Prostaglandin E_2 receptors in abdominal aortic aneurysm and human aortic smooth muscle cells

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Background: **Prostaglandin (PG) E_2 (PGE_2) appears to have a role in stimulating production of interleukin-6 (IL-6) and apoptosis of smooth muscle cells in diseased aortic tissue. These actions are mediated by cellular receptors for PGE_2 EP receptors.**

Objective: **This study was undertaken to identify EP receptors associated with production of IL-6 by aortic explants.**

Methods: **Biopsy specimens of abdominal aortic aneurysm were used for explant culture and preparation of messenger RNA. The presence of EP1, EP2, EP3, and EP4 receptors in tissue and cells was investigated with reverse-transcriptase polymerase chain reaction. IL-6 and cyclic adenosine monophosphate were measured with an enzyme-linked immunosorbent assay.**

Results: **PGE_2 or 11-deoxy-PGE_1 (EP 2/3/4 agonist) reversed partially the indomethacin suppression of IL-6 secretion from explant cultures, whereas butaprost (EP2 receptor agonist) and sulprostone (EP 1/3 receptor agonist) had no effect. Aortic biopsy specimens expressed EP2, EP3-III, and EP4 receptors. Aortic smooth muscle cells expressed EP2 receptor and four variants of EP3 receptor, ie, EP3-Ib, EP3-II, EP3-III, and EP3-IV, but PGE_2 did not stimulate secretion of IL-6. In contrast, PGE_2 or 11-deoxy-PGE_1 stimulated secretion of IL-6 from aortic macrophages.**

Conclusions: **In aortic explants, PGE_2 stimulates IL-6 secretion by activation of EP4 receptors, present in macrophages. (J Vasc Surg 2003;38:354-9.)**

Prostaglandin (PG) E_2 has a wide range of pathophysiologic effects, including contraction and relaxation of vascular smooth muscle, modulation of inflammatory processes, and connective tissue turnover.[1-3] PGE_2 is synthesized in high volume in diseased aorta, eg, with stenosis or aneurysm.[4-6] There is some evidence to suggest that PGE_2 and other cyclooxygenase products contribute to the pathologic processes that underlie expansion of abdominal aortic aneurysm (AAA). First, indomethacin inhibits expansion of experimental AAA by inhibition of cyclooxygenase 2 (COX2), with resultant decrease in PGE_2 and matrix metalloproteinase-9 (MMP-9).[6] Second, indomethacin suppresses secretion of inflammatory mediators from AAA tissue, effects that are partially reversible with exogenous PGE_2.[5] Third, nonsteroidal anti-inflammatory drugs may have some effect to reduce AAA expansion rates in human beings.[5] The actions of PGE_2 are mediated by an array of G protein–coupled receptors, with at least four subtypes, EP1, EP2, EP3, and EP4. Pharmacologic studies have indicated the presence of EP1, EP2, and EP3 receptors in vascular smooth muscle, although EP1 receptors are sparse in human tissue.[8,9] In addition, EP4 receptor messenger RNA (mRNA) has been detected in cultured human umbilical vein smooth muscle cells (SMC),[10] and both EP2 and EP4 receptors are present in inflammatory cells.[11] The EP3 receptor is the most complex, with alternate splicing potentially generating more than seven receptor subtypes.[9-11] These different EP3 receptor subtypes appear to be coupled to at least three second messenger systems, usually leading to inhibition of adenylyl cyclase.[9-11] The EP2 receptor is ubiquitous, and, like the EP4 receptor, couples to activation of adenylyl cyclase and increased cellular cyclic adenosine monophosphate (cAMP) concentration. In contrast, the EP1 receptor couples to phospholipase C.

