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Transcriptional Regulation of Basal Cyclooxygenase-2 Expression in Murine Lung Tumor-Derived Cell Lines by CCAAT/Enhancer-Binding Protein and Activating Transcription Factor/cAMP Response Element-Binding Protein

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ABSTRACT

Cyclooxygenase-2 (COX-2) is frequently expressed in cancer cells, contributing to tumor development. Most studies of COX-2 expression have examined artificially induced expression in noncancer cells rather than basal expression in cancer cells. Therefore, basal COX-2 expression and its regulation were examined in cell lines derived from a murine model of lung adenocarcinoma. The presence of COX-2 protein in these cells was demonstrated by Western analysis. COX-2 promoter activity was repressed by U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene], a mitogen-activated protein kinase kinase inhibitor, as well as SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole], an inhibitor of p38 mitogen-activated protein kinase, substantiating the involvement of these signal transduction pathways in the regulation of basal COX-2 expression. Retinoic acid also repressed promoter activity, yet increased activity significantly in one cell line after 18 and 30 h of treatment. Deletions of the murine COX-2 promoter revealed that the 5′ transcription factor binding sites were not required for basal expression, including the only nuclear factor-κB sites of the promoter. Site-directed mutagenesis of the 3′ C/EBP (CCAAT/enhancer-binding protein) sites inhibited promoter activity by 20 to 55%, while mutation of the 3′ ATF/CREB/AP-1 (activating transcription factor/cAMP response element-binding protein/activator protein-1) site inhibited activity by 70%. Mutation of the 3′ upstream stimulatory factor site did not affect promoter activity. Electrophoretic mobility shift assays indicated that the AP-1 transcription factor does not bind to the 3′ ATF/CREB/AP-1 site, leaving C/EBP and ATF/CREB as the major transcriptional regulators of basal expression of COX-2 in these lung tumor-derived cell lines and identifying new targets for the prevention/treatment of lung cancer through the modulation of COX-2 expression.

COX-2 catalyzes the rate-limiting step in the synthesis of prostaglandins, usually as part of the inflammatory response. This enzyme is expressed at low or undetectable levels in most tissues, but is induced rapidly in response to many types of signaling molecules such as growth factors and cytokines. COX-2 is frequently expressed in cancer cells and contributes to tumor progression via documented effects on the proliferation and apoptosis rates of tumor cells (reviewed by Fosslien, 2000). COX-2 expression also contributes to tumor development in vivo by increasing tumor cell invasiveness and the secretion of factors that regulate angiogenesis and host immune response (Huang et al., 1998; Tsujii et al., 1998; Rozic et al., 2001). Consequently, many studies have been undertaken examining the regulation of COX-2 expression. Unfortunately, most of these studies utilize cell lines that are not derived from cancer cells and do not have high endogenous levels of COX-2. COX-2 expression in these cell lines is induced by the administration of a growth factor, cytokine, or tumor promoter, and the mechanism that produces the subsequent increase in COX-2 expression is then analyzed (e.g., Jones et al., 1999; Chen at al., 2000, 2001; Subbaramaiah et al., 2000). In some cases, this analysis

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ABBREVIATIONS: COX-2, cyclooxygenase-2; NNK, 4-(methylnitrosamino)-1-(3-pyridal)-1-butanone; C/EBP, CCAAT/enhancer-binding protein; AP-1, activator protein-1; ATF, activating transcription factor; CRE, cAMP response element; CREB, CRE-binding protein; CREM, CRE modulator; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, MAP kinase kinase; NF-IL6, nuclear factor for interleukin-6 expression; NF-κB, nuclear factor κB; PCR, polymerase chain reaction; RA, all-*trans*-retinoic acid; SRB, sulforhodamine B; TBS, Tris-buffered saline; USF, upstream stimulatory factor; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole; PD98059, 2′-amino-3′-methoxyflavone.

provides insight into how COX-2 might be up-regulated in cancer cells or during the inflammatory response. However, the connection between this artificially induced expression and the in vivo up-regulation of COX-2 during the transition from normal cell to cancer cell is often not clear, with findings that frequently differ from one cell type to the next.

Only two studies have been conducted that examined the mechanisms responsible for basal, uninduced COX-2 expression in epithelial cancer cells (Kim and Fischer, 1998; Shao et al., 2000). Human colon and murine skin carcinoma-derived cell lines were used in these studies. The mechanisms regulating basal COX-2 expression in lung cancer, the leading cause of death due to cancer in the United States, have not been examined. The purpose of the current study was to identify the regulators of basal COX-2 expression in lung cancer cells and to determine whether these regulators differ from those involved in induced and basal expression in other cell types.

The model chosen for this study was the NNK-treated A/J mouse, a well characterized animal model of lung adenocarcinoma (Belinsky et al., 1992), the most common type of lung cancer. The adenocarcinomas in this model are thought to arise, in part, through NNK-induced mutation of the *Ki-ras* gene and consequent activation of Ras, an important growth-regulatory protein whose dysfunction is linked to the development of many human lung tumors. COX-2 protein and mRNA are frequently detected in human adenocarcinomas (Hida et al., 1998; Wolff et al., 1998; Watkins et al., 1999). Similarly, COX-2 is expressed in many of the NNK-induced murine lung tumors, particularly at the early stages of development (Wardlaw et al., 2000), and most significantly, COX-2 inhibitors repress tumor development in the lungs of NNK-treated mice (Rioux and Castonguay, 1998).

Cell lines derived from several A/J lung tumors were used in the current study. This study revealed that COX-2 protein is constitutively expressed in these cell lines and that basal COX-2 expression is regulated through the C/EBP and ATF/CREB transcription factor binding sites within the murine COX-2 promoter, possibly via the MEK/ERK and p38 MAP kinase signaling pathways. Therefore, the C/EBP and ATF-1/CREB-1 transcription factors could constitute additional targets for the prevention and/or treatment of lung cancer through the modulation of COX-2 expression.

Materials and Methods

Cell Lines. The CL13 and CL30 cell lines were derived from A/J mouse lung tumors induced by NNK. This murine model of lung cancer has been described in detail by Belinsky et al. (1992). The Spon4 cell line was derived from a spontaneously occurring A/J mouse lung tumor. Both induced and spontaneous A/J lung tumors are thought to arise from alveolar type II cells. All three tumor-derived cell lines are transforming and harbor mutations of the *Ki-ras* gene.

