Neoplasia • Vol. 7, No. 12, December 2005, pp. 1091–1103

www.neoplasia.com

Uroplakin Ib Gene Transcription in Urothelial Tumor Cells Is Regulated by CpG Methylation[1]

Prue Cowled, Irene Kanter*, Lefta Leonardos* and Paul Jackson*[†]

*Department of Surgery, The University of Adelaide, The Queen Elizabeth Hospital, Woodville, South Australia, Australia; [†]Oncology Research Centre, Prince of Wales Hospital, Randwick, NSW, Australia and Department of Medicine, University of New South Wales, Australia

Abstract

Uroplakin Ib is a structural protein on the surface of urothelial cells. Levels of uroplakin Ib mRNA are dramatically reduced or absent in many transitional cell carcinomas, but the molecular mechanisms responsible remain undetermined. Previously, we showed that loss of uroplakin Ib expression correlated with CpG methylation of Sp1/NFκB–binding motifs within the proximal promoter. In this study, we show that reporter activity was completely blocked by the methylation of three CpG pairs in this promoter region. Gel shift analysis using purified proteins or nuclear extracts showed that Sp1 and NFκB bound to motifs encompassing two of the three CpG pairs. Interestingly, the methylation of these two CpG sites did not prevent the binding of proteins to the promoter in gel shift analyses. Additionally, mutation of these two CpGs did not affect reporter activity, but mutation of 6-bp fragment spanning each CpG partially inhibited reporter activity, suggesting that these sites were functional. A requirement for both Sp1 and NFκB in regulating reporter activity was confirmed in transfection experiments using plasmids expressing individual proteins. Our data suggest that the methylation of specific CpG sites can silence the uroplakin Ib promoter, at least in part, by blocking the binding of Sp1 and NFκB, although other factors may be involved.

Neoplasia (2005) **7,** 1091–1103

Keywords: Methylation, transcriptional regulation, uroplakin Ib, Sp1, NFκB.

Introduction

Uroplakin Ib belongs to the tetraspanin family of membrane proteins [1–3] and forms plaque complexes with uroplakins Ia, II, and III along the asymmetric unit membrane of umbrella cells of the mammalian urothelium [4–6]. Formation of these plaques by specific interactions between the four uroplakins provides the luminal surface of the bladder with strength and flexibility, as well as an impermeable barrier to prevent urine invasion of underlying tissues. Uroplakin Ib is also required for the export of uroplakin III from the Golgi apparatus to form mature plaques on the apical surface of urothelial cells [7]. Because complexes of uroplakins Ib and III are essential for assembly of plaques on the mature fully differentiated urothelium, these data support the hypothesis that uroplakin Ib has a key functional role in driving the final stages of urothelial differentiation. Although initially considered to be entirely urothelium-specific, recent reports have suggested that uroplakin Ib mRNA is also expressed in the cornea and conjunctival epithelium [8], trachea, placenta, pancreas, and kidney [9] (UniGene Hs.2715 Bladder, Brain, Eye, Kidney, Lung, Muscle, Ovary, Pancreas, Placenta, Soft Tissue, Stomach, Tongue, and Uterus). However, given that a definitive biologic function for uroplakin Ib is yet to be determined, the importance of these observations is not clear.

Other tetraspanin proteins, including CD63, Co-029, and the leukocyte antigens CD9, CD53, CD37, CD82/KAI1, and Tapa-1, have been implicated in the growth regulation and activation of a wide range of cells, and it has been suggested that they represent a family of signal transduction molecules and adhesion- or motility-related receptors. Recent data have also stressed the importance of interactions between tetraspanin proteins and integrins for adhesion and signal transduction (reviewed in Refs. [10,11]). At least four tetraspanins (CD82/KAI1, KITENIN, CD63, and CD9) have roles in tumor progression. Loss of KAI1 expression is strongly correlated with advanced disease in many different cancer types, including bladder cancer [12], recurrence following initial treatment [13], and poor outcome [14]. Experimental studies have shown that overexpression of KAI1/CD82 in KAI1-negative colon, breast, and prostate cancer cell lines alters cell–cell and cell–matrix adhesion, and suppresses *in vitro* invasiveness and *in vivo* metastasis [15–17]. Similar results have been obtained for CD9 [18], and overexpression of CD63 in CD63-negative melanoma cells inhibited the growth and metastasis of transplanted tumors in nude mice [19], although more recent data have suggested that interpretation of data concerning the suppressive effect of CD63 may need to be treated with caution [20]. However,

Address all correspondence to: Prue Cowled, Department of Surgery, The University of Adelaide, The Queen Elizabeth Hospital, Woodville Road, Woodville, South Australia 5011, Australia. E-mail: prue.cowled@adelaide.edu.au

[1]This study was funded by a research grant from the Cancer Council of South Australia.
Received 20 May 2005; Revised 26 September 2005; Accepted 26 September 2005.

DOI 10.1593/neo.05364

molecular mechanisms leading to loss of tetraspanin expression in advanced cancer remain largely uncharacterized.

In a recent study [21], we provided evidence that methylation of a CpG island spanning the proximal promoter of the human *uroplakin Ib* gene was closely correlated with lack of uroplakin Ib mRNA expression in both clinical samples of transitional cell carcinoma (TCC) of the bladder and in TCC cell lines. Reactivation of uroplakin Ib mRNA expression in uroplakin Ib–negative TCC cell lines by 5-aza-2′-deoxycytidine further supported a regulatory role for methylation in gene transcription. Our preliminary data also indicated that loss of uroplakin Ib expression was correlated with methylation of CpG residues located within specific Sp1/NFκB– and Sp1-binding motifs in the core uroplakin Ib promoter, suggesting that these motifs played a key role in regulating transcription. In the current study, we further explored the importance of these binding motifs to the expression of the *uroplakin Ib* gene, and we have shown that Sp1 and NFκB were key determinants of uroplakin Ib transcription. Our data provide additional evidence that methylation of specific CpG residues silenced the *uroplakin Ib* gene during bladder cancer progression.

Materials and Methods

Cell Lines

The TCCSuP, J82, and T24 cell lines derived from TCCs and the colorectal carcinoma cell line SW480 were all obtained from ATCC (Rockville, MD). The colorectal carcinoma cell line LIM1215 was kindly supplied by Dr. R. Whitehead (Ludwig Institute for Cancer Research, Melbourne, Victoria, Australia). Dr. D. Leavesley (Royal Adelaide Hospital, Adelaide, South Australia, Australia) kindly provided the 5637 cell line. The HT1376, VM-Cub1, and VM-Cub3 cell lines were provided by Dr. Marc-Oliver Grimm (Department of Urology, Heinrich-Heine University, Dusseldorf, Germany). RT112 cells were obtained from Professor Pamela Russell (Oncology Research Centre, Prince of Wales Hospital, Sydney, Australia). Breast cancer cell lines (MDA-MB-231, MCF7, and MCF10A) were obtained from Dr. Sally Stephenson (Department of Haematology–Oncology, The Queen Elizabeth Hospital, Woodville, South Australia, Australia). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; pH 7.4) supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2.

Expression of Uroplakin Ib mRNA by Semiquantitative Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

RNA was isolated from cell lines using Trizol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. RT-PCR amplification of a 741-bp uroplakin Ib cDNA product was carried out as previously described [21]. To verify RNA quality and to ensure equal loading of cDNA into the reactions, levels of GAPDH were also analyzed. GAPDH was not amplified from the RNA template in the absence of reverse transcriptase (data not shown), demonstrating that neither genomic sequence nor the *GAPDH* pseudogene was amplified under these PCR conditions. Reaction products were separated on a 1.5% agarose gel at 100 V and then viewed using ethidium bromide.

Electromobility Shift Assay (EMSA)

Nuclear extracts from HT1376, RT112, and SW1710 cells were prepared by the Pierce NE-Per system (Pierce Endogen, Rockford, IL), as described by the manufacturer. Protein concentrations were determined by BCA assay (Pierce Endogen) before extracts were aliquoted and stored at −80°C. Single-stranded, complementary 25-mer oligonucleotides encompassing Sp1/NFκB–binding motifs and including CpG3 and CpG4 within the uroplakin Ib promoter [21] (see Figure 1*A* and Table 1), and oligonucleotides containing consensus-binding motifs for Sp1 and NFκB were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Oligonucleotides containing 5-Me cytosine at CpG3 and/or CpG4 were purchased from Geneworks (Adelaide, South Australia, Australia). All complementary oligonucleotides were annealed to form double-stranded probes. A double-stranded oligonucleotide containing a consensus AP1-binding motif was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Radioactive probes were generated by labeling a single-stranded promoter oligonucleotide with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ^{32}P]dATP (Geneworks), annealing the complementary oligonucleotide, and purifying through a Sephadex G25 Quickspin column (Roche Applied Science, Indianapolis, IN). Binding assays were performed for 10 minutes on ice using 6 μl of nuclear extract (6–10 μg of total protein, except for HT1376, which is 20 μg) in 30-μl reactions containing a binding buffer (10 mM HEPES, pH 7.8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, and 5% glycerol), 1 mM DTT, 0.25 μg of poly[dI:dC], and 0.2 μl of ^{32}P-labeled probe (13 fmol; >30,000 cpm/μl). In competition experiments, a cold competitor (×100 molar excess) was included with the nuclear extract for 10 minutes prior to addition of labeled probe. All gels were dried and examined by Phosphoimage analysis.