The pharmacologic tools for probing the roles of specific EP receptors are limited. Butaprost is a relatively selective EP2 receptor agonist; sulprostone is a relatively selective agonist for EP1 and EP3 receptors; and 11-deoxy-PGE_1 is an agonist for EP2, EP3, and EP4 receptors. We used these agonists to investigate the role of EP receptors in stimulating production of interleukin-6 (IL-6) in biopsy specimens from AAA, and we complemented these studies with investigation of EP receptor and mRNA expression. In addition, we listed these same agonists to investigate the EP

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receptors and their subtypes that modulate cAMP synthesis in aortic SMC. In vascular SMC cells, elevation of cAMP concentration is associated with inhibition of SMC proliferation.[12] In AAA there is evidence of apoptosis and irreversible loss of medial aortic SMC, and this process may be influenced by PGE_2.[5,13,14]

MATERIALS AND METHODS

Tissues and cells. Biopsy specimens of AAA were harvested, and explant cultures for analysis of IL-6 secretion and culture of SMC from the medial layer were established as described.[7] Explants were incubated with these reagents for 9 hours. Indomethacin and prostaglandins (PGE_2, 11-deoxy-PGE_1, butaprost, and sulprostone; all from Cayman Chemical Co, Ann Arbor, Mich), were prepared in ethanol, and controls included the same amount of ethanol; dexamethasone was obtained from Charing Cross Hospital Pharmacy (London, UK). Part of the biopsy sample was snap-frozen in liquid nitrogen for later isolation of mRNA. Human aortic SMC from normal aorta were obtained from Cascade Biologics (Portland, Ore) and used up to passage 6. All human aortic SMC were cultured in medium 231 (Cascade Biologics). Macrophages were isolated from aortic aneurysm biopsy specimens, as described previously,[5] yield 80 to 130,000 per gram of tissue wet weight. The HSB-2 cell line was obtained from the European Cell Culture Collection, and suspension cultures were maintained in RPMI 1640 supplemented with 10% fetal calf serum. Frozen placenta was a gift from Dr D. Sooranna (Imperial College). Genomic DNA, prepared from peripheral blood cells, was available from several patients with AAA. Local ethical approval was in place for use of all human tissue samples.

mRNA isolation and reverse transcription. mRNA was isolated from cells with the Micro-Fast Track mRNA Isolation kit (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. Tissue samples were ground to a powder in liquid nitrogen before using the same kit to isolate mRNA. First-strand complementary DNA (cDNA) was synthesized with the cDNA Cycle Kit (Invitrogen) according to the manufacturer's instructions. A 621 base pair (bp) fragment of the β-actin gene was amplified to confirm successful mRNA extraction and cDNA synthesis using the primer pair 5'-ATCATGTTTGAGACCTTCAA CACCCC-3' and 5'-CTTGATCTTCATTGTGCTGGG TGCCA-3'.

Polymerase chain reaction for EP receptors. Nested primers for the EP1, EP2, and splice variants of EP3 were designed with the freeware program Primers (Macintosh, Redmond, Calif). The primers used are described in Table I. mRNA from placenta was used to provide a positive control for EP1, EP2, EP3, and EP4 receptors and from HSB-2 cells as a positive control for EP3 variants.[11,15] All polymerase chain reactions (PCR) were performed in 10 mmol/L of Tris hydrochloride (pH 9.0), 50 mmol/L of potassium chloride, 0.1% Triton X-100, 1.5 mmol/L of magnesium dichloride, 200 μmol/L of deoxyribonucleoside triphosphate, 10 pmol of primer with 1 unit of Taq polymerase, in a total reaction volume of 50 μL (all reagents from Promega [Madison, Wis]). PCR conditions were denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, elongation at 72°C for 2 minutes, and final elongation at 72&degC for 5 minutes. Products were separated on 2% NuSieve (GTG 3:1)/1 × Tris-borate/EDTA gels containing 1.5 μg/mL of ethidium bromide and visualized with ultraviolet transillumination.

