Transcriptional Regulation of Cyclooxygenase-2 in Mouse Skin Carcinoma Cells

REGULATORY ROLE OF CCAAT/ENHANCER-BINDING PROTEINS IN THE DIFFERENTIAL EXPRESSION OF CYCLOOXYGENASE-2 IN NORMAL AND NEOPLASTIC TISSUES

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Many studies have suggested that overexpression of cyclooxygenase-2 (COX-2) contributes to the development of tumors in several tissues. COX-2 expression tends to be up-regulated in various types of tumors and transformed cell lines, and the overexpression of COX-2 is caused by enhanced transcription of the gene. In an attempt to characterize the signaling pathway leading to the overexpression of COX-2 in the mouse skin carcinoma cell line JWF2, we investigated cis- and trans-acting factors required for COX-2 expression and demonstrated a molecular mechanism by which COX-2 is expressed differentially in normal and neoplastic tissues. Two regions of the COX-2 promoter containing an E-box and nuclear factor IL6 site were identified as the positive regulatory elements through transient transfections with luciferase reporter vectors containing the various 5′-flanking regions of the promoter. Moreover, electrophoretic mobility shift assays and cotransfection experiments showed that upstream stimulatory factors and CCAAT/enhancer-binding proteins (C/EBPs) bind to the E-box and nuclear factor IL6 site, respectively, and functionally transactivate the COX-2 promoter. We also found that C/EBP isoforms are expressed differentially during mouse skin carcinogenesis, suggesting that overexpression of COX-2 in tumors may be caused by a change in C/EBP expression levels.

Prostaglandins (PGs) are involved in many normal and pathophysiological responses (1). As a rate-limiting enzyme in the synthesis of PGs, cyclooxygenase (COX) exists as two isoforms. COX-1 is thought to be involved in the housekeeping function of PGs, whereas COX-2, the inducible isoform of COX, is responsible for the rapid production of PGs in response to various external stimuli (2–4). Although COX-2 expression is typically repressed in most tissues except in the brain and renal cortex (5), various types of tumors and transformed cells tend to overexpress COX-2 constitutively (6–8). There is growing evidence that overexpression of COX-2 is associated with the tumorigenicity of cells. For example, rat intestinal epithelial cells stably transfected with a COX-2 expression vector exhibited altered adhesion properties and showed resistance to induced apoptosis (9). COX-2 overexpression in human colon cancer cells was associated with an increase in metastatic potential (10). Furthermore, nonsteroidal antiinflammatory drugs showed chemopreventive effects on colon cancer in several epidemiological studies (11–13). Finally, a COX-2-specific inhibitor suppressed polypl formation in mice possessing an adenomatous polyposis coli gene mutation (14).

It has been shown that up-regulation of COX-2 expression in tumors is caused by enhanced transcription (15). Even though several consensus sequences including NF-κB, SP1, NF-IL6, AP-2, PEA3, ATF/CRE, and E-box have been found on the 5′-flanking region of the COX-2 gene, so far NF-κB, NF-IL6, ATF/CRE, and E-box have been identified as the regulatory sequences involved in COX-2 induction in response to various stimuli in different species and cell types (16–24). COX-2 induction seems to be quite cell type- and agonist-specific, such that the same signal can elicit different responses. For instance, overexpression of v-src in mouse fibroblasts enhanced COX-2 expression (21), whereas it did not activate COX-2 transcription in rat mesangial cells (25). Several signal transduction pathways leading to the COX-2 induction by various stimuli have been suggested. Endothelin stimulated COX-2 expression through a tyrosine kinase signaling pathway in rat mesangial cells (26), and COX-2 expression was induced by v-src, platelet-derived growth factor, and serum through both the Ras/MEKK-1/JNK kinase/JNK/c-Jun and the Ras/Raf-1/MAPKK/ERK pathway in NIH3T3 cells (22, 23). There has been no report, however, suggesting which cis- and trans-acting factors are directly responsible for the expression of COX-2 in carcinoma cells and what the mechanism is by which COX-2 is expressed differentially in normal skin and tumors. In this study, we used the mouse skin squamous cell carcinoma JWF2 cells that constitutively overexpress COX-2 and identified the E-box and NF-IL6 sites on the murine COX-2 promoter as positive regulatory sequences. Furthermore, USFs and C/EBPs are identified as the transcription factors binding to those sites. Finally, we demonstrated that C/EBP isoforms are expressed differentially during mouse skin carcinogenesis, which may contribute to the overexpression of COX-2 in tumors.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse skin squamous cell carcinoma JWF2 cells (27) were grown in Eagle’s minimal essential medium supplemented with

