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Transcriptional Silencing of *Cyclooxygenase-2* by Hyper-methylation of the 5′ CpG Island in Human Gastric Carcinoma Cells[1]

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ABSTRACT

It has been well established that overexpression of *Cyclooxygenase-2* (*Cox-2*) in epithelial cells inhibits apoptosis and increases the invasiveness of malignant cells, favoring tumorigenesis and metastasis. However, the molecular mechanism that regulates *Cox-2* expression has not been well defined in gastric carcinoma. In this study, we examined whether the *Cox-2* expression could be regulated by hyper-methylation of the *Cox-2* CpG island (spanning from −590 to +186 with respect to the transcription initiation site) in human gastric carcinoma cell lines. By Southern analysis, we found that three gastric cells (SNU-601, -620, and -719) without *Cox-2* expression demonstrated hyper-methylation at the *Cox-2* CpG island. A detailed methylation pattern using bisulfite sequencing analysis revealed that all of the CpG sites were completely methylated in SNU-601. Treatment with demethylating agents effectively reactivated the expression of *Cox-2* and restored IL-1β sensitivity in the previously resistant SNU-601. By transient transfection experiments, we demonstrate that constitutively active *Cox-2* promoter activities were exhibited even without an exogenous stimulation in SNU-601. Furthermore, when the motif of the nuclear factor for interleukin-6 expression site, the cyclic AMP response element, or both was subjected to point mutation, the constitutive luciferase activity was markedly reduced. In addition, *Cox-2* promoter activity was completely blocked by *in vitro* methylation of all of the CpG sites in the *Cox-2* promoter region with *Sss*I (CpG) methylase in SNU-601. Taken together, these results indicate that transcriptional repression of *Cox-2* is caused by hyper-methylation of the *Cox-2* CpG island in gastric carcinoma cell lines.

INTRODUCTION

Cox-1 and Cox-2[3] produce the intermediate PGH_2 from which PGs, prostacyclin, and thromboxanes are derived (reviewed in Ref. 1). *Cox-1* appears to be constitutively expressed in most cell types and is associated with the maintenance of physiological functions. In contrast, *Cox-2*, first identified as an immediate early response gene, can be rapidly induced by growth factors, cytokines, and tumor promoters and is associated with inflammation (2–6). Recent reports have demonstrated that elevated *Cox-2* expression is also present in many types of tumors (7–12). The overexpression of *Cox-2* in intestinal epithelial cells inhibits butyrate-induced apoptosis or stimulates the production of angiogenic factors, which in turn increases metastatic potential (7, 8). The lack of *COX-2* expression results in decreased neoplastic growth and in the number of tumors that develop in $APC^{\Delta 716}$ knockout mice (9). Moreover, in gastric cancer, *Cox-2* overexpression is only limited to cancer tissues compared with the accompanying normal mucosa (10, 11), and the intensity of *Cox-2* expression is correlated with the metastatic involvement of the lymph nodes (12).

However, the possibility that *Cox-2* has distinct biological functions on cell cycle progression has been suggested recently (13). Overexpression of wild-type *Cox-2* in human vascular endothelial cells suppresses cell cycle progression at the S-phase, with a concomitant increase in G_0/G_1 population. The same results were obtained by the introduction of two *Cox-2* mutant constructs, possessing only peroxidase activity but without cyclooxygenase activity (14), suggesting *Cox-2*-mediated cell cycle arrest by a PG-independent mechanism. Therefore, the biological effect of *Cox-2* overexpression on cell cycle progression appears to be different in each cell type, and the mechanism by which such differences occurs remains to be elucidated.

Interestingly, it has been demonstrated that *Cox-2* overexpression is less frequent in gastric carcinomas with MSI than in those without MSI (15). This is consistent with a previous finding (16) of a significant reduction in *Cox-2* expression levels in colorectal cancers with defective MMR. MMR deficiency is strongly associated with hypermethylation of the *hMLH1* gene, and this type of epigenetic alteration is accompanied by a down-regulation of *hMLH1* expression in gastric cancer (17, 18) and colorectal cancer (19, 20). This type of epigenetic gene inactivation is not limited to *hMLH1*. DNA methylation is one of the predominant mechanisms for inactivating various genes during the tumorigenesis of gastric carcinoma (21–26). These findings led us to investigate whether *Cox-2* expression could be regulated by DNA methylation. Very recently, Toyota *et al.* (27) suggested that *Cox-2* expression is inhibited by the aberrant methylation of the exon 1-coding region of the *Cox-2* gene in a subset of colorectal tumors. However, we and others (21, 28) have shown that the methylation of a small number of CpG sites in the promoter region, rather than in the coding region, could down-regulate the promoter activity of *p16INK4a* in gastric and bladder carcinoma. In this regard and for an improved understanding of the transcriptional regulation of the *Cox-2* gene, it is also important to determine the critical region that down-regulates *Cox-2* expression by DNA methylation.

In this study, we show that the transcriptional silencing of *Cox-2* is strongly related with the methylation status of the 5′ CpG island of the *Cox-2* gene in human gastric carcinoma cell lines. Moreover, functionally active Cox-2 is reinduced by demethylation of the *Cox-2* promoter in gastric carcinoma cell lines.

MATERIALS AND METHODS

Cell Cultures. Six well-defined human gastric carcinoma cancer cell lines (SNU-484, -601, -620, -638, -668, and -719) were obtained from the Korean Cell Line Bank (Seoul, Korea; Ref. 29) and grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS (Hyclone Laboratories, Inc., Logan, UT) and gentamicin (10 μg/ml) in a 5% CO_2 humidified atmosphere. HCT-116, a human colon cancer cell line, was obtained from the American Type Culture Collection (Rockville, MD) and was grown in McCoy's 5A with 10% FBS. HELs have been described previously (30).

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[3] The abbreviations used are: Cox, cyclooxygenase; PG, prostaglandin; PGH_2, prostaglandin H_2; IL, interleukin; NF-κB, nuclear factor κB; NF-IL6, NF for IL-6 expression site; CRE, cAMP response element; MSI, microsatellite instability; MMR, mismatch repair; 5-aza-CdR, 5-aza-2′ deoxycytidine; HEL, human embryonic lung fibroblasts; FBS, fetal bovine serum; poly(A), polyadenylate.

