Prostaglandin endoperoxide H synthase-1 (PGHS-1) is expressed constitutively in murine NIH 3T3 cells and RAW 264.7 cells. PGHS-2 is inducibly expressed in these cells following stimulation with serum or bacterial lipopolysaccharide (LPS), respectively. Reverse transcription-polymerase chain reaction (RT-PCR) analysis established that a variety of G protein-linked and peroxisomal proliferator-activated prostanooid receptors are expressed in both of these cell types. The levels of the EP2 and EP4 prostaglandin E\(_2\) (PGE\(_2\)) receptors and the prostaglandin I\(_2\) receptor were changed in these cells by serum or LPS stimulation. Quantitative RT-PCR indicated that the mRNA for the murine EP4 receptor, the butaprost-insensitive PGE\(_2\) receptor that couples to G\(_\alpha_i\), increases 1.5–3-fold in response to serum (NIH 3T3) or LPS (RAW 264.7) with a time course approximating the induction of PGHS-2 expression. To study expression of the EP4 receptor we isolated the mouse EP4 receptor gene; the gene is 10 kilobase pairs (kb) in length and, like other known prostanooid receptor genes, contains three exons and two introns. The first intron is 0.5 kb and is located 16 base pairs (bp) downstream of the translational start site. This is a different location than that of the first introns of other prostanooid receptor genes. The second intron is located immediately following the sixth transmembrane domain at the same position as the second intron of the thromboxane A\(_2\) receptor, prostaglandin D\(_2\) receptor, prostaglandin I\(_2\) receptor, and one of the PGE\(_2\) (EP1) receptor genes. A major transcriptional start was detected at −142 bp upstream of the translational start. There are a variety of putative cis-acting elements within 1.5 kb upstream of the translational start site and within the first intron. Promoter analyses of the EP4 receptor gene promoter in RAW 264.7 cells indicated that there is a constitutive negative regulatory region between −992 and −928 bp, a constitutive positive region between −928 and −554 bp, and an LPS/serum-responsive region between −554 and −116 bp.

Prostaglandins and thromboxanes are biologically active metabolites of arachidonic acid formed by the sequential actions of prostaglandin endoperoxide H synthase-1 and -2 (PGHS-1 and -2)\(^1\) and specific prostaglandin and thromboxane synthases (1). Prostaglandins cause a variety of physiological actions including contraction and relaxation of smooth muscle, inhibition and stimulation of neurotransmitter release, inhibition of gastric acid secretion, inhibition of inflammatory mediator release, regulation of platelet aggregation, and control of water and salt reabsorption in the kidney (1). Prostanoids synthesized on the endoplasmic reticulum via the PGHS-1 biosynthetic system (2, 3) are transported to the outside of cells through a prostanooid transporter(s) (4). Following their exit from cells, these newly formed prostanooids mediate their effects through cell surface, G protein-linked receptors (5, 6). Prostanoids are also synthesized in association with the nuclear envelope, at least in part through the actions of PGHS-2, and in association with cell replication or cell differentiation (2, 3); these products may exert their effects through peroxisomal proliferator-activated receptors (i.e. PPAR\(\gamma\), members of the nuclear superfamily of receptors (7–11).

PGE\(_2\) is synthesized by the combined activities of PGHSs and PGE\(_2\) synthases (1, 12). There are four major PGE\(_2\) receptor subtypes of the G protein-linked receptor family called EP1 (13–15), EP2 (16, 17), EP3 (18–21), and EP4 (22–28) receptors. Each receptor appears to be coupled to a different G protein and downstream effector system (6, 27).

The nomenclature regarding EP2 and EP4 receptors has been somewhat confusing and has recently undergone some changes. Narumiya and co-workers (22) isolated a cDNA for a murine PGE\(_2\) receptor that coupled to the activation of adenylate cyclase and that they called the EP2 receptor. However, this receptor differed from the PGE\(_2\) receptor defined pharmacologically as the EP2 receptor in that it responded to butaprost only very weakly. More recently, a cDNA for a butaprost-sensitive PGE\(_2\) receptor was cloned (16); this latter receptor is the pharmacologically defined EP2 receptor, and that originally cloned by Narumiya and others is now called the EP4 receptor (22, 25, 26). mRNA for the mouse EP4 subtype has been detected in thymus, lung, spleen, ileum, and mastocytoma P-815 cells by Northern blotting analysis (22). The mouse EP4 receptor subtype is located on chromosome 15 (65).