The C10 cell line was kindly provided by Dr. Fred Tyson (National Institute of Environmental Health Sciences, Research Triangle Park, NC). This cell line was derived from normal BALB/c mouse lung tissue. C10 cells resemble alveolar type II epithelial cells; they are nontransforming and do not harbor any *ras* mutations (Malkinson et al., 1997).

All cell lines were cultured in Dulbecco's modified Eagles/F12 medium containing 5% fetal calf serum and 25 μg/ml gentamicin. Cells were maintained at 37°C in a humid atmosphere containing 5% CO_2.

Western Analysis. Protein extracts of cultured cells were prepared using mammalian protein extraction reagent (Pierce, Rockford, IL). Protein concentrations were determined by Bio-Rad protein assay (Hercules, CA). Cellular proteins (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then transferred to a polyvinylidene difluoride membrane by a tank-transfer system. The protein blots were incubated for 2 h at room temperature in a blocking solution composed of TBS, pH 7.6, plus 5% (w/v) dry milk (Bio-Rad). The blots were incubated with primary antibody at room temperature for 2 h or overnight at 4°C with antibody diluted 1:250 in TBS plus 3% (w/v) bovine serum albumin. The COX-2 antibody (C22420) was purchased from BD Biosciences (Franklin Lakes, NJ). Following incubation with the primary antibody, the blots were rinsed with TBS plus 0.1% Tween 20 and incubated for 1 h at room temperature with sheep anti-mouse IgG conjugated to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) diluted 1:3000 in TBS plus 3% milk. After rinsing with TBS plus Tween 20, the blots were incubated with enhanced chemiluminescent detection reagents (Amersham Biosciences) and exposed to film. The blots were stripped with Restore buffer (Pierce), and the analysis was repeated using rabbit anti-actin (1:500) (A2066; Sigma-Aldrich, St. Louis, MO) to verify loading of equal amounts of protein.

mRNA Quantitation. RNA was isolated from cultured cells using TRI reagent (Molecular Research Center, Cincinnati, OH), and COX-2 mRNA levels were quantitated by ribonuclease protection assay as described (Wardlaw et al., 2000).

Transfection/Luciferase Assay. Cells were grown to approximately 50% confluence in 6-well culture plates. The cells were rinsed with phosphate-buffered saline then incubated for 3 h with 1 ml of serum-free medium containing 2 μg of DNA, 10 μl of LipofectAMINE (Invitrogen, Carlsbad, CA), and 8.5 μl of PLUS reagent (Invitrogen). The DNA consisted of 1 μg of luciferase expression vector, with or without COX-2 promoter sequences, and 1 μg of pSV-β-galactosidase control vector (Promega, Madison, WI). After the 3-h incubation, 1 ml of serum-containing medium (10%) was added to each well. For the inhibitor studies, this medium also contained a 2× concentration of inhibitor in DMSO or DMSO only (0.1% in all wells). After 24 h, the cells were rinsed with phosphate-buffered saline and lysed with reporter lysis buffer (Promega). The cell debris was pelleted by centrifugation, and the supernatant was assayed for both luciferase and β-galactosidase activities. The luciferase assay was conducted by adding 100 μl of luciferase assay reagent (Promega) to 20 μl of cell extract in a 96-well plate. Reagent delivery, measurement of light production, and data processing were conducted via a MLX Microplate Luminometer and Dynex Revelation (version 4.06) software (Dynex Technologies, Chantilly, VA). The β-galactosidase activity was quantitated with a β-galactosidase assay kit (Promega). Due to the structure of the β-galactosidase promoter, β-galactosidase activity should remain constant regardless of treatment condition, making β-galactosidase activity a good indicator of well-to-well variations in vector uptake that may occur within cell lines. Thus, β-galactosidase activities were used to adjust the luciferase activities for these slight variations in transfection efficiency. All transfections were conducted in duplicate. Each duplicate was assayed in duplicate.

Promoter Analysis. The murine COX-2 promoter sequence was obtained from GenBank (accession no. M82862). The transcription factor binding sites within the promoter were located by means of the "public domain" version of MatInspector software, which utilizes a library of matrix descriptions to locate binding sites and assigns quality ratings to the resulting matches within a sequence (Quandt et al., 1995).

Promoter Deletions. Six PCR reactions were carried out using A/J mouse lung DNA as a template, generating one PCR product corresponding to the full-length murine COX-2 promoter and five shorter products. Each 5′ primer contained a *Mlu*I restriction site, and the 3′ primer contained a *Bgl*II site. The PCR products were

purified from an agarose gel (GeneClean kit; Qbiogene Inc., Carlsbad, CA). Promega's pGL2-Basic luciferase expression vector was digested with *Bgl*II, *Mlu*I, and calf intestinal alkaline phosphatase. The digested plasmid was purified with Wizard DNA Clean-Up Resin (Promega). Following digestion of the promoter fragments with *Bgl*II and *Mlu*I and a second gel purification, the promoter fragments and digested luciferase vector were ligated together with DNA ligase. A small amount of each ligation reaction was used to transform INVαF' chemically competent cells (TA cloning kit; Invitrogen). Plasmid was purified from individual bacterial colonies using a Wizard DNA Miniprep kit (Promega). Purified plasmid preparations were tested for the presence of insert by digestion with *Bgl*II and *Mlu*I followed by analysis of the digestion products on an agarose gel. Positive plasmid samples were subjected to DNA sequencing to confirm the presence of the COX-2 promoter insert as well as the correct nucleotide sequence.

Site-Directed Mutagenesis. MatInspector software (described above) was also used to determine the essential nucleotides within the C/EBP, ATF/CREB, and USF binding sites. At least two essential nucleotides were selected for mutation within each site. The ATF/CREB mutation also affected the AP-1 site found within the ATF/CREB site. The ATF/CREB site slightly overlapped the USF site, thus the mutations within these two sites were carefully chosen to not disrupt the other site.