Cloning and sequencing of fragments of the EP3 gene. To confirm the identity of each of the bands found after reverse transcription (RT) PCR for the EP3 receptor splice isoforms, the products were cloned into the pCR2.1 vector (Invitrogen), according to the manufacturer's instructions. After ligation and transformation, single colonies were picked and grown at 37°C for 16 hours in 2 mL of recommended medium with selective antibiotics. One milliliter of each liquid culture was used with no purification as the template in PCR with the internal EP3 primer pairs. After confirmation with gel electrophoresis that the clones contained fragments of the expected size, the plasmid DNA was purified (Wizard Plus Plasmid purification kits, Promega), and the inserts were cycle-sequenced with EP3-specific primers on an ABI373 automated sequencer (Applied Biosystems, Warrington, UK). After removal of plasmid sequences, the insert sequences were compared with the database EP3 splice isoform sequences to confirm identity with the best- fit algorithm.

Quantification of IL-6. IL-6 was measured with an enzyme-linked immunosorbent assay.[16]

Immunohistochemistry. Aortic aneurysm sections were prepared as described.[5] Sections were preincubated with biotin before sequential incubation with goat polyclonal antibodies to the EP4 receptors (Santa Cruz Biotechnology, Santa Cruz, Calif) at 1:50 dilution and biotinylated secondary antibody (Dako, Ely, England) at 1:250 dilution.

RESULTS

Secretion of IL-6 from aortic explants. After 96 hours in culture, aortic explants secreted large amounts of IL-6, 355 ± 41 ng/mL (n = 11). In the presence of indomethacin (10 μmol/L), this volume was reduced to 105 ± 16 ng/mL ($P < .001$; Table II). The simultaneous addition of exogenous PGE_2 or 11-deoxy PGE_1 partially reversed the indomethacin inhibition, whereas butaprost or sulprostone had no effect (Table II). In contrast, addition of dexamethasone completely suppressed IL-6 secretion.

Expression of EP2, EP3, and EP4 receptors in aortic aneurysm tissue. With the nested primers for EP1 and EP2 receptors, fragments of 482 and 121 bp, respectively, were expected. There was no evidence of the 482 bp band for the EP1 receptor in cDNA from placenta, aorta, HSB-2, or aortic SMC. A strong 121 bp band for the EP2 receptor was observed when cDNA from placenta or AAA biopsy specimens was amplified (Fig 1). Most cDNA, including that from placenta, HSB-2 cells, and SMC, also yielded a visible first-round product of 371 bp (primers

Table II. Interleukin-6 production by aortic explants

Explant in culture for 96 h	*n*	*IL-6 (ng/ml) (mean ± SEM)*	P
Control	11	355 ± 41	
+10 μmol/L indomethacin	11	105 ± 16	<.001*
+10 μmol/L indomethacin +PGE_2 (1 μM)	8	188 ± 13	<.025†
+10 μmol/L indomethacin +11-deoxyPGE_1 (1 μmol/L)	5	165 ± 21	<.05†
+10 μmol/L indomethacin +butaprost (1 μmol/L)	5	113 ± 18	<.5†
+10 μmol/L indomethacin +sulprostone (1 μmol/L)	5	92 ± 28	<.5†
+0.1 μmol/L dexamethasone	8	<10	<.001
+0.1 μmol/L dexamethasone +PGE_2 (1 μmol/L)	5	<10	No difference from +0.1 μmol/L dexamethasone

PGE, Prostoglandin E.
*Comparison with control.
†Comparison with +10 μmol/L indomethacin, Student paired *t* test.