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† The abbreviations used are: PG(s), prostaglandin(s); ATF, activation transcription factor; CEBP, CCAAT/enhancer-binding protein; CHOP-10, C/EBP homologous protein-10; CMV, cytomegalovirus; COX, cyclooxygenase; CRE, cyclic AMP response element; CREB, CRE-binding protein; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; β-gal, β-galactosidase; IL6, interleukin 6; JNK, c-Jun N-terminal kinase; MAPKK, mitogen-activated protein kinase kinase; MEKK-1, MAP kinase kinase kinase; NF-IL6, nuclear factor for IL6 expression; NF-κB, nuclear factor κB; TNF-α, tumor necrosis factor-α; TPA, 12-O-tetradecanoylphorbol-13-acetate; USF, upstream stimulatory factor.
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1% fetal calf serum at 37 °C in 5% CO₂.

Plasmids—The luciferase reporter vector (pT510L) containing the promoter region of the mouse COX-2 gene (−963/+70 from the transcription initiation site) was kindly provided by Harvey Herschman (University of California at Los Angeles) and was used as a template for unique restriction enzyme sites that were inserted within the series of five-deletion and internal deletion constructs was made. The COX-2 promoter insert was cut out from the template plasmid with BamHI or HindIII and XhoI digestion. Digested DNA fragments were gel purified using GeneClean II (BIO 101, Inc.) and then cut at the −756, −547, −203, −154, −68, and −50 positions by DraI, Asp-700, SphI, and HindIII, respectively. The vectorexpressing luciferase reporters were digested with XhoI and HindIII and inserted first at the XhoI site; the reaction was stopped by phenol/chloroform extraction, blunted end by Klenow, and religated. To produce internal deletion constructs, Δ−371/−69 and Δ−68/−51, pT510L was digested with Smal/XhoI and HindIII/BbrI, respectively. Then the DNA fragments containing specific promoter regions of COX-2 (−68/−70 and −963/−69) were ligated to the vector at the XhoI site or HindIII site. The subsequent reactions were the same as described above. For Δ−371/−51 construct, pT510L was digested with Smal/BbrI and blunt end ligated. All constructs were verified by dieoxy DNA sequencing. Plasmids expressing USF-1, 2 (p-USF1,2) were gifts from Michele Sawadogo (University of Texas, M. D. Anderson Cancer Center). C/EBP-homologous protein-10 (CHOP-10) expression vector was a gift from D. Ron (New York University). Plasmids expressing C/EBPs, β, and δ were generously provided by Steven McKnight (Tulakir Inc.).

Transient Transfection—Cells were plated in 35-mm dishes 40 h before transfection. 10 μg of luciferase reporter vector and 1.0 μg of pCMV-β-gal as a vector control per dish were transfected into cells at about 80% confluence by using the calcium phosphate precipitation method. In detail, the medium was changed 3 h before transfection and incubated for 5 h after DNA was added. To increase the transfection efficiency, glycerol shock (10% in 1× HEPES-buffered saline) was given to cells for 2 min. After washing with phosphate-buffered saline twice, cells were refed with 2 ml of fresh medium. To prevent COX-2 induction by serum in the medium, serum-free medium was added after 16 h of culture, and the cultures were incubated for an additional 24 h. Alternatively, 4 μg of reporter vector and 0.5 μg of pCMV-β-gal were complexed with 10 μl of Lipofectin (Life Technologies, Inc.) and transiently transfected into the cells following the manufacturer’s protocol. The amount of DNA/dish was made constant in cotransfection experiments by adding pCMV-β-gal reporter vector. Proteins were extracted according to the manufacturer’s protocol (Tropix). Luciferase and β-galactosidase activities were measured by a luminomet- ter from Tropix. Promoter activity was normalized by β-galactosidase activity.

