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Deletion of A-antigen in a human cancer cell line is associated with reduced promoter activity of CBF/NF-Y binding region, and possibly with enhanced DNA methylation of A transferase promoter

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Employing blood group A^- and A^+ clones derived from the same parental colonic cancer cell lines, we studied the molecular mechanism of deletion/ reduction vs. continuous expression of A antigen in A tumors, a crucial determinant of human tumor malignancy. A^- transferase mRNA level in one of the A^- clones (A^- SW480) was undetectable, while that in A^+ SW480 was strongly detectable by semiquantitative RT-PCR. Relatively lower ($\sim$1/3) transcript level was detectable in another A^- clone (A^- HT29) in comparison to A^+ HT29 by the same RT-PCR procedure, although none of these tumor cell lines showed detectable level of A transcript by Northern blotting or RNase protection methods. Therefore, subsequent studies were performed employing A^- vs. A^+ SW480 clones. Deletion of A transcript in A^- cells was not due to gene deletion, since Southern blot analysis showed equal presence of genomic DNA regardless of A^- vs. A^+ (SW480 or HT29) or B^+ (KATOIII) tumor cells. Two transcriptional control mechanisms leading to differences of A expression in SW480 cells are indicated.

i. Luciferase assay in A^- and A^+ SW480 cells showed that promoter activities of segments of 5′ flanking sequence of ABO gene reflected transcript levels in these cell lines. The enhancing activity of a 43 bp tandem repeat unit located between -3899 to -3618 was reduced in A^- compared to A^+ cells.

ii. Distinct differences in the pattern of CpG dinucleotide methylation were found in A^- vs. A^+ cells. Therefore, the methylation process of A promoter DNA may be another important factor controlling A activity in SW480 tumor cells.

Since proliferation and motility of tumor cells are associated closely with A expression, transcription control mechanism for expression of A transferase as described above may be of crucial importance in defining human tumor malignancy.

Keywords: **A− and A+ clones; promoter activity; CBF/NF-Y binding site; CpG island; methylation**

Introduction

Deletion or reduction of histo-blood group A or B antigen in various types of human cancer has been correlated with invasive and metastatic properties of tumor cells in numerous clinicopathological studies (for review see [1,2]). The molecular mechanism of this phenomenon is of crucial importance in defining human tumor malignancy, since deletion or addition of $\alpha1\rightarrow3$GalNAc (A determinant) or $\alpha1\rightarrow3$Gal (B determinant) to H, or expression of H (including Le^y or Le^b) may affect tumor cell motility, proliferation, and invasiveness through functional change of integrin receptors, CD44, and yet-unidentified membrane proteins or lipids. Evidence to support this concept has been provided recently by experiments with *A*, *B*, and *H* gene transfection [3–6].

In order to study the molecular mechanism of deletion/ reduction of A or B antigen in tumors, it is essential to compare A- or B-expressing (or -deleted) cells with parental cells having the same genetic background. We previously established A^- and A^+ tumor cell lines from two parental colonic cancer cell lines, HT29 and SW480 [6]. We undertook

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Dr. Iwamoto worked on this study during a sabbatical leave from Jichi Medical School, Japan, Sep 1997–Jun 1998.

the present study employing these A^- vs. A^+ cell lines to explore the molecular mechanism of deletion vs. persistence of A antigen.

The transcriptional regulation of ABO transferase gene was previously characterized based on minisatellite sequence located between −3899 to −3618 base pairs (bp) from the transcription initiation site. This region contains four tandem copies of a 43-bp repeat unit which encodes a binding motif for a transcription factor, CBF/NF-Y [7]. In this study, we attempted to resolve the mechanism for deletion of A antigen on SW480 cells, in order to clarify the molecular basis of A/B antigen disappearance in cancer cells.

Materials and methods

Cell culture and DNA transfection

SW480, HT29 and KATOIII cells were purchased from ATCC (Rockville, MD). A^+ and A^- cell populations from SW480 and HT29 cell lines were separated through a panning procedure from parental cell lines as described previously [6], and maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum. KATOIII cells were grown in RPMI 1640 with 20% fetal calf serum. Reporter plasmids (1.6 μg) were co-transfected with 0.4 μg of pXGH5 vector (Nichols Institute, San Juan Capistrano, CA) to normalize transfection efficiency to growth hormone levels. Transfection was performed in a 6-well culture plate with Lipofectamine (Life Technologies Inc, Gaithersburg, MD) according to manufacturer's instruction. Each transfection study was repeated at least three times.