Plasmids. *Cox-2* promoter constructs (−1432/+59, −327/+59, −220/+59, −124/+59, −52/+59, KBM, ILM, CRM, KBM + ILM, and Triple M) have been described previously (4, 6, 31). Human *Cox-2* cDNA (*PHS2Δ3′UTR*; Ref. 32) was generously provided by Dr. Stephen M. Prescott (University of Utah, Salt Lake City, UT). Human Cox-1 cDNA (33) was a kind gift from Dr. Timothy Hla (University of Connecticut Health Center, Farmington, CN). The *Cox-2-S516M* and *S516Q* mutant constructs (14) were the kind gifts of Dr. William Smith (Michigan State University, East Lansing, MI). pGL2-Basic control vector and pSV-β galactosidase control vector were purchased from Promega.

RNA Extraction and Northern Blot Analysis. Using a TRI REAGENT kit (Molecular Research Center, Cincinnati, OH) and an Oligotex mRNA kit (Qiagen, Valencia, CA), total or poly(A) RNA was isolated from 10^7 to 10^8 cultured cells according to the manufacturer's instructions. Total cellular RNA (20 μg) or 2 μg of poly(A) RNA were electrophoresed on a 1% agarose gel containing formaldehyde and transferred to nylon membranes (Schleicher & Schuell, Keene, NH) by capillary blotting. The blots were hybridized using a *Cox-2* and *Cox-1* cDNA for RNA expression, as described previously (26). *β-actin* cDNA signals were used as an internal control to determine the integrity of RNA and the equality of lane loading (30).

Western Blot Analysis. Cells were washed with ice-cold PBS and suspended in an extraction buffer [20 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM pepstatin A, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 10 μg/ml aprotinin, 0.5 mg/ml soybean trypsin inhibitor, and 1 mM benzamidine] on ice for 15 min. Lysates were cleared by centrifugation at 13,000 rpm for 20 min. Equal amounts of cell extracts (100 μg) were resolved on 10% SDS-polyacrylamide denaturing gels, transferred onto nitrocellulose membranes, and probed with antihuman Cox-1 and Cox-2 antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-á-tubulin antibody (Sigma Chemical Co., St. Louis, MO) was used as a loading control. Detection was performed using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Southern Blot Analysis. Genomic DNA was prepared using a standard SDS and proteinase K protocol (30). Samples of genomic DNAs (10 μg) were digested with excess restriction enzymes (New England Biolabs, Beverly, MA), as described in "Results." Double digestions were performed sequentially so that each restriction enzyme was in optimal incubation buffer, with a precipitation step in between (21). The digested DNA fragments were separated by electrophoresis on 1% agarose gels and blotted onto a nylon membrane. The *Cox-2* promoter probe, spanning −1432 to +59 with respect to the transcription initiation site (GenBank accession nos. *D28235* and *AF044206*), was prepared by digestion of *phPES2(−1432/+59)* plasmid (31) with *Kpn*I and *Hin*dIII. The gel-purified insert was labeled with [α-^{32}P]dCTP by random primer extension. After hybridization, the membranes were washed in a series of solutions as described (34) and exposed to X-ray film at −70°C.

Bisulfite Modification and Sequencing Analysis. A total of 2 μg of genomic DNA obtained from gastric carcinoma cells was modified by sodium bisulfite (35). Primary and secondary PCR reactions were carried out in 50-μl reaction mixtures under the following conditions: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 10 min. Primers Cox2-A1 (5′-TGT ATA TTG AAG GTA GTT ATT TTA T-3′) and Cox2-d2 (5′-ACC AAA TAC TCA CCT ATA TAA CT-3′) were used to generate the primary PCR product for regions A to D. To obtain products for sequencing, a secondary round of PCR was performed using this primary PCR product with specific PCR primer sets for each region. Primers Cox2-A1 and Cox2-a2 (5′-AAA CAC TTA ACT TCC TCT CCA A-3′) were used for secondary amplification of region A. Primers Cox2-B1 (5′-TTG GAG AGG AAG TTA AGT GTT T-3′) and Cox2-b2 (5′-ATC CCC ACT CTC CTA TCT AAT-3′) were used for region B. Primers Cox2-C1 (5′-ATT AGA TAG GAG AGT GGG GAT-3′) and Cox2-c2 [5′-TCT AAA AAC (A/G)TC TAA CTA TAA AAC T-3′] were used for region C, and primers Cox2-D1 [5′-AAG TGA G(C/T)G TTA GGA GTA (C/T)GT T-3′] and Cox2-d2 were used for region D. Secondary PCR products were gel-purified and cloned into a TA cloning vector (Invitrogen, Carlsbad, CA). Individual plasmid molecules were then sequenced using the ABI PRISM 377 DNA sequencer (Perkin-Elmer, Foster City, CA; Ref. 21).

5-Aza-CdR Treatments. Cells were seeded at a density of 1×10^6 cells/100-mm dish and allowed to attach over a 24-h period. 5-aza-CdR (Sigma Chemical Co.) was then added to a final concentration of 1, 5, or 10 μM, and the cells were allowed to grow for the times indicated in "Results." The same concentration of DMSO was also used as a control for nonspecific solvent effects on cells (21). At the end of the treatment period, the medium was removed, and the RNA and protein were extracted for Northern and Western analysis.

PGE_2 Production and Determination of Cox Activity. Cells were treated for 3 days with DMSO (0.1%) or 5-aza-CdR (10 μM) in complete growth medium containing 10% FBS. At the end of the treatment period, the culture media were collected to determine the amounts of PGE_2 secreted by the cells. For the Cox-2 catalytic activity measurement, cells were washed twice, and fresh mediums (without FBS) containing 30 μM of arachidonic acid (Sigma Chemical Co.) were added for 30 min at 37°C. The media were then collected for PGE_2 analysis. To determine whether 5-aza-CdR-induced alterations in PGE_2 concentration are mediated by a change in Cox-2 activity, NS-398 (50 μM; Cayman Chemical, Ann Arbor, MI) was added to cultures 30 min before the Cox-2 activity was determined. The amounts of PGE_2 released by these cells were measured using a PGE_2 enzyme immunoassay kit (Cayman Chemical) according to the manufacturer's instructions. The production of PGE_2 was normalized according to the number of adherent cells present in the culture at the time of sampling. Results are expressed as pg of $PGE_2/10^6$ cells ± SD.

Transient Transfection and Luciferase Assays. SNU-601 and HCT-116 were seeded at a density of 3×10^5/well in a 6-well dish and grown to 60–70% confluence in complete growth media containing 10% FBS. For each well, 2 μg of plasmid DNA and 0.5 μg of pSV-β galactosidase control vector were cotransfected into cells with Lipofectin (Life Technologies, Inc.) according to the manufacturer's instructions. After 6 h, complete medium (with 10% FBS) was added, and the cells were further incubated for 18 h. Cells were then serum starved by replacing the growth mediums with serum-free medium for an additional 24 h. Luciferase activities were measured using a TR717 Microplate Luminometer (Tropix, Inc.) with a Bioluminescent Reporter Gene Assay System (Tropix, Inc.) according to the manufacturer's instructions. A pGL2-Basic control vector without insert was used as a negative control in the transfection experiments. Luciferase activities were normalized using β-galactosidase.

