 to position −104. In keeping with the results obtained from truncated promoters generated from the 3′ end, shortening of the 73 bp sequence at the 3′ end produced promoter function that was significantly reduced. These results, therefore, again indicate that the core promoter function of *SPAN-XB* is contained within the 73 nucleotides at the 3′ end of the full length sequence.

Secondary regulatory function of specific CpG dinucleotide sequences

There are 11 CpG dinucleotides within the putative *SPAN-XB* promoter. We previously found that DNA methylation provides the primary regulatory mechanism for *SPAN-XB* gene expression. We also identified that hypomethylation at positions −310, −307, −299 and −221 strongly predicted for *SPAN-XB* expression, suggesting the involvement of these 4 CpG dinucleotides in the regulation of *SPAN-XB* gene expression through DNA methylation.[6] In the present study, we determined whether or not these CpG dinucleotides play any regulatory function in the *SPAN-XB* promoter independent of DNA methylation. To do so, a panel of mutant *SPAN-XB* promoters was generated by site-directed mutagenesis (Fig. 3*a*), sequentially mutating the cytosine or guanine of the CpG dinucleotides and determined for any changes in the function

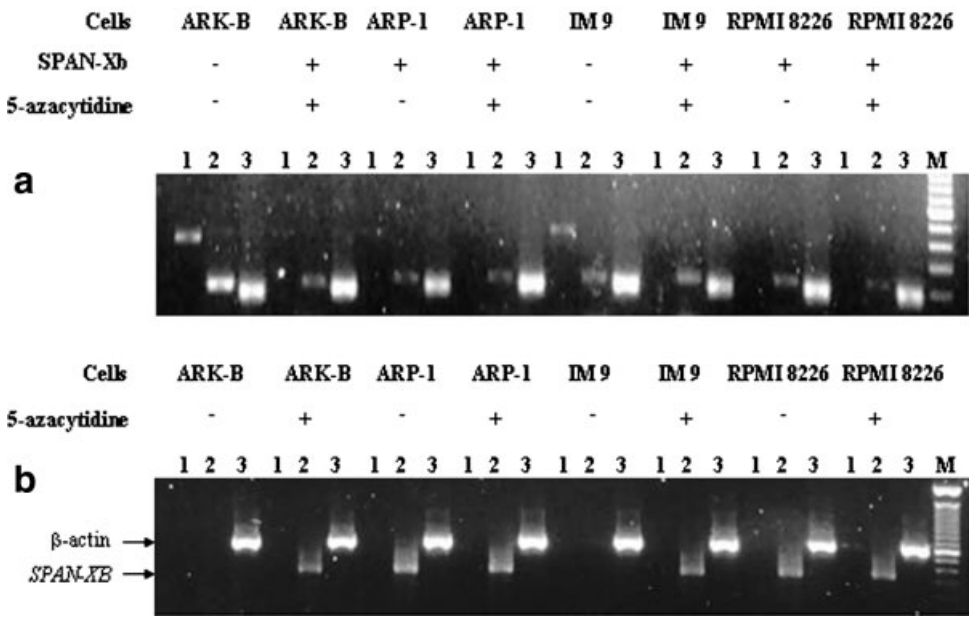

FIGURE 4 – Association between MeCP2 binding to *SPAN-XB* promoter and *SPAN-XB* expression in myeloma cell lines. Chromatin immunoprecipitation using antibodies directed at MeCP2 on nuclear preparations, followed by PCR amplification across the gene segments on the *SPAN-XB* promoter, showed (*a*) the association of the MeCP2 proteins with *SPAN-XB* promoters in myeloma cells that did not expressed SPAN-Xb and the dissociation of the MeCP2 from the *SPAN-XB* promoter after treatment of SPAN-Xb-negative cells with 5-azacytidine (M = molecular marker; Lane 1 = amplification for gene segment on the *SPAN-XB* promoter from position −310 to −104; Lane 2 = amplification for gene segment on the *SPAN-XB* promoter from position −225 to −104; Lane 3 = amplification for input DNA for the control gene, Clone 4). (*b*) Treatment of cells with 5-azacytidine also resulted in the expression of *SPAN-XB* in SPAN-Xb-negative myeloma cells (M = molecular marker; Lane 1 = PCR for *SPAN-XB* gene segment without RT; Lane 2 = RT-PCR for *SPAN-XB* gene segment; Lane 3 = RT-PCR for β-actin gene segment).