RNA Analysis by RNase Protection Assay, Northern Blot Hybridization, and RT-PCR

Poly A^+ RNAs of A^+ and A^- HT29, A^+ and A^- SW480, and KATOIII cells were extracted by PolyATract mRNA Isolation System (Promega, Madison, WI). DNA fragment from nt 867 to 1221 of the A transferase cDNA was subcloned in pBluescript (Stratagene, La Jolla, CA) and linearized with appropriate restriction enzyme. Antisense RNA probes were radiolabeled with T7 RNA polymerase (Promega). 5 μg of each polyA^+ RNA was hybridized with 20 Kcpm RNA probes at 55°C for 12 h. The hybridized samples were digested with RNase A/RNase T1 mixture at 30°C for 60 min. The products were precipitated and subsequently separated on a 5% sequencing gel. After electrophoresis, the gel was dried and exposed to X-ray film. Aliquots (5 μg) of poly A^+ RNA of each cell line were analyzed by Northern hybridization probed with A-transferase cDNA, followed by reprobing with actin cDNA.

For further quantification of the transcript level of A transferase in these five cell lines, semiquantitative RT-PCR was employed. 5 μg of total RNA of each cell line was reverse-transcribed by MMLV-RTase and the RT products were PCR-amplified with sense (GCCCCAGAAGTCTAATGCCAG, corresponding to nt 113 to 132) and antisense (CCCCCCCAGGTAGTAGAAATCGCCCTCGTCCTT, complementary to nt 769 to 801) primers and [^{32}P]dCTP. As an internal control of amplification in the individual tube, 10 fg of cDNA plasmid inserted with A^- transferase gene splicing isoform (200 bp longer than matured mRNA) was introduced in the PCR buffer. Reactions went for 24, 27, or 30 cycles, and products were subjected to electrophoresis on a 5% acrylamide gel. After electrophoresis, the gel was dried and exposed to X-ray film. Band intensities were quantitated by a densitometer.

Southern blot and methylation analysis

10 μg of genomic DNA was digested with sufficient units of EcoRI or HindIII, and resolved by electrophoresis on a 0.8% agarose gel. The DNA was transferred to a Zeta-Probe GT membrane (Bio-Rad, Hercules, CA), hybridized, washed, and exposed to film according to the manufacturer's instruction. The *Xho*I-*Sac*II DNA fragment of A transferase genomic DNA upstream from exon1 was labeled with ^{32}p-dCTP and used as probe. For methylation analysis, genomic DNA samples were digested with methylation-sensitive enzyme *Hpa*II or its methylation-insensitive isoschizomer *Msp*I. Fragments were resolved on 2% agarose gel and analyzed as above.

Construction of reporter plasmid

A DNA fragment containing the 5′-upstream sequence of the human ABO gene was subcloned into luciferase reporter plasmid, pGL3-basic (Promega). Serial 5′-deletion mutants were constructed as described previously [7]. The segmental deletion mutants were generated from the PN or pHiN (Fig. 3) construct using unique restriction enzymes followed by blunting with T4 DNA polymerase. A tandem repeat of 43-bp sequence located from −3843 to −3670 was isolated and linked by PCR, and ligated in the upstream of SN construct at *Nhe*I-*Xho*I site (Figs. 3, 4). Nucleotide sequences of the constructs were determined to rule out the possibility of additional mutations generated during PCR. The plasmids were purified by Qiagen purification kit (QIAGEN, Valencia, CA) and transfected into A^+ and A^- SW480 and KATOIII cells. 48 h after the start of transfection, cell lysates and cell supernatants were harvested. The expression of luciferase and human growth hormone proteins were assayed using Bertold Lumat luminometer (Bad Wildbad, Germany) and radio-immunoassay (Nichols Institute), respectively. For each sample, luciferase activity values were normalized to growth hormone levels to control for transfection efficiency. Normalized luciferase values were divided by values obtained for the empty vector (pGL3 basic; Fig. 3) or the core promoter construct (SN; Fig. 4).

Analysis of ABO gene promoter region methylation status

Genomic DNA was purified from A^+ and A^- SW480 sublines according to standard procedures [8] and digested with EcoRI

to reduce viscosity. Digested DNA was denatured and treated with sodium bisulfite under conditions that convert unmethylated cytosine residues to uracil [9,10]. A 741 bp sequence from the ABO transferase upstream region (Genbank accession #HSU22302; sequence corresponds to −717 to +24; where start of translation is +1) was amplified from 300 ng of bisulfite treated DNA using 5′ primer: CCTCTCCTGAGCTTCCTC and 3′ primer: CAGCGTCCGCAACACCTC. These primers were chosen to be minimally complementary to targets with CpG dinucleotides and should thus minimally bias the amplification reaction toward either bisulfite converted or non-converted DNA strands. PCR reactions used the elongase enzyme cocktail (Gibco/BRL) according to the manufacturer's instructions, supplemented with 13% DMSO. After an initial denaturation step at 95°C for 3 minutes; 45 cycles of 94°C for one minute, 54°C for 2 minutes and 68°C for 2 minutes were performed. PCR products were gel purified and sequenced directly, with both the 5′ and 3′ PCR primers, using the ABI Prism big dye terminator kit and the ABI 310 automated sequencer. Sequence electropherograms were visually inspected for conversion of C to T, or G to A (indicating cytosine conversion on the complementary strand).