Preparation of Nuclear Extracts—Nuclear extracts were prepared as described previously (28) with the following modifications. In detail, cells incubated in serum-free media for 24 h were washed with cold phosphate-buffered saline twice and scraped into 1 ml of cold phosphate-buffered saline. Cells were pelleted with microcentrifugation for 10 s and incubated in 2 packed cell volumes of buffer A (10 mM HEPES, pH 8.0, 0.5% Nonidet P-40, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 200 mM sucrose) for 5 min at 4 °C with flicking the tube. The crude nuclei were collected by microcentrifugation for 15 s; pellets were rinsed with buffer, resuspended in 1 packed cell volume of buffer B (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 400 mM NaCl, 0.2 mM EDTA, and 1.0 mM DTT), and incubated on a rocking platform for 30 min at 4 °C. Nuclei were clarified by microcentrifugation for 5 min, and the supernatants were diluted 1:1 with buffer C (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT), Protease inhibitors (1 μM phenylmethylsulfonyl fluoride, 50 μM of both aprotinin and leupeptin/ml) and phosphatase inhibitors (10 μM NaF, 10 μM β-glycerophosphate, 0.1 mM sodium orthovanadate, and 1 mM EDTA) were added to each type of buffer. Nuclear extracts were frozen in liquid N₂ and kept at −70 °C until use.

Electrophoretic Mobility Shift Assay (EMSA)—Synthetic oligonucleotides (Gensios) or restriction fragments containing the appropriate promoter region of the COX-2 gene were end labeled with γ[-32P]ATP by T4 polynucleotide kinase (Amersham Pharmacia Biotech). Assays were incubated 2 μg of nuclear extracts in 2 μl of binding buffer (4 mM Tris-HCl, 12 mM HEPES-KOH, pH 7.9, 60 mM KCl, 12% glycerol, 0.5 mM EDTA, and 1 mM DTT) containing 1 μg of poly(dI-dC) and 15,000 cpm of labeled probe for 25 min at room temperature. To assure the specific binding of transcription factors to the probe, the probe was chased by 50- and 100-fold molar excess of cold wild-type or mutant oligonucleotide. For the supershift experiment, antibodies (Santa Cruz Biotechnology) were incubated with nuclear extracts on ice for 30 min before adding to the binding reaction. Samples were then electrophoresed on 5% nondenaturing polyacrylamide gels (0.25×25 TBE (22.5 mM Tris borate, 0.5 mM EDTA) as running buffer), and the gels dried and subjected to autoradiography.

The NF-IL6 site and E-box, or both, were mutated to the −963 or −203 construct by unique site elimination method (29). Briefly, for E-box mutation, mE-box oligonucleotides were used both as a selection and a mutant primer. Clones resistant to the BbrI digestion were selected and sequenced. For NF-IL6 and double mutation, oligonucleotides containing the mutation at BglII site were used both as a selection and a mutant primer. The vectors and oligonucleotides were used both as a selection primer either on the wild-type or on the E-box mutation constructs. Clones resistant to the BglII digestion were selected and sequenced. The BMH 71-18 mutS strain (CLONTECH) was used for competent cells. Oligonucleotides used for mutagenesis are shown in Fig. 2. Site-specific mutations were confirmed by DNA sequencing.

RNA Isolation and Northern Analysis—Total RNAs were isolated from the epidermis of newborn mouse and JWF2 cells by using TRI REAGENT (Molecular Research Center, Inc.) according to the manufacturer’s protocol. 10 μg of RNA was separated on a formaldehyde-containing 1% agarose gel, transferred onto nylon membrane (Micron Separation, Inc.), and UV cross-linked to the membrane using a Stratagene (Stratagene) solution. Specific bands were detected by autoradiography.

Preparation of Whole Cell Extracts and Western Blot Analysis—Female SENCAR mice were used for tumor experiments. 12-O-Tetradecanoylphorbol-13-acetate (TPA, 1 μg in 0.2 ml of acetone) was applied topically to the dorsal side of shaved skin twice a week for 10 weeks, and the dorsal skins were used as hyperplastic skin, whereas the dorsal skins of untreated mice were used as normal skin. Skin homogenates were prepared by a Polytron PT10 homogenizer. The homogenates were sonicated with a Branson Sonifier 450 on ice, and centrifuged at 4 °C for 10 min. The supernatants were used for Western blot analysis. Protein lysates (25 μg) were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech). After being blocked with 5% nonfat dried milk in Tween/Tris-buffered saline, the blots were incubated with polyclonal antibodies for C/EBPβ (Santa Cruz Biotechnology) and monoclonal COX-2 antibody (Transduction Laboratory). Horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) were used for immunoblotting detection.

