

# Chapter 2

## Techniques Used to Study Regulation of Cyclooxygenase-2 Promoter Sites

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### Abstract

Cyclooxygenase-2 (COX-2), the rate-limiting enzyme for prostaglandin (PG) biosynthesis, plays a key role in inflammation, tumorigenesis, development and circulatory homeostasis. COX-2 expression is rapidly and sensitively regulated by various stimuli, and also its regulation is distinct among cell types at transcriptional and posttranscriptional levels. Therefore, it is important to consider these features of COX-2 expression in the reporter assays we describe in this chapter. Emphasis should be made with regard to two points. Firstly, COX-2 reporter assays should be evaluated by intrinsic COX-2 expression, such as RT-PCR, northern blotting, western blotting, or by PGE<sub>2</sub> measurement. Secondly, one must carefully choose several conditions in the reporter assays for experimental purposes.

**Key words:** Cyclooxygenase, Luciferase, Reporter, CRE, NF-IL6, NF-κB, *cis*-Acting element

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### 1. Introduction

Cyclooxygenase (COX) has two isoforms, COX-1 and -2. COX-1 is constitutively expressed in most cells, whereas COX-2 is largely absent but induced upon stimulation by inflammatory stimuli such as endotoxin, lipopolysaccharide (LPS), suggesting that COX-2 plays a critical role in inflammation. Growing evidence indicates that expression of COX-2 is differently regulated in different types of cells ((1–3), Fig. 1).

In COX-2 promoter sites, three *cis*-acting elements, NF-κB, NF-IL6 (also called as C/EBPβ) sites, and cyclic AMP response element (CRE), as well as TATA box are conserved among species (Fig. 2). The CRE is overlapped with E-box with which USF1 and USF2 are known to bind. The CRE in the COX-2 gene is not a symmetrical CRE such as the one found in the somatostatin gene, but an asymmetrical CRE found as a consensus sequence

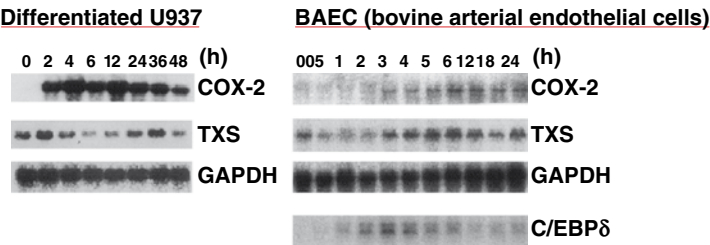


Fig. 1. Distinct expression of COX-2 mRNA by LPS between U937 cells and BAEC. Total RNAs were isolated from macrophage-like differentiated U937 cells and BAEC at the indicated times after LPS (10  $\mu$ g/ml) stimulation. RNAs (24  $\mu$ g) fractionated through formaldehyde-containing agarose gel electrophoretically were transferred to a nylon membrane and hybridized with a  $^{32}$ P-labeled COX-2, TXS (thromboxane synthase), PGIS (prostacyclin synthase), C/EBP $\delta$ , and GAPDH (glyceraldehydes-3-phosphate dehydroge-nase probes), respectively.