The selected mutations were introduced into the full-length COX-2 promoter/pGL2 luciferase construct using the Tranformer site-directed mutagenesis kit (BD Biosciences Clontech, Palo Alto, CA). In brief, the promoter-containing plasmid was denatured, a primer containing the desired mutation was annealed to the single-stranded plasmid, the double-stranded plasmid was regenerated with the addition of polymerase and ligase, and the new plasmid was amplified in repair-deficient bacteria. The probability of success was increased by the addition of a second primer that mutated a unique restriction site (*Bam*HI) in the promoter construct to a second unique restriction site (*Apa*I). The resulting plasmid population was then digested with the first restriction enzyme prior to cell transformation. Mutated plasmids were not cut and were therefore taken up by the cells at a much higher frequency than nonmutated (cut) plasmids. The amplified plasmids were purified and used to transform TOP10 chemically competent cells (TA cloning kit; Invitrogen). Plasmid was purified from individual colonies (five colonies per mutation). Purified plasmid preparations were tested for mutation by digestion with *Apa*I. Mutations were confirmed by DNA sequencing.

Electrophoretic Mobility Shift Assay. Crude nuclear extracts were prepared by the method of Schreiber et al. (1989) with slight modification. Pelleted cells were resuspended in 400 μl of cold hypotonic buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM DTT, plus 1 Mini Complete protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN) per 10 ml]. The cells were allowed to swell on ice for 15 min. The cells were lysed with the addition of 25 μl of 10% Nonidet P-40 and vigorous vortexing. The nuclei were pelleted by centrifugation and the supernatant discarded. The nuclei were then rocked vigorously for 15 min at 4°C in lysis buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM DTT, plus 1 Mini Complete protease inhibitor cocktail tablet per 10 ml). The debris was pelleted by centrifugation, and the supernatant (nuclear extract) was stored at −20°C.

To prepare the radiolabeled probe, single-stranded forward and reverse oligonucleotides [synthesized by Sigma-Genosys (The Woodlands, TX) were annealed by heating to 95°C and cooling slowly to room temperature in annealing buffer (10 mM Tris, pH 7.5, 50 mM $MgCl_2$). The double-stranded oligonucleotide (25 pmol) was then radiolabeled by incubating at 37°C for 1 h in 50 mM Tris, pH 7.5, 7.5 mM $MgCl_2$, 5 mM DTT, with 1.5 μg of bovine serum albumin, 20 units of T4 polynucleotide kinase, and 125 μCi [^{32}P]ATP (3000 Ci/mmol) in a total volume of 30 μl. Heating to 75°C for 10 min stopped the reaction. The labeled oligonucleotide was then purified with a MERmaid kit (Qbiogene Inc.). Specific activities were approximately 5000 cpm/μl.

The radiolabeled probe (approximately 0.4 ng) was incubated for 15 to 30 min at room temperature in 20 mM HEPES, pH 7.7, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, with 4.5 μg of bovine serum albumin, 1 μg of poly(dI·dC) (Sigma-Aldrich), and 5 to 15 μg of nuclear extract in a total volume of 30 μl. The binding reactions were loaded into the wells of a 3 to 4.5% 37.5:1 acrylamide/bisacrylamide, 0.5× Tris-borate-EDTA, 1.5-mm thick vertical gel and subjected to electrophoresis at 35 mA. The gel was transferred to filter paper, dried, and exposed to film. All supershift antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). When applicable, 2 μg of IgG (1 μl) was added to each binding reaction. The C/EBP oligonucleotide competitors (consensus and mutant), also purchased from Santa Cruz Biotechnologies, Inc., were added to the binding reaction in a 25× molar excess relative to the radiolabeled probe.

Results

Expression of COX-2 in Murine Lung Cancer Cell Lines. COX-2 protein levels in C10, Spon4, and CL13 extracts were measured by Western analysis (Fig. 1). COX-2 protein was detected in both lung tumor-derived cell lines, as well as in C10 cells. COX-2 protein expression was lowest in CL13 cells and highest in the C10 cells.

Effect of MAP Kinase Pathway Inhibitors on COX-2 Transcription. The effect of several MAP kinase pathway inhibitors on COX-2 mRNA levels (Fig. 2) and COX-2 promoter activity (Fig. 3) in C10, Spon4, and CL13 cells was determined. In both analyses, the effect of EGF treatment and consequent MAP kinase pathway activation was also determined. EGF treatment slightly increased COX-2 mRNA levels and COX-2 promoter activity in C10 cells, whereas no effect was seen in Spon4 and CL13 cells. These results correlate with their *Ki-ras* mutation status, whereby cells with an activating *Ki-ras* mutation should not respond to EGF receptor activation.

The MEK inhibitor PD98059 did not inhibit COX-2 mRNA expression or promoter activity; instead, a slight stimulation was seen in the C10 cells. However, the MEK inhibitor U0126 did inhibit COX-2 promoter activity at both concentrations tested in all three cell lines (Fig. 3). In addition, an inhibitor of p38 MAP kinase (SB202190) significantly repressed COX-2 promoter activity in C10 cells (Fig. 3). Repression of promoter activity by this compound in the Spon4 cells was less significant, with the CL13 cells displaying an intermediate level of repression.

The effect of RA on COX-2 mRNA expression in these three cell lines was also determined (Fig. 2). RA treatment resulted in a progressive decrease in COX-2 mRNA levels from 6 to

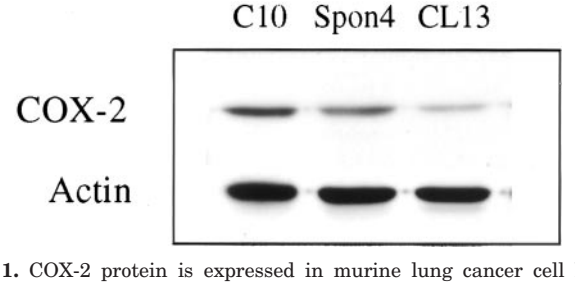

Fig. 1. COX-2 protein is expressed in murine lung cancer cell lines. Western blot analysis of 50-μg protein extracts from C10, Spon4, and CL13 cell lines. The blot was probed with specific antibodies against COX-2 and actin (loading control).