EP2-3F and EP2-2R). In contrast, no clear products were amplified from genomic DNA with these primers. The HSB-2 cell line was selected as a positive control for the EP3 receptor.[15] Amplification of cDNA from 10^6 HSB-2 cells, with nested primers (EP3-SF, EP3-1F, EP3-SR, EP3-2R) theoretically capable of detecting six splice variants of the EP3 receptor, yielded fragments of 108, 133, 134, and 227 bp (Fig 2). The 108, 134, and 227 bp fragments correspond to the sizes predicted for the EP3-III, EP3-IV, and EP3-1b splice variants, and the 133 bp variant probably corresponds to EP3-II. The identity of EP2, EP3-1b, EP3-II, and EP3-III bands were confirmed with sequencing. As expected, no bands were amplified from genomic DNA with these primers: intron 2 is very large.[11] With the nested primers (EP3-SF, EP3-1F, EP3-5R, EP3-6R) the predicted fragment of 108 bp for the EP3-III variant was amplified with cDNA isolated from placenta and from one of five AAA biopsy specimens (Fig 1). The EP4 receptor was identified as a 479 bp product with cDNA prepared from placenta and most (four of five) AAA biopsy specimens (Fig 1). Macrophages in the intima of aneurysm biopsy specimens stained positively for the EP4 receptor (Fig 2).

Cultured aortic SMC express EP2 and EP3 receptors. With the nested primers for EP1, there was no evidence for expression of EP1 in human aortic SMC from normal or aneurysmal aorta. With the nested primers for EP2 the 121 bp band was detected. Four splice variants of EP3 were detected (Fig 3), ie, EP3-III (108 bp), EP3-II (133 bp), EP3-IV (134 bp) and EP3-Ib (227 bp) with the nested primers shown in Table I. This latter isoform, EP3-1b, was detected only in cells cultured from normal aorta and not in cells cultured from aneurysmal aorta. The EP4 receptor was not detected.

Stimulation of IL-6 secretion from cultured aortic SMC. Unstimulated human aortic SMC from normal aorta did not secrete IL-6 into the culture medium (IL-6 < 0.5 pg/mL). After stimulation with IL-1β (5 ng/mL for 6 hours) the IL-6 concentration in the medium increased to 3.5 ± 0.6 pg/mL (n = 5; $P < .001$). In contrast, after stimulation of cells with PGE_2 (100 nmol/L) for 6 hours the IL-6 in the culture media remained at less than 0.5 pg/mL (n = 5). Similarly, IL-1β stimulation increased IL-6 secretion from human aortic SMC from aneurysmal aorta from less than 0.5 pg/μL to 3.8 ± 1.2 pg/mL (n = 3), but PGE_2 evoked no such increase.

Stimulation of aortic macrophages with EP receptor agonists. Isolated aortic macrophages (50,000 ± 5000) were cultivated for 48 hours in Cascade medium 231 (0.5 mL) containing 10 μm of indomethacin in the presence or absence of PGE_2, 11-deoxy-PGE_1, butaprost, or sulprostone. Addition of PGE_2 or 11-deoxy-PGE_1 increased IL-6 secretion (Table III).

DISCUSSION

The diverse actions of prostaglandins are a consequence of binding to specific cell-surface prostanoid receptors. The biologic functions mediated by PGE_2 may be particularly diverse, because there are four separate receptors, ie, EP1, EP2, EP3, and EP4.[8,9] Further complexity is provided by the numerous splice variants of the EP3 receptor, each of which may couple to a different cell-signaling pathway.[11] With this diversity of receptors, it is possible that specific receptor antagonists will provide safer, more selective drugs than cyclooxygenase inhibitors. PGE_2 is produced abundantly by the diseased aortic wall, and it may have a pivotal role in both the key cell and molecular mechanisms that drive aneurysm expansion, inflammation, and proteolysis.[17] For example, PGE_2 enhances secretion of IL-6, other cytokines, and matrix metalloproteinases by means of inflammatory cells.[15,17,18] In the diseased aortic wall, PGE_2 is synthesised principally through a COX2- dependent pathway. COX2 is widely expressed in aneurysm wall, particularly in macrophages and SMC.[5] Using the available selective EP receptor agonists, we have shown that the PGE_2–stimulated IL-6 production by aneurysm wall appears to require activation of the EP4 receptor. Expression of the EP4 receptor was confirmed at RT-PCR, and immunohistochemistry demonstrated staining of intimal macrophages. In contrast, SMC cultured from aneurysm wall did