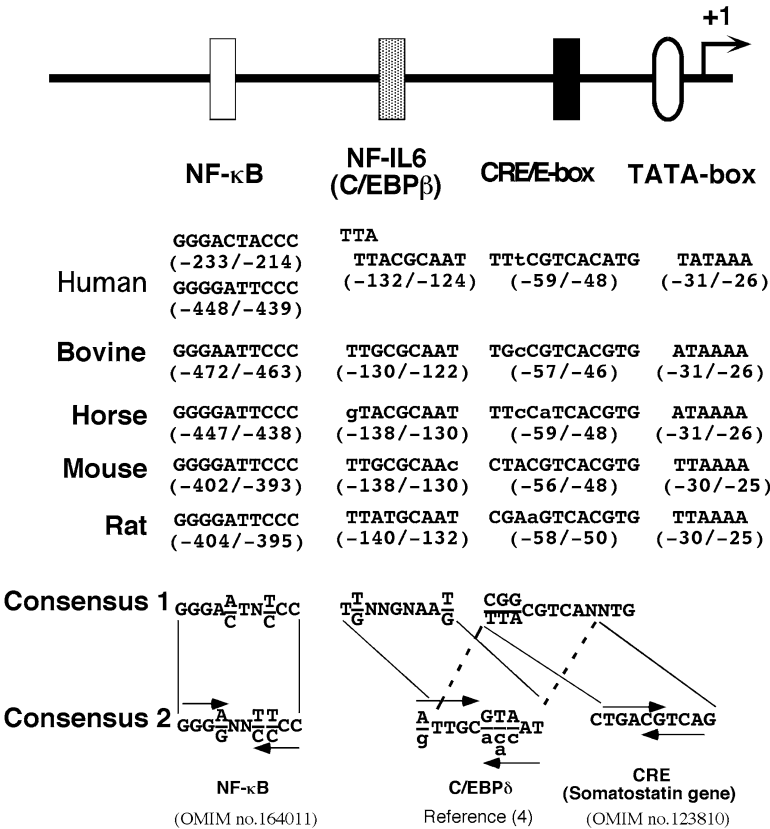


Fig. 2. Proximal promoter region of COX-2 gene. The diagrams show the potential response elements based on sequence similarities to consensual response elements. Distances are given as nucleotide positions relative to the transcriptional start site as +1.

for C/EBP $\delta$  (4), which will partly make possible the various expression patterns of COX-2. We constructed several COX-2 reporter vectors to study the roles of these *cis*-acting elements.

Fortunately, by using these reporter vectors, we and our collaborators have been showing that these *cis*-acting elements are differently involved in the COX-2 promoter activity in different cells ((5–18) (see [http://koto.nara-wu.ac.jp/kenkyu/Profiles/5/0000469/theses\\_e1.html](http://koto.nara-wu.ac.jp/kenkyu/Profiles/5/0000469/theses_e1.html) for more details) (see Notes 1 and 2). On the other hand, other *cis*-acting elements such as NF-AT sites (19) and PPRE (20) were reported in the COX-2 promoter region. Therefore, you may need to make your own reporters of COX-2 gene for your experimental purposes.

Transfection assays recently became more widely used. Various transfection reagents, reporter vectors, and luminometers are available from several suppliers. However, it is still difficult to perform transfection assays on some cell lines. Moreover, in the case of U937 cells, there exist two distinct cell types: one may be suitable for transfection assay but does not express COX-2, and the other type is difficult for the transfection assay but shows COX-2 induction by LPS after differentiation. In this case, we found that the nuclear receptor peroxisome proliferator activated receptor- $\gamma$  (PPAR $\gamma$ ) is only expressed in the latter type (10). Moreover, better transfection efficiency sometimes brings about more cell damage; for example, some types of cellular stress cause induction of COX-2. Therefore, one should measure COX-2 promoter activity without stimulation to determine whether COX-2 induction is due to stress or stimulation. In addition, measuring an induced promoter activity of COX-2 gene is also not easy because transfected cells need considerable time to recover from the damage of the transfection. Therefore, one should determine the appropriate conditions for the cell type in question by performing pilot experiments.

The method of reporter assays described below is for bovine arterial endothelial cells (BAEC). This method will be modified for various cell types by using different transfection reagents (see Note 3). It is also important to evaluate your luciferase assays, at least, by RT-PCR or northern blot analysis.