30 h in C10 cells. A progressive decrease was also observed in CL13 cells from 6 to18 h, slightly rebounding at 30 h. Interestingly, after a decrease at 6 h comparable to that seen in CL13 cells, COX-2 mRNA levels in the Spon4 cells increased significantly over control levels at the 18- and 30-h time points.

Murine COX-2 Promoter Analysis. The murine COX-2 promoter and five promoter segments representing sequential 5′ deletions of the promoter were ligated into a luciferase expression vector. The exact locations of the promoter deletions and the transcription factor binding sites within the murine COX-2 promoter are shown in Fig. 4, along with the corresponding luciferase activities of each promoter construct in the C10, Spon4, and CL13 cell lines. These data indicated that the 5′ to 3′ promoter deletions did not affect promoter activity up to nucleotide position −223 (i.e., the segment from −223 to +111 had full promoter activity). Consequently, one can conclude that the binding sites in the deleted regions are not required for basal COX-2 expression in these cell lines. These binding sites include several AP-1 sites, an SP-1 site, and the only NF-κB sites of the promoter. Subsequent deletions significantly repressed promoter activity. The segment from −112 to +111 had approximately 50% of the activity of the next longer segment. The shortest segment (−37 to +111) produced little promoter activity. These results suggested the possible involvement of the 3′ C/EBP, ATF/CREB, AP-1, and USF binding sites in the regulation of basal COX-2 expression in both the C10 cells and the two tumor-derived cell lines. Consequently, at least two essential nucleotides within each 3′ site were mutated by site-directed mutagenesis (Fig. 5A), and the effect of these mutations on COX-2 promoter activity was determined (Fig. 5B). Mutation of the first C/EBP site (−135) resulted in approximately 50% inhibition of promoter activity. Mutation of the second C/EBP site (−90) resulted in slightly less inhibition (20–45%). The most significant inhibition of promoter activity (approxi-

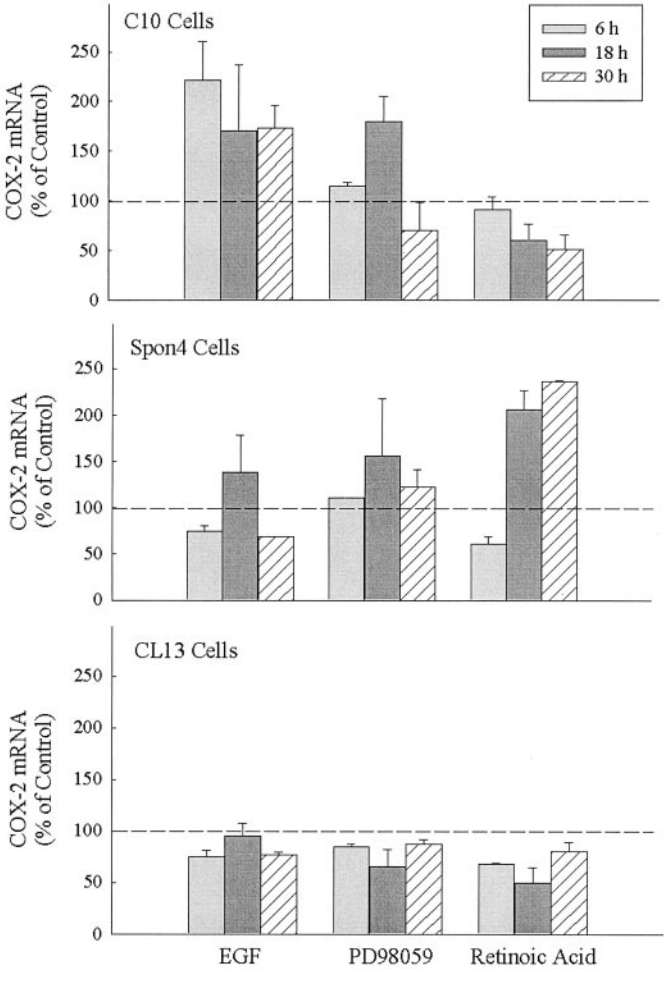

Fig. 2. Effects of Ras pathway activator and inhibitors on COX-2 mRNA levels. C10, Spon4, and CL13 cells were incubated for 24 h without serum. The culture medium was then replaced with medium containing 3 ng/ml EGF, 50 μM PD98059, or 1 μM all-*trans*-retinoic acid for 6, 18, or 30 h. All treatment media contained 0.03% DMSO. Control medium contained only DMSO. All treatments were conducted in duplicate. Following RNA isolation, COX-2 mRNA was quantitated by ribonuclease protection assay. Error bars represent the average of values obtained for duplicate treatments ± range.

