BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **244,** 143–148 (1998)
ARTICLE NO. RC988222

Transcriptional Role of the Nuclear Factor κB Site in the Induction by Lipopolysaccharide and Suppression by Dexamethasone of Cyclooxygenase-2 in U937 Cells

Hiroyasu Inoue and Tadashi Tanabe[1]

Department of Pharmacology, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565, Japan

Received January 30, 1998

Cyclooxygenase-2 (COX-2), an inducible isozyme of cyclooxygenase, is selectively expressed in response to lipopolysaccharide (LPS) and its expression is suppressed by the glucocorticoid dexamethasone (DEX) in the monocytic differentiated U937 cells. However, COX-2 mRNA was not detected nor induced by LPS before the cells differentiated. To study the transcriptional role of the NF-κB site (nucleotides −223 to −214) in the COX-2 gene, the luciferase reporter vector driven by the COX-2 promoter region (nucleotides −327 to +59) mutated at both the cAMP response element and the NF-IL6 site was stably transfected into U937 cells. The substantial luciferase activity observed in the undifferentiated cells was not induced by LPS. However, after the cells had differentiated, luciferase activity was induced by LPS and its induction was suppressed by DEX. Moreover, a protein tyrosine kinase inhibitor herbimycin A suppressed both the expression of COX-2 mRNA and the luciferase activity induced by LPS. These results suggest that the NF-κB site is involved in both the LPS-induced expression of the COX-2 gene and its suppression by DEX and herbimycin A in a differentiation-dependent manner.

Prostaglandin endoperoxide synthase (EC 1.14.99.1) known as cyclooxygenase (COX) plays a key regulatory role in the biosynthesis of prostanoids such as prostaglandins and thromboxane, and is an effective pharmacological target for nonsteroidal anti-inflammatory drugs such as aspirin and indomethacin (1, 2). Recent studies showed that there are two distinct COX isozymes encoded by separate genes: constitutive COX-1 and inducible COX-2. COX-2 is selectively expressed in response to various inflammatory stimuli such as lipopolysaccharides (LPS) and its induction is suppressed by the glucocorticoid dexamethasone (DEX). Therefore, COX-2 is thought to play a critical role in the production of prostanoids especially in inflammatory responses. U937 cells are committed to the macrophage branch of the myeloid lineage and can be induced, by a variety of agents including phorbol 12-myristate 13-acetate (PMA), to mature from a promonocytic to a monocytic stage of development (3). We found that U937 cells begin to express COX-2 during monocytic differentiation (4) and that the production of thromboxane A_2 in response to LPS and its suppression by DEX are predominantly regulated by the expression of COX-2 (5). However, COX-2 mRNA is not observed nor induced by LPS before the cells differentiated. Therefore, U937 cells provide a good model for studying the transcriptional regulation of the COX-2 gene in response to differentiation and inflammatory stimuli.

In the human COX-2 gene, consensus sequences of NF-κB site, NF-IL6 site and cyclic AMP response element (CRE) are found at nucleotides −223 to −214, −132 to −124 and −59 to −53, respectively (6, 7). We have reported that the CRE plays an important role for expression of the human COX-2 gene in the monocytic differentiated U937 cells (4). Other cis-acting elements are also important for the expression of the gene because the region including the NF-κB site and the NF-IL6 site without the CRE shows weak but significant promoter activity (4). However, we could not observe the LPS-induced promoter activity of COX-2 gene probably due to cell damage caused by electroporation used for transient transfection of the reporter vectors (unpublished results). On the other hand, the 5′-flanking region (−327 to +59) of the human COX-2 gene shows

[1] To whom correspondence should be addressed. Fax: +81-6-872-8092. E-mail: tanabe@ri.ncvc.go.jp.

Abbreviations used: COX, cyclooxygenase; DEX, dexamethasone; CRE, cyclic AMP response element; NF-IL6, nuclear factor for interleukin-6 expression; NF-κB, nuclear factor κB; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; dbcAMP, dibutyryl cAMP; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

0006-291X/98 $25.00

promoter activity inducible by LPS and PMA in bovine arterial endothelial cells (8). However, redundant cooperation among the CRE, NF-IL6 site and NF-κB site of the COX-2 gene makes it difficult to analyze the effect of each element. NF-κB, the prototype of a family of dimeric transcription factors, regulates genes that participate in immune and inflammatory responses (9). To evaluate the role of the NF-κB site (−223/−214) of the COX-2 gene in U937 cells, the luciferase reporter vector driven by the human COX-2 promoter region (−327/+59) mutated at both the CRE and NF-IL6 site was stably transfected into U937 cells. Here, we describe the transcriptional role of the NF-κB site for COX-2 expression in U937 cells.