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## 2. Materials

### 2.1. Cell Culture and Cell Lysis

1. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with or without 10% fetal bovine serum (FBS, see Note 4). Store at 4°C.
2. Phosphate buffered saline (PBS) for cell culture: 137 mM of NaCl, 2.7 mM of KCl, 4.2 mM of Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM of KH<sub>2</sub>PO<sub>4</sub>. The final pH should be adjusted at 7.4. Store at 4°C.
3. Trypsin (0.25%) and EDTA (0.02%) solution (cell culture tested, sterile-filtered). Store at –20°C. The working solution (0.05% trypsin, 0.01% EDTA) is freshly prepared and stored at 4°C within several days.

4. LPS (from *Escherichia coli* serotype 055: B5) is dissolved at 1 mg/ml in tissue culture grade water, sterile-filtered and stored at 4°C. Aliquot and store at -80°C for long-term storage.
5. Reporter lysis buffer (Promega Cat. # 3971) is used for cell lysis. However, the manufacturing company does not reveal the composition of the buffer. So, if you do not want to use this buffer, the following buffer will also work: 25 mM of glycylglycine (pH7.8), 15 mM of MgSO<sub>4</sub>, 4 mM of EGTA, 1%(v/v) of Triton X-100, 1 mM of dithiothreitol (DTT, add immediately before use). Store at room temperature.
6. Cell scrapers.
7. 24-well cell culture dishes. Lower transfection efficiency may need larger culture dishes such as 6-well or 12-well.

## **2.2. Transfection Reagents**

1. Reporter vectors. Human COX-2 reporter vectors pHES2 (-327/+59) and (-1,432/+59) are available from our laboratory upon request (5). pSVβ-gal (Promega), an expression vector for β-galactosidase under control of SV40 promoter, is used for normalization of transfection. pEGFP-C1 (Clontech), expression vector for GFP (green fluorescent protein) is used for the confirmation of transfection efficiency before the reporter assay (optional). It is essential to obtain high quality plasmid DNAs for transfection assays. We usually use a plasmid purification kit from QIAGEN (see Note 5). Each plasmid DNA is dissolved in 0.1× TE (10 mM of Tris-HCl, pH 7.6, and 1 mM of EDTA), which is carefully prepared using endotoxin-free tissue culture grade water. Concentration of each DNA solution is kept at 1 mg/ml. Aliquot and store at -80°C (for long-term storage) or at 4°C (for short-term storage).
2. Trans IT-LT-1 (Mirus, Madison, WI). This lipid-mediated transfection reagent is suitable for BAEC, but not for other cells such as HEK293 cells.
3. Sterile Polystyrene Tubes (12×75 mm).

## **2.3. Reporter Assays**

1. Luciferase assay reagent (Promega E3971). Store aliquots at -80°C. In order to avoid over three times of freezing/thawing, determine the appropriate aliquot size in your experiments (in our case, 3 ml).
2. β-galactosidase assay reagent: 80 mM of sodium phosphate buffer, pH7.3, 102 mM of 2-mercaptoethanol, 9 mM of MgCl<sub>2</sub>, 8 mM of CPRG (β-d-galactopyranoside, add immediately before use). Store this reagent without CPRG at room temperature. CPRG is dissolved at 400 mM in tissue culture water and stored as a stock solution aliquoting at -80°C.

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### 3. Methods

#### **3.1. Preparation of Cells for Transfection**

1. In a 24-well tissue culture plate, seed  $2 \times 10^4$  cells per well in 0.5 ml of DMEM containing 10% FBS.
2. Incubate the cells at 37°C in a CO<sub>2</sub> incubator until the cells are 60–70% confluent. This will usually take 18–24 h.