In experiments using purified transcription factors, recombinant human Sp1 and human NFκB (p50) were obtained from Promega (Madison, WI). In each experiment, 900 ng of Sp1 or 2.2 μl of p50 was used. Reactions were performed as described above, except that competitors were used at ×100 to ×300 molar excess.

Cloning of the Uroplakin Ib Reporter Construct

A 246-bp fragment (Figure 1*A*; −152 to +94) [22] spanning the uroplakin Ib proximal promoter and exon 1 was cloned into the pGL3-Basic reporter plasmid (Promega), which contained a *firefly luciferase* reporter gene (Figure 1*B*). This fragment of the uroplakin Ib promoter has previously been shown to be sufficient for the transcription of a reporter gene [22].

The uroplakin Ib promoter fragment was amplified from 50 ng of genomic DNA isolated from the peripheral blood of a normal volunteer, using 0.5 μM of each primer (sequences are given in Table 2), 2 mM $MgCl_2$, 200 μM dNTPs, and 1.25 U of High-Fidelity PfuUltra Taq Polymerase (Stratagene, La Jolla, CA) in a total volume of 50 μl. Amplification was achieved in

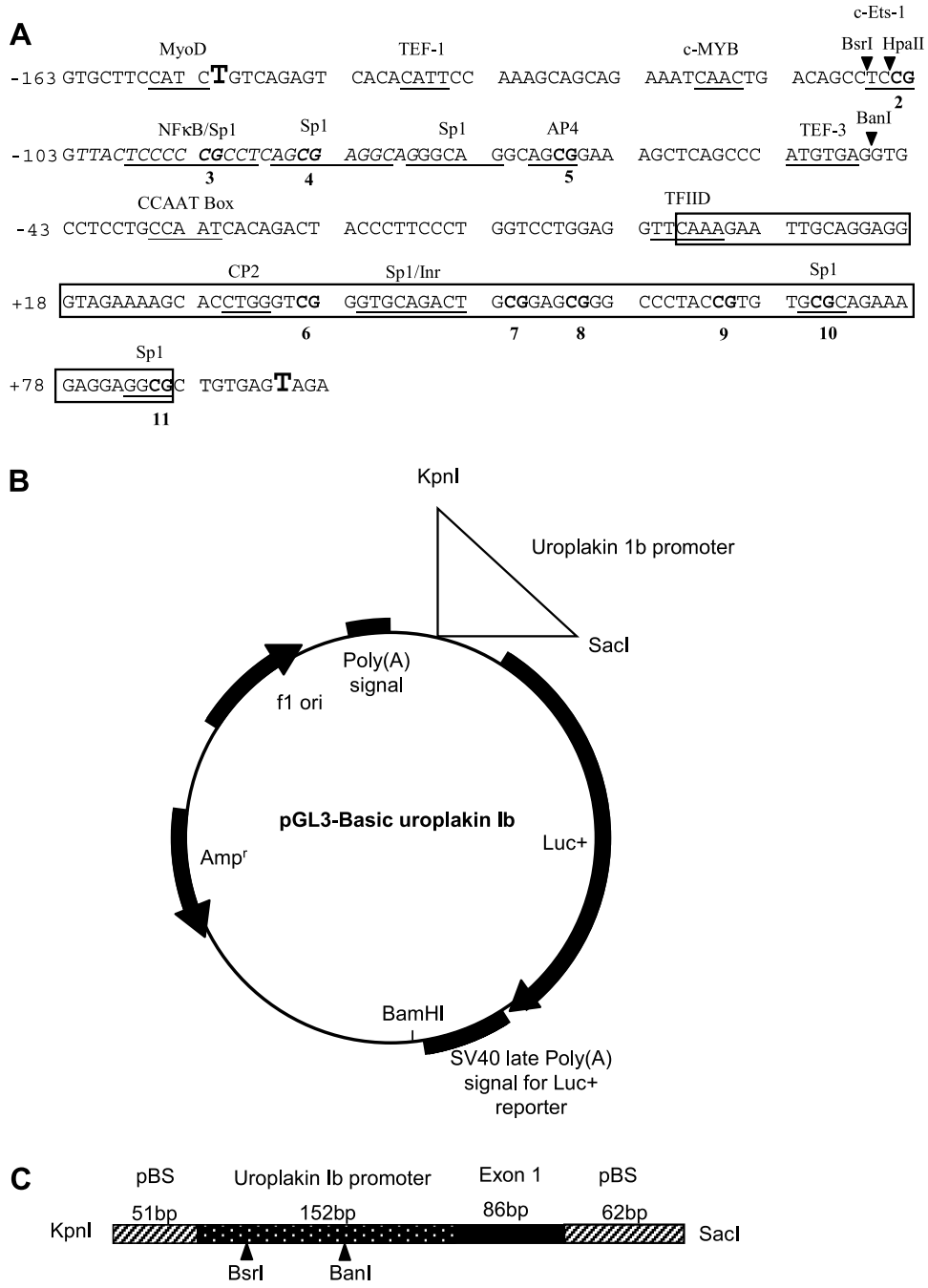

Figure 1. *Structure and functional features of the proximal promoter region and exon 1 of the* uroplakin Ib *gene. (A) The CpG pairs are marked in bold type and numbered 2 to 11, with the numbering shown below the sequence [21]. The Ts in bold and larger type represent the beginning and end of the primer sequences used to amplify and clone the promoter fragment for subsequent cloning in the reporter vectors and thus define the fragment of the promoter and exon 1 examined in these studies. The sequence in italics defines the location of the oligonucleotides used in EMSA experiments. Exon 1, as defined in Ref. [22], is boxed. The underlined sequences represent core sequences for putative transcription factor–binding sites, as determined by transcription factor prediction programs TESS and MatInspector [21]. A potential CCAAT box is also marked. The locations of restriction sites used to excise fragments for* in vitro *methylation and cloning are also marked. (B) Diagram of the uroplakin Ib pGL3-Basic construct used for luciferase reporter assays. (C) Structure of the promoter insert cloned into pGL3-Basic for reporter assays. PBS represents short sequences derived from pBlueScript II KS(+). The locations of restriction sites used to excise fragments for* in vitro *methylation and cloning are marked.*

an Eppendorf Mastercycler using the following protocol: 94°C for 5 minutes; then 34 cycles of 94°C for 1 minute, 61°C for 1 minute, and 72°C for 3 minutes; followed by a final extension of 72°C for 5 minutes.

PCR DNA product was electrophoresed through a low-melting-point agarose gel, excised, and then purified using a Qiaquick gel extraction kit (Qiagen, Clifton Hills, Victoria, Australia), according to the manufacturer's instructions. The purified fragment was then TA-cloned into the plasmid vector pGEM-T Easy (Promega). Because the restriction enzyme recognition sites in pGEM-T Easy were not compatible with those in the multiple cloning site in pGL3-Basic, the promoter fragment was first subcloned into pBlueScript II KS(+) (Stratagene) to introduce appropriate restriction sites for the

Table 1. Oligonucleotide Sequences Used for EMSA Experiments.

Wild-type uroplakin Ib promoter	TTACTCCCCCGCCTCAGCGAGGCAG
Mutation 1	TTACTCCCC**TT**CCTCAGCGAGGCAG
Mutation 2	TTACTCCCCCGCCTCAG**TT**AGGCAG
Mutation 3	TTACTCCCC**TT**CCTCAG**TT**AGGCAG
5Me-CpG3	TTACTCCCC(5-Me)CGCCTCAGCGAGGCAG
5Me-CpG4	TTACTCCCCCGCCTCAG(5-Me)CGAGGCAG
5Me-CpG3 and 5Me-CpG4	TTACTCCCC(5-Me)CGCCTCAG(5-Me)CGAGGCAG

The 5′–3′ sequences are shown. CpG residues 3 and 4, respectively, are underlined, and mutated residues are in bold.

final step of the cloning. The fragment was excised from pGEM-T Easy by *Eco*RI (New England Biolabs) digestion then cloned into pBlueScript II KS(+) at the *Eco*RI site before being excised from this construct using *Kpn*I and *Sac*I. This fragment was finally cloned into *Sac*I/*Kpn*I–digested pGL3-Basic (Figure 1*C*). Correct insertion of the promoter fragment into the reporter and verification of the correct promoter sequence were ensured by direct sequencing; its structure is shown in Figure 1*B*.