***In Vitro* Methylation Assay.** *In vitro* methylation assays were carried out according to the methods described by Robertson and Ambinder (36) and Kudo (37). Briefly, *Cox-2* promoter constructs (−1432/+59, −327/+59, and −220/+59) were incubated overnight with three units of *Sss*I (CpG) methylase (New England Biolabs)/μg of plasmid DNA in the presence (methylated) or absence (mock-methylated) of 1 mM *S*-adenosylmethionine, as recommended by the manufacturer. After phenol extraction and ethanol precipitation, equal amounts (2 μg) of methylated and mock-methylated reporter constructs were transiently transfected into SNU-601, and luciferase activities were examined as described above. Individual reactions were monitored by digestion with *Hpa*II or *Hha*I restriction enzymes.

Data Analysis. Results are representative of at least three independent experiments performed in triplicate and are presented as the means ± SD. Comparisons between groups were made using Student's paired *t* test.

RESULTS

Loss of Cox-2 Expression in Some Gastric Carcinoma Cells. Among the six gastric carcinoma cell lines, three cells (SNU-484, -638, and -668) expressed Cox-2 mRNA and protein, whereas the remaining three (SNU-601, -620, and -719) did not (Fig. 1*A*). Upon the addition of IL-1β (3, 5), Cox-2 mRNA was also induced after 2 h in SNU-484 and -638 (Fig. 1*B*). But treatment with IL-1β had no effect on *Cox-2* expression in SNU-601. Compared with the variable expression of *Cox-2*, all of the cell lines expressed relatively equal amounts of Cox-1 mRNA and protein.

Determination of the Methylation Status of the *Cox-2* Gene by Southern Analysis. The *Cox-2* promoter region, from −590 to +186 with respect to the transcription initiation site (GenBank accession no. *D28235*), contains 51 methylatable CpG bp and satisfies the estab-

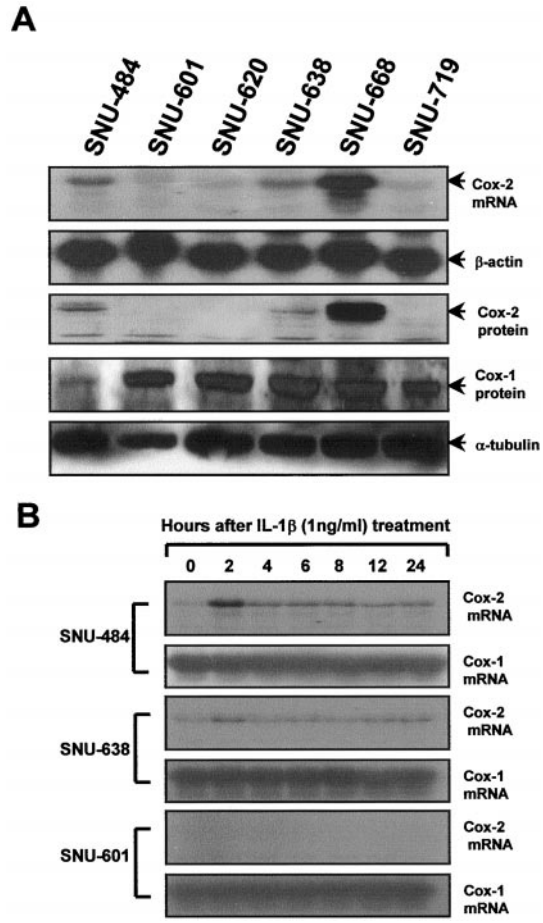

Fig. 1. Expression of Cox-2 mRNA and protein in gastric carcinoma cell lines. *A*, total RNA (20 μg/lane) and protein (100 μg/lane) were prepared from gastric carcinoma cells for Northern and Western blot analysis, as described in "Materials and Methods." *B*, IL-1β-mediated Cox-2 mRNA induction. Cells were plated in 100-mm dishes and grown to 60–70% confluence in culture mediums containing 10% FBS. Cells were made quiescent by incubation in serum-free medium for 24 h and further incubated for various periods with IL-1β (1 ng/ml), and total RNA was prepared.

lished definition of a CpG island (776 bp fragments with a G + C content $>$ 0.57 and an observed/expected presence of CpG $>$ 0.8; Refs. 38, 39). These findings led us to investigate whether the hypermethylation of the *Cox-2* CpG island could regulate *Cox-2* expression in gastric carcinoma cell lines.

A restriction map of the *Cox-2* CpG island is shown in Fig. 2*A*. Genomic DNAs from gastric carcinoma cell lines and HELs were treated with *Eco*RI. This resulted in a 4.3-kb fragment containing the promoter region of *Cox-2*, which was then digested with a methylation-sensitive restriction enzyme, namely *Hpa*II, *Hae*II, or *Hha*I. The genomic DNA of HEL was digested into a smaller fragment of approximately 1.5 kb, which suggested that the recognition sites for the three methylation-sensitive restriction enzymes are free of methylation (Fig. 2*B*). The digestion patterns obtained from SNU-484 and SNU-668 were consistent with that of HEL. However, three cell lines (SNU-601, -620, and -719) without *Cox-2* expression showed resistance to digestion with the three methylation-sensitive restriction enzymes, indicating that heavily methylated CpG-rich regions are present in the *Cox-2* CpG island. In the case of the SNU-638 with low *Cox-2* expression, a methylation-protected 4.3-kb fragment coexisted with a 1.5-kb band, showing that the methylation status of the *Cox-2* CpG island is somewhat heterogeneous in SNU-638. The existences of 2-kb fragments may indicate that some CpG sites located near the downstream of exon 1 are not completely methylated in SNU-601, -620, and -719. These findings strongly suggest that the methylation status of the *Cox-2* CpG island is related with the transcriptional silencing of *Cox-2* in gastric carcinoma cell lines.