MATERIALS AND METHODS

Cell culture. U937 cells were supplied by the Japanese Cancer Research Resources Bank and maintained in suspension in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (Flow Laboratories, Irvine, Scotland), 50 μM 2-mercaptoethanol, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 in air. For differentiation, U937 cells were plated at 4×10^6 cells/ml in the medium containing 100 nM PMA (Sigma) and allowed to adhere for 48 h, after which they were fed with PMA-free medium and cultured for 24 h prior to use. LPS (from *Escherichia coli* serotype 055: B5) was obtained from Sigma and used at concentration of 10 μg/ml in the medium.

Plasmid construction. The construction of the reporter vector for the human COX-2 gene phPES2(CRM, ILM) has been described previously (4). A triple mutant construct phPES2(Triple M) was prepared by the same method as phPES2(CRM, ILM). Briefly, the PCR product obtained using the primer set P1 and P8 (5′-GGTcccgggCACTCTCCTGTCTGATCCCTC-3′, originating from the complementary sequence −246/−217 with an *Xma*I recognition sequence in the NF-κB site) was inserted into the *Xma*I site of phPES2(−218/+59, CRM, ILM). The construct phPES2(−218/+59, CRM, ILM) was prepared by the same method as phPES2(−223/+59) using primer P9 (5′-GTGcccgggACCCCCTCTGCTCCCAAATTG-3′, originating from the sequence −220/−191 with an *Xma*I recognition sequence in the NF-κB site) instead of P2 and phPES2(CRM, ILM) as the template (8). The oligonucleotide primers were synthesized on an Applied Biosystems 391 DNA synthesizer. Each construct was verified by DNA sequence analysis.

RNA blot analysis. RNA blot analysis was performed as described previously (6). Total RNA was isolated according to the acid guanidinium thiocyanate procedure (10). Twenty four micrograms per lane of total RNA was used for electrophoresis. The cDNA probes used were the 1.5-kb entire insert of pHEPSII17 for COX-2, and the PCR product corresponding to nucleotide positions 61-950 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6). The mRNA levels were calculated on the basis of hybridization signals measured by a Fujix Bio-image analyzer BAS 2000 (Fuji Photo Film Co., Tokyo).

DNA transfections. To isolate long-term stable transfectants, a total of 5×10^6 U937 cells were cotransfected with 10 μg of the luciferase expression vector phPES2(CRM, ILM) or phPES2(Triple M) and 1.5 μg of pCB6 which contains a neomycin-resistant gene by electroporation at 250 V and 960 μF in 250 μl of the complete medium, using a Bio-Rad Gene Pulser. Cells were selected by adding 400 μg/ml G418 (Gibco BRL, Gaithersburg, MD) to the complete culture medium after 2 days of the electroporation. After 2 weeks of selection, G418-resistant cells were expanded and maintained in culture medium containing 400 μg/ml G418. Stable cells were pooled to randomize the effects of variation in integration sites. Integration of each luciferase expression vector was confirmed by direct DNA sequencing of PCR products using the primers GL-1 and GL-2 (Promega) and DNA extracted from pooled transfectants as a template. Luciferase activity of soluble cell extracts was measured for 10 s as reported previously using a luminometer (Berthold). Activity was normalized to the total protein measured using a BCA protein assay kit (Pierce, Rockford, IL) (4).

Nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared as described previously (11). The following oligonucleotides were made using a DNA synthesizer: 5′-GAGAGTGGGGACTACCCCCTCT-3′, containing the consensus NF-κB sequence of the human COX-2 gene as indicated by underlining (PES2KB); 5′-GAGAGTGcccgggACCCCCTCT-3′, containing a six-point mutation (indicated by lower case letters) within the consensus CRE sequence by introducing a *Xma*I site (PES2KBmut). Complementary oligonucleotides synthesized separately were annealed in 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol. The annealed oligonucleotides were phosphorylated at the 5′-end with [γ-^{32}P]ATP (110 TBq/mmol, Amersham) and T4 polynucleotide kinase. Electrophoretic mobility shift assays using synthesized oligonucleotides were carried out as described previously (12). In the supershift experiments, the antibodies raised against components of NF-κB such as p50 and Rel B (Santa Cruz, CA) and p65 (Serotec, Oxford, United Kingdom) were added to the binding reaction mixtures, and the mixtures were incubated for 30 min at room temperature before the probe was added.