Transient Transfections

Transfections were performed using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. To control for transfection efficiency, the pSV2CAT plasmid, which constitutively expresses high levels of chloramphenicol acetyl transferase (CAT), was cotransfected into cells with a reporter construct. Briefly, 4 μg of total plasmid was diluted into 250 μl of DMEM; 5 μl of Lipofectamine 2000 reagent diluted in 250 μl of DMEM was added; and the mixture incubated for 20 minutes. The transfection mixture was then added to the wells of a six-well plate containing nearly confluent cells and then incubated for 24 hours. Protein lysates were prepared using a lysis buffer from the CAT ELISA kit (Roche Applied Sciences), according to the manufacturer's instructions. Analysis of luciferase and CAT activity was performed as described below.

Luciferase and CAT Reporter Assays

Cells were lysed and uroplakin Ib promoter activity was assessed using the Luciferase assay system (Promega). Cell lysate (50 μl) was assayed and luminescence was measured in an AutoLumat Plus LB 953 Luminometer (Berthold Technologies, Bad Wildbad, Germany). The results were normalized to protein concentration, as measured by Bradford assay using Protein Assay Reagent (BioRad, Hercules, CA).

CAT activity in cell lysates was assayed by ELISA using a CAT ELISA kit (Roche Molecular Biochemicals), according to the manufacturer's instructions. The absorbance was measured at 405 nm using a Microplate reader (model 680; BioRad).

In Vitro *Methylation of Uroplakin Ib Promoter Sequences Using Sss1 Methylase*

The uroplakin Ib promoter fragment was excised from within the pGL3-Basic/uroplakin Ib reporter construct by *Sac*I and *Kpn*I digestion, purified, and then treated with *Sss1* methylase (2 U enzyme/10 μg DNA) to methylate all 10 CpG sites. To confirm methylation, an aliquot of the methylated promoter fragment was incubated with the methylation-sensitive restriction enzyme *Hpa*II (New England Biolabs), which cleaves the fragment into two, only if the CpG sites are unmethylated (the location of the *Hpa*II site is shown in Figure 1*A*).

The fully methylated uroplakin Ib promoter fragment was then added to the *Kpn*I/*Sac*I–linearized pGL3-Basic plasmid and religated by overnight incubation with T4 DNA ligase. The ligation mixture was then transfected directly into RT112 and HT1376 cells (which both express endogenous uroplakin Ib mRNA) and T24 and VMCub3 cells (which lack uroplakin Ib expression), along with pSV2CAT. Luciferase and CAT activities were then assayed 24 hours after transfection. To control for possible bias introduced by the cloning process on transcriptional activation, a promoter fragment was processed in parallel with the methylated promoter fragment above, with the single exception that *Sss1* methylase was omitted. This fragment was then ligated into pGL3-Basic and transfected as above.

Table 2. Sequences of PCR Primers.

Uroplakin Ib RT-PCR	TGTTCGTTGCTTCCAGGGCCTGC AGTAGAACATGGTACCCAGGAGAACC
GAPDH control	CCACCCATGGCAAATTCCATGGCA TCTAGACGCAGGTCAGGTCCACC
Cloning of uroplakin Ib promoter fragment	TGTCAGAGTCACACATTCCAAAG ACTCACAGCGCCTCCTCTTTC
Plasmid-specific primers for sequencing and PCR	CTAGCAAAATAGGCTGTCCCC CTTTATGTTTTTGGCGTCTTCCA
Mutagenesis cloning	
Plasmid-specific primers	CTAGCAAAATAGGCTGTCCCCAGTGCAAGTGCAGG
	CTTTATGTTTTTGGCGTCTTCCATGGTGGCTTTAC
(a) CG to TA	GAAATCAACTGACAGCCTC**TA**GTTACTCCCCTACCTCAGC
CpG2	CGCTGAGGATGGGGAGTAATAGGAGGCTGTCA
CpG3 and CpG4	CCTCCGGTTACTCCCC**TA**CCTCAG**TA**AGGCAGGGCAGGCAGCG
	CGCTGCCTGCCCTGCCTTACTGAGGTAGGGGAGTAACCGGAGG
(b) 6-bp mutations	CCTCCGGTTACTCCTT**TA**TTTCAGCGAGGCAGGGCAGGCAGCG
CpG3	CGCTGCCTGCCCTGCCTCGCTGAAATAAAGGAGTAACCGGAGG
CpG4	CCTCCGGTTACTCCCCCGCCTCTT**TA**TTGCAGGGCAGGCAGCG
	CGCTGCCTGCCCTGCAATAAAGAGGCGGGGGAGTAACCGGAGG

The underlined sequences represent the mutated sequences, and the residues in bold mark the location of CGs.

To determine if methylation of CpG3 and CpG4 specifically played a role in regulating the transcription of the uroplakin Ib promoter, a smaller 61-bp fragment of the promoter spanning both these sites (as well as CpG5) was excised from the uroplakin Ib *Kpn*I/*Sac*I promoter fragment by digesting with *Bsr*I and *Ban*I restriction enzymes (locations of the restriction sites are shown in Figure 1*A*). The *Bsr*I recognition site was first created by site-directed mutagenesis at CpG2 in the normal promoter sequence, which changed the second C to A, thereby introducing the required *Bsr*I site (Figure 1*A*). This 61-bp fragment was then methylated *in vitro* with *Sss1* methylase as above and religated firstly with the other two promoter fragments and then into pGL3-Basic, linearized with *Sac*I and *Kpn*I. The ligation mixture was then transfected directly into cell lines as above. A control nonmethylated reporter construct was also prepared exactly as described above, but with the omission of *Sss1* methylase.

Site-Directed Mutagenesis

To ascertain the functional importance of Sp1- and NFκB-binding sites within the 246-bp uroplakin Ib promoter fragment, the sequence was mutated at CpG3 and CpG4, which span the consensus-binding sites for these transcription factors. These sites were mutated either individually or together, using a PCR-based site-directed mutagenesis approach. The pGL3-Basic/uroplakin Ib construct was used as a template in PCRs using primers carrying the desired mutation, thus inactivating the Sp1- and/or NFκB-binding site. Mutagenesis reactions were carried out to introduce either the short (CG to TA) or the longer (six bases around the CpG site) mutation. Sequences of primers are given in Table 2 and show the location of mutated bases. PCR conditions included 15 mM $MgCl_2$ and primer concentrations of 2 ng/μl. Amplification was achieved with the proofreading Taq Polymerase PfuTurbo (Stratagene) and with conditions of 95°C for 15 minutes, followed by 30 cycles of 96°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 7 minutes. Three reactions were performed to obtain the full-length mutated product, with the first PCR amplifying the sequence upstream to the mutation and with a second reaction amplifying the sequence downstream of the mutation. A final PCR was then carried out using outer primers only and the products of the two previous reactions as templates. After amplification, the product was digested with *Sac*I and *Kpn*I and electrophoresed through low-melting-point agarose; the band of appropriate size (251 bp) was excised and purified using the Qiaquick gel purification kit (Qiagen), before religation into *Sac*I/*Kpn*I–digested pGL3-Basic plasmid. Direct sequencing was performed to confirm the successful introduction of desired mutations.

Results

Transcriptional Activity of the Uroplakin Ib Reporter Construct in Bladder Cancer and Non-bladder Cell Lines

We have previously reported that methylation of the uroplakin Ib promoter in TCC cell lines and in clinical samples of TCC is associated with silencing of uroplakin Ib expression, and that this silencing correlates with the methylation of two specific CpG residues (CpG3 and CpG4) within the proximal region of the uroplakin Ib promoter [21] (Figure 1*A*). To obtain more direct evidence for the importance of proximal promoter methylation in the control of uroplakin Ib expression, we initially generated a reporter plasmid (pGL-Basic/uroplakin Ib) containing a 241-bp fragment (residues −152 to +94, including CpG3 and CpG4) spanning the proximal promoter, exon 1, and part of intron 1 (Figure 1, *A*–*C*). A similar-sized fragment of the uroplakin Ib promoter has previously been shown to support transcription in a variety of cell types after transient transfection [22]. Our uroplakin Ib reporter construct was transfected into several bladder cancer cell lines, which we have previously shown [21] to normally express clearly detectable uroplakin Ib mRNA (RT112 and HT1376; mRNA levels in RT112 > HT1376) or which do not have detectable uroplakin Ib mRNA (T24, J82, VMCub3, 5637, and TCCSup) and whose levels of uroplakin Ib mRNA reflected the methylation status of the uroplakin promoter (summarized in Figure 2*A*). Transfection results presented in Figure 2*A* clearly show that uroplakin Ib reporter activity reflected endogenous uroplakin Ib expression. Reporter activities were highest in RT112 and HT1376, with activity in RT112 that is almost five-fold higher than in HT1376. Reporter activities in cell lines that do not normally express detectable uroplakin Ib mRNA were at least four-fold lower than in HT1376, suggesting that this 241-bp promoter fragment contains sequences important for the determination of endogenous uroplakin Ib mRNA levels. This was confirmed by examining reporter activity in a series of non-urothelial cell lines (Figure 2*B*). These cell lines did not express endogenous uroplakin Ib mRNA (summarized in Figure 2*B*) and induced only very low levels of luciferase activity (2- to 10-fold lower than in HT1376; Figure 2*B*).