#### **3.2. Transfection and Cell Lysis**

1. Prepare the following solutions A and B in sterile polystyrene tubes. This is an example of transfection for 24 wells.
2. Solution A: Dilute 32.5  $\mu$ l (0.8  $\mu$ l  $\times$  26) of Trans-IT-LT-1 into 650  $\mu$ l of DMEM without FBS, mix by gentle pipetting, and incubate at room temperature for 15 min.
3. Solution B: Dilute 7.8  $\mu$ g (0.3  $\mu$ g  $\times$  26) of a luciferase reporter vector pGV-PES2(–327/+59) and 1.6  $\mu$ g of  $\beta$ -galactosidase reporter vector pSV $\beta$ -gal (0.06  $\mu$ g  $\times$  26) into 650  $\mu$ l of DMEM without FBS (i.e. 25  $\mu$ l per well). Prepare for 26 wells not for 24 wells to avoid shortage of the solutions, and mix by gentle pipetting. It is important that the medium contains no antibiotics such as penicillin and streptomycin. If you also use optional reporter vector such as pEGFP-C1, each DNA amount should be 0.8  $\mu$ g each for pSV $\beta$ -gal and pEGFP-C1 to keep total DNA amounts constant (see Note 6).
4. Add solution B to solution A, mix gently, and incubate at room temperature for 15 min.
5. Add the mixed solutions A and B (50  $\mu$ l per well), dropwise to the cells in complete growth medium in Subheading 3.1. Gently rock the dish back and forth and from side to side to distribute the DNA/lipid complexes evenly.
6. Incubate for 5 h, replace 0.5 ml of the fresh DMEM containing 10% FBS and incubate for an additional 24 h (see Note 7).
7. Add LPS (final working concentration 10  $\mu$ g/ml), and incubate for an additional 5 h (see Note 8). Other Stimuli can be used along with or without LPS.
8. Observe the transfected cells with pEGFP-C1 expression vector by using a fluorescence microscope. If expression of GFP is detected in over 30% of transfected cells, it will be good for transfection assays for BAEC.
9. Remove the growth medium from the wells. Wash the cells once with PBS, being careful not to dislodge any of the cells. Remove as much of the final PBS wash as possible.
10. Add 100  $\mu$ l per well of reporter lysis buffer to cover the cells. Rock the dish slowly several times to ensure complete coverage of the cells.

11. Scrape all areas of the dish, then tilt the dish and scrape the cell lysate to the lower edge of plate. Take care to scrape down all visible cell debris. Transfer the cell lysate to a microcentrifuge tube with a pipette and place the tube at  $-80^{\circ}\text{C}$  for at least 15 min (see Note 9).
12. After thawing the cell lysate at room temperature, vortex the tube for 15 s, then centrifuge in a microcentrifuge ( $11,000\times g$ ) for 15 s at room temperature. Transfer the supernatant to a fresh tube.
13. Mix 10  $\mu\text{l}$  of cell extract with 50  $\mu\text{l}$  of Luciferase Assay Reagent in sample tubes at room temperature (see Note 10).
14. Place the reaction mixture in a luminometer. Measure the light produced for 10 s. Assay each reaction at the same time interval after addition of the sample to assay reagent (see Note 11).
15. Mix 2  $\mu\text{l}$  of cell extract (room temperature) with 100  $\mu\text{l}$  of  $\beta$ -galactosidase assay reagent on a microtiter plate. As a control, mix 2  $\mu\text{l}$  of Reporter Lysis Buffer (room temperature) with 100  $\mu\text{l}$  of  $\beta$ -galactosidase assay reagent. Incubate samples at room temperature for 5–30 min.
16. Read the absorbance at 580 nm with a microtiter plate reader (see Note 12).
17. Calculate the normalized luciferase activity by data obtained from steps 14 and 16. For example, when the measured values in step 14 are LucA1, LucA2, and LucA3 (without stimulus X) and LucB1, LucB2, and LucB3 (with stimulus X), respectively, and the corresponding measured values in step 16 are bGalA1, bGalA2, and bGalA3 (without stimulus X) and bGalB1, bGalB2, and bGalB3 (with stimulus X), respectively, firstly, we obtain the values LucA1/bGalA1, LucA2/bGalA2, and LucA3/bGalA3, and LucB1/bGalB1, LucB2/bGalB2, and LucB3/bGalB3, then calculate the means and standard deviations for A and B, respectively. If these means and standard deviations are expressed by  $M(A)$ ,  $SD(A)$ ,  $M(B)$ , and  $SD(B)$ , respectively, the following values are expressed as

$$\begin{aligned} &\text{Normalized luciferase activity A (without stimulus X)} \\ &= 1 \pm SD(A)/M(A) \end{aligned}$$

$$\begin{aligned} &\text{Normalized luciferase activity B (with stimulus X)} \\ &= M(B)/M(A) \pm SD(B)/M(A) \end{aligned}$$