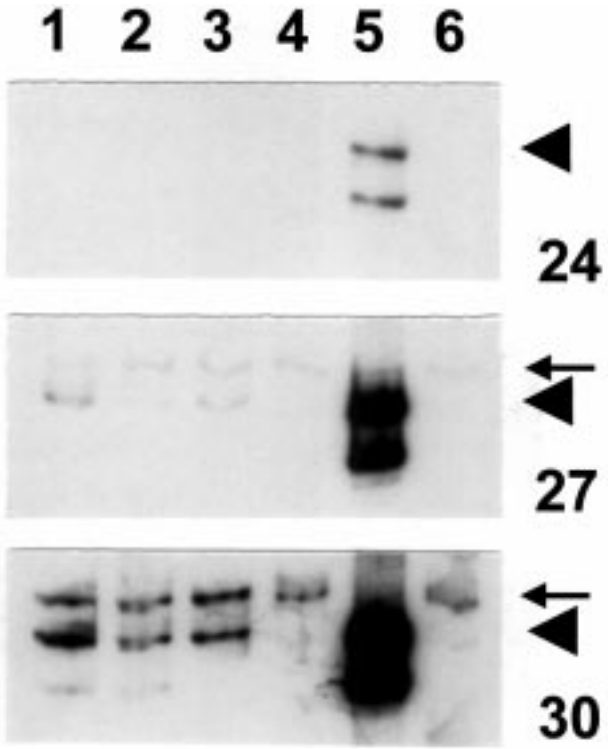

Figure 1. Transcript levels of ABO transferases analyzed by RT-PCR. Five μg total RNA from the cell lines (lane 1 to 5) were reverse-transcribed into cDNA with oligo-dT (19), and subsequently amplified by polymerase chain reaction with 10 fg internal control (thin arrows). Lane 1, A^+ HT29. Lane 2, A^- HT29. Lane 3, A^+ SW480. Lane 4, A^- SW480. Lane 5, KATOIII. Lane 6, yeast transfer RNA. Numbers at lower right of three panels [24, 27, 30] indicate number of PCR cycles. In A^- HT29 and A^- SW480 cells, ABO transferase transcripts are reduced or not detected, respectively, compared to A^+ cells (arrow heads). Note that the internal control for KatoIII (lane 5) is not amplified due to the overwhelming amount of A-transferase mRNA in this cell line. Slight differences in internal control amplification levels for HT-29-A^+ and -A^- (lanes 1 and 2) and SW480-A^+ and -A^- (lanes 3 and 4) are most likely attributed to the presence of splice variant transcripts in A^+ but not A^- lines. This is supported by RNase protection assay experiments, which detect trace amounts of splice variant transcripts in A^+, but not A^- lines (data not shown).

Results

mRNA levels of A^- vs. A^+ clones of colonic cancer cell lines HT29 and SW480

Levels of A transferase mRNA in A^- and A^+ cell lines from either HT29 or SW480 cells were too low to be detected by Northern blot or RNase protection analysis with A transferase cDNA probe, although control actin blotting level was strongly detectable (data not shown). Semiquantitative RT-PCR was used to detect A transferase mRNA in A^- vs. A^+ HT29 or SW480 cells, employing KATOIII as control. 10 fg of cDNA clone containing a splice variant (200 bp larger) was added to each amplification reaction as an amplification control. A strong band corresponding to A transferase mRNA was

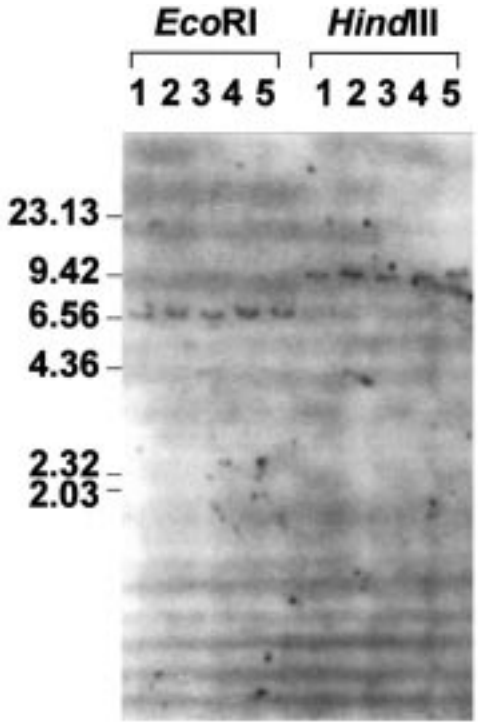

Figure 2. Southern blotting analysis. Ten μg per lane of genomic DNA from A^+ HT29 (lane 1), A^- HT29 (lane 2), A^+ SW480 (lane 3), A^- SW480 (lane 4) and KATOIII (lane 5) cells were digested with sufficient units of restriction enzymes as indicated. DNA fragments were resolved by electrophoresis on a 0.8% agarose gel. Size markers (λ phage HindIII digested) are shown to the left.