In Vitro *Methylation of the Uroplakin Ib Promoter Silences Transcription*

To assess the effects of methylation on the activity of the uroplakin Ib reporter, in preliminary experiments, the entire pGL3-Basic/uroplakin Ib reporter plasmid was methylated *in vitro* with *Sss1* methylase and then transfected into RT112, HT1376, or T24 cells. Although methylation by this method completely silenced luciferase activity, a similar result was obtained using a methylated pGL3-Control vector, which constitutively expressed luciferase from an SV40 promoter (data not shown), suggesting that methylation of nonspecific CpG sites within the plasmid itself was affecting transcriptional activity. To overcome this problem, an alternative approach was used [23], whereby the 241-bp uroplakin Ib promoter fragment was excised from pGL3-Basic/uroplakin Ib by *Sac*I and *Kpn*I digestion, and then methylated *in vitro* using *Sss1* methylase. This reaction resulted in the methylation of only the 10 CpG pairs within the promoter fragment. This methylated promoter was then incubated with *Sac*I/*Kpn*I–linearized pGL3-Basic/uroplakin Ib and T4 ligase, and the entire ligation mixture was transfected directly into cell lines described above. As control, cells were also transfected with a ligation mixture containing an unmethylated promoter fragment that was prepared in parallel, but without

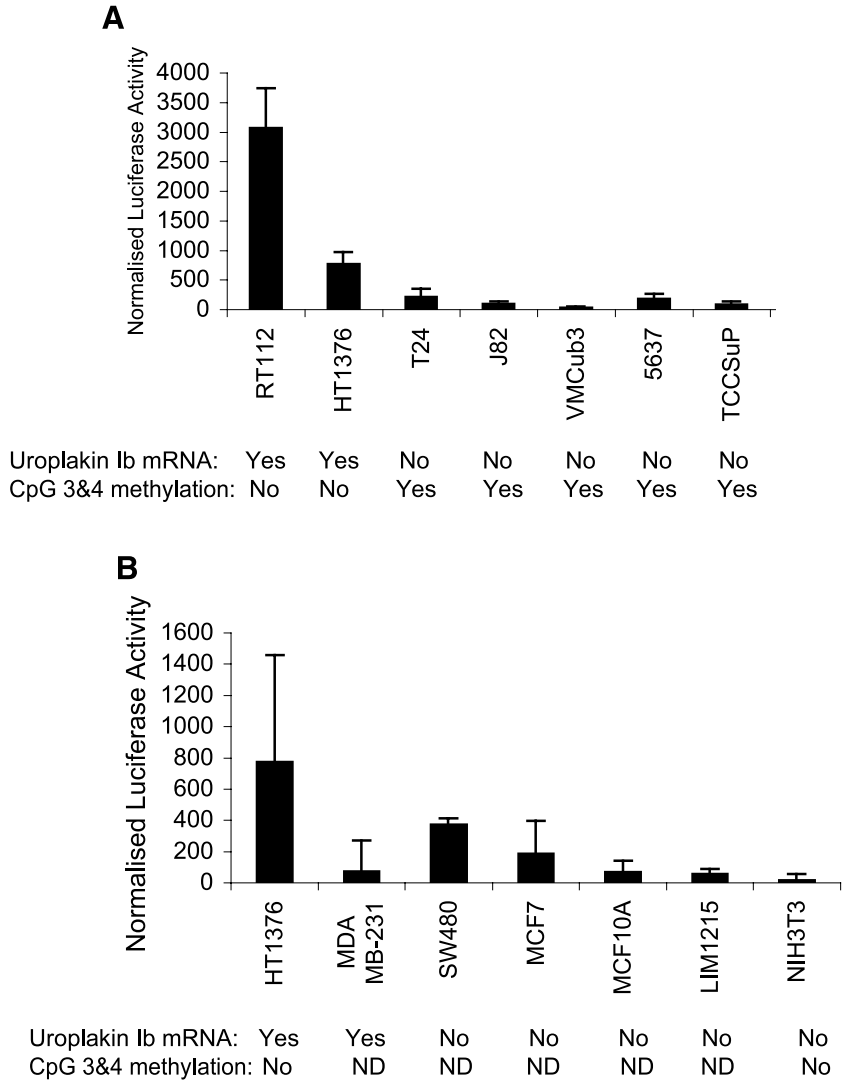

Figure 2. *Luciferase activity in a range of cell lines demonstrating a close correlation between levels of transcriptional activity and expression of uroplakin Ib mRNA. Luciferase reporter activity was normalized against the expression of CAT and total protein, as determined by Bradford assay. Results are expressed as means of three independent experiments ± SEM. (A) Uroplakin Ib reporter activity was assayed in seven TCC lines correlated with endogenous expression of uroplakin Ib, as determined by qualitative RT-PCR. The endogenous methylation status of CpG3 and CpG4, as reported in our previous study [21], also correlated with the expression of uroplakin Ib mRNA and high levels of reporter activity. (B) Urothelial specificity of the expression of the uroplakin Ib reporter construct in TCC lines RT112 and HT1376 and in five non-urothelial cancer cell lines. The highest levels of expression were detected in the urothelial cells, and all other cell lines showed minimal reporter activity. The methylation status of CpG3 and CpG4 in cell lines HT1376 and NIH3T3 [21] is also shown. ND, not determined.*

Sss1 methylase treatment. All cells were harvested 24 hours after transfection and luciferase activity was determined. Results summarized in Figure 3*A* clearly show that methylation of the promoter fragment dramatically inhibited luciferase reporter activity by >99% in RT112 cells and by 97% in HT1376 cells. In addition, the very low level of reporter activity obtained from T24 cells was also inhibited by approximately 92% following promoter methylation.

To more directly assess the importance of specific CpG residues, a short 61-bp fragment encompassing CpG3 to CpG5 (Figure 1*A*) was methylated *in vitro*, religated into pGL3-Basic/uroplakin Ib, and transfected into cells, as described above. Results presented in Figure 3*B* show that methylation of the promoter fragment containing only three CpG sites was as effective as methylation of the full promoter fragment in inhibiting luciferase activity in RT112 and HT1376 cells. However, in contrast to the inhibition of reporter activity observed when the complete promoter was methylated, there was little effect on very low levels of reporter activity in T24 cells. Taken together, these data suggested that CpG3, CpG4, and CpG5 are important for methylation-mediated silencing of high levels of transcription and provided experimental support to our previous study, which indicated the importance of CpG3 and CpG4 in regulating transcription [21].

Transcription Factor Binding to the Core Region of the Uroplakin Ib Promoter

Because methylation of CpG pairs 3, 4, and 5 correlated with loss of endogenous uroplakin Ib mRNA expression [21] and because *in vitro* methylation of CpG3, CpG4, and CpG5 similarly inhibited activity from the uroplakin Ib promoter in a transient transfection assay system, we investigated which transcription factors might have binding sites affected by

methylation of these CpG residues. The region of the uroplakin Ib promoter chosen for initial examination contains potential binding motifs for Sp1 and NFκB (Figure 1*A*) that surround CpG3 and CpG4. A potential binding motif for AP4 encompasses CpG5, but this sequence is not present in the oligonucleotide probe under analysis. Gel shift analysis with a radiolabeled oligonucleotide probe encompassing promoter residues −150 to −126 (Table 1) was used to first examine if purified recombinant human Sp1 and NFκB (p50) were capable of binding. Several complexes formed with rhSp1 (Figure 4*A*, *lane 2*), and specificity was confirmed by competition with an excess cold consensus Sp1-binding motif, but not by a consensus AP1-binding motif (Figure 4*A*, *lanes 3–5*). A single shifted band was observed using rp50 (Figure 4*B*, *lane 2*). Interestingly, formation of this complex was only weakly competed by a ×300 molar excess cold competitor (Figure 4*B*, *lane 3*), consistent with technical comments from the manufacturer's instruction manual (Promega Technical Bulletin TB110). However, there was full competition by a consensus p50-binding motif and no competition by an AP1 motif (Figure 4*B*, *lanes 4* and *5*). These data suggested that Sp1 and NFκB (p50) were capable of binding a region of the uroplakin Ib promoter encompassing CpG pairs 3 and 4. To verify that the CpG sites bound relevant transcription factors, EMSA was also carried out using rhSp1 and rhp50 and with oligonucleotides containing mutations at the CpG sites (sequences in Table 1). However, mutation of the CpGs did not affect factor binding (data not shown).

