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CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF THE P2Y$_2$R GENE PROMOTER

A Thesis presented to the Faculty of the Graduate School
University of Missouri-Columbia

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

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by

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF THE $P2Y_2R$ GENE PROMOTER

Presented by Nishant Jain

A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance.

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LIST OF ABBREVIATIONS:

ABC: ATP-binding cassette

ADP: adenosine 5'-diphosphate

ATP: adenosine 5'-triphosphate

$[Ca^{2+}]_i$: concentration of cytoplasmic free calcium

C/EBPβ: CCAAT enhancer binding protein beta

DAG: diacylglycerol

EC: endothelial cells

ECM: extracellular matrix

EGR1: early growth response element-1

EGFR: epidermal growth factor receptor

E-NPP: ecto-nucleotide pyrophosphophatase/phosphodiesterase

E-NTPDase : ecto-nucleoside 5'-triphosphate diphosphohydrolase

ERK1/2: extracellular-signal regulated kinases 1/2

FAK: focal adhesion kinase

GPCR: G protein-coupled receptor

HCAEC: human coronary artery endothelial cells

IP3: inositol-1, 4, 5- trisphosphate

JNK: Jun amino-terminal kinases

MAP kinase: mitogen-activated protein kinase

MAP kinase kinase: mitogen activated protein kinase kinase

2-MeSATP: 2-methylthioadenosine 5'-triphosphate

MyT1: myelin transcription factor 1

NAD^+: nicotinamide adenine dinucleotide

NCBI: National Center for Biotechnology Information

NDPK: nucleoside diphosphokinase

PAGE: polyacrylamide gel electrophoresis

PDGFR: platelet-derived growth factor receptor

PI3K: phosphatidylinositol 3-kinase

PIP2: phosphatidylinositol-4, 5-bisphosphate

PKC: protein kinase C

PLA_2: phospholipases A_2

PLC: phospholipases C

PMA: phorbol 12-myristate 13-acetate

Pyk2: proline-rich tyrosine kinase 2

RACE: rapid amplification of cDNA ends

RGD: arginine-glycine-aspartic acid

RGE: arginine-glycine-glutamic acid

RRR: relative response ratio

SH3: Src homology domain 3

SH2: Src homology domain 2

SMC: smooth muscle cells

TESS: transcription element search system

UDP: uridine 5'-diphosphate

UTR: 5'- untranslated region

UTP: uridine 5'-triphosphate

VCAM-1: vascular cell adhesion molecule-1

WHN: winged helix nude

CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF THE $P2Y_2R$ GENE PROMOTER

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ABSTRACT

Extracellular nucleotides can bind to the $P2Y_2R$ and modulate proliferation and migration of smooth muscle cells, which is known to be involved in intimal hyperplasia that accompanies atherosclerosis and post-angioplasty restenosis. Moreover, the $P2Y_2R$ is up-regulated in vascular smooth muscle cells and endothelial cells in response to tissue injury. These findings suggest that the $P2Y_2R$ is a potential target for the pharmacological control of progression of atherosclerosis and post-angioplasty restenosis. However, the mechanisms governing $P2Y_2R$ up-regulation remain unknown.

In this study, we have cloned a 2071 bp 5'-flanking region of the $P2Y_2R$ gene in a reporter vector and carried out a serial deletion analysis. The deletion of a 175 bp region completely abolished promoter function and results further indicate that the $P2Y_2R$ gene promoter uses an array of positive and negative response elements in the regulation of gene expression. Furthermore, other results show that the cytokine IL-1β may be involved in down-regulation of $P2Y_2R$ activity in human coronary artery endothelial cells. Further studies will potentially lead to the identification of novel pathways involved in the regulation of $P2Y_2R$ gene expression, information that might be useful to suppress neointimal hyperplasia in atherosclerosis and the restenosis of angioplasty.