Fine Mapping of Methylation Status of *Cox-2* by Bisulfite-Modification. To obtain more precise information about methylation status, high sensitivity mapping of the methylated cytosine was carried out by bisulfite modification (35). As shown in Fig. 3, *Cox-2* CpG island was divided into four regions (A to D) as defined by the PCR primers sets used to amplify bisulfite-modified genomic DNA. After the amplification of each region, PCR products were cloned, and individual plasmids were sequenced, as described in "Materials and Methods." SNU-484 was found to be hypo-methylated at the proximal 5′-end of the CpG island (64% of 15 CpG sites in region A and 30% of 10 CpG sites in region B), but methylated CpG sites were not detected in regions C and D (Fig. 3). However, all of the 51 CpG sites within regions A to D were completely methylated in SNU-601. Interestingly, SNU-638 was heavily methylated in regions C and D (82% of 12 CpG sites and 60% of 14 CpG sites, respectively), which was similar to SNU-601 (90% and 81%, respectively). On the other hand, the methylation status of region B (30%) of SNU-638 was much different from that of SNU-601 (93%). These results may suggest that hypo-methylation in region A may not block transcriptional initiation of *Cox-2* and that hyper-methylation of regions C and D is necessary but not sufficient to confer methylation-dependent repression of *Cox-2* in the presence of a hypo-methylated region B.

Treatment of 5-Aza-CdR Reinduced Functionally Active Cox-2 in SNU-601. The demethylating agent 5-aza-CdR (40, 41) was used to investigate whether the loss of methylation could induce *Cox-2* expression in nonexpressing cells. Three gastric cell lines were treated with 10 μM 5-aza-CdR for 3 days, and then *Cox-2* expression levels and Cox-2 enzymatic activity were analyzed, as described in "Materials and Methods" (Fig. 4). Treatment with 5-aza-CdR had little effect on *Cox-2* expression and Cox-2 activity in SNU-484. However, it is noteworthy that the addition of 5-aza-CdR resulted in the superinduction of the mRNA and protein level of Cox-2 in SNU-601. Consistent with elevated Cox-2 expression, PGE_2 production and Cox-2 enzymatic activity were also significantly higher after treatment with 5-aza-CdR, as compared with that achieved by DMSO treatment, in SNU-601 (Fig. 4, *B* and *C*). 5-aza-CdR-induced Cox-2 activity was completely abolished by NS-398 pretreatment, a selective inhibitor of Cox-2 (20). Similar to SNU-601, treatment of 5-aza-CdR elevated *Cox-2* expression and enzymatic activity in SNU-638 with heterogeneous Cox-2 methylation status. Cox-2 mRNA was also detected after treatment with 5-aza-CdR in SNU-620 and SNU-719 (data not shown). Taken together, these results suggest that the lack of *Cox-2* expression is attributable to a block of transcription caused by the methylation of the *Cox-2* CpG island and that functionally active Cox-2 can be activated by 5-aza-CdR in SNU-601.

Treatment with Demethylating Agent Caused Time-dependent Re-expression of *Cox-2* in SNU-601. To further elucidate the kinetic mechanism of 5-aza-CdR-mediated *Cox-2* induction, SNU-601 was exposed to increasing concentrations of 5-aza-CdR for 5 days. The induction of *Cox-2* was detected at a low dose of 1 μM 5-aza-CdR (Fig. 5*A*). SNU-601 was then monitored at various times after treatment with 5 μM 5-aza-CdR. Cox-2 mRNA and protein were easily detected between 48 and 72 h of treatment (Fig. 5*B*). Under demethylated conditions, the addition of IL-1β caused a rapid and sustained up-regulation of Cox-2 mRNA, which was maximally increased 2-fold after 2 h and remained elevated for 6 h (Fig. 5*C*). This induction of Cox-2 mRNA was associated with an increase in Cox-2 protein, which significantly increased 4 h after stimulation with IL-1β and lasted for 6 h. Therefore, demethylation by 5-aza-CdR resulted in