To examine complexes formed in cells, nuclear extracts from HT1376 and RT112 were analyzed because they both expressed high levels of endogenous uroplakin Ib mRNA. Three major complexes (A, B, and C) were detected in HT1376 extract (Figure 5*A*, *lane 2*). Formation of all complexes was dramatically reduced by competition with excess unlabeled probe (Figure 5*A*, *lane 3*). The involvement of Sp1 and NFκB was tested by competition with an excess of unlabeled oligonucleotide carrying consensus-binding motifs for either factor or a consensus motif for AP1. All complexes strongly competed with the consensus Sp1 motif, but not with the consensus AP1 motif (Figure 5*A*, *lanes 4* and *6*). In contrast, the consensus NFκB motif had no effect on any of the complexes (Figure 5*A*, *lane 5*). In RT112 extracts, two major

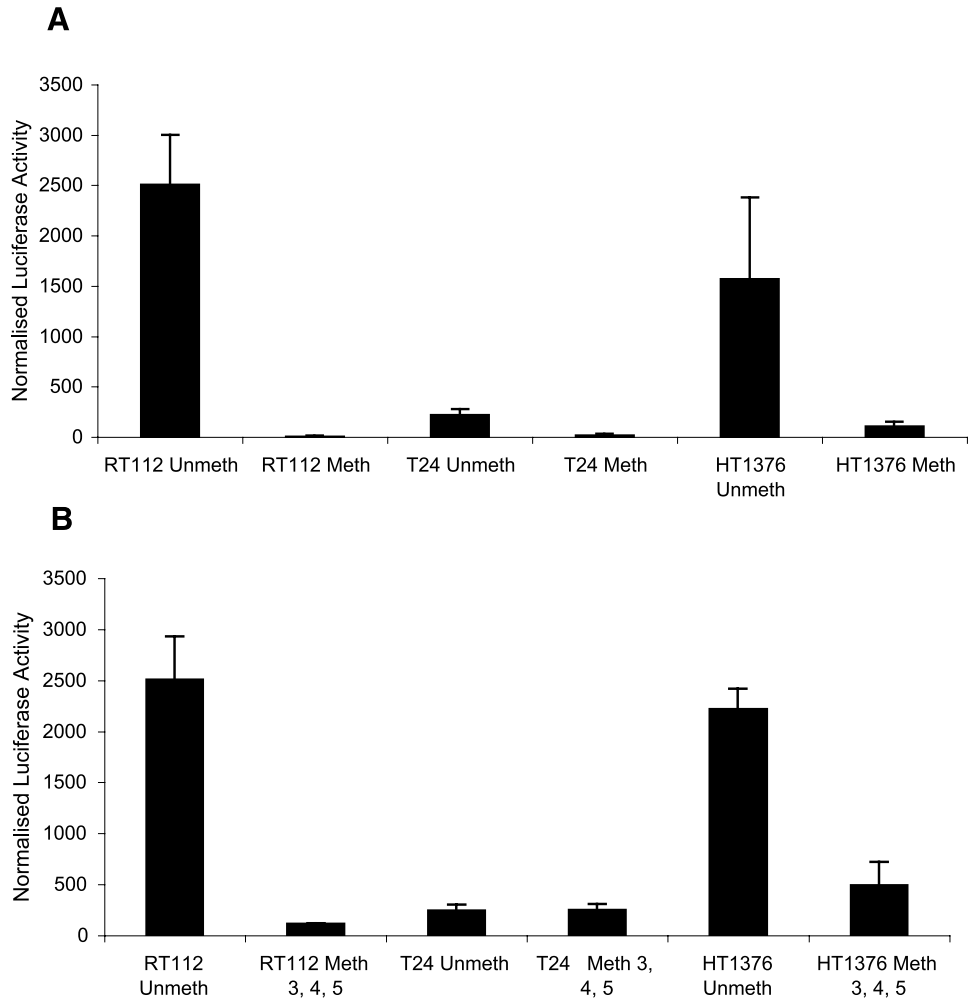

Figure 3. In vitro *promoter methylation silences transcriptional activity of the uroplakin Ib construct. (A) All 10 CpG sites in the uroplakin Ib promoter fragment were methylated* in vitro *using Sss1 methylase before ligation into pGL3-Basic. Luciferase reporter activity was almost completely abolished in all cell lines following transient transfection with the methylated construct. (B) A short fragment of the uroplakin Ib promoter containing only CpG3, CpG4, and CpG5 was methylated* in vitro. *Luciferase reporter activity was similarly inhibited in cells following transfection with the methylated construct.*

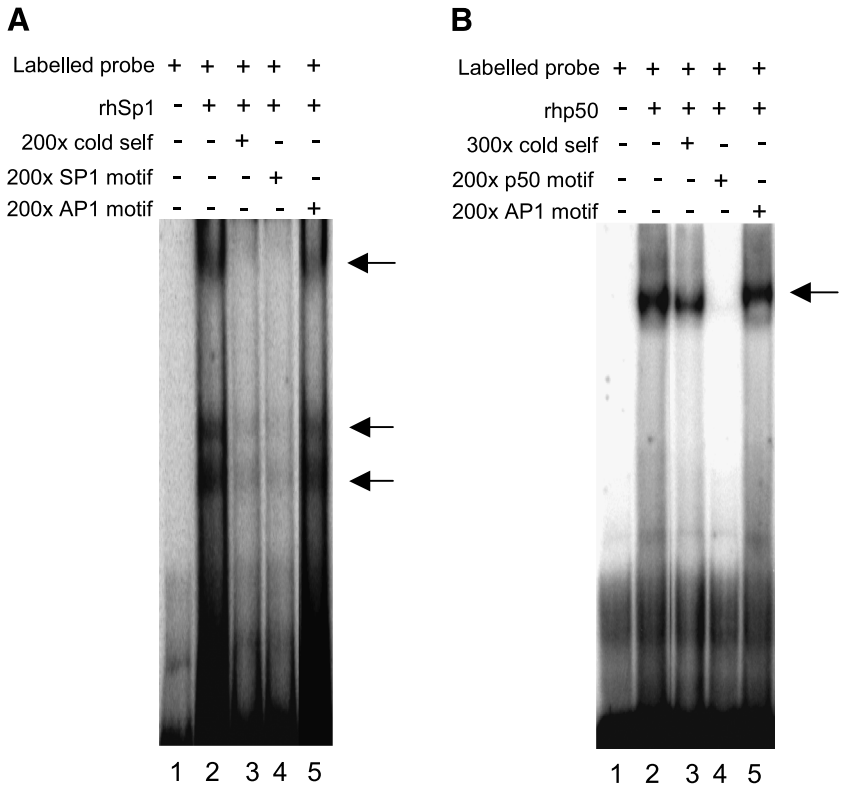

Figure 4. *Binding of Sp1 and the NFκB subunit p50 to the uroplakin Ib promoter. (A) Binding of rhSp1 to the uroplakin Ib oligonucleotide. Three bands, indicated by arrows, were identified. (B) Binding of rp50 to the uroplakin Ib oligonucleotide, with one specific band indicated by an arrow.*

complexes (D and E) were present (Figure 5*B*, *lane 2*), the formation of which was dramatically inhibited by competition with unlabeled oligo (Figure 5*B*, *lane 3*). Interestingly, consensus Sp1 and NFκB motifs each completely blocked the formation of the uppermost complex (complex D; Figure 5*B*, *lanes 4* and *5*), suggesting that both Sp1 and NFκB were required for the formation of this complex. However, these two proteins had no effect on the lower complex (complex E), suggesting that another unidentified protein was also capable of binding this region of the uroplakin Ib promoter. Again, there was no competition for complex formation with the AP1 consensus motif (Figure 5*B*, *lane 6*). These data suggested that in cells expressing high levels of endogenous uroplakin Ib mRNA, at least two possible scenarios might exist. In cells exemplified by HT1376, only Sp1 bound this promoter sequence; however, in cells exemplified by RT112, Sp1, NFκB, and an unidentified protein were all binding. Moreover, our data suggested cooperative binding by Sp1 and NFκB. This raised the possibility that the higher levels of endogenous uroplakin Ib mRNA and uroplakin Ib reporter activity in RT112 *vs* HT1376 might reflect a cooperative effect of binding by both Sp1 and NFκB—together with the presence of a third protein—to this region of the uroplakin Ib promoter.