In studies designed to relate expression of the two PGHS isozymes to that of G protein-linked and PPAR\(\gamma\) prostanooid receptors in NIH 3T3 and RAW 264.7 cells, we used RT-PCR to determine which receptors are present in both quiescent (serum-starved) and serum- or bacterial lipopolysaccharide (LPS)-

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\(^1\) The abbreviations used are: PGHS, prostaglandin endoperoxide H synthase; PG, prostaglandin; EP, prostaglandin E\(_2\) receptor; DP, prostaglandin D\(_2\) receptor; IP, prostaglandin I\(_2\) receptor; TP, thromboxane A\(_2\) receptor; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; CS, calf serum; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; bp, base pair(s); kb, kilobase; LPS, lipopolysaccharide; PPAR\(\gamma\), peroxisomal proliferator-activated receptor \(\gamma\); FP, prostaglandin F\(_{2\alpha}\) receptor.
**EP4 Receptor Gene**

stimulated cells. Both EP2 and EP4 receptor mRNA levels were increased by cell stimulation, whereas IP receptor mRNA levels were decreased. We describe here investigations on the structure of the EP4 receptor including the isolation and characterization of the EP4 receptor gene and the transcriptional regulation of this gene.

**EXPERIMENTAL PROCEDURES**

**Materials**—A fXII1 mouse genomic library was purchased from Stratagene. [α-32P]dATP, [α-32P]dCTP, [α-32P]dGTP, and [α-32P]dATP were obtained from DuPont NEN. pGKL lucerase basic plasmid, pSV-β-galactosidase plasmid, a luciferase assay system, and a β-galactosidase assay kit were purchased from Promega. Taq polymerase, SuperScript I reverse transcriptase, and oligonucleotide primers were synthesized by the Michigan State University Macromolecular Structure and Sequencing Facility.

**Cell Culture and RNA Isolation**—Murine NIH 3T3 cells were cultured in DMEM containing 2% FCS and 8% CS in a water-saturated 7% CO2 atmosphere. To stimulate NIH 3T3 cells, a DNA transfected 3T3 cells was performed as described previously (29). Murine RAW 264.7 cells were cultured in DMEM containing 10% FCS in a water-saturated 5% CO2 atmosphere. Stimulation of the cells was performed by adding LPS (200 ng/ml, final concentration) to the culture medium. NIH 3T3 cells and RAW 264.7 cells were isolated by scraping the cells from the culture dishes with a rubber policeman and then sedimenting the cells by centrifugation at 500 × g for 5 min. Total RNA was isolated using Trizol reagent according to the instructions of the manufacturer.

**PCR Detection of Murine Prostanoid Receptor mRNAs**—Mouse lung and kidney were minced and homogenized with Trizol reagent, and total RNA was isolated as described above. Reverse transcription with oligo(dT) priming was used to generate cDNAs from 10 μg of total RNA extracts of serum-starved and serum-stimulated NIH 3T3 cells and control and LPS-stimulated RAW 264.7 cells using reverse transcriptase (SuperScript II) and the protocol of Life Technologies, Inc. The following primers were used for PCR amplification of the resulting cDNAs: 5′-GAGTACTTGCGCTCAGGAGGAGC-3′ (nucleotides 954–972), and WS 157, 5′-CGCGGGTGGTGTCTTGACT-3′ (nucleotides 1347–1366); and WS 168, 5′-GTCCTAGATAAGTG-3′ (nucleotides 446–465) for the mouse EP4 receptor cDNA; and WS 224, 5′-CATGGTTGAGCTGACTTTATTG-3′ (nucleotides 258–276), and WS 233, 5′-TTCCCAGCTGACTTTATTG-3′ (nucleotides 655–673) were synthesized. PCR amplification was performed using a Perkin-Elmer Gene-Amp PCR system 9600. Amplified fragments were separated by electrophoresis on a 1.5% agarose gel.

**Isolation of the EP4 Receptor Gene from a fXII1 Mouse Genomic Library**—Two sets of primers (WS 170, 5′-CCCAGTGTTCTATACGCA-3′ (nucleotides 954–972) and WS 157, 5′-GGAGGTTGTCGCTTTGACT-3′ (nucleotides 1347–1366); and WS 168, 5′-CTCCTGAGATATG-3′ (nucleotides 446–465)) for the mouse EP4 receptor were isolated from positive phage plaques and sequenced by the Michigan State University Macromolecular Structure and Sequencing Facility.