RESULTS

Involvement of NF-κB site in promoter activity induced by LPS. To evaluate the role of the NF-κB site (−223/−214) of the human COX-2 gene, U937 cells were stably transfected with the luciferase reporter vector phPES2(CRM, ILM) (Fig. 1A) which is driven by the human COX-2 promoter region (−327/+59) mutated at both the CRE (−59/−53) and the NF-IL6 (−132/−124) site. As shown in Fig. 1B, the luciferase activity of differentiated cells transfected with phPES2(CRM, ILM) (2.0×10^3 RLU/μg protein) was increased 5-fold by LPS. In contrast, the luciferase activity of undifferentiated transfected cells, which was 6.5-fold of that of the differentiated cells, was not induced by LPS. Treatment with dibutyryl cAMP (dbcAMP), a cell-permeable and stable derivative of cAMP, has been reported to potentiate the promoter activity induced by LPS in U937 cells transfected with a reporter vector driven by the promoter of the interleukin 1β gene (13). Therefore, we used dbcAMP as an enhancing agent for LPS-stimulation signals. As shown in Fig. 1B, dbcAMP potentiated the promoter activity induced by LPS from 5-fold to 10-fold, but dbcAMP alone did not induce promoter activity in the differentiated cells. On the other hand, the luciferase activity of cells transfected with phPES2 (Triple M), which is driven by the human COX-2 promoter mutated at the CRE, NF-IL6 and NF-κB sites (Fig. 1A), was 0.6% that of cells transfected with phPES2 (CRM, ILM) and close to that of non-transfected cells even after stimulation with LPS and dbcAMP. COX-2 mRNA in transfected cells after differentiation was not induced by dbcAMP alone but did increase by about 18-fold in the presence