If methylation of CpG3 and CpG4 prevented the binding of transcription factors to the promoter and was a key factor in determining levels of uroplakin Ib mRNA, we reason that these factors might still be capable of binding our uroplakin Ib promoter probe even in cells in which endogenous uroplakin Ib mRNA levels are very low due to methylation of the endogenous promoter because these transcription factors might still be present. To test this possibility, we performed gel shift analysis using nuclear extracts from SW1710 cells, which do not express detectable levels of uroplakin Ib mRNA and in which the region of the endogenous uroplakin Ib promoter is methylated [21]. In this case, two complexes were formed (Figure 5*C*), and competition experiments generated results similar to those obtained in RT112 cells, suggesting that Sp1, NFκB, and the unidentified protein were binding the uroplakin Ib promoter sequence.

Because the role of CpG methylation in regulating transcriptional activity was clearly demonstrated in the reporter experiments described above, we then examined the effect of CpG methylation on transcription factor binding to the core region of the uroplakin Ib promoter. Oligonucleotides were synthesized with 5-methyl cytosine incorporated at either CpG3, CpG4, or both sites. Methylated oligos were used at $\times$100 molar excess to compete with the binding of nuclear extracts to the ^{32}P-labeled wild-type oligo probe. Figure 5*D–F* shows results and demonstrate that the methylated oligos were able to compete effectively to prevent the binding of complexes to the uroplakin Ib promoter. This result suggests that, in contrast to the highly inhibitory effect of methylation on reporter activity, methylation of CpG3 and CpG4 did not inhibit the binding of complexes to the promoter.

Site-Directed Mutagenesis (CG to TA) of CpG2, CpG3, and CpG4 Does Not Affect Uroplakin Ib Reporter Activity

Because methylation of CpG3 and/or CpG4 silenced reporter activity and formed part of the binding motifs for Sp1 and NFκB, we initially changed both CG pairs to TA by site-directed

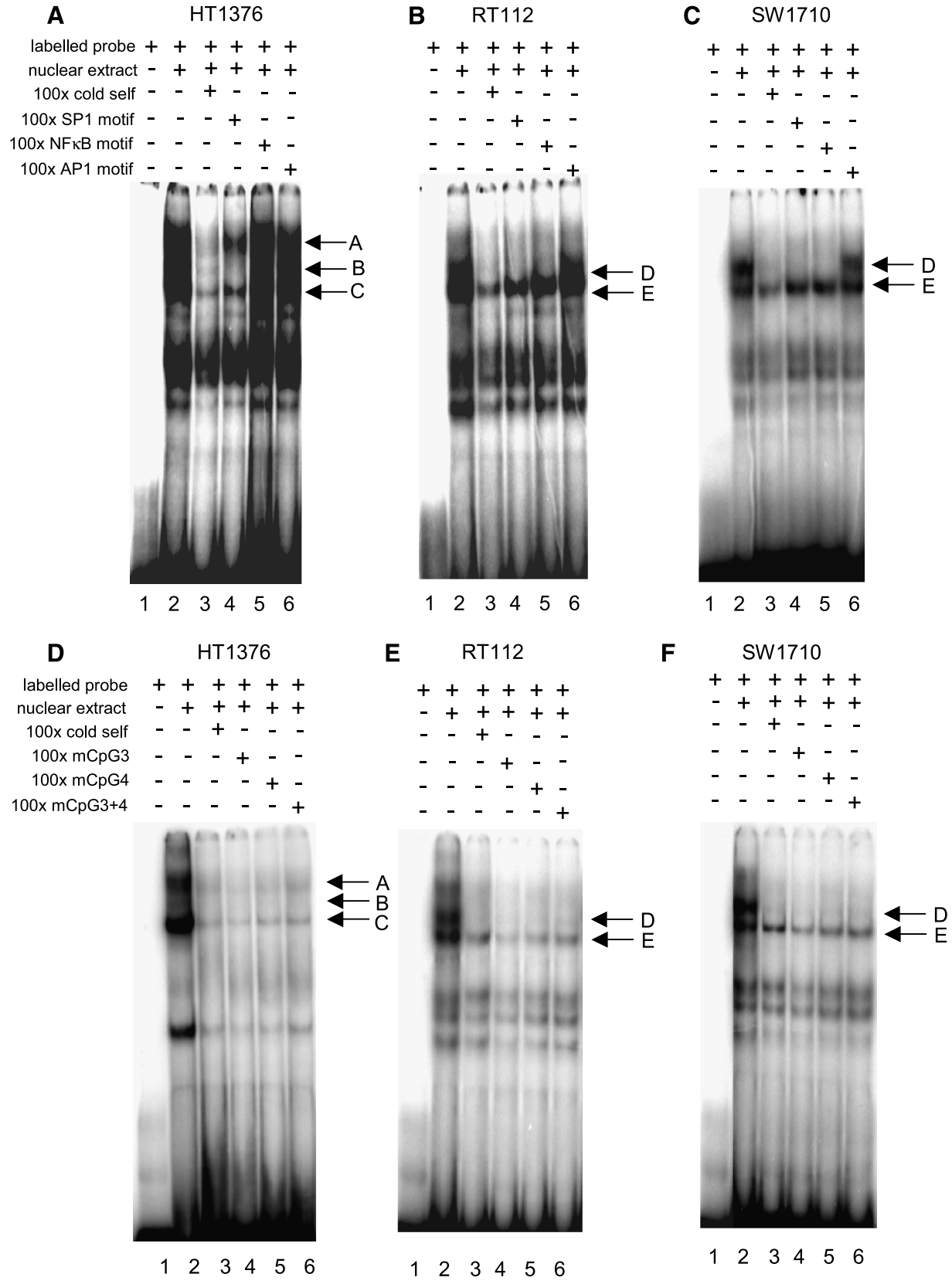

Figure 5. *Binding of TCC nuclear extracts to the uroplakin Ib promoter. (A–C) Gel shift analysis of nuclear extracts from HT1376, RT112, and SW1710 cells using a ^{32}P-labeled uroplakin Ib oligonucleotide and indicated probes and competitors. Specific bands are indicated with arrows. (D–F) Gel shift analysis of nuclear extracts from HT1376, RT112, and SW1710 cells, respectively, using a ^{32}P-labeled uroplakin Ib oligonucleotide with competitor probes methylated at CpG3, CpG4, or both CpG3 and CpG4.*

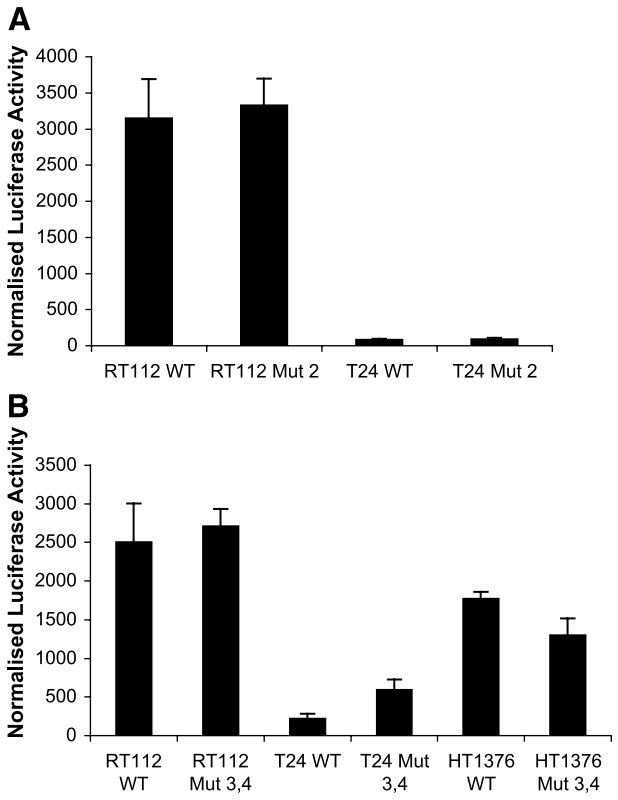

Figure 6. *Effect of site-directed mutagenesis (converting CG to TA at CpG2, CpG3, and CpG4) on the transcriptional activity of the uroplakin Ib reporter construct. (A) Mutagenesis of CpG2 had no effect on the transcriptional activity of the uroplakin Ib promoter when the mutated construct was transfected into TCC cell lines. (B) Mutagenesis of both CpG3 and CpG4 had no effect on luciferase activity when transfected into TCC cells.*

mutagenesis. As negative control, we also mutated CpG2 to TA because methylation of this site did not correlate with loss of endogenous uroplakin Ib mRNA [21]. As anticipated, mutation of CpG2 had no effect on the activity of the reporter after transfection into RT112 and T24 cells (Figure 6*A*). However, we were surprised to discover that the activities of reporters in which CpG3 and CpG4 were mutated to TA were also unaffected (Figure 6*B*). One possible explanation for these data is that methylation of CpG3 and CpG4 generates a steric block to the binding of Sp1 and NFκB, which is not achieved by the simple mutation of CG to TA (which still permits the binding of these factors). In an alternative strategy, a 6-bp fragment flanking either CpG3 or CpG4 was mutated (sequences are shown in Table 2) to ensure that the Sp1- and NFκB-binding motifs were completely disrupted. Data presented in Figure 7 show that mutation of either CpG3 or CpG4 each caused an almost 30% reduction in luciferase activity, suggesting that each motif contributed to transcriptional activity.