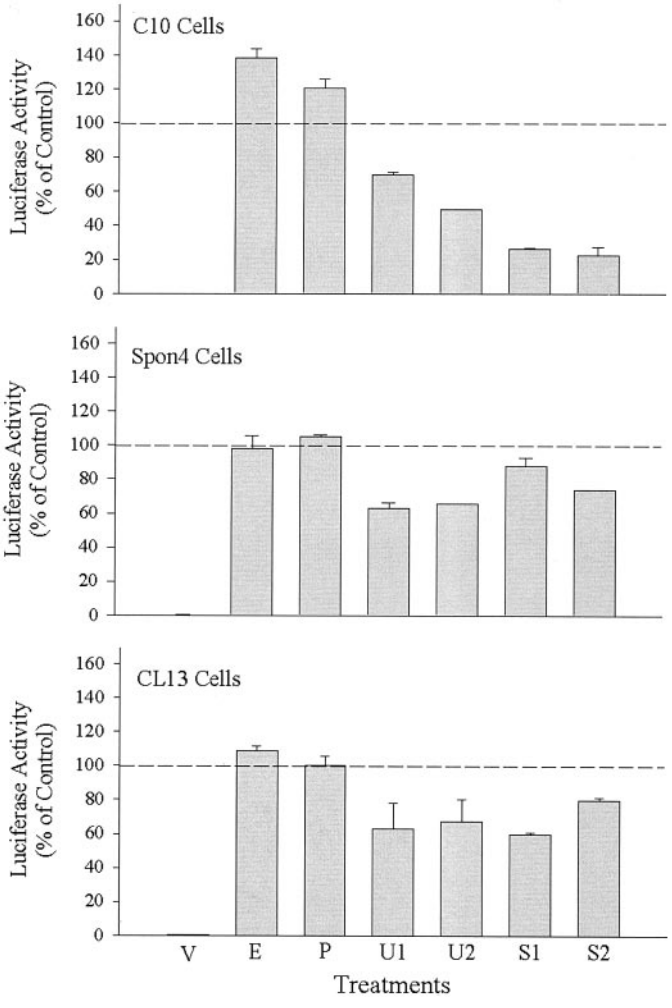

Fig. 3. Effects of Ras pathway activator and inhibitors on murine COX-2 promoter activity. C10, Spon4, and CL13 cells were transfected with a luciferase expression vector containing the full-length murine COX-2 promoter or a promoterless vector (V), along with a β-galactosidase expression vector. After approximately 3 h, the cells were treated with 50 ng/ml murine EGF (E), 50 μM PD98059 (P), 1 μM (U1) or 5 μM (U2) U0126, or 2 μM (S1) or 10 μM (S2) SB202190. All treatment media contained 0.1% DMSO. Control medium contained only DMSO. All treatments were conducted in duplicate. After 24 h, luciferase and β-galactosidase activities were quantitated. β-Galactosidase activities were used to correct luciferase activities for variations in transfection efficiency. Error bars represent the average of values obtained for duplicate treatments ± range.

mately 70%) was seen with mutation of the ATF/CREB/AP-1 binding site, whereas mutation of the USF site had no inhibitory effect on promoter activity. The ability of the USF site mutations to disrupt USF binding was confirmed by EMSA and is discussed below. These results supported the possible involvement of the C/EBP, ATF/CREB, and AP-1 transcription factors, but not the USF transcription factor, in the regulation of basal COX-2 expression in all three cell lines.

EMSAs were then conducted to confirm the binding of proteins to the transcription factor binding sites implicated in controlling basal COX-2 expression by the promoter deletion and site-directed mutagenesis studies. The radiolabeled probes used matched the binding sites and surrounding nucleotide sequence within the murine COX-2 promoter. For example, probe 1 was identical to the promoter sequence containing the first C/EBP site at −135; probe 2 matched the region containing the second C/EBP site at −90; and probe 3 corresponded to the promoter region encompassing the ATF/CREB, AP-1, and USF sites near −50.

The incubation of probe 1 with crude nuclear extract from the three cell lines produced several shifted bands indicating the binding of proteins to this promoter sequence (Fig. 6). Only one band was common to all four cell lines. The intensity of the other bands varied with the amount of bovine serum albumin present in the reaction (data not shown). Addition of excess unlabeled probe almost completely eliminated the common band, whereas the other bands were less affected (data not shown). The addition of a C/EBP antibody that recognizes all C/EBP isoforms produced at least one supershifted band (Fig. 6, arrows) for each cell line (two with C10 and Spon4 extracts). These supershifted bands could be eliminated by the addition of a 25× molar excess of a commercially available oligonucleotide corresponding to the C/EBP consensus sequence. A mutated version of this oligonucleotide had no such effect. These competitor oligonucleotides had little apparent effect on protein binding in the absence of antibody. A fourth cell line (CL30) was included in the EMSAs for comparison. This cell line is similar to the CL13 cell line but has a higher level of COX-2 mRNA expression (Wardlaw et al., 2000). Similar overall results were obtained with probe 2 (Fig. 7). Taken together, these results support a role for C/EBP in the regulation of basal COX-2 expression in these cell lines.

Protein binding to probe 3 is shown in Fig. 8. Probe 3 contains the ATF/CREB, AP-1, and USF binding sites. As seen in Fig. 8, A to C, multiple shifted bands resulted from the incubation of probe 3 with extracts from all four cell lines. Figure 8A shows a single supershifted band resulting from the addition of an antibody that recognizes ATF-1, CREB-1, and CREM-1, indicating that one or more of these proteins bind to this regulatory region. Figure 8B shows a lack of any supershifted bands upon the addition of an antibody that recognizes the Jun proteins (c-Jun, Jun B, and Jun D), an essential component of the AP-1 transcription factor. The activity of this antibody was confirmed by Western analysis, revealing the expression of Jun proteins in these cells (data not shown). c-Jun expression was also detected in these cells using a second, more specific antibody (data not shown). These results suggest that AP-1 is not involved in the regulation of basal COX-2 expression in these cell lines. Finally, an EMSA was conducted using probe 3 and an antibody against USF-1. Interestingly, a single supershifted band resulted from the addition of this antibody, suggesting that USF-1 is binding to this region even though mutagenesis studies indicated that the USF site was not necessary for COX-2 promoter activity. The efficacy of those USF site mutations at preventing USF-1 binding was confirmed by introducing the same mutations into probe 3. When unlabeled mutated probe 3 was used as a competitor, there was no loss of the USF-1 antibody-induced supershift, indicating that even though mutated probe 3 was present in 25× molar excess, USF did not bind this mutated sequence to any appreciable extent.

Discussion

These studies substantiate a critical role for the C/EBP and ATF/CREB transcription factors as key regulators of basal COX-2 transcription. The presence of shifted bands that were not supershifted by the addition of C/EBP and ATF/CREB antibodies suggests that additional proteins may also bind to these regulatory regions within the COX-2 promoter. The