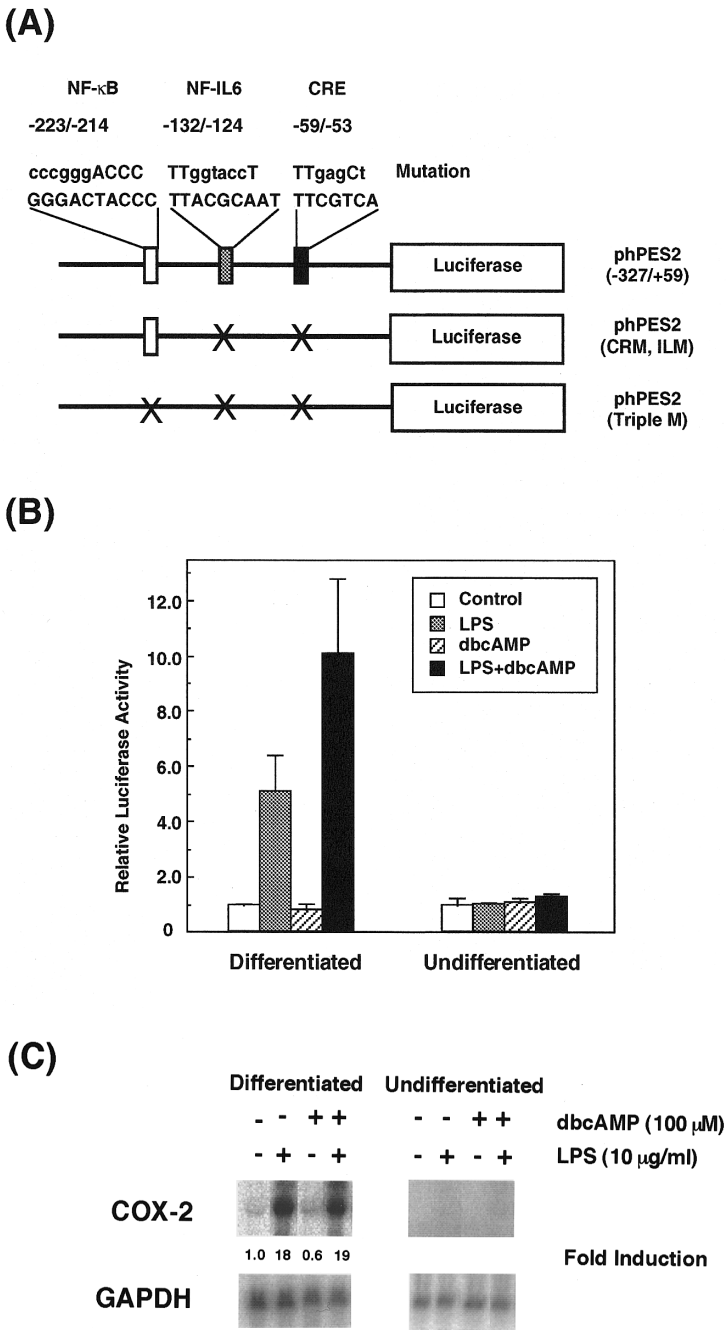

FIG. 1. Expression of COX-2 mRNA and the luciferase activity of stably transfected U937 cells with reporter vectors phPES2(CRM, ILM). (A) Schematic representation of reporter vectors containing the 5′-flanking region of the human COX-2 gene used in this study. The NF-κB site (open box), NF-IL6 site (shaded box) and CRE (closed box) are indicated with their sequences, together with mutations (lower case letters represent the changed nucleotides). Distances are given as nucleotide positions relative to the transcriptional start site as +1. Transfected U937 cells in both the PMA-differentiated and undifferentiated states were incubated for 7 h (B) or 5 h (C) with either no stimulant, 10 μg/ml LPS, 100 μM dbcAMP, or a combination of 10 μg/ml LPS and 100 μM dbcAMP. (B) The cells were then harvested, lysed and assayed for luciferase activity. Results are represented as fold increases in luciferase activity (RLU) per microgram protein relative to each control (Undifferentiated control cells = 1.3×10^4 RLU/μg protein, Differentiated control cells = 2.0×10^3 RLU/μg protein). Experiments were carried out in triplicate. The data are presented as means ± standard deviations. (C) Twenty micrograms of each total RNA was fractionated by electrophoresis through a formaldehyde-containing agarose gel. The fractionated RNAs were transferred to a nylon membrane and hybridized with a ^{32}P-labeled COX-2 probe. The blot was stripped in boiling 0.1% SDS and rehybridized with a ^{32}P-labeled GAPDH probe. Relative amounts of COX-2 mRNA normalized with those with GAPDH mRNA were measured using a Fujix Bio-image analyzer BAS 2000 and are represented as fold induction relative to the control.

of LPS independently of the presence of dbcAMP, whereas it was not detected in undifferentiated cells even after these treatments (Fig. 1C). From these results, the NF-κB site (−223/−214) alone shows promoter activity inducible by LPS in differentiated U937 cells, which may contribute to the induction of human COX-2 mRNA.

Involvement of the NF-κB site in the suppression of promoter activity by DEX and herbimycin A. We have previously shown that pretreatment of differentiated U937 cells with DEX before stimulation with LPS reduces the COX-2 mRNA level (5), which agrees with a report that DEX represses LPS-stimulated thromboxane A_2 biosynthesis in differentiated U937 cells (14). On the other hand, protein tyrosine kinase inhibitors such as herbimycin A suppress COX-2 induction by LPS and other stimulants, suggesting that tyrosine phosphorylation is a part of the signal transduction mechanism that mediates the induction of COX-2 (15-19). Therefore, we examined the role of the NF-κB (−223/−215) site in the suppression of the LPS-induced COX-2 mRNA by DEX and herbimycin A. In cells transfected with phPES2(CRM, ILM), the induction of COX-2 mRNA by LPS or a combination of LPS and dbcAMP was suppressed by treatment with DEX and herbimycin A (Fig. 2A). Moreover, the luciferase activity of the transfected cells induced by LPS or a combination of LPS and dbcAMP was suppressed in a dose-dependent manner by DEX and herbimycin A (Fig. 2B). These results suggest that the NF-κB site (−223/−214) is involved in the transcriptional suppression of the COX-2 gene by DEX and herbimycin A in the differentiated U937 cells.