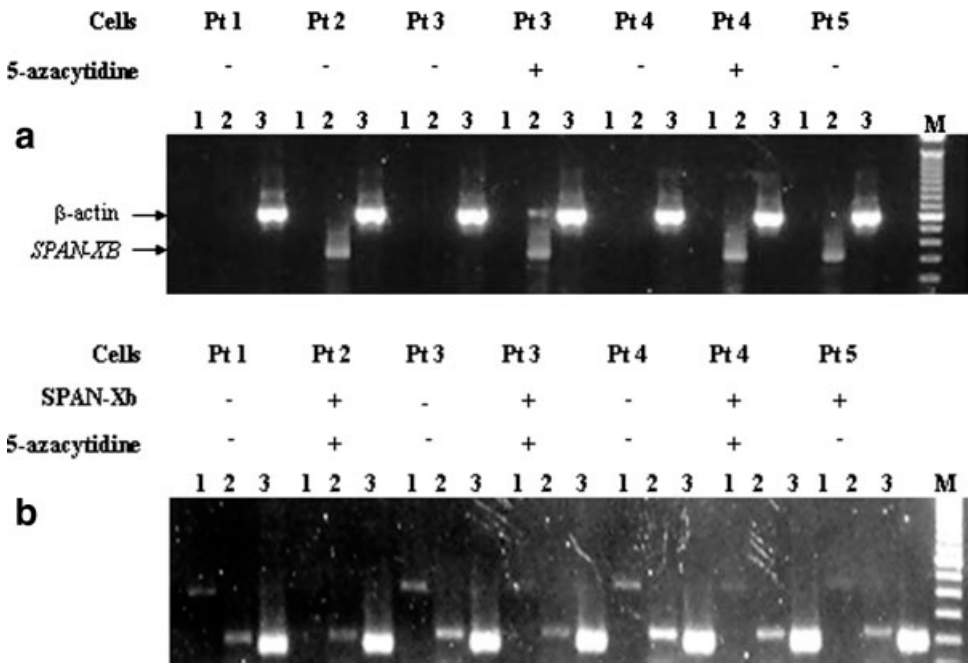

FIGURE 5 – Association between MeCP2 binding to *SPAN-XB* promoter and *SPAN-XB* expression in fresh CD138-enriched myeloma cells from 5 patients. (*a*) RT-PCR analysis of RNA derived from myeloma cells showed that Patients 2 and 5 were SPAN-Xb-positive and Patients 1, 3 and 4 were SPAN-Xb-negative (M = molecular marker; Lane 1 = PCR without RT; Lane 2 = RT-PCR for *SPAN-XB* gene segment; Lane 3 = RT-PCR for β-actin gene segment). (*b*) Chromatin immunoprecipitation using antibodies directed at MeCP2 on nuclear preparations, followed by PCR amplification across the gene segments on the *SPAN-XB* promoter, showing the association of the MeCP2 proteins with *SPAN-XB* promoters in myeloma cells that did not expressed SPAN-Xb and the dissociation of the MeCP2 from the *SPAN-XB* promoter after treatment of SPAN-Xb-negative cells with 5-azacytidine (M = molecular marker; Lane 1 = amplification for gene segment on the *SPAN-XB* promoter from position −310 to −104; Lane 2 = amplification for gene segment on the *SPAN-XB* promoter from position −225 to −104; Lane 3 = amplification for the control gene, Clone 4).

of the mutant promoters by CAT analysis. Mutation of these 4 CpG dinucleotides resulted in either increases or decreases of the promoter function when compared to the wild-type *SPAN-XB* promoter (Fig. 3*b*), suggesting their secondary regulatory function in the control of *SPAN-XB* gene expression. Mutation at the other CpG dinucleotides also altered the promoter function, indicating that, although DNA methylation at these CpG dinucleotides may not be involved in the regulation of gene expression, these CpG dinucleotide sequences also play important secondary regulatory role in *SPAN-XB* gene expression.