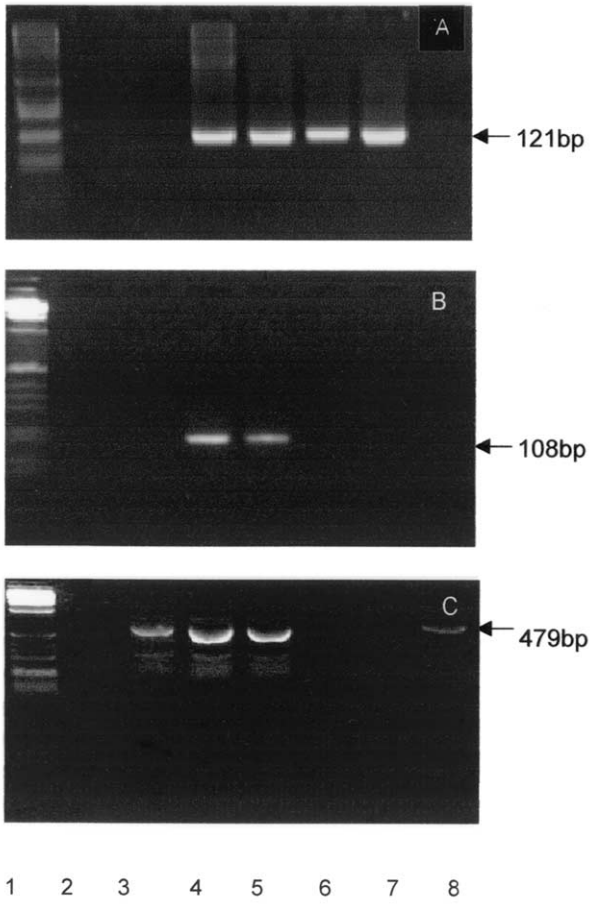

Fig 1. Expression of EP2, EP3-III, and EP4 in abdominal aortic aneurysm (AAA) biopsy specimens, showing placenta, aortic biopsy tissue, and human aortic smooth muscle cell results in three separate gels. Each gel has molecular weight markers in lane 1 and a water control in lane 2. **A,** EP2: lane 3, placenta; lane 4, genomic DNA; lane 5, placenta; lanes 6 through 8, samples from AAA biopsy specimens. **B,** EP3: lanes as in **A. C,** EP4: lanes 3 through 6, samples from AAA biopsy specimens; lane 7, genomic DNA; lane 8, placenta.

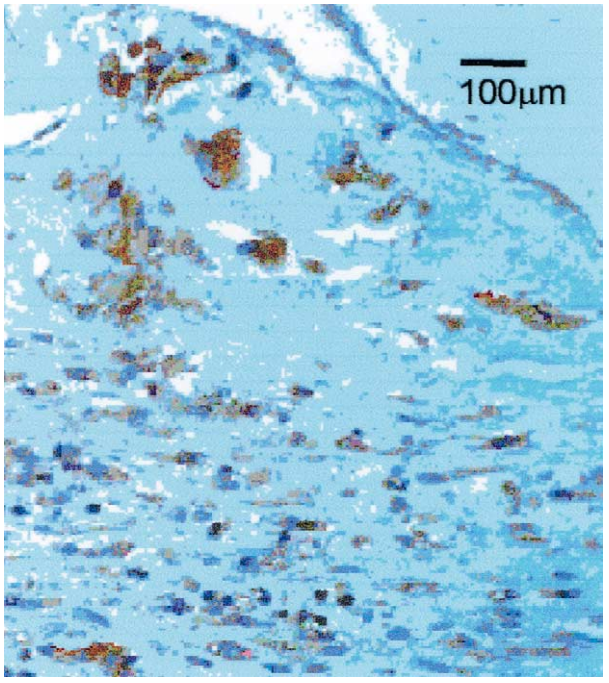

Fig 2. Expression of EP4 receptors in aortic aneurysm biopsy specimen. Section through an aneurysmal wall, with lumen in top right-hand corner. Large brown staining cells (macrophages) are present predominantly in the atherosclerotic intima. Section counterstained with hematoxylin.

