Identification of a cis-Regulatory Element for Δ12-Prostaglandin J2-induced Expression of the Rat Heme Oxygenase Gene*

We recently reported that Δ12-prostaglandin (PG) J2 caused various cells to synthesize heme oxygenase, HO-1 (Koizumi, T., Negishi, M., and Ichikawa, A. (1992) Pros- taglandins 43, 121–131). Here we examined the molecular mechanism underlying the Δ12-PGJ2-induced HO-1 synthesis. Δ12-PGJ2 markedly stimulated the promoter activity of the 5′-flanking region of the rat HO-1 gene from −810 to +101 in rat basophilic leukemia cells. From functional analysis of various deletion mutant genes we found that the Δ12-PGJ2-responsive element was localized in a region from −690 to −660, containing an E-box motif, which was essential for the Δ12-PGJ2-stimulated promoter activity. When the region containing the Δ12-PGJ2-responsive element was combined with a heterologous promoter, SV40 promoter, in the sense and antisense direction, the element showed an enhancer activity in response to Δ12-PGJ2. Gel mobility shift assays demonstrated that Δ12-PGJ2 specifically stimulated the binding of two nuclear proteins to the E-box motif of this region. These results indicate that Δ12-PGJ2 induces the expression of the rat HO-1 gene through nuclear protein binding to a specific element having an E-box motif.

Eicosanoids are oxygenated metabolites of arachidonic acid, and are regarded as modulators of cellular functions in various physiological and pathological processes (1). Eicosanoids are oxygenated metabolites of arachidonic acid, and are regarded as modulators of cellular functions in various physiological and pathological processes (1). Eicosanoids are classified into two groups, conventional eicosanoids and cyclopentenone-type prostaglandins (PGs) according to their mechan-isms of action. Conventional eicosanoids, such as PGE2 and PGD2, act on a cell surface receptor to exert their actions, and the molecular structures of their receptors have been revealed recently (2). Cyclopentenone PGs, such as Δ12-PGJ2 and PGA2, have no cell surface receptor, but are actively transported into cells and accumulated in nuclei, where they act as potent inducers of cell growth inhibition and cell differentiation (3). The actions of cyclopentenone PGs are attributed to the synthesis of the various proteins induced by them, such as heat shock proteins (HSPs) (4, 5), γ-glutamylcysteine synthetase (6), collagen (7), gadd 153 (8), and heme oxygenase (9). In contrast to conventional eicosanoids, the molecular characterization of cyclopentenone PG actions has been hardly carried out.

Heme oxygenase is one of the most prominent proteins induced by Δ12-PGJ2, and it is a key enzyme in heme catabolism, oxidatively clearing heme to yield biliverdin, iron, and carbon monoxide (10). The biological functions of this enzyme are the production of biliverdin as a physiological antioxidant and the conservation of the iron (11). Furthermore, carbon monoxide produced on the enzymatic degradation of heme has been suggested as a function as a neural messenger (12). Two isoforms of heme oxygenase, HO-1 and HO-2, have been identified (13). HO-2 is constitutively expressed, while HO-1 is drastically induced in response to a variety of stresses, including heavy metals, heat shock, and UV irradiation (14). We previously found that Δ12-PGJ2 preferentially induced the synthesis of HO-1 in various cells involved in the reticuloendothelial system, in which active degradation of heme by HO-1 takes place during inflammation (9, 15). In order to elucidate the mechanism underlying Δ12-PGJ2-induced protein synthesis, we examined the effect of Δ12-PGJ2 on the promoter activity of the HO-1 gene. We report here that Δ12-PGJ2 induces the expression of the rat HO-1 gene through nuclear protein binding to a specific Δ12-PGJ2-responsive element, located 660 base pairs upstream from the transcription initiation site.