Association between MeCP2 protein binding to promoter sequence and SPAN-XB gene expression

We previously demonstrated by bisulfite conversion and sequence analysis the association between methylation at specific CpG dinucleotides with repression of *SPAN-XB* gene.[6] Here, we extended our study to determine whether or not the methylated cytosine binding protein (MeCP2) interacts with the *SPAN-XB* promoter gene in myeloma cells. Four myeloma cell lines were used: ARK-B, ARP-1, IM 9 and RPMI 8226 cells. Using the anti-MeCP2 antibodies in chromatin immunoprecipitation assay on nuclear extracts from these cells, we found that the PCR products across 2 DNA segments within the *SPAN-XB* promoter from the 2 cell lines that did not express SPAN-Xb (ARK-B and IM 9 cells) were consistently significantly much higher than those that expressed SPAN-Xb (ARP-1 and RPMI 8226 cells) (Fig. 4*a*), indicating the association of more MeCP2 protein with the promoter sequence in cells such as ARK-B and IM 9 that did not express *SPAN-XB* gene. Control DNA amplification for input DNA for the ubiquitous gene (Clone 4)[7] produced identical amount of PCR products. Treatment of myeloma cells that did not express SPAN-Xb with the hypomethylating agent, 5-azacytidine (2 μM), resulted in the dissociation of MeCP2 protein from the promoter sequence (Fig. 4*a*) and subsequent expression of *SPAN-XB* gene (Fig. 4*b*).

We next determined if the association between MeCP2 protein and repression of *SPAN-XB* also occurred in fresh CD138-enriched myeloma cells from 5 patients. We first determined SPAN-Xb expression by RT-PCR[2] and found that myeloma cells from 2 of the 5 patients expressed SPAN-Xb (Fig. 5*a*). MeCP2 protein was also found to be associated with the *SPAN-XB* promoter in myeloma cells that did not express SPAN-Xb (Fig. 5*b*). Treatment of 2 of the 3 SPAN-Xb-negative myeloma cells with 5-azacytidine resulted in SPAN-Xb expression (Fig. 5*a*) and the dissociation of the MeCP2 protein from the promoter sequence (Fig. 5*b*). These results, therefore, raise the likelihood that the MeCP2 protein physically interacts with the promoter sequence and modulates the silencing of *SPAN-XB* gene expression, in cooperation with histone deacetylase complex and other components.

Discussion

Most tumor antigens currently being studied are cellular molecules that are not essential for the neoplastic process or the survival of the tumor cells. As a result, heterogeneous expression of tumor antigens is commonly seen, even within individual tumor specimens. This poses one of the greatest obstacles to successful tumor immunotherapy. It is not uncommon to observe that successful tumor immunotherapy targeting a specific antigen is only to be followed by tumor relapse due to the emergence of antigen-negative variant tumor cells. Despite this, most attentions have focused on improving the delivery of the tumor antigens to antigen presenting cells to enhance immune responses. Very little has been focused on dissecting the mechanisms regulating antigen expression. We reasoned that identification of the mechanisms regulating the expression of the target genes may provide insights into approaches that upregulate target gene expression to circumvent antigen deficiency within individual tumor specimen and improve tumor cytotoxicity, reduce the opportunity for the development of antigen-negative variants and increase the applicability of the vaccine.

We previously identified SPAN-Xb as a novel CT antigen in hematologic malignancies, including MM.[2] However, the antigen was only expressed in around 30% of these patients, limiting the applicability of this antigen for tumor vaccine candidate. Further study indicated that promoter methylation was the primary regulatory mechanism for *SPAN-XB* gene expression and that SPAN-Xb expression could be upregulated by 5-azacytidine.[6] In the present study, we set out to further dissect the *SPAN-XB* promoter to determine the core sequence of the promoter and the roles of the specific CpG dinucleotides within the promoter sequence in providing secondary regulatory control of SPAN-Xb expression.