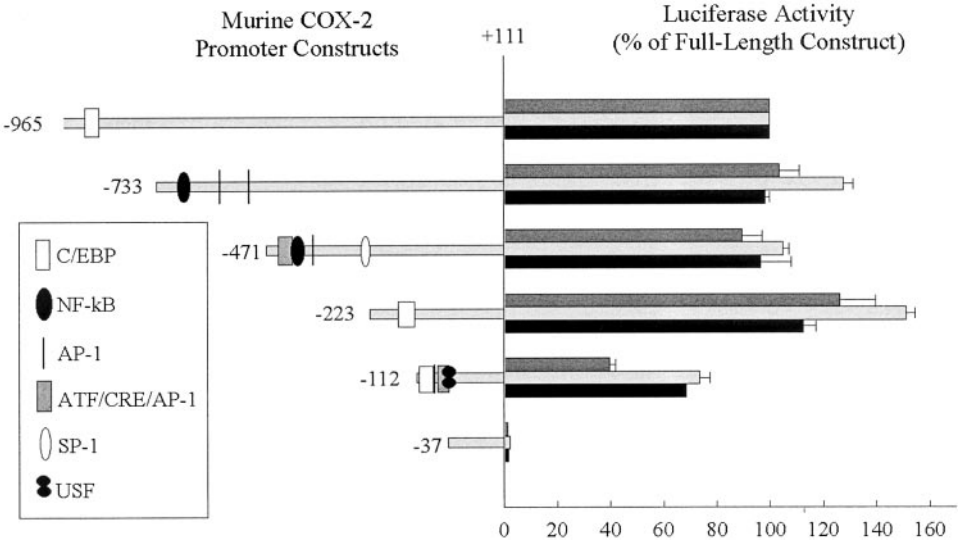

Fig. 4. 5′ transcription factor binding sites are not required for basal COX-2 promoter activity in murine lung cancer cells. The indicated promoter sequences were prepared by PCR amplification of A/J lung DNA. Individual sequences were ligated into a luciferase expression vector, then transfected into C10 (medium gray bar), Spon4 (light gray bar), and CL13 (black bar) cells. All transfections were conducted in duplicate. After 24 h, luciferase and β-galactosidase activities were quantitated. β-Galactosidase activities were used to correct luciferase activities for variations in transfection efficiency. Error bars represent the average of values obtained for duplicate transfections ± range.

inhibition of COX-2 activity significantly decreased cell growth in vitro supporting the involvement of this pathway in the A/J mouse lung tumor model. In addition, findings from our study further substantiate a role for the Ras and p38 MAP kinase signal transduction pathways in the regulation of the basal expression of this gene.

EGF stimulated COX-2 expression in only the C10 cell line, suggesting that COX-2 expression can be induced in this cell line by Ras activation. A similar response in the Spon4 and CL13 cell lines was not expected since Ras is already constitutively active. In contrast, inhibitors of both the MEK/ERK and p38 MAP kinase pathways affected COX-2 expression in all three cell lines. p38 MAP kinase is involved in induced COX-2 expression in other epithelial cell lines (Chen et al., 2001; Guo et al., 2001; Kulkarni et al., 2001), but has not been linked to the modulation of endogenous expression in epithelial cancer cells.

RA-induced retinoic acid receptor activation can inhibit the activity of the AP-1 transcription factor that is frequently activated by MAP kinase signaling pathways. This direct effect of RA treatment may explain the rapid decrease in COX-2 mRNA expression in all three cell lines after 6 h of treatment. Activated retinoic acid receptors also bind to specific response elements in the promoters of many genes, up-regulating their transcription. One or more of the resulting gene products could then act directly or indirectly on the COX-2 promoter to affect transcription. Such a response would likely require more time, and this may explain the up-regulation of COX-2 mRNA expression in Spon4 cells at the later time points. Although other studies have demonstrated the inhibition of COX-2 expression in epithelial cell lines by retinoids (Mestre et al., 1997; Kanekura et al., 2000; Merritt et al., 2001), this is the first instance of RA-induced up-regulation of COX-2 expression in an epithelial cell line.

Sequential 5′ deletions of the COX-2 promoter revealed that the 5′ transcription factor binding sites were not required for basal expression of COX-2. These sites included several AP-1 sites, an SP-1 site, and the only NF-kB sites of

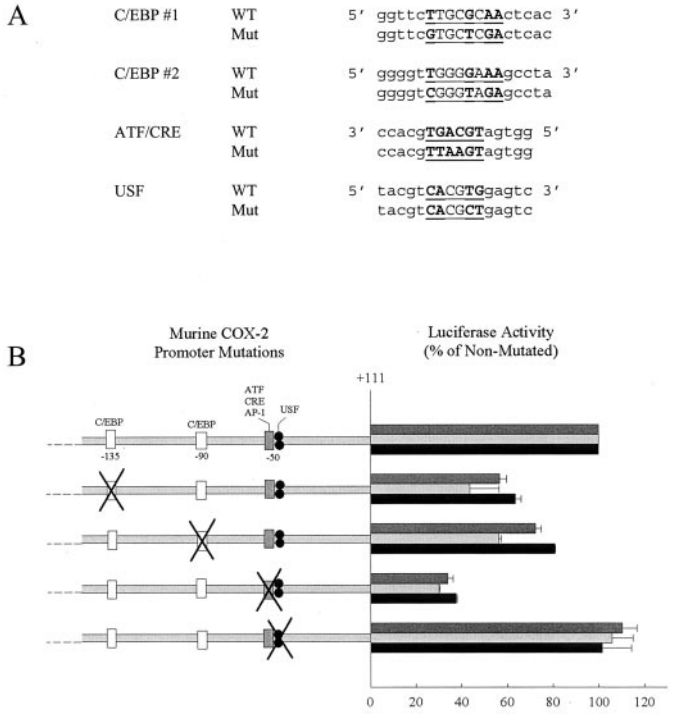

Fig. 5. 3′ C/EBP and ATF/CRE/AP-1 sites are required for full murine COX-2 promoter activity. The regions of the murine COX-2 promoter containing the 3′ C/EBP, ATF/CREB/AP-1, and USF binding sites are shown with the critical nucleotides in bold type. Below the wild-type sequences (WT) are the sequences containing the mutations (Mut) introduced by the site-directed mutagenesis of a luciferase expression vector containing the full-length murine COX-2 promoter (A). The nonmutated and mutated expression vectors were transfected into C10 (medium gray bar), Spon4 (light gray bar), and CL13 (black bar) cells. All transfections were conducted in duplicate. After 24 h, luciferase and β-galactosidase activities were quantitated. β-Galactosidase activities were used to correct luciferase activities for variations in transfection efficiency. Error bars represent the average of values obtained for duplicate transfections ± range (B).