EXPERIMENTAL PROCEDURES

Materials—Δ12-PGJ2 was a generous gift from Teijin Ltd. (Tokyo, Japan). Δ12-PGJ2 was kindly supplied by Dr. M. Suzuki of Gifu University. PUC000CAT was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Other agents were obtained from commercial sources, as follows: γ,γ′-PATP (3,000 Ci/mmol) and [32P]dCTP (3,000 Ci/mmol), DuPont NEN; [14C]1-deoxychloramphenicol (54 mCi/mmol), Amersham; PGE2, PGA1, PGF2α, PGA2, PGD2, PGE1, and PAGA1, Funakoshi Pharmaceuticals (Tokyo, Japan); and staurospo-rycin, acetyl-CoA, and hemin, Sigma. The sources of other materials are given in the text.

Rat basophilic leukemia (RBL)-2H3 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 4 mM glutamine, 0.2 mg/ml streptomycin, and 100 units/ml penicillin under humidified air containing 5% CO2 at 37 °C.

Northern Blots—Total RNA from RBL-2H3 cells was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (16), and 5 μg of each RNA was separated by electrophoresis on a 1.2% agarose gel, transferred to a nylon membrane (Hybond-N, Amersham Corp.), and hybridized with a 32P-labeled fragment, corresponding to exon 3 of the rat HO-1 gene. The same filter was rehybridized with 32P-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA probe (Clontech). Hybridization was carried out at 68 °C in 6× SSC, and the filter was washed at 68 °C in 2× SSC. The filter was autoradiographed with x-ray film (Fuji RX). The radioactivity was determined with a Fuji BAS 2000 imaging analyzer (Fuji Film Co., Tokyo).

Construction of Plasmid DNA—The 5′-flanking region of the rat HO-1 gene (nucleotide residues −810 to +101, relative to the transcription start site) was obtained by means of the polymerase chain reaction (PCR) from genomic DNA prepared from RBL-2H3 cells, as described.

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1 The abbreviations used are: PG, prostaglandin; RBL, rat basophilic leukemia; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; HSE, heat-shock element; MRE, metal-responsive element; HSF, heat shock factor; HSP, heat shock protein; PAGE, polyacrylamide gel electrophoresis. **
Previously (17). The PCR primers with restriction enzyme sites (BamHI for the forward primer and Sall for the reverse one) were designed according to the published sequence of the rat HO-1 gene (18). The BamHI/Sall fragment of the PCR product was first constructed into pBluescript II (Stratagene), and then the Sad/KpnI insert was constructed into pUC8/CAT to generate plasmid RH0810, containing the 5'-flanking region of the HO-1 gene, upstream of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. The sequence of the cloned 5'-flanking region was confirmed by sequence analysis using the diexodeoxy nucleotide chain termination method.

Successive deletion plasmids as to the 5'-flanking region, RH0600 (−600 to +101), RH0320 (−320 to +101), RH0270 (−270 to +101), RH0200 (−200 to +101) and RH0810 (−810 to −600), were also generated by PCR using the respective forward primers with a BamHI site. To prepare a deletion plasmid without region (−600 to −270), two DNA fragments (−810 to −600, and −270 to +101) were prepared by PCR and then sequentially constructed into pUC8/CAT. For the functional analysis using a heterologous promoter, the 5'-flanking region from −810 to −600 was inserted into pCAT promoter vector (Promega), which carries the SV40 promoter upstream from the CAT gene, and the DNA fragment from −810 to −600 was located upstream from the SV40 promoter, in the sense (pCAT-H0a) and antisense direction (pCAT-H0b).

Transfection and CAT assay—RBL-2H3 cells were transiently transfected with the plasmid DNA constructs by the DEAE-dextran method (19). After the cells (4 × 10⁶ cells/assay) had been incubated for 30 min at 37 °C in 0.5 ml of serum-free Dulbecco's modified Eagle's medium containing 8 μg of plasmid DNA and 20 μg of DEAE-dextran (Pharmacia Biotech Inc.), they were incubated for 10 min at 37 °C with a dimethyl sulfoxide-hypertonic solution (28 mM Tris-HCl, pH 7.5, containing 0.4 M sucrose, 8% polyethylene glycol 4000, 84 mM NaCl, and 10% dimethyl sulfoxide). They were then cultured for 2 days in DME/10% foetal calf serum.