**Prostanoid Receptors in Murine NIH 3T3 cells and RAW 264.7 Cells**—Prostaglandin endoperoxide H synthase-1 (PGHS-1) is

**RESULTS**

Prostanoid Receptors in Murine NIH 3T3 cells and RAW 264.7 Cells—Prostaglandin endoperoxide H synthase-1 (PGHS-1) is
expressed constitutively in both murine NIH 3T3 cells and RAW 264.7 cells; a second isozyme, PGHS-2 is induced as an immediate early gene when quiescent, serum-starved murine NIH 3T3 cells are treated with phorbol esters or serum (29, 35, 36) or when RAW 264.7 cells are treated with endotoxin (i.e. bacterial LPS (37)). We used RT-PCR to determine which G protein-linked and PPARγ prostanoid receptors are expressed in quiescent and serum-stimulated 3T3 cells and in control and LPS-stimulated RAW 264.7 cells, because these receptors might be expected to mediate downstream events associated with constitutive and/or inducible prostanoid production in these cells. PCR primers for the various receptors were developed, which permitted us to perform the amplification steps at relatively high temperatures, and such that convenient, unique restriction endonuclease sites were present near the midpoints of the amplified fragments. As summarized in Fig. 1, both quiescent and serum-stimulated 3T3 cells express EP4, FP, and IP receptor mRNA but lack detectable EP1, EP2, EP3, or TP receptor mRNA. Quiescent and LPS-stimulated RAW 264.7 cells express EP2, EP3, EP4, and IP receptor mRNA but lack EP1, FP, and thromboxane receptor mRNA. PPARγ receptor mRNA was present in RAW 264.7 but not in NIH 3T3 cells (data not shown); the levels of PPARγ in RAW 264.7 cells were unchanged by LPS treatments. We were unable to develop appropriate PCR primers for the murine DP receptor. The results of these initial RT-PCR experiments also indicated that the levels of mRNA for three of the prostanoid receptors were changed by serum and/or LPS stimulation. In both NIH 3T3 cells and RAW 264.7 cells, EP4 receptor mRNA levels were increased by cell stimulation, whereas IP receptor mRNA levels were decreased; in RAW 264.7 cells, EP2 receptor mRNA levels increased.

The EP4 receptor is the butaprost-insensitive PGE₂ receptor, which functions through G₂ to activate adenylyl cyclase (22, 25, 26, 28). Small but consistent increases in EP4 mRNA appeared to occur upon serum stimulation of 3T3 cells and LPS stimulation of RAW 264.7 cells (Fig. 1). A “three-band” quantitative RT-PCR method was developed to measure EP4 receptor mRNA levels (38), and this procedure was applied to both quiescent and serum-stimulated murine NIH 3T3 cells and control and LPS-treated murine RAW 264.7 cells; the three-band method is a modification of the two-band quantitative RT-PCR method developed by Clontech Laboratories, Inc. The results of experiments performed with RAW 264.7 cells are shown in Fig. 2. Briefly, the following cDNAs were amplified: (a) a 528-bp β-actin cDNA generated by reverse transcription of β-actin mRNA present in the total RNA fraction from RAW 264.7 cells; (b) a 423-bp EP4 receptor cDNA generated by reverse transcription of EP4 receptor mRNA present in the total RNA fraction from RAW 264.7 cells; and (c) a 330-bp...

**Fig. 1. Expression of prostanoid receptors in murine NIH 3T3 cells and RAW 264.7 cells.** Total mRNA was isolated from serum-starved 3T3 cells, 3T3 cells treated for 3 h with 10% FCS, and RAW 264.7 cells treated for 3 h with endotoxin (i.e. bacterial LPS), and prostaglandin receptor (EP1, EP2, EP3, EP4, FP, IP, and TP) mRNAs were amplified by RT-PCR using receptor-specific oligonucleotide primers as detailed under “Experimental Procedures.” *Upper panel*, +, detectable PCR fragment observed; −, no PCR fragment observed. *Lower panels*, EP4, EP2, and FP PCR fragments, respectively, generated by RT-PCR from total RNA from mouse lung, mouse kidney, quiescent 3T3 cells (3T3−), serum-stimulated 3T3 cells (3T3+), control RAW 264.7 cells (RAW−), or LPS-treated RAW 264.7 cells (LPS+).