detectable in A^+ SW480, but was not detectable in A^- SW480. This contrast was clearly seen after PCR cycle 27 (Fig. 1, lane 3 vs. 4 in panels marked "27" and "30"). This band was significantly reduced in A^- HT29 cells as compared to A^+ HT29 (lane 1 vs. 2 in panels "27" and "30"). The intensity of the A^+ bands in both HT29 and SW480 at PCR cycle 30 was similar to that of KATOIII at PCR cycle 24 (lanes 1 and 3 in panel "30" vs. lane 5 in panel "24"). Since the contrast of level of mRNA for A transferase was very clear in A^- vs. A^+ SW480, further studies were performed using these cell lines, as described below.

Genomic DNA level as indicated by southern blotting

Reduction or deletion of transcript for A transferase as indicated above, particularly near-deletion of transcript in A^- SW480, may be ascribable to deletion of the A transferase gene in these tumor cells. However, Southern blot analysis showed bands of equivalent intensity regardless of transcript levels found in A^- or A^+ HT29 or SW480 cells, or control KATOIII cells (Fig. 2). Therefore, reduction or deletion of A transcript is not ascribable to change of genomic DNA in these tumor cell lines.

Transcriptional activities of ABO transferase promoter in A^+ and A^- SW480 and KATOIII cells

The serial 5′-deletion constructs were transiently transfected, together with an internal control plasmid, PXGH5, which expresses human growth hormone. For each sample, luciferase activity values were normalized to human growth hormone levels to control for differences in transfection efficiency. To