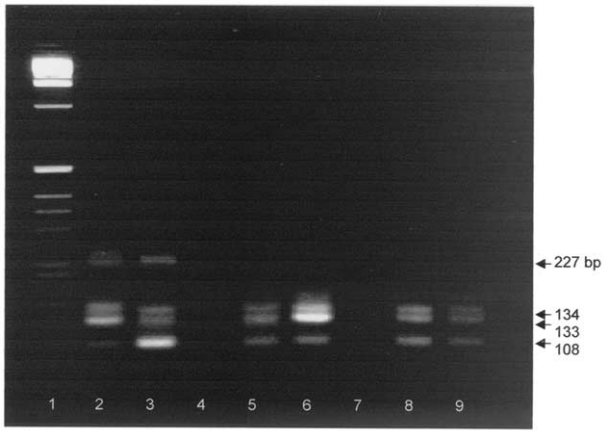

Fig 3. Expression of EP3 variants in aortic smooth muscle cells, showing range of variants in a single gel. Lane 1, molecular weight markers; lane 2, HSB-2 cells; lane 3, normal human aortic smooth muscle cells; lane 4, water control; lanes 5 through 6, human aortic smooth muscle cells cultured from abdominal aortic aneurysm (AAA) biopsy specimens; lane 7, genomic DNA; lanes 8 and 9, human aortic smooth muscle cells cultured from AAA biopsy specimens.

not respond to PGE_2 with IL-6 secretion and did not express the EP4 receptor mRNA. Therefore it appears likely that activation of EP4 receptors in other cell types, including macrophages, is the principal mechanism underlying PGE_2 stimulation of IL-6 production by aortic aneurysm wall.

Aortic explants remain viable for up to 1 week in culture, and provide opportunity to study the complex architecture of the aortic wall.[18] The pharmacology of EP receptor agonists in human beings has shown that butaprost is relatively selective for the EP2 receptor and that sulprostone is relatively selective for the EP3 receptor, because EP1 receptors are scant in human beings. 11-Deoxy-PGE_1 is primarily an agonist for both EP2 and EP4 receptors, with lower specificity for EP3 receptors.[19] Of these agonists, only 11-deoxy-PGE_1 partially reversed indomethacin inhibition of IL-6 production by aortic explants or isolated macrophages, to indicate an EP4 receptor

Table III. Interleukin-6 production by macrophages isolated from aortic wall

Condition	*+10 μmol/L Indomethacin*	*IL-6 (ng/mL) (mean ± SD)*
Control	No	53 ± 16
Control	Yes	22 ± 9
+1 μm PGE_2	Yes	40 ± 12
+1 μm 11-deoxy-PGE_1	Yes	36 ± 9
+1 μm Butaprost	Yes	19 ± 14
+1 μm Sulprostane	Yes	16 ± 5

Experiments were performed with cells isolated from three different aneurysm biopsy specimens, with a single well for each condition providing duplicate samples for IL-6 analysis. With n = 3, no statistical comparisons were made.
IL-6, Interleukin-6; *PGE,* prostaglandin E.

pathway. We used fixed saturating concentrations of agonists, and did not use a range of concentrations to determine their efficacy (median effective concentration). There were two reasons for this: limited amounts of biopsy material and the wide range of IL-6 production by explant cultures reflecting pathobiologic variation. However, EP4 receptor was expressed in AAA tissue, together with EP2 and EP3-III, and EP4 protein was associated with macrophages in the aortic wall. In contrast, SMC cultured from AAA wall did not express EP4 receptor mRNA and did not secrete IL-6 in response to PGE_2. This supports our suggestion that EP4 receptors are important in mediating the IL-6 response to PGE_2 in the aneurysm wall.