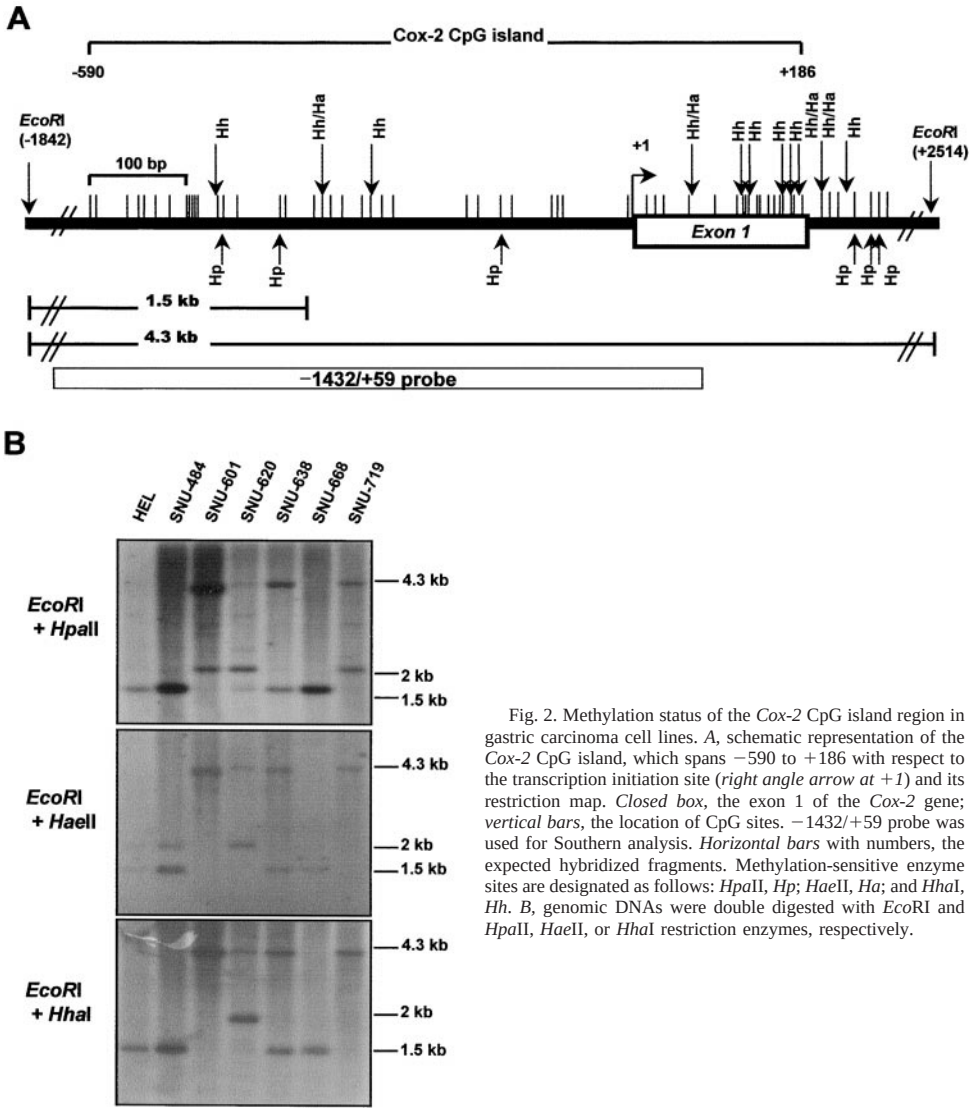

Fig. 2. Methylation status of the *Cox-2* CpG island region in gastric carcinoma cell lines. *A*, schematic representation of the *Cox-2* CpG island, which spans −590 to +186 with respect to the transcription initiation site (*right angle arrow at +1*) and its restriction map. *Closed box*, the exon 1 of the *Cox-2* gene; *vertical bars*, the location of CpG sites. −1432/+59 probe was used for Southern analysis. *Horizontal bars* with numbers, the expected hybridized fragments. Methylation-sensitive enzyme sites are designated as follows: *Hpa*II, *Hp*; *Hae*II, *Ha*; and *Hha*I, *Hh*. *B*, genomic DNAs were double digested with *Eco*RI and *Hpa*II, *Hae*II, or *Hha*I restriction enzymes, respectively.

the time-dependent reactivation of *Cox-2* and restored responsiveness to IL-1β in previously resistant SNU-601.

Constitutively Active Cox-2 Promoter Activity Was Detected in SNU-601 without Exogenous Stimulus. If the hyper-methylated *Cox-2* CpG island causes the transcriptional silencing of *Cox-2* expression, SNU-601 could be sensitized to the *Cox-2*-stimulating signal by the introduction of the unmethylated *Cox-2* CpG island. Recently, Kutchera *et al.* (42) demonstrated that the transiently transfected *Cox-2* promoter was constitutively active in the HCT-116 colon cancer cell line without exogenous stimuli. In this study, we also transiently transfected the *Cox-2* promoter constructs (−1432/+59; Fig. 6) into HCT-116 and SNU-601 and incubated the cells in a serum-starved state for 24 h to eliminate any interference by undefined substances within the serum. The relative *Cox-2* promoter activities were determined by luciferase assay and were normalized to the β-galactosidase activity. The results presented in Fig. 6*B* show remarkable constitutively active *Cox-2* promoter activities exhibited in SNU-601 despite serum starvation. Interestingly, the *Cox-2* promoter activities of SNU-601 were greater (approximately 7-fold) than those of the HCT-116.

Next, to elucidate the critical region of the *Cox-2* promoter responsible for the constitutively active *Cox-2* promoter activity, we introduced a series of *Cox-2* deletion constructs (−1432/+59, −327/+59, −220/+59, −124/+59, and −52/+59) into SNU-601 under the same serum-starved conditions, and the relative *Cox-2* promoter activities were determined (Fig. 6*C*). The *Cox-2* promoter activity was highest when the −327/+59 promoter construct was used. As the *Cox-2* promoter was shortened, lower basal activities were realized. Thus, the −52/+59 construct exhibited an approximate 95% relative decrease in *Cox-2* promoter activity compared with the −327/+59 construct. These results suggest that the constitutive *Cox-2* promoter activities were derived using the *cis*-acting elements located in the *Cox-2* promoter region, between nucleotides −327 and −53. In this region, three *cis*-acting elements (NF-κB, NF-IL6, and CRE) exist, which are known to be involved in cytokine-mediated promoter activity in the human *Cox-2* gene (4, 6, 31, 43). To precisely define which of these *cis*-acting elements is involved in the constitutively active *Cox-2* promoter activity, transient transfections were performed in SNU-601 using site-specific mutant *Cox-2* promoter constructs. As shown in Fig. 6*D*, the introduction of ILM (−327/+59 construct in which the NF-IL6 site had been mutagenized) and CRM (mutated at the CRE site) construct significantly decreased basal *Cox-2* promoter activity compared with the wild-type −327/+59 construct. However, the KBM construct (mutated at the NF-κB site) had no effect on *Cox-2* promoter activity. The *Cox-2* promoter activity derived from the double mutant construct (KBM + ILM; mutated at both the

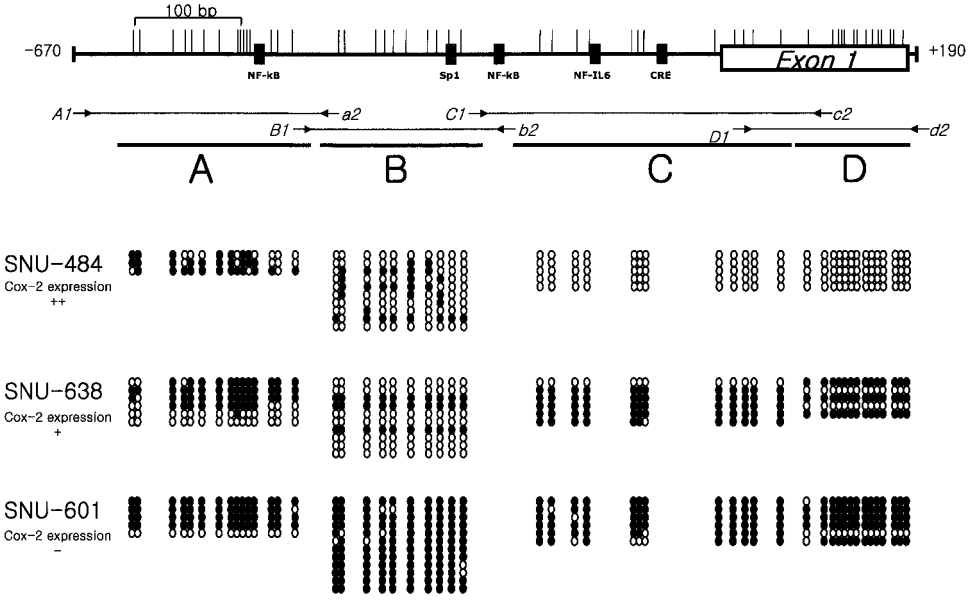

Fig. 3. Genomic sequencing data of the *Cox-2* CpG island in gastric carcinoma cell lines. *Cox-2* CpG island was divided into four regions (*A–D*) defined by the specific primer sets used for PCR amplification. The approximate locations and directions of the primers for each region are indicated by *arrows* denoted according to the particular segments to be amplified. Methylation status of 51 CpG sites of Cox-2-positive cells (SNU-484 and -638) and Cox-2-negative cells (SNU-601) were compared. Each *row of circles* represents a single plasmid cloned and sequenced from PCR products generated from amplification of bisulfite-treated DNA. ○, unmethylated cytosines; ●, methylated cytosines.