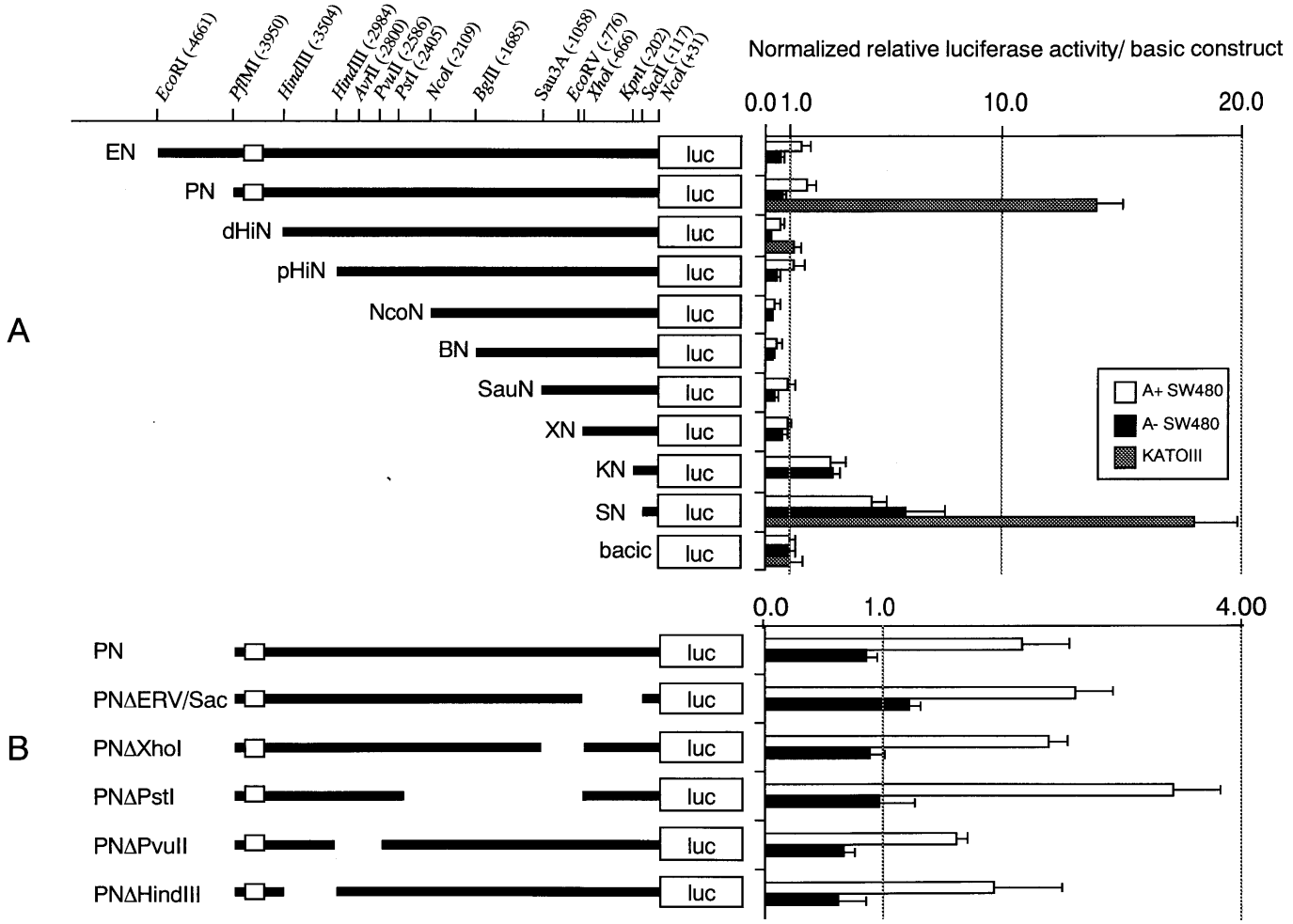

Figure 3. Luciferase assay. The 5′ flanking sequence of ABO transferase gene was inserted upstream of luciferase gene open reading frame. The serial 5′ deletion mutants (A) and the segmental deletion mutants (B) were constructed by using unique restriction sites indicated on the map. The open box indicates the minisatellite sequence which has been previously identified as an enhancer sequence. Plasmids were transfected into A^+ SW480, A^- SW480, and KATOIII cells by lipofection. To normalize for transfection efficiency, a referential plasmid (pXGH5; encoding human growth hormone) was co-transfected in all experiments. The ratio of luciferase unit to human growth hormone in each sample was normalized with the value of basic luciferase reporter vector and served as a measure of normalized luciferase value. The full length (EN) construct and PN construct shows weak positive activity in A^+ SW480 cells. Distinctive increase of luciferase activity is observed between dHiN and PN constructs in KATOIII cells.

compare promoter activities among cell lines, normalized luciferase values for various constructs were divided either by values for the empty vector (pGL3 basic) or core promoter (SN). The profile of luciferase values in KATOIII cells almost follows the data reported by Kominato et al. [7]. Promoter activities of the full length (EN) and the region between *Pfl*MI and distal *Hind*III fragment, which includes the minisatellite containing CBF/NF-Y binding motif, were weak but still significantly higher for A^+ SW480 than for A^- SW480 (Fig. 3A, EN, PN), reflecting A transferase mRNA levels in A^+ vs. A^- cells. This region showed highest activity in KATOIII (Fig. 3A, PN). The promoter activity of *Sac*II/*Nco*I construct was high for KATOIII, but was not significantly different for A^- vs. A^+ SW480 cells (Fig. 3A, SN). Thus, the region between *Pfl*MI and distal *Hind*III contains promoter activity which reflects A transferase transcript levels in A^+ vs. A^- SW480 cells.

In order to identify a responsible element for down regulation, five segmental deletion mutants were made from the PN construct. The promoter activity of PN in A^+ SW480 was twice that in A^- SW480 cells. This ratio was maintained, even if PstI, EcoRV/SacII, PvuII, XhoI or HindIII fragments were deleted from construct PN (Fig. 3B). This suggests that the DNA fragment from PflMI to distal HindIII includes the element responsible for the reduction of A transcript in A^- SW480 cells, but that it is not contained within the deleted regions. For further analysis, repressive sequences were removed and the core promoter was joined with variable lengths of upstream DNA fragments. The resulting growth hormone normalized luciferase values were divided by that of core promoter construct, SN. When the minisatellite sequence was directly joined to the core promoter (NFY/SN), the promoter activities of SN were enhanced in all three cell lines (Fig. 4). However, the enhancement in A^- SW480 cell was one-third that of A^+ SW480, which reflects the clear difference in mRNA levels of SW480 cells (Fig. 4). The flanking sequence of the minisatellite attenuated the enhancing activities to one-tenth of the NFY/SN construct in all cell lines, and diminished the enhancing activity in A^- SW480 cells below the level of SN (Fig. 4, PNΔHindSac). Although the DNA fragment from proximal HindIII to AvrII did not enhance the core promoter activity, the fragment increased the activity of PNΔHindSac in A^+ SW480 and KATOIII cell lines when it was inserted between the minisatellite and the core promoter. Conversely, it decreased promoter activity in A^- SW480 cells (Fig. 4, PNΔHinΔAvrSac).

Although transient transfection studies with a reporter gene suggest a clear difference in promoter activity of A^+ vs. A^- SW480 cells, differential binding of transcription factor to the 43 bp repetitive sequence has not been clearly identified. A probe containing the CBF/NF-Y site showed specific binding to this repetitive sequence, but the specific bands were essentially of equal intensity in both A^+ and A^- cells, as well as in B-positive KATOIII cells (data not shown). Recently, similar results were reported for the G-CSF responsive myeloperoxidase promoter CBF/NF-Y binding site [11] (see Discussion).