RESULTS

Murine COX-2 Promoter Contains Two Positive Regulatory Regions—Because COX-2 is an immediate early gene and its expression is regulated at the transcriptional level (15), first we decided to identify the cis-acting elements responsible for the expression of the COX-2 gene. Transient transfection experiments with a series of deletion-requiring region deletion constructs showed the involvement of two positive regulatory sequences for the expression of COX-2 (Fig. 1). Deletion of the region spanning from −202 to −135 resulted in a significant decrease in promoter activity (40% of the full-length activity). The deletion disrupts the consensus NF-IL6 sequence (TTGCG/CCAA) located at −138/−131. It has been shown that this NF-IL6 site is one of the regulatory elements involved in COX-2 expression.
induced by various agents (17, 24). Further deletion of the COX-2 promoter (−67/−51) led to the complete loss of promoter activity. The ATF/CRE site overlapping the E-box (CAGT-CACGTTG) is located at −56/−48. Internal deletion constructs, Δ−371/−69, Δ−371/−135, and Δ−68/−50 also confirmed the presence of two positive regulatory elements on the murine COX-2 promoter. Interestingly, the −203 construct showed almost the same promoter activity as the full-length construct (−963/+70). In previous studies, the short construct (−371/−70) was also more responsive to TPA, serum, and platelet-derived growth factor compared with the full-length construct (−963/+70) (30, 31).

We next investigated whether the two regions are functional binding sites for particular transcription factors. DNA fragment −203/−70 was labeled and subjected to the competition assay with various oligonucleotides corresponding to the consensus sequences found on the COX-2 promoter region (Fig. 2). Even though multiple complexes were formed, the upper complexes were competed out by ATF/CRE E-box oligonucleotides (Fig. 3, fourth lane from left), and the bottom complex was competed out by NF-IL6 oligonucleotides (Fig. 3, sixth lane). The DNA fragment (−68/−70) containing ATF/CRE E-box competed out the upper complexes (Fig. 3, seventh lane). This result indicates that the ATF/CRE-E-box region is the major protein binding site within the −68/+70 region.

**USFs Are the Proteins Binding to the ATF/CRE-E-box Site**—Because ATF/CRE overlaps with the E-box, it was first necessary to determine which site is responsible for protein binding. It has been shown that the ATF/CRE site is essential for the COX-2 expression induced by v-src, serum, and platelet-degenerated growth factor in NIH3T3 cells (21–23), whereas the E-box was required for COX-2 expression in rat ovarian granulosa cells in response to various hormones (18). When nuclear extracts of JWF2 cells were incubated with the radiolabeled ATF/CRE E-box oligonucleotides in the presence of wild-type, mutant, and consensus oligonucleotides of CREB (32) or E-box-binding protein USF (33), the protein-DNA complexes were completely competed out by consensus USF oligonucleotides (Fig. 4A, seventh lane), whereas consensus ATF/CRE oligonucleotides had no significant effect on the binding (Fig. 4A, sixth lane). The supershift experiment using antibodies further confirmed that there are no detectable ATF/CRE-binding proteins in the complex. Preimmune serum had no effect on the binding (Fig. 4B, third lane). Complete supershift was achieved by USF-1-specific antibody (Fig. 4B, fourth lane), and USF-2 antibody partially supershifted the complex (Fig. 4B, fifth lane).

Because USFs are basic helix-loop-helix proteins that bind to a target sequence as either a homo- or heterodimer, the complexes appear to be composed of USF-1 homodimer and USF-1/USF-2 heterodimer. We also tested antibodies for several ATF/CRE-binding proteins such as CREB, ATF-2, and c-Jun, which have been reported previously to bind to the mouse ATF/CRE-E-box (21, 22), and E-box-binding protein, c-Myc (Fig. 4B, sixth through ninth lanes). None of them had any effect on the complexes. Moreover, when USF-1 and 2 were overexpressed ectopically by a cotransfection experiment, COX-2 promoter activity increased in a dose-dependent manner (data not shown).