CHAPTER 1: INTRODUCTION

I. Historical Perspective

The seminal work of Drury and Szent-Gyorgi in 1929 described the involvement of extracellular nucleotides and nucleosides in potent biological actions in mammalian heart and blood vessels; they showed that intravenous injection of purines into whole animals induced a decrease in heart rate and arterial blood pressure, dilation of coronary blood vessels and inhibition of intestinal movements. Furthermore, Holton suggested a role for adenosine 5'-triphosphate (ATP) as a transmitter in the nervous system by using the firefly luciferase assay to show that ATP is released during antidromic stimulation of sensory nerves in sufficient quantities to produce vasodilatation of rabbit ear arteries [1,2]. Eleven years later, the neurotransmitter-like activity of ATP was demonstrated in non-adrenergic, non-cholinergic nerves supplying the gut [3]. This led to the controversial proposal of the "purinergic-neurotransmission" hypothesis which stated that the intracellular metabolite ATP or a related nucleotide can act as a neurotransmitter when released from nerves in the gut and bladder [4]. Although the concept of extracellular nucleotides as signaling molecules met with resistance initially, it has now been firmly established that ATP acts as a co-transmitter along with classical neurotransmitters (e.g., acetylcholine) in both the peripheral and the central nervous systems, and that purine nucleotides are powerful extracellular messengers in non-neuronal cells, including exocrine, endocrine, secretory, endothelial, bone, immune and inflammatory cells [5]. Early investigations into the effects of adenosine were particularly made on heart and

vasculature [2,6]. Initial studies on the effects of extracellular UTP also focused on its cardiovascular effects [2].

II. Extracellular Release of Nucleotides

Earlier, little was known about the sources and release of nucleotides in mammalian cells [7]. The development of sensitive assays that utilized a cell-surface targeted luciferase as an ATP sensor enabled the more accurate measurement of the concentrations of extracellular nucleotides in the pericellular environment of cells [8]. These methods have shown that extracellular nucleotides are released constitutively by many cell types under unstimulated conditions. Stimuli such as stress or mechanical stimulus can lead to increased levels of extracellular nucleotides. It has been postulated that cells maintain a steady-state equilibrium between release and ecto-metabolism of extracellular nucleotides [9].

ATP and adenosine 5'-diphosphate (ADP) are released from sympathetic nerves, activated platelets, erythrocytes, cardiac tissue, mast cells, endothelial cells, and smooth muscle cells [10-12]. The main source of intraluminal ATP is likely to be endothelial cells, and its release can be measured during conditions such as changes in the rate of blood flow and hypoxia in amounts sufficient to activate endothelial cell G protein-coupled P2Y purinoceptors [13]. It also has been shown that ATP is released from vesicles in urothelial and endothelial cells in the ureter leading to acute inflammation [14]. The effects of ATP are often counteracted by its degradation product adenosine. Levels of the pyrimidine nucleotide uridine 5'-triphosphate (UTP) increase in human plasma in response to alterations in blood flow [15,16] and animal studies have shown that UTP release occurs in heart during cardiac ischemia, most likely from cardiomyocytes and to a lesser extent

from red blood cells and endothelial cells [17]. Several lytic and non-lytic mechanisms for the release of UTP and ATP have been proposed. Tissue damage and cell lysis can cause an increase in extracellular nucleotide levels. Nucleotide release mechanisms associated with excitable and secretory tissues include vesicular exocytosis in ureter epithelium [18] and exocytotic granule release [19]. Other mechanisms include transport by ATP-binding cassette (ABC) proteins, stretch-activated channels and voltage-activated channels (which are still controversial) [14,20]. Apart from these mechanisms, additional ATP transporters have been hypothesized to exist in murine cells [21].