DNA methylation of promoter region

The core promoter sequence of ABO gene has high CpG dinucleotide content ($\sim$82%), and qualifies as a CpG island

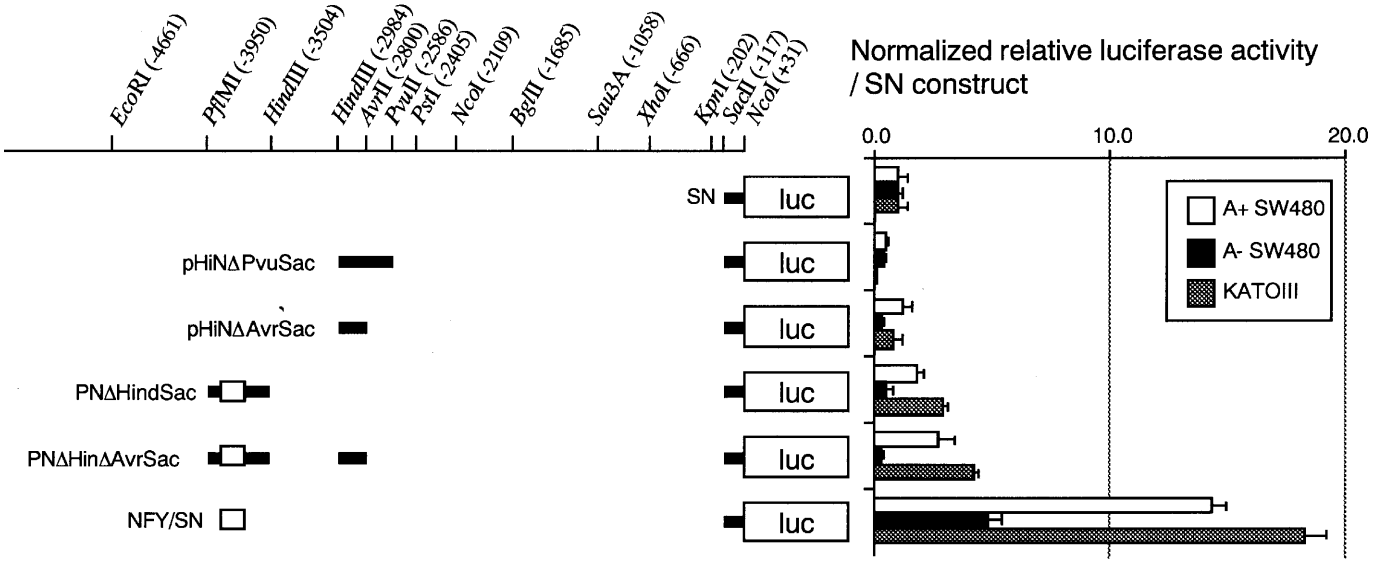

Figure 4. Luciferase assay with segmental deletion mutants. In order to identify the responsible element for the down regulation, repressive sequences were removed and the core promoter was joined with variable lengths of upstream DNA fragments. The luciferase values were normalized to that of the core promoter construct, SN, determined from an independent transfection. While the minisatellite sequence enhances the promoter activities of SN in all three cell lines, the enhancement in A^- SW480 cell is smaller than that of A^+ SW480 and KATOIII cells (NFY/SN).

[12]. CpG islands are typically located in 5′ upstream regions of certain genes and often contribute to transcriptional regulation of the gene. CpG island methylation is associated with transcriptional silencing. Promoter methylation may therefore contribute to transcriptional regulation. No difference was detected, however, between the methylation pattern of the ABO transferase promoter region in A^+ and A^- SW480 cells using the Southern blot technique (data not shown). This was probably due to difficulty in detecting the small fragments produced and the minority of CpG sites that show methylation differences (see below). We subsequently used the more sensitive methylation specific PCR technique [9,10] and found multiple methylation differences (Fig. 5). The differences observed fit the expected pattern (methylated in A^- SW480 and un- or hypomethylated in A^+ SW480 cells) for the sense strand, but were roughly equally divided among the above