Reactions were started by the addition of the test agents. After incubation for the indicated times, cellular extracts were prepared by four cycles of freezing and thawing. The CAT assay was performed as described previously (20). Cellular extracts containing equal amounts of protein for each condition were prepared from the cells. Following electrophoresis, the wet gel was stained with an ABI 391 DNA synthesizer. The sequences were analyzed using the diexodeoxy nucleotide chain termination method.

To identify the cis-regulatory element for Δ12-PGJ2-induction of a 5'-flanking region of the HO-1 gene by Δ12-PGJ2, we examined the effect of Δ12-PGJ2 on the expression of the HO-1 gene in RBL-2H3 cells by Northern blot analysis. As shown in Fig. 1A, after a 0.5-h lag time, Δ12-PGJ2 gradually increased the mRNA level of HO-1. This stimulation of expression of HO-1 mRNA preceded the Δ12-PGJ2-induced HO-1 protein synthesis and HO-1 activity (9). A Δ12-PGJ2 concentration dependence caused marked accumulation of the mRNA, the maximum being reached at 10 μM (Fig. 1B), and this concentration dependence was consistent with those of Δ12-PGJ2-induced HO-1 protein synthesis and HO-1 activity (9). On the other hand, the mRNA level of glyceraldehyde-3-phosphate dehydrogenase did not change.

In RBL-2H3 cells, the time course and concentration dependence of the Δ12-PGJ2-induced HO-1 protein synthesis and HO-1 activity (9) were determined as described previously (22). The 5'-end-labeled fragment from −960 to −660 was incubated with the nuclear extract under standard gel mobility shift assay condition. Following electrophoresis, the wet gel was irradiated for 20 min using a UV transilluminator (254 nm; 1700 micro-watt/cm²) at a distance of 12 cm of the UV source. The autoradiograph was scanned using an Amplifier (Fuji RX).
ulation of the promoter activity for various PGs. As shown in Fig. 5, RHO810, containing both HSE and MRE, had completely lost the 

PGJ2 responsiveness, indicating that HSE or MRE is not the 

Δ12-PGJ2-responsive element. RHO270, without either HSE or MRE, showed basal promoter activity without Δ12-PGJ2 re-

sponsiveness, but RHO-0, without the 5'-flanking region, lost basal promoter activity, indicating that the region from –270 to 0 is required for the basal promoter activity of HO-1 gene. Whereas the region (–810 to –600) itself did not show the basal promoter activity, this region (–270 to +101) regained the Δ12-PGJ2-stimulated promoter activity, suggesting that this region contains an enhancer-like element. These results indicate that Δ12-PGJ2 stimulates the expression of the HO-1 gene through neither HSE nor MRE, but through an element located between –810 and –600. On the other hand, heat shock stimulated the promoter activity of either RHO600 or RHO320, but failed to stimulate the activity of the HSE-deleted mutant gene, RHO270 (data not shown).

Enhancer Function of the Δ12-PGJ2-responsive Element—To examine whether the region from –810 to –600 has an enhancer function, we constructed another fusion gene with a heterologous promoter, SV40 promoter (Fig. 6). Δ12-PGJ2 stimulated the CAT activity by about 7-fold in the cells transfected with pCAT-HO-s or pCAT-HOa containing the region from –810 to –600 in the sense or antisense orientation upstream from the SV40 promoter. This establishes that Δ12-PGJ2-responsive element has an enhancer function.

Point Mutation of the E-box Motif in the Δ12-PGJ2-responsive Element—To identify more precisely the region responsible for the stimulation by Δ12-PGJ2 and to detect nuclear protein binding to the region, we performed a gel mobility shift assay using various sized DNA fragments, from –810 to –600. We could not find potential binding sites for so far known transcription factors in the region from –810 to –600, but this region contained the consensus E-box motif, CANNTG (~673 to ~688), for a large family of putative transcription factors, containing a basic helix-loop-helix domain (28). Fig. 7A shows the result of protein-DNA complex formation using a DNA fragment of the region from –690 to –660, containing the E-box motif. Δ12-PGJ2 markedly induced two nuclear protein-DNA complexes with different mobilities (l and II). These complexes were displaced by double-stranded DNA fragments of –690 to –660, but not by either the single-stranded fragments or the E-box motif-mutated fragment, indicating that the nuclear pro-
protein synthesis. DNA complexes form, respectively. Bands with different molecular weight, 80,000 and 24,000, as analysis of the photolabeled complexes showed radioactive irradiation. As shown in Fig. 7, the element containing a E-box motif, in RBL-2H3 cells.