**C/EBPs Bind to the NF-IL6 Site of the COX-2 Promoter**—It has been reported that the NF-IL6 site plays an important role in COX-2 expression induced by TNF-α in a mouse osteoblastic cell line (24) and by TPA and lipopolysaccharide in bovine...
arterial endothelial cells (17). Because C/EBP, a basic leucine-zipper DNA-binding protein, has been shown to bind to the consensus NF-IL6 site (TTGCGCAA) from 138 to 131, the DNA-protein complexes were subjected to the competition and supershift experiments with consensus C/EBP oligonucleotides (34) and each C/EBP isoform-specific antibody. The complexes were competed out by the C/EBP consensus oligonucleotide (Fig. 5A, fifth lane). Antibodies against C/EBPβ and δ supershifted significantly or cleared the complexes (Fig. 5B, fifth and sixth lanes), but the C/EBPa-specific antibody caused only a slight supershift (Fig. 5B, fourth lane). Considering that C/EBP should form a dimer with the same family member or with other proteins for binding to the target DNA, we looked for the presence of other transcription factors known to form dimers with the C/EBP family. Antibodies specific for ATF-2, the p50 and p65 subunits of NF-κB, E2F, and CREB, had no effect on binding (data not shown). Because the oligonucleotide used for EMSA also contains a possible AP-2 site (24), we investigated the binding of AP-2. An AP-2-specific antibody also failed to supershift or clear the complexes (data not shown).

C/EBPs Transactivate the COX-2 Promoter Activity in an...
Isoform-specific Manner—It is evident that C/EBPs are the predominant proteins binding to the NF-IL6 site from the supershift experiments. To assess whether these proteins actually regulate the transcription of the COX-2 gene, C/EBP expression vectors were transfected into JWF2 cells along with the −203 COX-2 reporter vector. Promoter activity was increased significantly by overexpressed C/EBPβ (2.6-fold), whereas C/EBPα showed only a slight transactivation effect, and overexpressed C/EBPβ decreased the promoter activity (Fig. 6A). A similar result was reported previously for the human COX-2 promoter (17). To investigate further whether COX-2 transcription is mediated by endogenous C/EBPs, the CHOP-10 expression vector was transfected into JWF2 cells along with the reporter vector. CHOP-10, one of the C/EBP family members, lacks the transactivation and DNA binding domains while possessing intact dimerization domains (35). It therefore can function as a dominant-negative C/EBP. COX-2 promoter activity decreased with increasing amounts of CHOP-10 expression vector, confirming the important role of C/EBPs in COX-2 transcription (Fig. 6B).

NF-IL6 Site and E-box Overlapping with ATF/CRE Are Functional cis-Acting Elements for COX-2 Expression—To understand the role of the NF-IL6 site and the E-box on the expression of COX-2 in the context of the intact COX-2 promoter, site-specific mutations were introduced in either the E-box, NF-IL6, or both sites (Fig. 7). The mutation at the NF-IL6 site alone, both on the −963 and −203 construct, decreased the promoter activity significantly (47% and 28% of the −963 construct, respectively). Mutation of the E-box alone on both constructs resulted in a more dramatic decrease in promoter activity (27% and 14% of the −963 construct, respectively). When both sites were mutated, there was an almost complete loss of promoter activity, especially with the −203 construct (4% of the −963 construct). These data are in concordance with the result obtained in Fig. 1. Thus, these results suggest that the murine COX-2 promoter requires both sites for full activity. Compared with the mutations on the −203 construct, the mutation effect was more or less moderate on the full-length construct, indicating the presence of other positive regulatory element(s) between −963 and −203 base pairs. It was reported that the NF-κB site on the murine COX-2 promoter (−401/−393) plays a role in induction of the gene by TNF-α (24).

C/EBP Isoforms Are Expressed Differentially and Bind to the NF-IL6 Site in Normal Skin and Tumor Cells—To investigate whether there is any difference in the protein binding pattern between normal and transformed cells at the ATF/CRE-E-box and NF-IL6 site, supershift experiments were performed with the nuclear extracts isolated from normal mouse epidermis and JWF2 cells. The two cell types showed the same binding profile between normal and transformed cells at the ATF/CRE site, NF-IL6 site, and NF-IL6 site, supershift experiments were performed with the nuclear extracts isolated from normal mouse epidermis and JWF2 cells. The two cell types showed the same binding profile isolated from normal mouse epidermis and JWF2 cells. The two cell types showed the same binding profile.