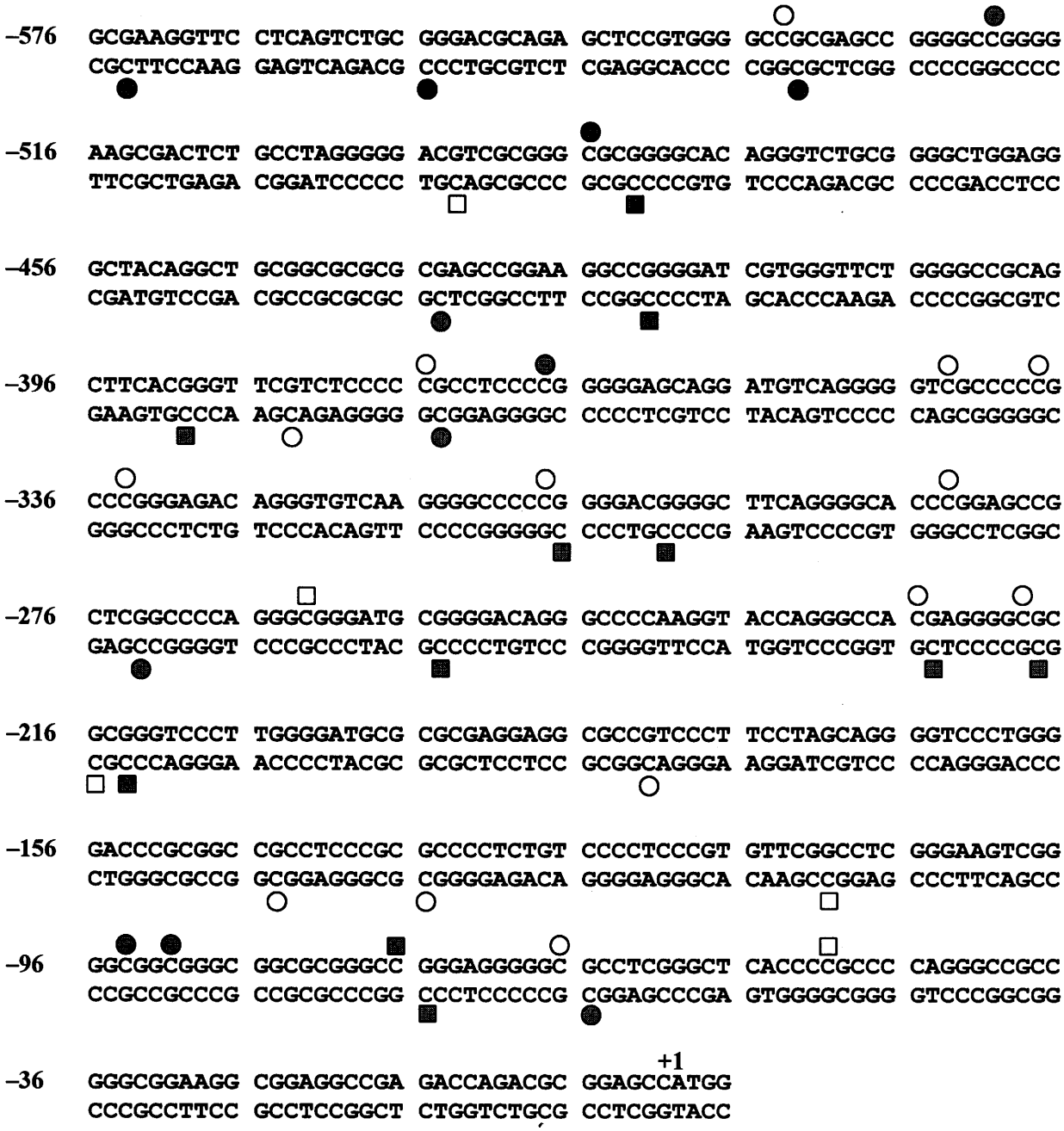

Figure 5. Summary of ABO transferase gene promoter region CpG methylation pattern differences between A^+ and A^- SW480 cell sublines. Circles mark cytosine residues of CpG dinucleotides that are unmethylated in A^+SW480, but methylated in A^- SW480; squares denote the opposite result. Filled circles or squares: difference was observed in at least two of the three experiments. Open squares or circles: difference was observed in one experiment. +1: start of translation.

pattern and the opposite pattern for the antisense strand. For the sense strand, only 1 of the 68 CpG sites tested (1.5%) showed unmethylated status in SW480 A^- in two experiments, while 13 of 68 (19%) did so in SW480 A^+ (Fig. 6).

Discussion

Among various types of aberrant glycosylation often observed in human cancer that affect the degree of malignancy, deletion or reduction of histo-blood group A or B, and/or enhanced expression of H (or Le^y/Le^b), have been correlated with patient survival rate [2]. In order to examine the molecular mechanism underlying this phenomenon, one should compare two tumor cell populations with different degree of A or B expression derived from the same tumor, rather than unrelated, randomly-selected cell lines derived from different tumors. Great effort has been spent on selection of such comparable tumor cell lines. Paired A^+ and A^- cell lines have been isolated from various colonic carcinomas. Among these, A^+ vs. A^- lines from SW480 were used throughout the present study, focused on the promoter activity of A transferase gene.

Several mechanisms are known for the downregulation of gene transcript levels in cells: (i) deletion of the gene [13], (ii) *de novo* mutation which affects the regulation of transcription [14], (iii) downregulation of transcription through dysregulation of trans-activating factors [15], (iv) methylation of cis regulatory sequence [16], and (v) enhanced turnover of mRNA [17]. Results of the present study indicate that deletion or reduction of A antigen expression in tumor cells is associated with downregulation of promoter activity, based on a defined 43 bp repetitive sequence element (i.e., mechanism iii), although the transcription factor involved in this down-regulation is not clearly identified. The present study also indicates a possibility that methylation of cis regulatory sequence at the proximal promoter region could be a cause of downregulation of A transcription (i.e., mechanism iv). The possibility of mechanisms i and ii was ruled out in the present study, and the possibility of mechanism v is difficult to determine because transcript level for A^+ cell line is extremely low.