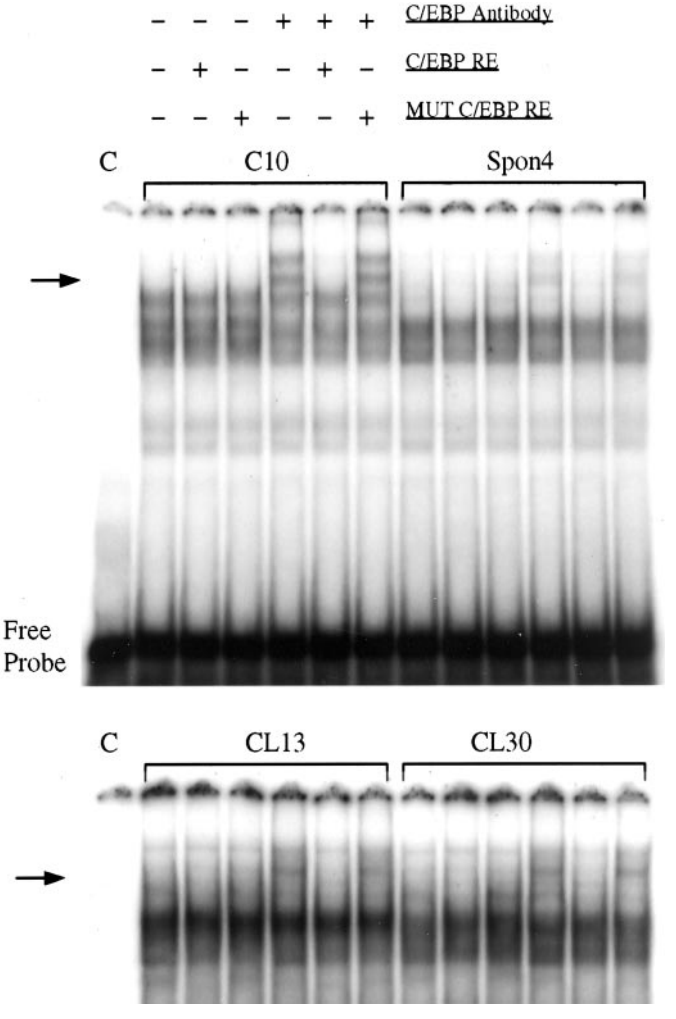

Fig. 6. Proteins bind to the COX-2 promoter region containing the −135 C/EBP site, including C/EBP transcription factor(s). An oligonucleotide probe containing the −135 C/EBP site of the murine COX-2 promoter plus surrounding sequence (−154 to −121) was radiolabeled and used as a probe in an electrophoretic mobility shift assay. The probe was incubated with crude nuclear extracts prepared from C10, Spon4, CL13, and CL30 cell lines (lane 1 of each set). Unlabeled, commercially available C/EBP-response element (RE) oligonucleotides (consensus and mutated) were added to the incubation mixture in a 25× molar excess over radiolabeled probe (lanes 2, 3, 5, and 6 of each set). The protein-DNA complexes were supershifted (arrows) by the addition of an antibody that recognizes all C/EBP isoforms (lanes 4–6 of each set). The top panel shows the full autoradiograph with free probe at the bottom. The bottom panel shows only the upper portion of the autoradiograph (the lower portion of the autoradiograph looked the same as shown in the top panel). The "C" lane in both panels is a control incubation lacking nuclear extract.

the promoter. COX-2 is frequently up-regulated in cells in response to treatment with activators of NF-κB (Chen et al., 2000; Subbarayan et al., 2001; Weaver et al., 2001). Although this mechanism is likely to be involved in the up-regulation of COX-2 as part of the inflammatory response and may be involved in COX-2 up-regulation in some cancer cells, the results reported here suggest that NF-κB is not involved in the aberrant expression of COX-2 in lung cancer cells.

Site-directed mutagenesis of the 3′ transcription factor binding sites indicated a role in COX-2 regulation for all sites except the USF site. Further studies of the 3′ C/EBP and ATF/CREB/AP-1 sites indicated that AP-1 is also not involved in the basal expression of COX-2 in these cell lines. This was unexpected due to the initial inhibitory effects of RA treatment and the frequent involvement of AP-1 in the induction of COX-2 expression by exogenous factors in other epithelial cell lines (Subbaramaiah et al., 2000; von Knethen and Brüne, 2000; Guo et al., 2001). Thus, RA treatment must have repressed COX-2 transcription through a mechanism independent of AP-1.

The elimination of USF and AP-1 as likely regulators of COX-2 expression, and the supporting data from the promoter deletion, mutagenesis, and EMSA studies identified the C/EBP and ATF/CREB factors as the major transcriptional regulators of basal COX-2 expression in A/J lung tumor-derived cell lines. These results are corroborated in part by the two previously published studies on the regulation of constitutive COX-2 expression in epithelial cancer cells. In the first, Shao et al. (2000) reported that both the NF-IL6 (C/EBP) regulatory element and CRE were responsible for the regulation of COX-2 transcription in human colon carcinoma cells. Similarly, Kim and Fischer reported the involvement of the NF-IL6 (C/EBP) sites and C/EBP transcription factors (all isoforms) in the transcriptional regulation of COX-2 in mouse skin carcinoma cells (Kim and Fischer, 1998). The C/EBP isoforms were differentially expressed during progressive stages of skin carcinogenesis, supporting a relationship between C/EBP levels and COX-2 expression. Although the mechanisms regulating COX-2 transcription in cancer cells appear to differ between cell types, these two studies, combined with our results, reveal similarities in the mechanisms regulating basal COX-2 transcription in epithelial cancer cells.