NF-κB and NF-IL6 sites) was similar to that of the ILM construct, demonstrating that the NF-κB site is not involved in constitutive *Cox-2* promoter activity in SNU-601. Finally, a triple mutant construct (Triple M; mutated at the NF-κB, NF-IL6, and CRE sites) completely abolished basal *Cox-2* promoter activity. These results suggest that constitutively active *Cox-2* promoter activity was caused by using the NF-IL6 and CRE elements existing in the *Cox-2* promoter region even without an added stimulus in SNU-601.

In vitro methylation analysis (36, 37) was performed to examine whether constitutively active *Cox-2* promoter activity was inhibited by the methylation of *Cox-2* CpG island. pGL2-Basic control vector (a total 256 CpG sites are located in the vector sequences) and a series of *Cox-2* promoter deletion constructs, −1432/+59, −327/+59, and −220/+59 (including 47, 19, and 12 CpG sites, respectively), were methylated with *Sss*I (CpG) methylase and transiently transfected into SNU-601. CpG methylation of the pGL2-Basic control vector reduced the luciferase activity to 50%. However, compared with that of the unmethylated vector, CpG methylation of the *Cox-2* promoter entirely abolished its activity (Fig. 6*E*). Taken together, our findings strongly suggest that the transcriptional silencing of *Cox-2* expression was caused by hyper-methylation of *Cox-2* CpG island in SNU-601.

DISCUSSION

In the present study, we provide experimental evidence that the constitutively active *Cox-2* promoter activity, which is induced by using the NF-IL6 and CRE elements, is completely blocked by heavy methylation of the *Cox-2* CpG island in gastric carcinoma cells.

It has been suggested recently by Toyota *et al.* (27) that *Cox-2* has an atypical CpG island that begins just after the transcriptional start site and that encompasses 350 bp near the exon 1-coding region of the *Cox-2* gene. They also reported that methylation closest to its transcription initiation site is limited to neoplastic tissues and is associated with a loss of *Cox-2* expression in colorectal cancer. However, in this study, we have shown that *Cox-2* CpG islands are present more extensively than they expected. The *Cox-2* promoter region from −590 to +186 (which contains 51 CpG sites with a G + C content of 0.578 and an observed/expected presence of CpG of 0.8) with respect to the transcriptional start site clearly meets the established criteria for a CpG island (Fig. 2*A*). To elucidate the importance of this CpG island, we compared the methylation status of this CpG island with *Cox-2* expression in gastric carcinoma cell lines. By Southern and bisulfite-modification sequencing analysis, we have shown that three gastric cells, the *Cox-2* expression of which was not observed, display hyper-methylation at this *Cox-2* CpG island. The restoration of *Cox-2* expression and IL-1β sensitivity by 5-aza-CdR treatment (40, 41) provides a molecular mechanism that may explain the loss of *Cox-2* expression in some gastric cell lines with methylation at the *Cox-2* CpG island region. In addition, here we show for the first time that the reinduced Cox-2 protein retains its functionally active enzymatic activity, *i.e.*, its cyclooxygenase and peroxidase activities (44), and thus metabolizes arachidonic acid into PGs (1), indicating that the demethylation of the *Cox-2* CpG island can successfully recover its function. The significance of the methylation status of this CpG island is further supported by our observations that a *Cox-2* promoter construct, which is inactive in normal cells without exogenous stimulation (4, 5), is constitutively active in SNU-601 even under serum-depleted conditions. Especially, the NF-IL6 and CRE elements may be involved cooperatively in the transcriptional regulation of *Cox-2* in SNU-601, even in the absence of any exogenous stimuli. A number of recent reports (45–48) also described a pivotal role of these sites in *Cox-2* gene expression in a wide range of cells, in response to a variety of stimuli. On the other hand, *Cox-2* promoter activity is almost completely abolished by the introduction of CpG-methylated *Cox-2* promoter, which is similar to results obtained in investigations of *p16INK4a* (28) and *p14ARF* (49). These results strongly suggest that if hyper-methylation of this *Cox-2* CpG island did not exist, SNU-601 might have expressed high steady-state levels of Cox-2 mRNA and protein and elevated PGE_2 biosynthesis (12). However, further evaluation is required to determine whether the relationship between the methylation status of this CpG island and the loss of *Cox-2* gene expression is valid in primary gastric carcinoma tissues, because no methylation-positive case was detected among eight cases examined in this study (data not shown).

Other workers and ourselves have demonstrated previously that the *de novo* methylation of only a small number of CpG sites in the promoter region can down-regulate the promoter activity of *p16INK4a* in bladder (28) and gastric carcinomas (21). In the case of *Cox-2*, we were unable to identify this type of methylation "hot spot," which is a critical region capable of inhibiting the transcriptional