**Fig. 2. Quantitation of EP4 receptor mRNA by RT-PCR.** Total RNA was isolated from RAW 264.7 cells that had been cultured in DMEM containing 10% FCS and then treated with LPS for the indicated times. The RNA was used as a template to generate cDNAs for β-actin and the EP4 receptor using reverse transcriptase. The resulting cDNAs (from 0.25 μg of total RNA) were mixed with the indicated concentrations (denoted as M₃−M₄) of an EP4 MIMIC DNA standard and the appropriate concentrations of β-actin and EP4 receptor oligonucleotide primers and subjected to coamplification by PCR as detailed under “Experimental Procedures.” The upper band (528 bp) in all of the lanes is the β-actin PCR fragment amplified from the cDNA using β-actin-specific primers. The middle band (423 bp) in all of the lanes is the EP4 receptor PCR fragment amplified from the cDNA using EP4 receptor-specific primers. The bottom band (330 bp) in all of the lanes is the EP4 MIMIC DNA standard amplified using EP4 receptor-specific primers. M₃−M₄ represent initial EP4 MIMIC DNA concentrations (in amol): M₃, 250 × 10⁻⁹; M₄, 125 × 10⁻⁹; M₅, 62.5 × 10⁻¹⁰; M₆, 31.25 × 10⁻¹⁰; M₇, 15.63 × 10⁻¹⁰; M₈, 7.81 × 10⁻¹⁰; M₉, 3.91 × 10⁻¹⁰; M₁₀, 1.95 × 10⁻¹⁰. The arrows above each panel denote those lanes for β-actin DNA and EP4 receptor DNA that have the same intensity as the EP4 MIMIC DNA.
The EP4 Receptor Gene

**Exon-intron junction structure of the mouse PGE2 receptor EP4 sub-type.** The EP4 receptor cDNA and the locations of the exons within the cDNA are shown by open and closed boxes, respectively. Locations of restriction enzyme cleavage sites within the gene are shown for BamHI (B), EcoRI (E), HindIII (H), Kpn1 (K), SacI (S), and Xhol (X). Locations of the ATG and TAG initiation and stop codons are indicated. Orientation of the genomic clones (\(\alpha\)FIXEP4-3-1 and \(\alpha\)FIXEP4-3-2) isolated from the \(\alpha\)FIXII library are shown under the gene.

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The first intron is located 16 bp downstream and the second intron is 951 bp downstream of the translational start site (ATG).

**Table I**

| Exon | Intron | Exon |
|------|--------|------|
| 16   |        |      |

**EP4 Receptor Gene Promoter Analysis**—We made five different constructions containing various EP4 promoter sequences coupled to luciferase cDNA and expressed these by transient transfection of RAW 264.7 cells (Fig. 6). Under both serum-starved and serum/LPS-stimulated conditions (Fig. 6, dark and hatched bars), luciferase activity increased in going from pGLog4-1 to pGLog4-4, suggesting that between −4200 and −992 there are negative regulatory elements in the promoter. In contrast, luciferase activity was decreased when the EP4 receptor promoter region between −928 and −554 (i.e., pGLog4-4 versus -5) was removed, indicating that this part of the promoter contains positive regulatory sites. After serum/LPS stimulation (Fig. 6, hatched bars), luciferase activities increased between 20 and 90% with all of the constructs. When the luciferase activities observed with the control pGL3 plasmid are subtracted from the luciferase activities obtained with the other constructs, the differences between nonstimulated and stimulated conditions become much more obvious, with increases ranging from 1.2- to 2.7-fold over the pGL3 control. Most notably after subtracting the values for the pGL3 control, the luciferase activity in pGLog4-5 was stimulated 2.7-fold by serum/LPS. These results suggest that there are positive regulatory elements responsive to serum/LPS stimulation between −554 and −116.