The C/EBP and ATF/CREB transcription factors may constitute new targets for the down-regulation of COX-2 expression in cancer cells. In support of this approach, a recent

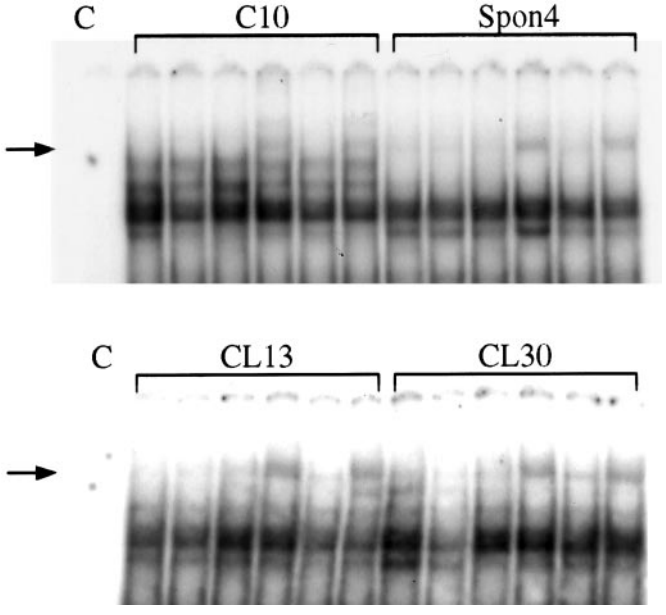

Fig. 7. Proteins bind to the COX-2 promoter region containing the −90 C/EBP site, including C/EBP transcription factor(s). An oligonucleotide probe containing the −90 C/EBP site of the murine COX-2 promoter plus surrounding sequence (−104 to −75) was radiolabeled and used as a probe in an electrophoretic mobility shift assay. Incubation conditions for each lane are identical to those described in the legend to Fig. 6. Only the upper portion of each autoradiograph is shown. The "C" lane in both panels is a control incubation lacking nuclear extract.

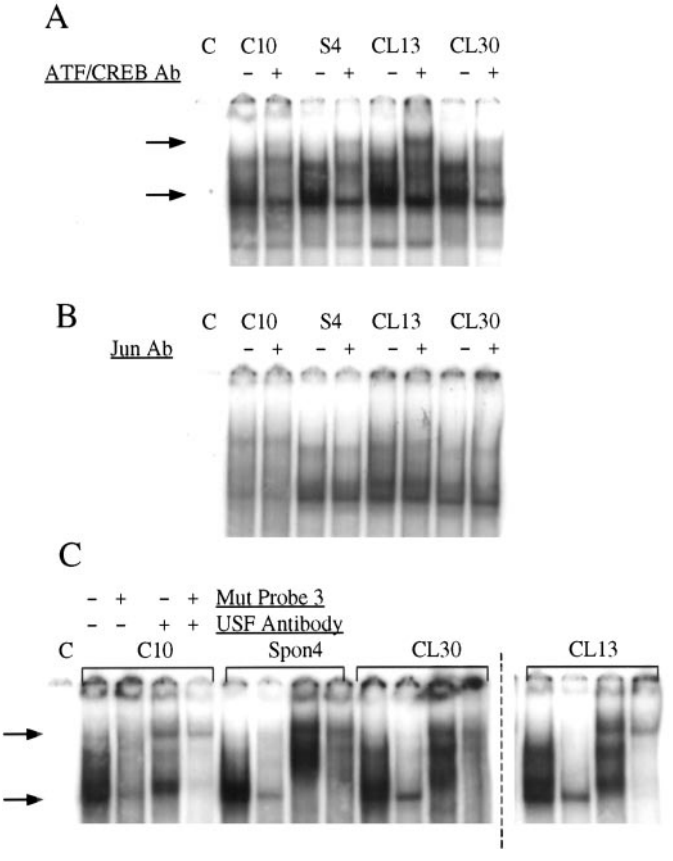

Fig. 8. Proteins bind to the COX-2 promoter region containing the ATF/CREB/AP-1 and USF binding sites, including ATF-1/CREB-1 and USF-1, but not AP-1. An oligonucleotide probe containing the 3′ ATF/CRE/AP-1 region of the murine COX-2 promoter (−72 to −38) was radiolabeled and used as a probe in an electrophoretic mobility shift assay. The probe was incubated with crude nuclear extracts prepared from C10, Spon4, CL13, and CL30 cell lines (A and B, "−" lanes; C, lane 1 of each set). The "C" lane in all three panels is a control incubation lacking nuclear extract. An antibody specific for ATF-1, CREB-1, and CREM-1 was included in the incubation mixture ("+" lanes), producing a supershifted band (lower arrow in "−" lanes to upper arrow in "+" lanes) (A). An antibody specific for c-Jun, Jun B, and Jun D was included in the incubation mixture ("+" lanes). No supershifted bands are apparent (B). An antibody specific for USF-1 was included in the incubation mixture (lane 3 of each set) producing a supershifted band (lower arrow/lane 1 to upper arrow/lane 3). An unlabeled probe, identical to the labeled probe except for the presence of the USF-specific mutations shown in Fig. 5, was added to the incubation mixture in a 25× molar excess over radiolabeled probe. In the absence of antibody, one band is not diminished by the presence of the unlabeled, mutated competitor. In the presence of antibody, the supershifted band is not diminished by the presence of the competitor, indicating a lack of affinity between USF-1 and the mutated response element (C).

study suggests that the anti-inflammatory effects of aspirin and salicylate may be due to the inhibition of C/EBPβ binding to the COX-2 promoter (Saunders et al., 2001). Salicylate did not inhibit tumor necrosis factor-α-induced binding of NF-κB to the COX-2 promoter, disproving a mechanism suggested by others to account for the inhibition of prostaglandin synthesis by this compound after it was shown not to inhibit COX activity (Mitchell et al., 1993). This might explain the efficacy of nonsteroidal anti-inflammatory drugs as lung cancer preventives even though NF-κB does not appear to be involved in the aberrant expression of COX-2 in lung cancer cells. Thus, salicylate-mediated inhibition of C/EBP binding could be an example whereby prostaglandin synthesis (and subsequent inflammation or tumorigenesis) is inhibited by targeting an effector of COX-2 transcription rather than targeting COX-2 enzyme activity itself. The results reported here indicate that a second target for repression of prostaglandin synthesis is the ATF/CREB binding site. CRE decoy oligonucleotides have recently been shown to inhibit gene expression and tumor growth in vitro and in vivo in a broad spectrum of cancer cells, without adversely affecting normal cell growth (Cho-Chung et al., 2000). Thus, the inhibition of COX-2 expression, and possibly the expression of other growth-regulatory genes, by this method appears to be a promising approach for the prevention and/or treatment of lung cancer and other epithelial cancers.

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