COX-2 expression and differential expression of C/EBP isoforms during mouse skin carcinogenesis (Fig. 10). A dramatic increase in COX-2 expression was observed from tumors at 10 weeks of promotion and older. The expression of C/EBPα protein was restricted to normal skin. C/EBPβ, however, increased
as tumors became more malignant. The C/EBPβ level also increased slightly. These results suggest that differential expression of C/EBP isoforms may contribute to the constitutive overexpression of COX-2 in neoplastic tissues.

**DISCUSSION**

COX-2 expression is highly up-regulated in various types of tumors. Recently, it has been reported that overexpression of COX-2 in tumors may be regulated at the transcriptional level (15). In NIH3T3 cells, induction of COX-2 by v-src was mediated via JNK at an ATF/CRE site (−56/−48) (22). In virally and oncogene-transformed mammary epithelial cell lines, transformation was related to the increase of COX-2 transcription, and the ATF/CRE site was also a major regulatory region (8). In this study, we used murine skin squamous cell carcinoma JWF2 cells, which harbor an H-ras mutation and constitutively overexpress COX-2. In an attempt to elucidate the signaling pathway leading to the expression of COX-2, we first investigated *cis*- and *trans*-acting factors involved in the transcription of this gene and demonstrated that the E-box and NF-IL6 sites are two major regulatory sequences. It was surprising that only E-box-binding proteins were detectable at the ATF/CRE-E-box by EMSA because there has been no previous report showing the direct involvement of E-box in the transcription of COX-2, except in rat ovarian granulosa cells (18). When mutations were introduced at the various positions of the ATF/CRE-E-box and their effects on protein binding tested, the oligonucleotides having mutations at the last 2 bases of the E-box (CGTCACGGT) could not compete away the complex. The mutations, however, had little effect on the promoter activity (data not shown). The mutations at these bases were also reported to have no effect on the induction of COX-2 by serum, platelet-derived growth factor, or v-src (22, 23). When the first 3 bases of the E-box, which also contain the ATF/CRE core sequence, were mutated (CGTACAGTG → CGTACAGTG), the primer activity was decreased dramatically, and the complex was not competed out by molar excess of cold oligonucleotides. The oligonucleotide having mutations at the first 2 bases (CGTACAGTG → ATTACAGTG), which was described previously as an ATF/CRE-specific mutation (21), still competed out the complex. In addition, the DNA-protein complexes were not affected by the molar excess of cold consensus CREB oligonucleotides. Based on these data, the first 3 bases of the E-box appear to be the more crucial binding sites for the catalytic domain of USFs. Another approach such as methylation interference assay, however, should provide more information on which bases are actual contact sites for the transcription factor. Previous studies have suggested that the binding of USFs to E-box in the absence of MgCl₂ in binding buffer would be nonspecific (36). Because the binding buffer we used for EMSA contains no MgCl₂, we also investigated the effect of MgCl₂ on the binding profile at the ATF/CRE-E-box. Even in the presence of 5 mM MgCl₂, only USFs were detectable at the ATF/CRE-E-box by using antibodies, indicating that the binding reaction was specific (data not shown). In fact, several E-box-binding proteins were also detected at ATF/CRE-E-box on the murine COX-2 promoter (21) even though they do not appear to be important in mediating the response. Interestingly, when the oligonucleotide containing the murine ATF/CRE-E-box was incubated with the nuclear extract from rat ovarian granulosa cells, the DNA-protein complex contained CREB, even though USFs were reported to be the only detectable proteins at the site of the rat E-box (18). Therefore, the proteins binding to ATF/CRE-E-box of murine COX-2 appear to be cell type- as well as species-specific. It is still not clear how such a ubiquitously expressed protein as USF can regulate the transcription of COX-2, an immediate early response gene. Nevertheless, several studies have shown that USFs can regulate cell- or tissue-specific gene expression in various ways. For instance, the regulation of rRNA gene transcription was dependent on the composition of USF dimers (37). Ectopic overexpression of USF-1 or USF-2 as homodimers inhibited transcription, whereas the overexpressed USF-1-USF-2 heterodimer activated transcription. Moreover, the expression of many cell type-specific genes is regulated by the cooperation of ubiquitous USFs and cell-specific transcription factors. For instance, the expression of calcitonin/calcitonin-related peptide gene was regulated by USFs and cell-specific transcription factor, octamer-binding protein 2 (OB2) (38). A recent study has shown the presence of several other USF isoforms in addition to the classical 43-kDa USF-1 and 44-kDa USF-2, and each isoform displayed different transactivation efficiencies (39). Even though the catalytic mechanisms of USFs are not well understood and the USF level does not change significantly in response to external stimuli, it is quite possible that USFs might be activated by phosphorylation or more probably through the interaction with other transcription factors. In fact, USF-2 was first referred to as Fos-interacting protein (FIP) (40).