Cotransfection of Expression Plasmids for Sp1 and NFκB Family Members and the Uroplakin Ib Reporter

The data described above illustrate that rhSp1 and rhp50 can each bind to the uroplakin Ib oligonucleotide probe and that disruption of the relevant CpG sites by methylation or mutagenesis inhibited transcriptional activity of the reporter construct. Cotransfection experiments were then carried out to verify a functional role for Sp1 and NFκB in reporter activities. Cell lines were cotransfected with combinations of the uroplakin Ib reporter construct and plasmids expressing Sp1, NFκB family proteins (p50, p65, and c-Rel), and luciferase activity, determined as above.

Results illustrated in Figure 8*A* show that, in RT112 cells, cotransfection of Sp1 alone with the uroplakin Ib reporter caused a small but consistent two-fold enhancement of luciferase activity, whereas cotransfection of NFκB members (p50 alone, p50 and p65, or p50 and p65 with c-Rel) had little effect on reporter activity. However, when Sp1 was included in cotransfections with p50, p65, or c-Rel, there was marked enhancement of uroplakin Ib reporter activity. The highest levels of luciferase activity (×6.0 basal reporter activity) were observed when cells were cotransfected with the uroplakin Ib reporter and Sp1 plus p65 plus c-Rel. These data suggested that a combination of Sp1 and NFκB might be required for the highest levels of activity from the uroplakin Ib promoter. We extended our studies to examine the effects of Sp1 and NFκB family members on uroplakin Ib reporter activity in TCC cell lines that do not normally express uroplakin Ib mRNA. Results similar to those in RT112 were obtained for the transfection of Sp1 alone, in 5637 cells (Figure 8*A*), and in J82, VMCub3, and TCCSuP cells (Figure 8*B*). In contrast to RT112, transfection of NFκB proteins was also able to cause modest enhancement (about two-fold) of luciferase activity in J82, VMCub3, and TCCSuP cells. However, the inclusion of Sp1 again resulted in further elevation of reporter activity. In particular, the combination of Sp1 plus p65 plus c-Rel increased luciferase activity (by 9.4-fold in 5637 cells, by 22-fold in VMCub3 cells, by 3.2-fold in TCCSuP cells, and by 4.6-fold in J82 cells). Thus, enhanced transcriptional response to cotransfection of Sp1 with NFκB occurred in all cells, irrespective of their capacity to express endogenous uroplakin Ib mRNA (Figure 2). These data were consistent with our ideas that methylation of the uroplakin Ib promoter inhibits transcription by blocking the binding of Sp1 and NFκB to the promoter and also

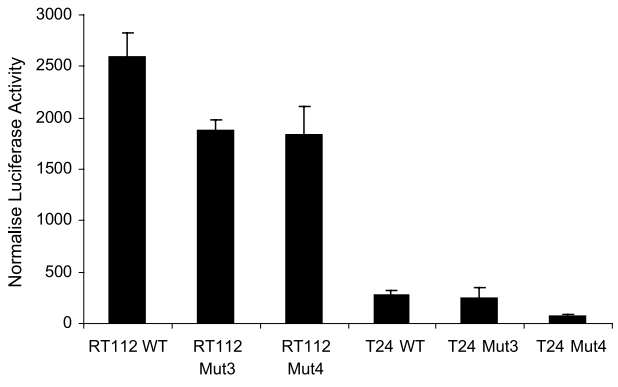

Figure 7. *Effect on the transcriptional activity of site-directed mutagenesis of a 6-bp sequence spanning either CpG3 or CpG. The sequence spanning CpG3 or CpG4 was subjected to site-directed mutagenesis, and luciferase reporter activity was assessed following transfection into RT112 or T24 cells. Luciferase activity in RT112 cells was inhibited by approximately 30% by mutation of either site, but there was no effect on low levels of luciferase activity in T24 cells.*

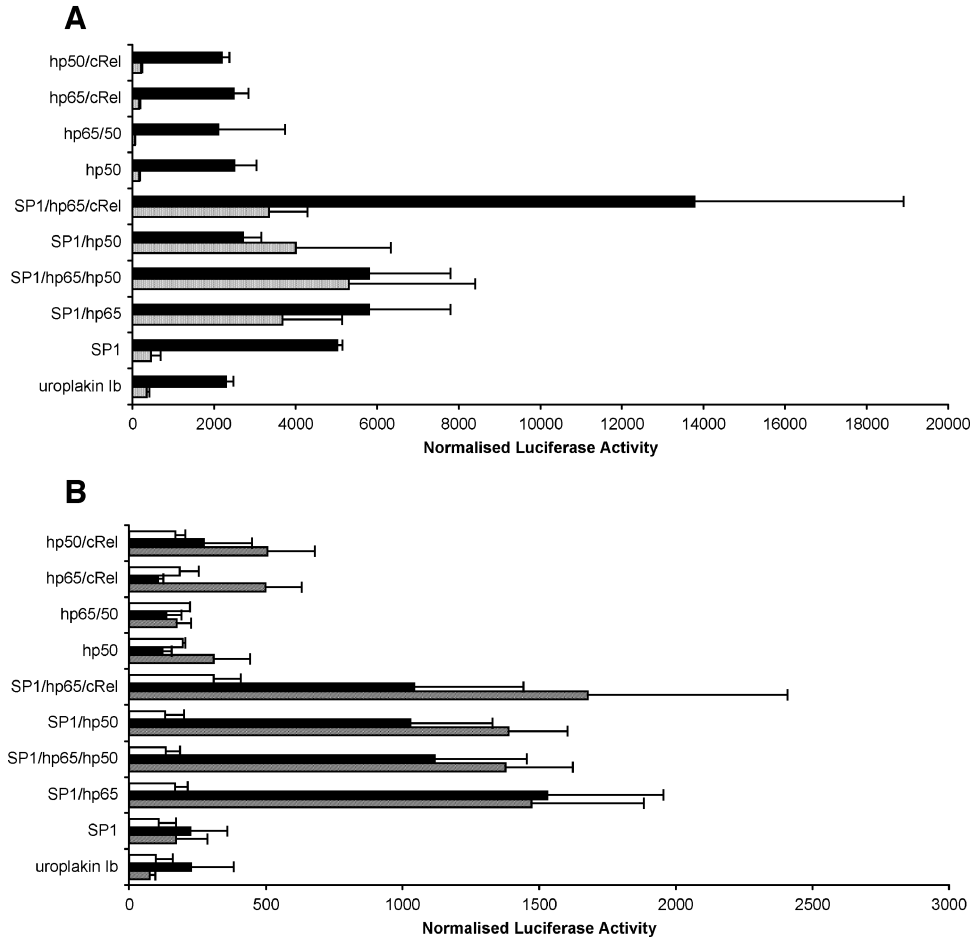

Figure 8. *Cotransfection of the uroplakin Ib reporter construct and plasmids constitutively expressing Sp1 or NFκB family proteins (p50, p65, or c-Rel). (A) (■) RT112 cells; (▩) 5637 cells. (B) (■) J82 cells; (▨)VMCub3 cells; (□) TCCSuP cells.*

suggested that expression of different NFκB family members might play a role in determining normal levels of uroplakin expression in different cell lines.

Discussion

This study has provided direct experimental evidence that regulation of uroplakin Ib transcription is mediated by CpG methylation at specific sites in the proximal promoter region. Transcriptional activation also requires the involvement of both Sp1 (CpG3 and CpG4) and NFκB family proteins (CpG3), which bind to the motifs encompassing these CpG pairs. Methylation of CpG3 to CpG5 completely inhibits luciferase activity, suggesting that these sites are vital for transcription from the reporter construct. Although CpG5 is not part of a motif for Sp1 or NFκB binding, due to lack of useful restriction sites, it is not technically possible to methylate only CpG3 and CpG4. Thus, we cannot currently rule out the possibility that CpG5 might also be involved in transcriptional regulation.

To verify that these CpG sites were functional in mediating transcriptional activity, site-directed mutagenesis was carried out by mutating CG to TA at both CpG3 and CpG4. Surprisingly, these small mutations did not affect luciferase activity, but a 6-bp mutation spanning either CpG3 or CpG4 reduced activity by about 30%, suggesting that methylation of cytosine residue induces steric hindrance to transcription factor binding. Because transcriptional activity was not completely inhibited, this result suggests that multiple sites in the promoter have collaborative functions in inducing transcriptional activity. Because methylation of CpG3 to CpG5 will completely inhibit transcription, regulation of the transcriptional activity of the reporter construct must reside in a combination of these three sites. It is not yet clear if the methylated CpGs directly inhibit the binding of transcription factors or if methylated CpGs recruit methyl CpG–binding proteins, which subsequently interfere with the binding of transcription factors to the sites [24].