Electrophoretic mobility shift assay targeting the NF-κB site. To examine the nuclear factors that bind to the NF-κB site (−223/−214) of the COX-2 gene, we performed an electrophoretic mobility shift assay using the oligonucleotide PES2κB (−230/−209) as a probe (Fig. 3). A DNA-protein complex, which was inhibited by the unlabeled oligomer PES2κB but not by the mutated oligomer at the NF-κB site (PES2κBmut), was detected using nuclear extracts from differentiated U937 cells and was enhanced by stimulation with LPS. The amount of this complex was barely changed by treatment with DEX and herbimycin A. Supershift assays using antisera against p50, p65 and Rel B indicated that this protein complex contained the p50 and p65 subunits of NF-κB. Moreover, no effect of DEX or herbimycin A on this protein complex formation was confirmed by the supershift assay using antisera against p50, p65 and Rel B (data not shown).

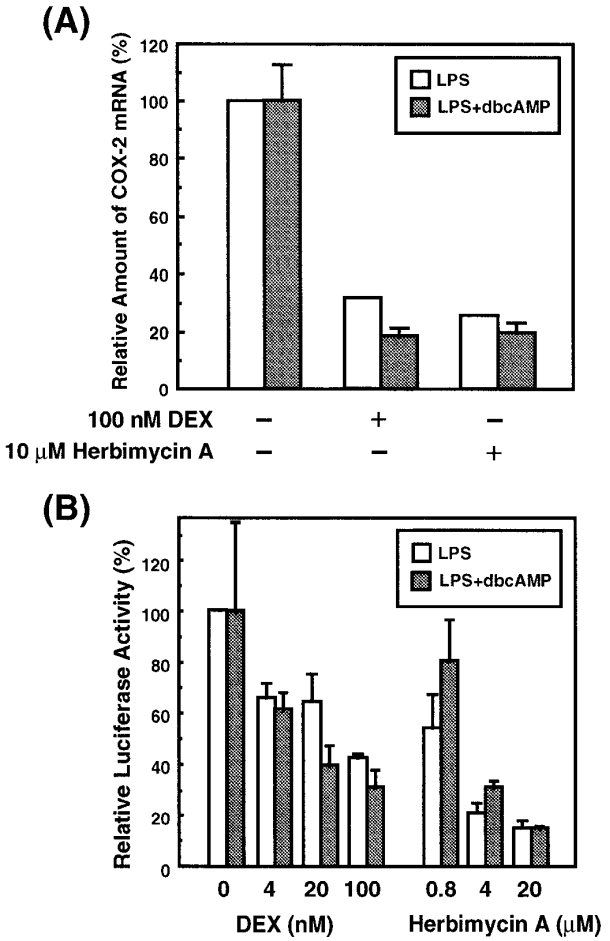

FIG. 2. Effects of DEX and herbimycin A on expression of COX-2 mRNA and the luciferase activity of differentiated U937 cells transfected with phPES2(CRM, ILM). PMA-differentiated U937 cells transfected with phPES2(CRM, ILM) were incubated for 5 h (A) or for 7 h (B) with 10 μg/ml LPS or a combination of 10 μg/ml LPS and 100 μM dbcAMP in the presence or absence of DEX, herbimycin A at the indicated concentrations. (A) Twenty micrograms of each total RNA was analyzed as described in Fig. 1C. Relative amounts of COX-2 mRNA were measured using a Fujix Bio-image analyzer BAS 2000, normalized with those with GAPDH mRNA and indicated taking the value stimulated with LPS and dbcAMP as 100%. (B) The cells were then harvested, lysed and assayed for luciferase activity. Results are presented as % of luciferase activity per microgram protein determined with cells stimulated with LPS and dbcAMP as 100%. Experiments were carried out in triplicate. The data are presented as means ± standard deviations.