We also identified C/EBPs as the transcription factors binding to the NF-IL6 site on COX-2 promoter. Inoue *et al*. (17) demonstrated that the NF-IL6 site, in cooperation with the CRE, is the regulatory sequence responsible for the induction of human COX-2 by TPA and lipopolysaccharide, and the response was mediated by C/EBPβ. C/EBPβ (NF-IL6) was also identified as the major protein binding to the NF-IL6 site in mouse osteoblastic cells treated with TNF-α (24). Therefore, it
appears that COX-2 induction by one or more C/EBP family members would be an important regulatory mechanism in the acute phase response. It is hard to generalize the functions of C/EBPs in the regulation of transcription because their expression is quite cell type- and differentiation stage-specific. Even the same C/EBP isoform displays opposite effects depending on the cell type (41, 42). C/EBPα, however, is known to be involved in the regulation of cell proliferation and differentiation. Many studies have shown that C/EBPα can induce cell growth arrest in various cell types. For example, C/EBPα expression is reduced in preneoplastic nodules compared with the surrounding liver tissues (43). C/EBPα overexpression inhibits hepatocyte proliferation and also suppresses colony growth in mouse fibroblasts (44, 45). Furthermore, human hepatoma cell lines transfected with an inducible C/EBPα expression vector showed decreased tumorigenicity (46). Unlike C/EBPα, C/EBPβ and δ are implicated primarily in the regulation of genes involved in inflammation and cell proliferation and tend to be up-regulated during the acute phase response (47, 48). Many studies showed either the transition of protein binding from C/EBPα to C/EBP β and δ or a change in the relative expression level between C/EBPα mRNA and C/EBP β and δ messages when genes are activated. For instance, in mammary epithelial cells and hepatocytes primed by the agents causing acute phase response, C/EBPβ and δ were predominantly expressed, whereas the level of C/EBPα was quite low (47, 49, 50). In contrast, while the C/EBPα level increased, C/EBPβ and δ levels were down-regulated in terminally differentiated adipocytes and normal hepatocytes (45, 51). Alam et al. (49) also reported the dramatic induction of C/EBPδ mRNA by lipopolysaccharide without a change of the C/EBPα level in various tissues. In mouse skin treated with TPA, C/EBPβ and δ mRNA levels were increased while C/EBPα level was not changed. In our study, C/EBPβ and δ were identified as the major C/EBP isoforms, whereas C/EBPα was barely detectable both in JWF2 cells and tumors. In agreement with these results, when rat granulosa cells were stimulated by various hormones, COX-2 promoter activity was induced by the combination of increased C/EBPδ mRNA and decreased C/EBPα transcript (19).