In our previous study [21], we identified several putative transcription factor–binding motifs in the uroplakin Ib proximal promoter, including Sp1 at CpG3 and CpG4, and a putative NFκB site directly adjacent upstream of CpG3 (six of nine matches for consensus NFκB). The current study examined the ability of a double-stranded uroplakin Ib promoter oligonucleotide containing these motifs to bind to proteins in nuclear extracts from TCC cells and to recombinant Sp1 and p50. Initial EMSA experiments demonstrated that recombinant Sp1 and NFκB (p50) both bind to the sequence, although we did not test if the other NFκB family members can also bind to the oligonucleotide. When nuclear

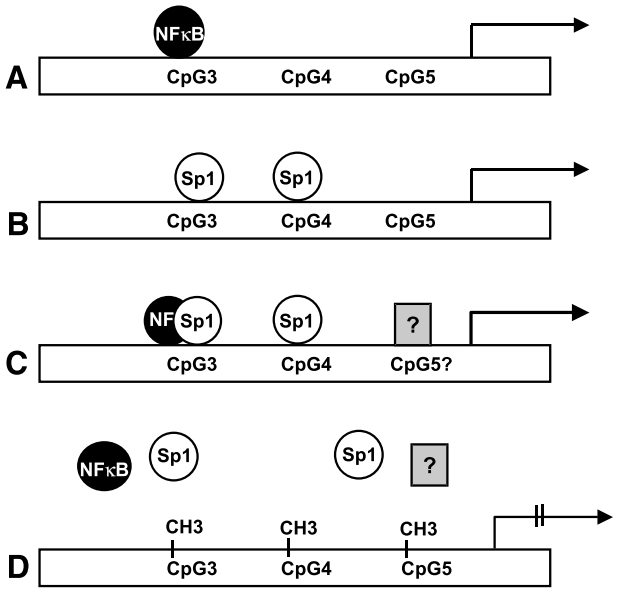

Figure 9. *Proposed mechanisms regulating uroplakin Ib transcription from specific CpG sites in the proximal promoter. (A) Binding of NFκB to CpG3 induces minimal levels of transcriptional activation. (B) Binding of Sp1 to CpG3 and CpG4 allows low levels of transcription. (C) Both Sp1 and NFκB family members binding to CpG3 and CpG4 can act in synergy to induce high levels of transcription. High-level activity may also require the involvement of another as-yet-identified factor binding to CpG3 and CpG4 and a factor binding to CpG5. (D) Methylation of CpG3, CpG4, and CpG5 prevents the binding of Sp1 and NFκB, an unidentified factor, and a factor binding to CpG5, thus blocking transcription.*

extracts were subjected to EMSA analysis, complexes were detected from cells expressing uroplakin Ib and showed evidence for both Sp1 and NFκB binding to the oligonucleotide because consensus motif oligonucleotides were only each partially able to inhibit complex formation.

The same complexes were also formed in nuclear extracts prepared from cells that do not express endogenous uroplakin Ib mRNA and contain methylated CpG motifs in the promoter [21]. These data suggest that lack of uroplakin Ib expression in these cells is not due to the absence of relevant transcription factors, but may be due to CpG methylation preventing transcription factor access to the motif. This is also suggested by the fact that mutation of the CpGs in the oligonucleotide did not affect factor binding because there was still sufficient sequence remaining for the motif to be recognized.

At least two patterns of transcription factor binding appear to be present in nuclear extracts and may be a reflection of the levels of reporter activity in their respective cells. In HT1376 cells, binding of Sp1 only was detected and the NFκB consensus oligo did not compete in binding to the ^{32}P-labeled oligonucleotide. Sp1 may potentially bind to either CpG3 or CpG4 and complexes may be formed if Sp1 binds to either possible motif; these would be of the same size. Larger complexes would be formed if Sp1 binds simultaneously to both motifs; this pattern can be observed in Figure 5*A*. In RT112 and SW1710 cells (Figure 5, *B* and *C*), it appears that Sp1 and NFκB are both present in complexes forming on this sequence because competition with either consensus motif completely abrogates complex formation. These data also suggest that both proteins are essential for complex formation. Other examples of cooperative functions for NFκB and Sp1 in gene activation have been reported in the literature [25]. The cotransfection experiments discussed above have provided further direct functional evidence that both transcription factor families are required for an effective transcription from the reporter construct. This correlates well with the presence of consensus-binding sites for both Sp1 and NFκB in the sequence encompassing CpG3 and suggests that this site might be the major regulatory motif in this sequence.

Interestingly, gel shift data using nuclear extracts from RT112 and HT1376 cell lines also provided evidence for the binding of an unknown protein to this region of the uroplakin Ib promoter. Binding of this protein to either Sp1 or NFκB was not competed with by oligos, suggesting that this protein binds independently of these latter two proteins. Conceivably, this protein might function to facilitate functional synergy between Sp1 and NFκB, although our transfection data suggest that a third protein is not essential for the cooperative activation of transcription by Sp1 and NFκB.

The current study demonstrated that cell lines that expressed significant levels of endogenous uroplakin Ib RNA had the highest levels of transcriptional activity from the reporter construct. There was also very little activity supported in non-urothelial lines, suggesting that this promoter sequence might be regulating the urothelial specificity of the expression of uroplakin Ib. If control of urothelial specificity resides in this sequence, this knowledge could be important in allowing the design of tissue-specific vectors for gene therapy approaches to treating TCC. Such an approach has shown promise in studies using the uroplakin II promoter to target urothelial tissue by an adenoviral vector. This vector caused a significant regression of RT4 bladder cancer xenografts in mice [26]. However, other evidence suggests that this strategy is likely to be less successful if the uroplakin Ib promoter were used for gene therapy vectors, as expression of uroplakin Ib is not strictly urothelial-specific. As an example, the colorectal carcinoma cell line SW480 supports moderate luciferase activity that is approximately half that of the urothelial line HT1376 (Figure 2*B*). A large number of non-urothelial uroplakin Ib expressed sequence tags have also been identified, and the Unigene entry HS271580 (as of May 9, 2005) currently reports 96 ESTs from tissues as diverse as the eye, lung, brain, ovary, and pancreas, both in purified islets and in pancreatic adenocarcinoma. Abundant amounts of uroplakin Ib mRNA and protein have also been detected in the corneal epithelium, although its function in this context is not clear [27].

The sequence analyzed in the current study, although vital for the positive regulation of the expression of uroplakin Ib, is likely to be only partially responsible for the control of endogenous uroplakin Ib mRNA expression. A recent study from Olsburgh et al. [22] suggested that there were inhibitory motifs directly upstream of the proximal promoter sequence analyzed in our current study. In their study, Olsburgh et al. identified a sequence at −628 bp, which was closely homologous to a putative TGF-β1 inhibitory element. The proximal

region analyzed in the current study is an activating region, but control of levels of endogenous uroplakin Ib mRNA is likely to be more complex.

In conclusion, our data demonstrate that transcriptional activity of the uroplakin Ib reporter is regulated by CpG methylation at CpG3, CpG4, and CpG5. Transcription is also controlled by both Sp1 and NFκB family proteins, which bind to their putative motifs encompassing CpG3 and CpG4 but may also bind to an unidentified factor in this sequence and to a factor binding to a sequence involving CpG5. As illustrated in Figure 9, Sp1, NFκB, and the unidentified factors may be required to activate significant levels of transcription from the luciferase reporter. However, if the reporter is methylated at CpG3, CpG4, and CpG5, binding of the factors may be sterically hindered and transcriptionally blocked. Future studies will determine if these factors are also important for the regulation of uroplakin Ib expression in clinical samples of TCC and will identify the molecular mechanisms by which these tumors frequently lose expression of uroplakin Ib during tumor progression.

Acknowledgements

We thank Lor Wai Tan (Department of ENT Surgery, The Queen Elizabeth Hospital) for methodological advice and discussion; Sally Stephenson (Department of Haematology–Oncology, The Queen Elizabeth Hospital) for providing the breast cancer cell lines; and Pamela Russell (Oncology Research Centre, Prince of Wales Hospital) for providing the TCC cell lines. We would also like to thank Merlin Crossley (Department of Microbiology and Molecular Biology, University of Sydney, Sydney, Australia) for help with gel shift assays. Plasmid vectors expressing p50 (pCMV-p50) and p65 (pCMV-p65) were obtained from Celine Gelinas (Rutgers University, Newark, NJ); Sp1 (pEVR2-Sp1) was obtained from Phillipe Galera (Faculty of Medicine, University of Caen, Caen, France); and c-rel (pRcCMV-c-rel) was obtained from Frances Shannon (John Curtin School of Medical Research, Canberra, Australia).

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