DISCUSSION

Using U937 cells transfected with phPES2(CRM, ILM) and phPES2(triple M) we have suggested that the NF-κB site (−223/−214) is involved in the promoter activity induced by LPS and the suppression of its induction by DEX and herbimycin A. Remarkably, LPS-induction of the luciferase activity was not observed in undifferentiated cells (Fig. 1B). On the other hand, the amount of the LPS-induced DNA-protein complex containing the p50 and p65 was barely changed by DEX or herbimycin A. Therefore, the suppression of COX-2 gene expression by DEX may be regulated not in the step of the binding of a transcription factor NF-κB to

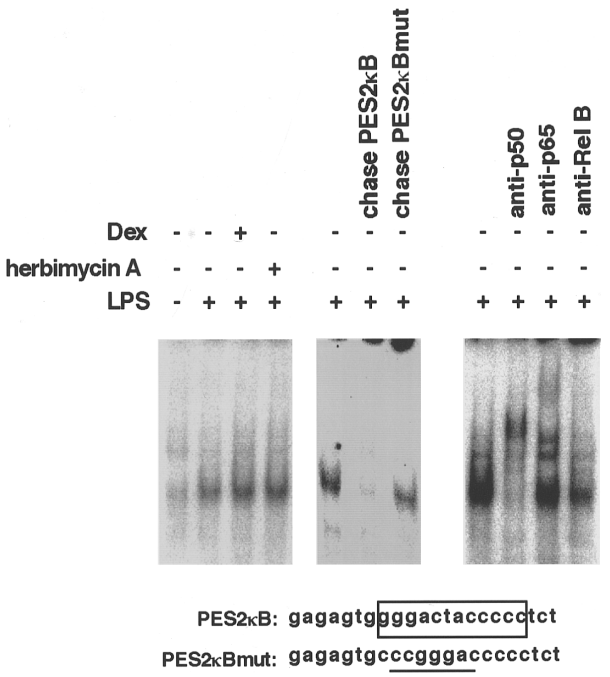

FIG. 3. Electrophoretic mobility shift assay targeting the NFκB site. A ^{32}P-labeled probe PES2κB was incubated with the nuclear extract (1 μg protein) of differentiated U937 cells treated with or without 10 μg/ml LPS, 100 nM DEX or 20 μM herbimycin A. Cold chase experiments were performed with a 30-fold molar excess of a competitor oligonucleotide as indicated. Anti-NF-κB p50, anti-NF-κB p65, or anti-Rel B antibody was incubated with the nuclear extracts.

the NF-κB site but rather through the step of the transcriptional modulation after its binding.

There are two discrepancies between the expression of COX-2 mRNA and the luciferase activity of cells transfected with phPES2(CRM, ILM). First, the luciferase activity was about 6.5-times higher than that of the differentiated transfected cells (Fig. 1B), whereas no COX-2 mRNA was observed in the undifferentiated cells (Fig. 1C). Secondly, the induction of the luciferase activity by treatment with LPS or a combination of LPS and dbcAMP was 5-fold or 10-fold, respectively (Fig. 1B), whereas the induction of COX-2 mRNA in the differentiated cells was almost same (about 18-fold) (Fig. 1C). These discrepancies may be explained by the involvement of other cis-acting elements. Especially, a differentiation-dependent involvement of the NF-κB site in the expression of the COX-2 gene may require activation of other cis-acting elements in U937 cells. As these candidates, involvement of the CRE and NF-IL6 site (4, 8, 20–22) as well as the NF-κB site (22–25) was reported on the COX-2 expression. Previously, we have reported that the CRE (−59/−53) is important for expression of the human COX-2 gene in differentiated U937 cells and that a nuclear protein binds to the CRE during differentiation (4). And that, overexpression of a CRE binding protein decreased the promoter activity of the COX-2 gene in NIH3T3 cells (18) and bovine endothelial cells (unpublished results). However, cross-talk among CRE, NF-IL6 and NF-κB sites in expression of COX-2 gene still remains to be elucidated because we have not obtained stable transfectants with the luciferase reporter vector driven by the wild-type COX-2 promoter region at present.

In summary, the NF-κB site is involved in the LPS-induced expression of the COX-2 gene and its suppression by DEX in U937 cells in a differentiation-dependent manner. However, other cis-acting elements such as the CRE and the NF-IL6 site are also involved in the transcriptional regulation of the COX-2 gene. Thus, further studies of the cross-talk among the NF-κB site and other cis-acting elements are necessary to understand the regulation of the COX-2 gene expression.