Even though we identified C/EBPα as a major isoform binding to the NF-IL6 site in normal mouse skin where COX-2 is barely expressed, it is unlikely that C/EBPα itself functions as a bona fide repressor on the COX-2 promoter. Rather, it may inhibit transcription of COX-2 indirectly by occupying the NF-IL6 site, thus preventing the other C/EBP isoforms from binding to the DNA. When, however, cells are stimulated, the C/EBPα level is decreased and could be displaced by C/EBPβ or δ, the levels of which are increased during cell proliferation or the acute phase response. Because of their transient inducible
nature, the C/EBPα or δ level seems to decline very rapidly after stimulation in normal tissue (52, 53). In that sense, it is likely that up-regulation of C/EBPβ, and especially δ expression in tumors, may provide a continuous signal to activate COX-2 transcription. The important role of the NF-IL6 site and C/EBP proteins in the constitutive expression of COX-2 has been demonstrated in pancreatic islet-derived cells where, as in tumors, COX-2 expression was highly up-regulated under basal condition, whereas COX-1 was not detected (54). Moreover, the NF-IL6 site was responsible for the COX-2 promoter activity, and the DNA-protein complexes at the NF-IL6 site were supershifted by NF-IL6 protein (C/EBPβ)-specific antibody, but not by C/EBPα-specific antibody. Therefore, it appears that both the NF-IL6 site and C/EBPs may be required for the constitutive expression of COX-2.

Interestingly, C/EBPα and β mRNAs can produce different translational products by a leaky ribosomal scanning mechanism (55). Although some of them function as an activator, others repress the transcription of target genes (56, 57). Because C/EBPs can form a dimer with various transcription factors including C/EBP itself, the ratio of activator to repressor in certain isoforms may also be important in determining the transcription rate. In fact, C/EBPβ was present both in normal tissue and tumor cells. It would be also interesting to investigate whether different forms of C/EBPβ (repressor versus activator) bind to the NF-IL6 site in both cell types. In addition, previous studies reported that phosphorylation of C/EBPs can affect their transactivational capacities and binding affinities to the target DNA sequence (34, 58). Phosphatase inhibitors such as okadaic acid and sodium orthovanadate potentiated the promoter activity regulated by C/EBPβ (47). We also observed similar effects of phosphatase inhibitors on the COX-2 promoter activity, suggesting that phosphorylation of C/EBPs by certain kinases may be important also in inducing COX-2 promoter activity. Finally, Yin et al. (59) reported TNFα-induced translocation of C/EBPβ and δ from cytosol to nucleus in hepatocytes without a significant change in mRNA or protein level. Therefore, regulation of gene expression by C/EBPs seems to be very intricate and fine tuned in normal tissues. It is highly possible however that the balance or ratio among several factors including isoforms, activator/repressor, extent of phosphorylation, and the cellular distribution of each isoform may be disrupted or changed in tumors, thereby leading to the up-regulation of many genes including COX-2. In fact, expression of many genes involved in cell proliferation tends to be up-regulated during carcinogenesis (60). Therefore, it will be worthwhile to investigate whether expression of those genes can be up-regulated by certain C/EBPs. Insulin-like growth factor-I is one of the most potent mitogens, and its expression is increased during mouse skin carcinogenesis (61). Interestingly, it has been reported that PGE2, a major product of COX-2, induces insulin-like growth factor-I expression in osteoblastic cells, which is mediated by C/EBPδ (62).

We were unable to demonstrate directly how two different classes of transcription factors binding to the ATF/CRE-E-box and NF-IL6 site interact with each other. Because the mutation on either site alone decreased the promoter activity significantly, the COX-2 promoter appears to require both sites for full promoter activity. Timchenko et al. (63) showed the possible interaction between USF and C/EBPδ in the autoregulation of C/EBPα gene, where overexpression of C/EBPα resulted in the enhanced binding of USF. Direct interaction between centromere-binding factor-1, a counterpart of USF, and Met-4, Met-28, basic leucine zipper proteins, has been reported in yeast (64). Furthermore, direct physical interaction between a member of the helix-loop-helix proteins, MyoD, and the leucine zipper protein, Jun, was also reported (65). However, how USFs and C/EBPs interact with each other on the COX-2 promoter and activate transcription remains to be elucidated.

Although many studies have addressed the role of COX-2 expression in tumor development, the molecular mechanism regulating expression in tumors has not been elucidated previously. Therefore, our findings may have many implications with regard to understanding the mechanism by which COX-2 is overexpressed in neoplastic tissues. First, we identified both cis- and trans-acting factors involved in COX-2 expression in mouse skin carcinoma cells. Second, we demonstrated that overexpression of COX-2 during mouse skin carcinogenesis is correlated with the increase of C/EBPδ level and decrease of C/EBPα expression. Further studies will be directed toward elucidating the upstream components of the signaling pathway leading to the overexpression of COX-2 in tumors.

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