The results indicate that the region (−690 to −660) contains the Δ12-PGJ2-responsive element and that the E-box motif in this element is essential for this stimulation of the promoter activity.

**Discussion**

We demonstrated here that Δ12-PGJ2 drastically induced HO-1 mRNA through a Δ12-PGJ2-specific cis-regulatory element, containing a E-box motif, in RBL-2H3 cells. Δ12-PGJ2 induces the synthesis of a variety of proteins. Among the Δ12-PGJ2-induced protein synthesis, the mechanism for the synthesis of HSP has been well characterized. A wide range of external stress stimuli, including heat shock, heavy metals, amino acid analogues, and oxidizing agents, drastically induce the expression of the HSP gene through activation of HSF, which binds to HSE located in the 5′-flanking sequence of the HSP gene (29). Δ12-PGJ2 induces the expression of the HSP gene, and this induction is also mediated by HSF activation, as well as the above mentioned stimuli (26, 27). We here showed that Δ12-PGJ2 stimulated the promoter activity of the 5′-flanking region of the HO-1 gene and then induced HO-1 mRNA in RBL-2H3 cells (Figs. 1 and 2). Although the 5′-flanking region contains HSE, HSE is not necessary for the Δ12-PGJ2-stimulated promoter activity (Fig. 5), but this stimulation requires the specific region (−690 to −660) containing the E-box motif (Fig. 7), indicating that Δ12-PGJ2 induces the gene expression through a specific element other than HSE. On the other hand, heat shock stimulated the promoter activity of RH0810, but this stimulation was completely abolished on removal of HSE, indicating that heat shock induces this gene expression through HSE. Furthermore, other stimuli, such as hemin and arsenite, did not stimulate the promoter activity of RH0810 or showed only very low stimulation (Fig. 4B). These findings demonstrate that this element specifically responds to Δ12-PGJ2 activation. Thus, the element could be referred to as the Δ12-PGJ2-responsive element, and this is the first example of a cis-regulatory element showing a Δ12-PGJ2-specific response. Among various PGs, the stimulation of the promoter activity of HO-1 is specific for Δ12-PGJ2 and Δ7-PGA1 (Fig. 4A). The stimulation was not observed with A type cyclopentenone PGs as well as conventional PGs. Generally, the biological actions of PGA1 and PGA2 are much weaker than those of Δ12-PGJ2 and Δ7-PGA1 (3). A characteristic of cyclopentenone PGs, such as Δ12-PGJ2 and PGA1, is that they contain α,β-unsaturated ketones, which are very susceptible to nucleophilic addition reactions with thiols, and are essential for the actions of the PGs (30, 31). PGA1 or PGA2 forms a mononconjugate with a thiol (32, 33), but Δ12-PGJ2 or Δ7-PGA1 can form a bisconjugate with two thiols (30). Furthermore, the binding of PGA1 to synthetic polymer-supported thiols as the model of thiol-containing proteins is reversible, but that of Δ12-PGJ2 or Δ7-PGA1 is irreversible (34). An irreversible bisconjugate with thiol groups of proteins may be required for the stimulation of the HO-1 promoter activity, or the stimulation by PGA1 or PGA2 might be marginal in the detection of the stimulation of the activity of CAT.