ACKNOWLEDGMENTS

We thank Tamiko Sugimoto, Setsuko Bandoh and Satoko Honda for technical assistance and Drs. Chieko Yokoyama, Toshihisa Hatae, Tomoko Yabuki, Takeo Nishimori, and Manabu Shimonishi for discussion. This work was supported in part by grants from the Ministry of Health, and Welfare and the Ministry of Education, Science, Culture and Sports of Japan and also by Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan.

REFERENCES

1. Herschman, H. R. (1996) *Biochim. Biophys. Acta.* **1299,** 125–140.
2. DeWitt, D. and Smith, W. L. (1995) *Cell* **83,** 345–348.
3. Hass, R., Bartels, H., Topley, N., Hadam, M., Köhler, L., Goppelt-Strübe, M., and Resch, K. (1989) *Eur. J. Cell Biol.* **48,** 282–293.
4. Inoue, H., Nanayama, T., Hara, S., Yokoyama, C., and Tanabe, T. (1994) *FEBS Lett.* **350,** 51–54.
5. Nanayama, T., Hara, S., Inoue, H., Yokoyama, C., and Tanabe, T. (1995) *Prostaglandins* **49,** 371–382.
6. Kosaka, T., Miyata, A., Ihara, H., Hara, S., Sugimoto, T., Takeda, O., Takahashi, E., and Tanabe, T. (1994) *Eur. J. Biochem.* **221,** 889–897.
7. Appleby, S. B., Ristimaki, A., Neilson, K., Narko, K., and Hla, T. (1994) *Biochem. J.* **302,** 723–727.
8. Inoue, H., Yokoyama, C., Hara, S., Tone, Y., and Tanabe, T. (1995) *J. Biol. Chem.* **270,** 24965–24971.
9. Baeuerle, P. A., and Baltimore, D. (1996) *Cell* **87,** 13–20.
10. Chomczynski, P., and Sacci, N. (1987) *Anal. Biochem.* **132,** 6–13.
11. Andrews, N. C., and Faller, D. V. (1991) *Nucleic Acids Res.* **19,** 2499.
12. Inoue, H., Baetge, E. E., and Hersh, L. B. (1993) *Mol. Brain Res.* **20,** 299–304.
13. Gray, J. G., Chandra, G., Clay, W. C., Stinnett, S. W., Haneline, S. A., Lorenz, J. J., Patel, I. R., Wisely, G. B., Furdon, P. J., Taylor, and Kost, J. D. (1993) *Mol. Cell. Biol.* **13,** 6678–6689.
14. Bienkowski, M. J., Petro, M. A., and Robinson, L. J. (1989) *J. Biol. Chem.* **264,** 6536–6541.
15. Geng, Y., Blanco, F. J., Cornelisson, M., and Lotz, M. (1995) *J. Immunol.* **155,** 796–801.

16. Blanco, A., Habib, A., Levy, T. S., and Maclouf, J. (1995) *Biochem. J.* **312,** 419–423.
17. Chanmugam, P., Feng, L., Liou, S., Jang, B. C., Boudreau, M., Yu, G., Lee, J. H., Kwon, H. J., Beppu, T., Yoshida, M., Xia, Y., Wilson, C. B., and Hwang, D. (1995) *J. Biol. Chem.* **270,** 5418–5426.
18. Wong, W. Y., and Richards, J. S. (1992) *Endocrinology* **130,** 3512–3521.
19. Stroebel, M., and Goppelt, S. M. (1994) *J. Biol. Chem.* **269,** 22952–22957.
20. Sirois, J., and Richards, J. S. (1993) *J. Biol. Chem.* **268,** 12199–12206.
21. Xie, W., and Herschman, H. R. (1995) J. Biol. Chem. **270,** 27622–27628.
22. Yamamoto, K., Arakawa, T., Ueda, N., and Yamamoto, S. (1995) *J. Biol. Chem.* **270,** 31315–31320.
23. Schmedtje, J. F. Jr., Ji, Y. S. , Liu, W. L., DuBois, R. N., and Runge, M. S. (1997) *J. Biol. Chem.* **272,** 601–608.
24. Newton. R., Kuitert. L. M., Bergmann, M., Adcock, I. M., and Barnes, P. J. (1997) *Biochem. Biophys. Res. Commun.* **237,** 28–32.
25. Crofford, L. J., Tan, B., McCarthy, C. J., and Hla, T. (1997) *Arthritis Rheum.* **40,** 226–36.