The minisatellite containing CBF/NF-Y binding sites showed transcriptional promoting activity in extracts from not only KATOIII but also A^+ and A^- SW480 cells. Recently, Orita et al. (11) reported similarly perplexing results for a CBF/NF-Y site in the myeloperoxidase (MPO) promoter. They found that this site was responsible for MPO promoter activation upon stimulation of myeloid cells with G-CSF, but found no difference in abundance of bound NF-Y in G-CSF-stimulated vs. -unstimulated cells. These authors suggested that the transcriptional activation function of CBF/NF-Y is not determined simply by binding to the promoter, but possibly by post-translational modification of the factor as is found for C-EBP [18] and NF-IL6 [19]. A similar mechanism might explain our results for A^+ and A^- SW480 cells.

Anomalous methylation of gene promoter CpG islands is often found in cancer cells, and may be an explanation for epigenetic changes in tumor gene expression [20]. The results from three separate methylation specific PCR experiments (Figs. 5, 6) show a distinct differential pattern of unmethylated CpG sites in A^+ vs. A^- SW480 cells. For the majority of CpG sites that could be analyzed by these experiments, both cell lines showed full methylation (73% of sense strand sites and 65% of antisense strand sites). In most cases where differences could be seen, specific CpG sites were hypomethylated in A^+ SW480 and fully methylated in A^- SW480. This is particularly true for the sense strand, where 15 out of 18 differentially methylated CpG sites fit this pattern. For the antisense strand, sites with differential methylation were roughly evenly divided between those showing the above pattern and those showing the opposite pattern. Interestingly, of the 15 CpG sites showing hypomethylation in A^- SW480 and full methylation in A^+ SW480, 12 occurred on the antisense strand. The strand specific CpG site methylation pattern difference seen in Fig. 5 has, to our knowledge, not been described before. The significance of this observation is presently unclear.

The results differ from typical promoter methylation studies where active promoters are fully unmethylated and inactive promoters are fully methylated (reviewed in [16]). These studies typically compare cell lines or tissues that show clear positive/negative expression of the studied gene. This study has examined cells that differ only minutely in mRNA

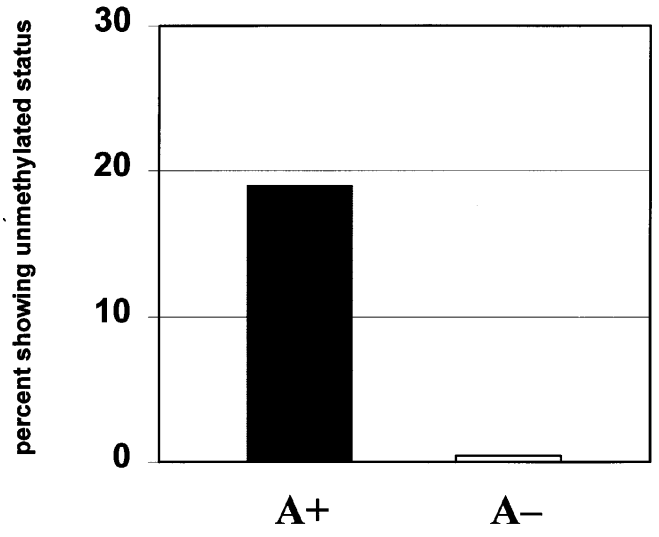

Figure 6. Differences in CpG sites showing methylation at C in promoter region DNA of SW480 A^+ vs. A^- cells. CpG sites showing unmethylated C in two experiments. Dark column: percentage showing unmethylated status in A^+ cells, in two separate experiments (13 of 68 CpG sites). White column: percentage showing unmethylated status in A^- cells, in two separate experiments (1 of 68 CpG sites). Note that unmethylated C, convertible to U by sodium bisulfite, is significantly higher in CpG sites in A^+ than in A^- cells.

expression (detectable only by RT-PCR in A^+ SW480 cells) and, therefore, may represent a higher resolution view of key CpG sites determining lack of expression vs. low level expression. The results described here are similar to those of Hsieh [21] in demonstrating a role of methylation density *per se* in determining gene expression.

Acknowledgments

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Note added at proof: While this paper was in press, it was reported that methylation of promoter region of *A* and *B* transferase genes is associated with low level of *A*/*B* antigen expression. However, the study used randomly-selected, genetically unrelated cell lines (Kominato Y, Hata Y, Takizawa H, Tsuchiya T, Tsukada J, Yamamoto F, *J Biol Chem* **274**(52): 37240–50, 1999).

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