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## 4. Notes

1. Human COX-1 and COX-2 reporter vectors are available for your request (7, 9; e-mail to H.I.). Luciferase activity of the COX-2 reporter is higher than that of the COX-1 reporter,

whereas COX-2 mRNA expression is much lower than COX-1 mRNA without stimulation in several cells. This discrepancy comes from COX-2 instability sequences in the 3'-untranslated region, whose reporter vector is also available (13).

2. We constructed the COX-2 reporter vectors using pGL-2 (Promega). We also constructed COX-2 reporter vectors using pGL-3 (Promega), which encodes a stable luciferase gene. However, induction of the latter type is lower than that of the former type. We also constructed COX-2 reporter vectors using several GFPs as a reporter; however, we could not measure the good dynamic range of FACS analysis using the GFP compared with the luciferase activity in the induction experiment. Experimenters should also pay attention to other assays such as Chromatin immunoprecipitation and gel-shift assays to evaluate transfection assays.
3. There are several transfection methods. In our case, we usually use lipid-mediated transfection reagents because of convenience and stable results in spite of their higher cost. In transfection into BAEC, firstly, we used a lipofectin (Invitrogen) (7), and then changed to Trans-IT-LT1 (Mirus) (9). On the other hand, in transfection into HepG2 and HEK293 cells, we use a lipofectamin (Invitrogen). Recently, many transfection reagents are available. You could carefully choose the reagents for your experiments. At that time, you had better check an induction of luciferase activity by stimulants, not basal luciferase activity without stimulants because COX-2 promoter may be activated by the reagents themselves due to their potential damaging effects to transfected cells (see Note 4).
4. Choice of a good FBS is very important for COX-2 transfection assays. We usually choose a suitable serum for COX-2 reporter assay. As an example, Fig. 3 shows different induction of the COX-2 promoter activity by LPS in BAEC cultured with four different lots of FBS. In this case, we chose Lot #2 because of lower luciferase activity without LPS and higher activity with LPS. Fig. 3a, b show luciferase activities derived from pHES2 (-327/+59) with and without normalization of  $\beta$ -galactosidase activities derived from pSV- $\beta$  gal, respectively.
5. A special plasmid DNA purification kit which removes endotoxin is available from QIAGEN. Although this may be more effective, we have observed no difference in the results of reporter assays using plasmid DNA prepared by normal and special kits.
6. If you cotransfect an expression vector, such as PPAR $\alpha$ , with COX-2 reporter vector, the total amount of plasmids remains constant. In this case, for example, 3.9  $\mu$ g PPAR $\alpha$  expression vector, 3.9  $\mu$ g COX-2 reporter, 0.9  $\mu$ g pSV- $\beta$ -gal, and 0.9  $\mu$ g pEGFP-C1 are used (10). If you investigate the effects of an expression vector for X protein on COX-2 promoter, you