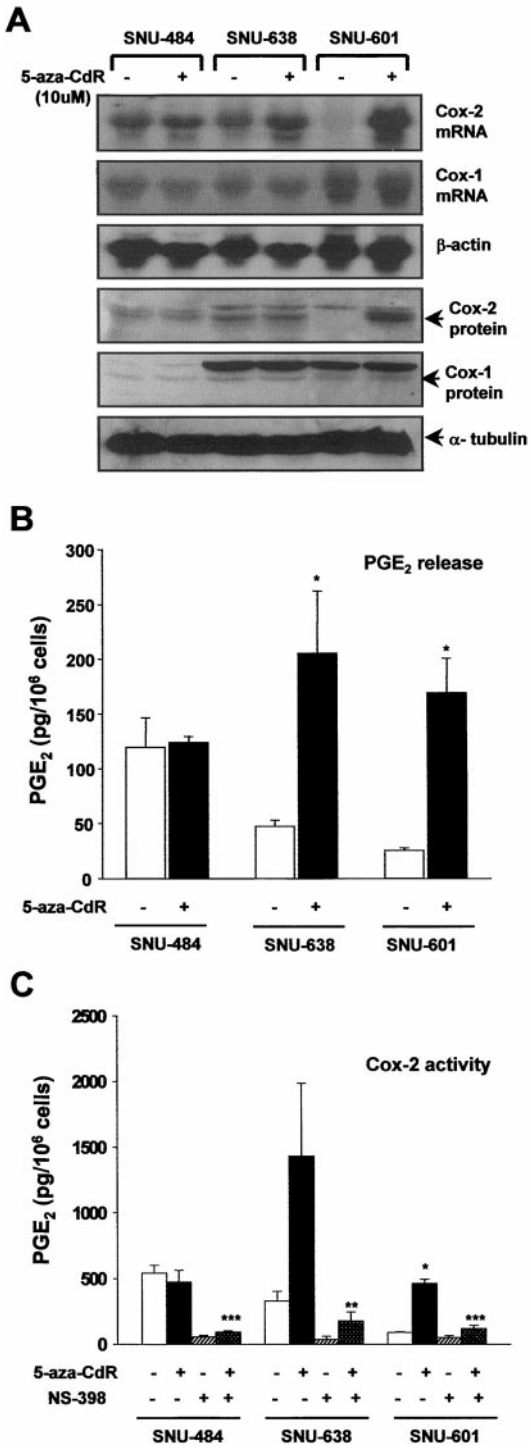

Fig. 4. Restoration of *Cox-2* expression and Cox-2 activity after 5-aza-CdR treatment. Cells were treated for 3 days with DMSO (vehicle; 0.1%) or 10 μM of 5-aza-CdR, as indicated. *A*, total RNA and cell lysates were isolated for detection of the re-expressed levels of *Cox-2*. Under identical experimental conditions, supernatants were collected for PGE_2 release measurement (*B*), and the Cox-2 activity (*C*) was determined as described in "Materials and Methods." *Columns*, means; *Bars*, SD ($n = 3$). (*, $P < 0.01$ *versus* DMSO-treated control; ** and ***, $P < 0.05$ and $P < 0.01$ *versus* NS-398-untreated control, respectively).

initiation of *Cox-2* by the methylation of only a small number of specific CpG sites. Dense methylation at the 3′-end of the *Cox-2* CpG island (region C and D; Fig. 3) is necessary but not sufficient for the complete inhibition of *Cox-2* expression under hypo-methylation conditions at the 5′-end of the *Cox-2* CpG island (region B).

What is the functional significance underlying the methylation-mediated transcriptional loss of *Cox-2* in gastric carcinoma cells? To the best of our knowledge, this type of epigenetic gene inactivation is only restricted to tumor-suppressor genes during tumorigenesis (reviewed in Refs. 50, 51). Moreover, accumulating evidence indicates that *Cox-2* overexpression is associated with gastric cancer (10, 12), so that the loss of *Cox-2* expression may provide a disadvantage for cancer cell survival, which may in turn detrimentally affect the development of gastric cancer. Interestingly, it was reported recently that the overexpression of *Cox-2* is less frequent in colorectal (16) and gastric cancer cells (15) with MSI rather than in cells without MSI. Because there is a strong link between hyper-methylation of the *hMLH1* gene and the MSI phenotype in gastric (17, 18) and colorectal cancer (19, 20), this raises the possibility that *Cox-2* CpG methylation is associated with the MSI phenotype caused by a defective MMR (52). However, no significant correlation between *Cox-2* methylation and the MSI phenotype was found in the gastric carcinoma cells examined in

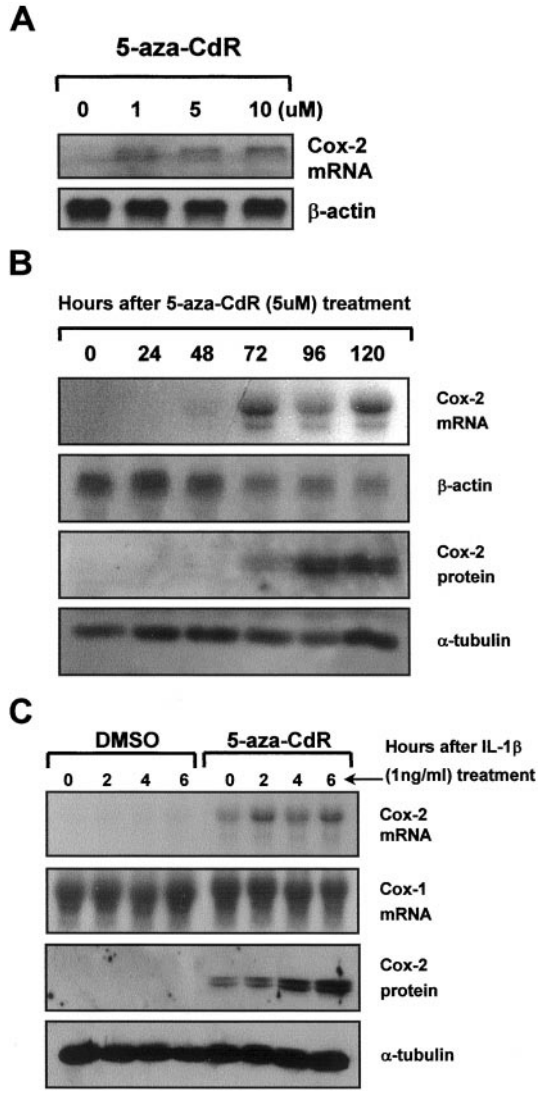

Fig. 5. Time-dependent re-expression of *Cox-2* gene and restoration of IL-1β-sensitivity after 5-aza-CdR treatment. *A*, SNU-601 was treated with 1, 5, or 10 μM of 5-aza-CdR for 5 days and poly(A) RNA were isolated. *B*, 5 μM of 5-aza-CdR was added to SNU-601, and total RNA or proteins were prepared at the indicated times. *C*, SNU-601 were treated with 5-aza-CdR (10 μM) or DMSO (0.1%) for 48 h, and cells were made quiescent by incubation in serum-free medium for 24 h and further incubated for various periods with IL-1β (1 ng/ml), and the total RNA or proteins were prepared.