**DISCUSSION**

There appear to be two distinct prostaglandin biosynthetic systems that can coexist in cells (2, 47, 48). One system uses...
the constitutive PGHS-1 as the initiating enzyme and functions primarily in the ER to produce prostanoids that act extracellularly through G protein-linked receptors (5, 6) to mediate well-known physiological "housekeeping" effects of prostanoids (2, 3). A second system, involving the inducible PGHS-2, appears to produce prostanoids that act, at least in part, at the level of the cell nucleus (2, 3), perhaps through nuclear receptors such as PPARα (7–11) in the early stages of cell replication or differentiation. In the work described in this report, we first used RT-PCR to identify those G protein-linked and PPARα prostanoid receptors that are expressed in quiescent and activated murine NIH 3T3 and RAW 264.7 cells. This led us to examine the structure and regulation of expression of the gene for the EP4 receptor, the butaprost-insensitive, PGE2 receptor which is coupled to Gs. Overall, these studies are potentially important in determining which receptors are involved in mediating prostanoid responses associated with cell replication and differentiation.

Both NIH 3T3 cells and RAW 264.7 cells expressed mRNAs for several G protein-linked prostanoid receptors. PPARα was expressed in RAW 264.7 cells but not NIH 3T3 cells. Assuming that at least some of the prostanoids formed via PGHS-2 act on nuclear targets in association with cell replication, the finding that PPARα was present only in RAW 264.7 cells suggests that this receptor is not uniformly involved in mediating nuclear actions of prostanoids. The EP4 receptor gene was found to contain three exons and two introns. A similar overall structure has been found for TP (51), EP1 (52), DP (53), and IP (54) prostanoid receptor genes.

FIG. 4. Nucleotide sequences of the promoter and first intron of the mouse PGE2 EP4 receptor gene. Potential cis-acting elements are boxed, and the names of the elements are indicated under the box. The cis-acts elements found in the antisense sequence are denoted with italic letters. A, nucleotide sequence of the promoter of the EP4 receptor gene. The transcriptional start site determined by primer extension analysis (Fig. 5) is indicated with an asterisk above the sequence. The positions of nucleotides are numbered beginning with the translational start site ATG and shown on the right. B, nucleotide sequence of the first intron sequence. Correspondence with the GT-AG rule is denoted with underlines. The numbering of the nucleotides is shown on the left counting from the intron start site.

FIG. 5. Primer extension analyses of EP4 receptor mRNA. Primer extension analyses are shown in the left three lanes of each panel and were performed using total RNA from quiescent (Starved) or serum-stimulated (Stimulated) murine NIH 3T3 cells and the primer WS 309 or WS 310 as detailed under "Experimental Procedures." Sequence ladders are shown to the right of extension analyses and are labeled GATC at the top. Nucleotides at transcriptional initiation sites are indicated by circles and arrows, and the positions of the transcriptional start sites (relative to the ATG translational start site) are shown on the left of each panel next to the circles.
In all of these cases, including that of the EP4 receptor, the second intron occurs at the end of the putative sixth transmembrane domain. However, the first intron of the EP4 receptor gene is located within the coding region of the receptor cDNA, whereas the first introns for other prostanoid receptor genes characterized to date are found on the 5′-side of the ATG translational start sites (51–54). In addition, the first intron is relatively small (0.5 kb) in the case of the EP4 receptor gene. DP, EP2, EP4, and IP receptors are classified in the same phylogenetic cluster, CL-1, whereas TP and EP1 receptors are in cluster CL-2, and EP3 receptors are in cluster CL-3 (55). EP4 receptors are divided from other receptors of the CL-1 cluster at an early evolutionary stage. The overall organizations of the DP and IP receptor genes are more similar to the TP receptor gene than to the EP4 receptor gene. Thus, the EP3 receptor gene in cluster CL-3 may have the same structure as other previously characterized prostanoid receptor genes, and only the EP4 receptor gene (and perhaps the EP2 receptor gene) may have a structure different from that of other prostanoid receptor genes.

Although EP4 receptor mRNA appears to be expressed in many cells and tissues, 1.5–3-fold increases in EP4 receptor mRNA did occur in response to both serum (3T3 cells) and LPS (RAW 264.7 cells), indicating that the EP4 receptor gene is inducible. In the case of RAW 264.7 cells, which have the cell surface monocyte CD14 antigen, the LPS/serum LPS-binding protein complex binds to CD14 and elicits intracellular signals (57). When we examined the expression and induction by serum/LPS of five different constructs of the EP4 receptor gene promoter in murine RAW 264.7 cells, a maximal increase in luciferase activity was observed with the pGLep4-5 construct containing nucleotides −116 to −554 bp. There are Sp1 and AP2 elements and an E-box in this region. Although Sp1 sites are essential for CD14 expression (58), we have not resolved which specific response element(s) is essential for EP4 receptor gene expression.

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