Using a panel of truncated promoter sequences, we first showed that the 73 bp at the 3′ end of the full-length promoter, from position −176 to position −104 of the translation start site, were able to function as a promoter as efficiently as the full-length promoter. Truncations involving shortening of the sequence from the 3′ end of the full length promoter all resulted in loss of promoter activity. We previously demonstrated that DNA methylation was the primary mechanism regulating *SPAN-XB* gene expression. We also identified 11 CpG dinucleotides within the full length promoter sequence. Since DNA methylation occurs at the CpG dinucleotides, we were, therefore, rather surprised the 73 nucleotides that exhibited full promoter function at the 3′ end of the full length promoter did not contain any CpG dinucleotide. On the basis of these results, we conclude that the core sequence of the promoter resides within these 73 nucleotides and that the regulatory element of the promoter resides in the sequences 5′ of the core sequence.

In addition to showing that DNA methylation was the primary regulatory mechanism for *SPAN-XB* gene expression, we also identified, by bisulfite conversion and sequence analysis, that hypomethylation at positions −310, −307, −299 and −221 strongly predicted for *SPAN-XB* expression, suggesting the involvement of these 4 CpG dinucleotides in the regulation of *SPAN-XB* gene expression through DNA methylation. In order to dissect DNA methylation from the roles specific CpG sequences play in the regulation of *SPAN-XB* gene expression, we carried out oligonucleotide-directed mutagenesis of these individual CpG nucleotides to generate a panel of mutant *SPAN-XB* full length promoters. Mutation of these CpG nucleotides further affected *SPAN-XB* promoter, either by enhancing or reducing its function when compared to the wild type full length *SPAN-XB* promoter. This occurred in the setting of demethylated promoter sequence, suggesting that, although promoter methylation is the primary regulatory mechanism of *SPAN-XB* promoter, specific CpG nucleotides exert secondary effects on the core sequence to modulate gene expression, although it is also possible that the other nucleotides within this regulatory element of the promoter sequence may also influence the promoter function.

Methyl-CPG-binding proteins (MBDs) are an essential part of the epigenetic machinery. They constitute a link between DNA methylation and histone modification in processes leading to stable repression of gene repression.[8] On the one hand, they bind both to methylated DNA and to DNA methyltransferases; on the other hand, they also bind directly to histone deacetylases.[9] Hence, MBDs can be viewed as a molecular bridge between 2 key epigenetic events. MeCP2 is one of the best characterized MBD, which can bind as little as a single methylated CG nucleotide, but higher number of methylated residues may increase the binding[10]; it associates with the Sin3A histone deacetylase complex and recruits the HDAC1 and HDAC2 to the promoter chromatin.[11,12] Histone deacetylation suppresses gene expression by removing the acetyl groups from histones, which leads compact and transcriptionally inactive chromatin.[13] Having demonstrated previously the association between promoter DNA hypomethylation and *SPAN-XB* gene expression, we therefore determined whether or not MeCP2 interacts with *SPAN-XB* promoter. Using the chromatin immunoprecipitation technique with antibodies directed at MeCP2, we showed the specific binding of MeCP2 to the promoter sequence, and this association correlates with SPAN-Xb repression. Treatment of myeloma cells that did not express SPAN-Xb with the hypomethy-

lating agent (5-azacytidine) resulted in the dissociation of the MeCP2 protein with the *SPAN-XB* promoter and subsequent *SPAN-XB* gene expression. Taking into account our previous finding[6] of the role of DNA methylation in gene regulation, these results suggest that silencing of the *SPAN-XB* gene in myeloma cells by DNA methylation may be mediated through MeCP2 interaction with these CpG dinucleotides within the promoter sequence. Obviously, gene repression *via* MeCP2 independent of these CpG dinucleotides may also occur, since chromatin compaction and gene repression can occur in the absence of DNA methylation.[14] If MeCP2 association with the *SPAN-XB* promoter results in gene repression, it may be possible to use DNA methylation and HDAC inhibitors in myeloma, not only to induce tumor cell apoptosis[15–17] but also to upregulate *SPAN-XB* gene expression to enhance the efficacy of a SPAN-Xb-based tumor vaccine.

In conclusion, we have demonstrated in the present study that *SPAN-XB* promoter consists of a core sequence and a regulatory element; the core element resides within the 73 bp at the 3′ end of the full length promoter. *SPAN-XB* gene expression by the core sequence is regulated in myeloma cells by specific CpG nucleotides and associated with MeCP2 binding to the promoter sequence.

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