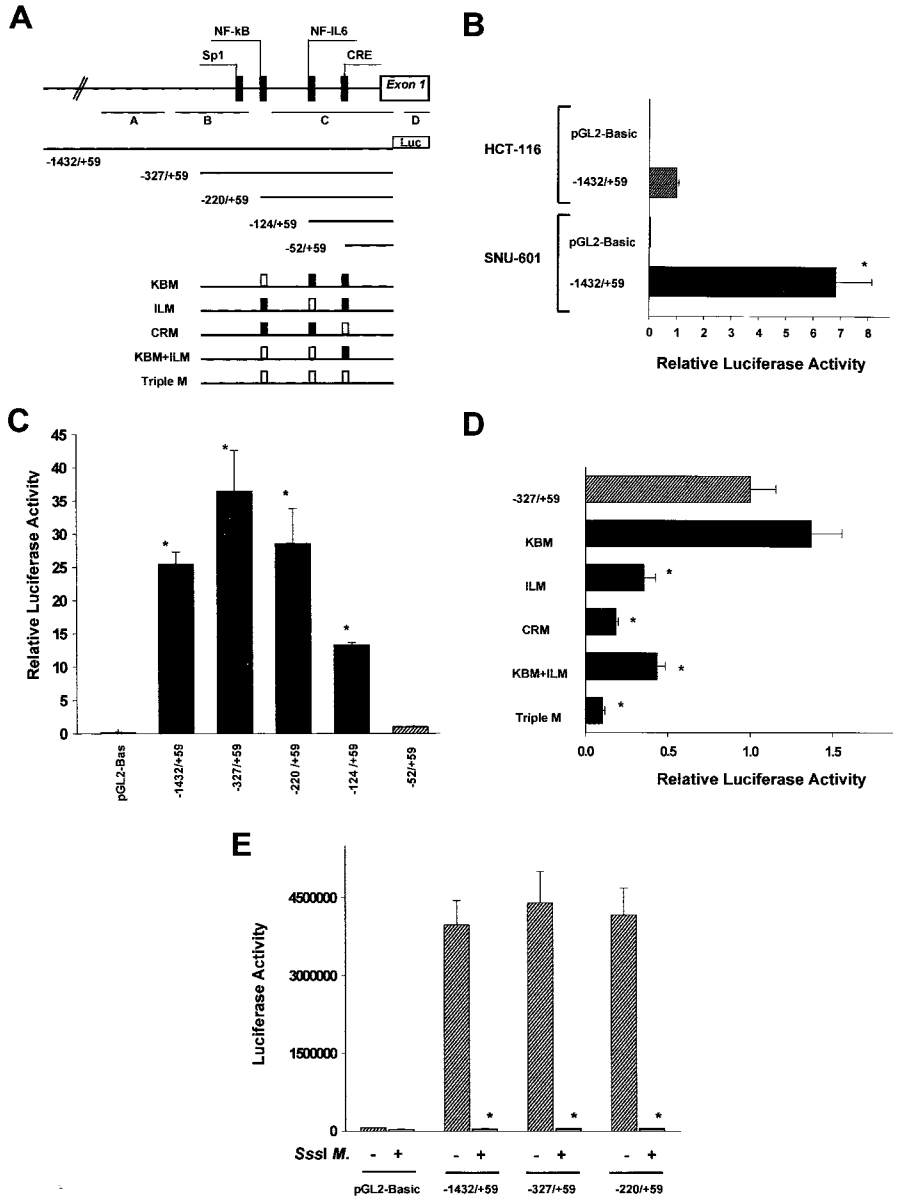

Fig. 6. Constitutive *Cox-2* promoter activity in SNU-601 without exogenous stimuli. *A*, 5′-regulatory region of the human *Cox-2* gene. The *Cox-2* promoter deletion and site-specific mutant constructs are presented schematically. The sequences that serve as binding sites for transcription factors (■) or mutated sites (□) are labeled accordingly. As described under "Materials and Methods," cells were transfected with *Cox-2* promoter constructs or pGL2-Basic control vector together with pSV-β galactosidase vector. After transfection, cells were maintained in a serum-starved state for 24 h before cell lysates were analyzed. Expression was assessed as luciferase activity and was normalized to the β-galactosidase activity. *B*, *Cox-2* promoter activity of SNU-601 was compared with that of HCT-116. Under the same conditions, SNU-601 was transfected with a series of *Cox-2* promoter deletions (*C*) or mutant constructs (*D*). *E*, complete inhibition of *Cox-2* promoter activity by *in vitro* CpG methylation. The *Cox-2* promoter constructs (−1432/+59, −327/+59, and −220/+59) and pGL2-Basic control vector were methylated *in vitro* with *Sss*I (CpG) methylase. Methylated (■) or mock-methylated (▨) constructs (2 μg) were transiently transfected into SNU-601. Luciferase activity represents data that have been normalized with β-galactosidase activity. *Columns*, means; *bars*, SD ($n = 5$ for *B* and *D*; and $n = 3$ for *C* and *E*). (*, $P < 0.01$ comparing ▨ *versus* ■).

this study (53), indicating that MSI may not be involved in the mechanisms responsible for *Cox-2* methylation in gastric carcinoma cells. Although Yamamoto *et al.* (15) found that SNU-638 with the replication error phenotype+ did not express Cox-2 protein, the results of the present study show that low levels of Cox-2 mRNA and protein are expressed in SNU-638. The heterogeneous methylation status of the *Cox-2* CpG island in SNU-638 may go some way toward explaining the difference between the above findings.

Recently, Trifan *et al.* (13) reported that the overexpression of *Cox-2* cDNA or mutant *Cox-2* constructs suppressed cell cycle progression at the S-phase, with a concomitant increase in the G_0/G_1 population in human vascular endothelial cells. This implies that the overexpression of *Cox-2* may induce G_0/G_1 growth arrest by an uncharacterized nonprostanoid-dependent signaling pathway. To elucidate the effects of *Cox-2* overexpression on the cell cycle, in this study, SNU-601 was transiently transfected with wild-type *Cox-2* cDNA (PHS2Δ3′UTR; Ref. 32) or the two mutant *Cox-2* cDNAs (S516M and S516Q, which possess only peroxidase activity, are without cyclooxygenase activity, and, therefore, mimic aspirin-treated Cox-2; Ref. 14). Cell cycle distributions were then analyzed by DNA staining with propidium iodide followed by fluorescence-activated cell sorting analysis. Neither the wild-type *Cox-2* cDNA nor the two mutant types had any effect on cell cycle progression in SNU-601 (data not shown). However, we were unable to exclude the possibility that this lack of responsiveness may result from the genetic and/or epigenetic alternation of other gene(s) that may contribute to the proliferation of SNU-601. Indeed, it is important to point out that the transcriptional initiation of *p16INK4a* (21, 26), *TIMP-3* (22), and *TGF-β type I receptor* gene (23) in SNU-601 is also blocked by hyper-methylation of the 5′ CpG island in these genes. Thus, the functional significance of the down-regulation of *Cox-2* during carcinogenesis merits further elucidation.

In this study, we demonstrate that the transcriptional silencing of *Cox-2* is caused by the hyper-methylation of the *Cox-2* CpG island and results in the inhibition of the binding of a certain transactivator that essentially uses the NF-IL6 and CRE elements in SNU-601. Although the biological functions associated with the presence of methylation-mediated transcriptional silencing of the *Cox-2* gene are not clear, the DNA methylation of the *Cox-2* CpG island is a new mechanism for the down-regulation of *Cox-2* expression in gastric carcinoma cell lines.

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