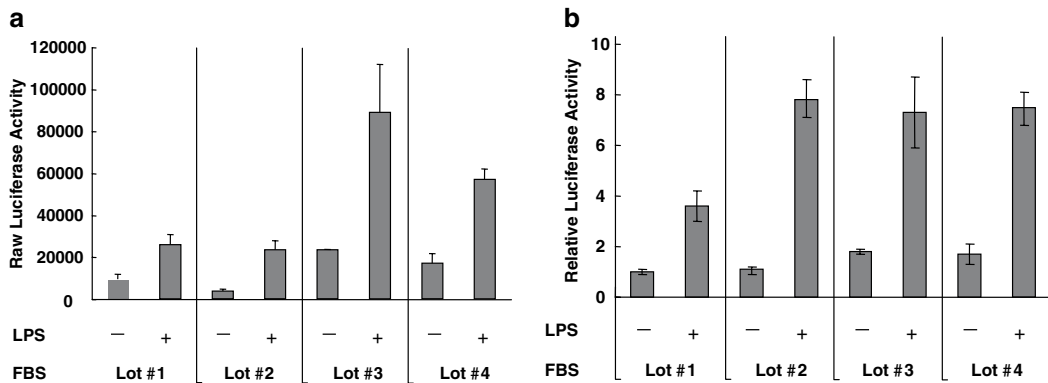


Fig.3. Effects of different lots of FBS on COX-2 promoter activity. BAEC were transiently transfected with phPES2(-327/+59) together with pSV- $\beta$ gal used as an internal control for the transfections. Following transfection, the cells were incubated for 5 h with no stimulant or with LPS (1  $\mu$ g/ml) in the presence of four different lots of FBS. The cells were then harvested, lysed, and assayed for both luciferase and  $\beta$ -galactosidase activities as described. Results are represented as raw luciferase activities (a) or relative luciferase activities (b) obtained by dividing the normalized luciferase activity in lot #1.

should be using the same expression vector without gene for X protein as a negative control.

7. Incubation time before adding stimulants, such as LPS, should be determined by each preliminary experiment. In our case, induction of COX-2 promoter activity is not observed in the incubation for 5 h because transfected cells are exposed to the stimuli of the transfection itself and will not be ready to respond to stimulants. In the case of MC3T3E1 cells (21), an incubation of 72 h before adding stimulants is needed for good induction of the luciferase. According to our experience, longer incubation time is better for measuring the induced luciferase activities, but not for measuring the basal luciferase activities.
8. Incubation time after adding stimulants such as LPS should also be determined by performing preliminary experiments. We recommend a time-course experiment for each stimulant. Stimuli should be dissolved in DMSO or ethanol. Stock solutions should be concentrated 1,000 times more than the final concentration used in the culture medium in order to dilute DMSO and ethanol significantly.
9. Luciferase in the cell extract is not stable even if you store it at  $-80^{\circ}\text{C}$ . On the other hand, a freezing/thawing cycle provides better extraction of cells. We recommend measuring the luciferase activity using freshly prepared samples after a freezing/thawing cycle. If you use the stored sample, you should measure all samples in the set because a luciferase activity measured on one day is different from that measured on another day.



10. Measuring the luciferase activity is sensitive to temperature. It is important to keep the samples at room temperature. The luminometer should be turned on at least 15 min before use.
11. Luciferase activity of each transfected sample should be at least three times the negative control without nontransfected cell extract. Dynamic range and linearity of luciferase activity should be determined by each luminometer.
12. The absorbance of each transfected sample should be at least three times the negative control without nontransfected cells. Dynamic range and linearity of  $\beta$ -galactosidase activity should be determined by each plate reader. In general, dynamic range of  $\beta$ -galactosidase activity is much narrower than that of luciferase activity.

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## Acknowledgments

We would like to thank Ms. Haruka Takeuchi, Mariko Hotta, Tomoko Tsukamoto, and Tomomi Matsuyama for their technical assistance. This work was supported by Grant-in-Aid for scientific research from Ministry of Education, Culture, Sports, Science and Technology (Houga18650213, B19300250).

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Cyclooxygenases

Methods and Protocols

Ayoub, S.S.; Flower, R.J.; Seed, M. (Eds.)

2010, X, 218 p. 38 illus., Hardcover

ISBN: 978-1-58829-953-6

A product of Humana Press