We also noted complete suppression of IL-6 secretion from aneurysm wall explants treated with dexamethasone. Dexamethasone suppresses COX2 expression, but appears to have variable effect on IL-6 expression, depending on cell type.[20-23] Dexamethasone also may suppress expression of EP receptors, but evidence for this is weak.[24] Other evidence indicates the presence of a positive feedback loop, in which PGE_2 stabilizes COX2 mRNA through an EP4-dependent mechanism.[25] However, exogenous PGE_2 did not reverse dexamethasone suppression, and therefore experiments with synthetic agonists were not conducted.

The most elegant approaches to determine the different pathobiologic effects of the four subtypes of EP receptors have been based on use of genetically modified animals. For example, in an experimental murine model of rheumatoid arthritis, deletion of EP1, EP2, or EP3 receptors did not alter severity of the disease process.[26] However, EP4 receptor–deficient mice demonstrated less severe disease, together with marked reduction in serum IL-6 concentration.[26] Others have shown that the EP4 receptor mediates IL-6 secretion in monocyte cell lines.[23]

Our findings support the importance of EP4 receptors in inflammatory processes in the AAA wall and indicate a more selective route for therapeutic intervention than nonsteroidal anti-inflammatory drugs, in developing a therapeutic strategy to limit the growth of small aneurysms.

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Table I, online only. Primers designed for amplification of EP receptors

Primer	*Sequence*	*Sequence position*	
EP1-1F	ATG GTC TTC TTC GGC CTG T	422-440	Exon]
EP1-2R	AAA GAA GGT GGA GGC CGA A	901-883	Exon]
EP1-3F	TGG TGC TGC GTC TGT ACA CT	354-373	Exon]
EP1-4R	TAG TCG TTG GGC CTC TGG TT	1306-1287	3′ UTR]
EP2-1F	GAA ACC TCT TCC CGA AAG GA	1018-1037	Exon]
EP2-2	TGA AAT CCG ACA ACA GAG GAC	1161-1141	Exon]
EP2-3F	GCC TGC AAC TTC AGT GTC ATT	793-813	Exon]
EP2-4R	CGC ATT AGT CTC AGA ACA GGA G	1136-1115	Exon]
EP3-SF	CTT AAT AGC TGT TCG CCT GG	983-1002	Exon 2
EP3-1F*	CTG AAC CAG ATC TTG GAT CC	1008-1027	Exon 2
EP3-SR	GGC AGA AAG GCA GGT TTT A	1464-1446	Exon 1 C
EP3-2R*	TCA TGG AGC TTC CAG TGA TG	1491-1472	Exon 1 C
EP3-3	GAA GTT GGA AAC TCC TGG	1722-1702	Exon 4
EP3-4	TCC TCC AAT CAT CAT CTG TGA	1674-1654	Exon 4
EP3-5R	TAC GAA TGG CAG ACT CAA CA	1632-1613	3′ UTR
EP3-6R	GAT AAT GAG ATA GGC TGC CCT	1497-1478	Exon 1 C
EP3-7R	TCC AGA GAT GCA CTG CAT T	1783-1765	3′ UTR
EP3-8R	GCA GTC TTG GCA ATT CTG A	1725-1707	3′ UTR
EP4-F†	AGA CAC CTG GTG CTT CAT CG	885-904	Exon
EP4-R†	GGT CTA GGA TGG GGT TCA CA	1363-1344	Exon

EP1, Base positions refer to Genbank accession number L22647.
EP2, Base positions refer to Genbank accession number NM000956.
EP3, Base positions refer to Genbank accession numbers D38297 (EP3-3 and EP3-4, EP3-5R, and EP3-6R); X83862.1 (EP3-7R and EP3-8R).
EP4, Base positions refer to Genbank accession number D28472.
*Primers designed by Kotani et al.[9]
†Primers designed by Paul et al.[10]