The 5′-flanking region of the HO-1 gene contains a number of DNA sequences of potential regulatory elements. The 5′-flanking region of the rat HO-1 gene up to position −600 contains several potential binding sites for different transcription factors: a transcription factor, Sp1, a positive regulator for the control of amino acid synthesis (GCN4), a heat shock transcription factor, and a metal-dependent transcription factor (18). The proximal promoter region within 149 base pairs of the upstream sequence of the mouse HO-1 gene contains several sequence elements for AP-1, AP-4, C/EBP, and c-MycMax/
USF, and is required for basal promoter activity (35). Several NF-kB and AP-2-like binding sites have been found in the 5'-flanking region of the human HO-1 gene up to position -500 (36). However, the more upstream region of rat HO-1 (−810 to −600), containing the Δ12-PGJ<sub>2</sub>-responsive element, has not yet been reported to contain potential binding sites for so far known transcription factors. Thus, the Δ12-PGJ<sub>2</sub>-responsive element is a novel cis-regulatory element and plays an important role in HO-1 gene expression. Recently, the heme oxygenase transcription factor, an essential transcription factor, was shown to interact with the cis-acting element (−51 to 35), located just upstream of the TATA box of the rat HO-1 gene, and this binding is essential for the basal expression of the HO-1 gene in rat glioma cells (37). In RBL-2H3 cells, the proximal 5'-flanking region (−270 to +101) containing the heme oxygenase transcription factor binding element and TATA box showed the basal promoter activity. The proximal promoter region, containing the heme oxygenase transcription factor binding element and TATA box, is essential for the basal promoter activity. Whereas the region (−810 to −600) containing the Δ12-PGJ<sub>2</sub>-responsive element itself did not show the basal promoter activity, this region (−810 to −600), when combined with the proximal 5'-flanking region (−270 to +101), enhanced the promoter activity of the region (−270 to +101) in a Δ12-PGJ<sub>2</sub>-dependent manner (Fig. 5). Furthermore, this region (−810 to −600) enhanced activity of the exogenous promoter, SV40 promoter (Fig. 6). The Δ12-PGJ<sub>2</sub>-responsive element thus appears to act as an enhancer.

HO-1 mRNA was increased more than 30-fold by Δ12-PGJ<sub>2</sub> (Fig. 1), although the magnitude of stimulation of CAT activity was 5-fold (Fig. 3). Such a difference may simply represent that the fusion genes lack the additional element required for the maximal stimulation by Δ12-PGJ<sub>2</sub>. Alternatively, this may be due to a limitation of transient expression assays; namely, integration of the fusion genes into the genomic DNA is required for the maximal stimulation.

Using UV cross-linking, we identified two nuclear proteins, the Δ12-PGJ<sub>2</sub>-responsive factors, which specifically bind to the Δ12-PGJ<sub>2</sub>-responsive element, their apparent molecular weight being 80,000 and 24,000 as DNA complex form, indicating that two different proteins bind to the element in response to Δ12-PGJ<sub>2</sub> (Fig. 7B). The Δ12-PGJ<sub>2</sub>-responsive element contains an E-box motif, and this motif is essential for the Δ12-PGJ<sub>2</sub>-responsive factor binding to this element and for the Δ12-PGJ<sub>2</sub>-stimulated promoter activity (Fig. 7, A and C). The transcription factors which bind to the E-box motif contain a helix-loop-helix domain and an adjacent basic amino acid region, and a variety of transcription factors, such as MyoD, c-myc, and Myf-5, belong to this family (38). In RBL-2H3 cells, the Δ12-PGJ<sub>2</sub>-responsive factors appear to belong to a family of basic helix-loop-helix type transcription factors.

Most cells synthesize and subsequently release PGD<sub>2</sub>, which is converted into Δ12-PGJ<sub>2</sub> by serum albumin during the process of inflammation (39). The Δ12-PGJ<sub>2</sub> produced stimulates the transcription of the HO-1 gene in various cells, such as basophils, fibroblasts, and vascular endothelial cells, which are actively involved in the inflammatory response, and the reticuloendothelial system is the site at which the active degradation of heme by HO-1 may take place during inflammation. The induction of HO-1 by Δ12-PGJ<sub>2</sub> may play an important role in the fate of heme liberated during inflammation.

In summary, we here identified the Δ12-PGJ<sub>2</sub>-specific cis-regulatory element responsible for the expression of the rat HO-1 gene. This study will contribute not only to understanding of the HO-1 gene expression mechanism but will also facilitate elucidation of the molecular mechanisms of cyclopentenone PG actions.

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