The levels of extracellular nucleotides depend both on release mechanisms and the activities of cell-surface enzymes that metabolize nucleotides, such as soluble and membrane-bound ecto-nucleotidases that are expressed in most cell types [22]. A variety of different ecto-enzymes can participate in degradation and interconversion of extracellular nucleotides. These include the E-NTPDase (ecto-nucleoside 5′-triphosphate diphosphohydrolase; previously known as ecto-apyrase, NTPase or E-ATPase) family that hydrolyzes nucleoside 5′-triphosphates to diphosphates and then to monophosphates, the E-NPP (ecto-nucleotide pyrophosphatase/phosphodiesterase) family that hydrolyzes phosphodiester bonds, the ecto-5′-nucleotidase and alkaline phosphatase families that hydrolyze terminal phosphates of nucleoside 5′-monophosphates to yield the corresponding nucleoside, and the nucleotide converting enzyme ecto-NDPK (ecto-nucleoside diphosphokinase) and adenylate kinase that phosphorylate adenosine 5′-monophosphate [22]. E-NPP isoenzymes can hydrolyze either purine or pyrimidine nucleotides, as well as dinucleotides and uridine 5’-diphosphate-conjugated sugars (UDP-sugars). E-NPP can hydrolyze ATP into AMP and pyrophosphate (PP_i); 3’, 5’-cyclic

AMP (cAMP) into AMP; ADP into AMP and inorganic phosphate (P_i); and NAD^+ (nicotinamide dinucleotide) into AMP and nicotinamide mononucleotide. Ecto 5'-nucleotidase is the major enzyme involved in the formation of adenosine in the extracellular space. Ecto-NDPK catalyzes the reversible transphosphorylation of nucleoside 5'-diphosphates into nucleoside 5'-triphosphates such as ATP + UDP to ADP + UTP [23-28]. Alkaline phosphatase activity alone can catalyze the complete sequential hydrolysis of a nucleoside 5'-triphosphate to the corresponding nucleoside [22]. Alkaline phosphatase also can hydrolyze PP_i [29,30].

III. Classification of P2 Receptors

Extracellular nucleotides and their metabolites act through two classes of receptors termed P1 receptors, whose ligand is adenosine, and P2 receptors, whose ligands include ATP, ADP, UTP, UDP and UDP-glucose. There are two different categories of P2 receptors: G protein-coupled P2Y receptors, and P2X ligand-gated ion channels (Table I). Eight P2Y receptor subtypes and seven P2X receptor subtypes have been cloned. The 8 human P2Y receptors can be grouped into two structurally distinct subfamiles: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ in one group that couples to phospholipase C, whereas $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ belong to a group that couples to adenylate cyclase. In the case of $P2Y_{11}$, two signaling pathways can be activated, adenylyl cyclase as well as phospholipase C [31]. Seven P2X receptor subunits ($P2X_{1-7}$) have been identified, and these subunits can form a variety of homo- and heterotrimeric channels. All of these P2 receptors have been characterized pharmacologically and accepted as valid members of the P2 receptor family [32,33] .

Table 1: P2 Receptors: Agonist Potency and Signal Transduction Mechanisms.

Receptor Subtype	Rank Order of Agonist Potency	Signal Transduction
$P2Y_1$	2-MeSADP > ADP > 2-MeSATP > ATP	$PLC^3/IP_3/Ca^{2+}$
$P2Y_2$	UTP = ATP > Ap_4A > ATPγS	$PLC^3/IP_3/Ca^{2+}$
$P2Y_4$	UTP > UTPγS >> ATP, UDP	$PLC^3/IP_3/Ca^{2+}$
$P2Y_6$	UDP = 5-Br-UDP >> UTP > 2-MeSADP	$PLC^3/IP_3/Ca^{2+}$
$P2Y_{11}$	ATPγS = BzATP > ATP > 2-MeSATP	$PLC^3/IP_3/Ca^{2+}$
$P2Y_{12}$	2MeSATP = 2MeSADP > ADP	Adenylate Cyclase
$P2Y_{13}$	2MeSATP = 2MeSADP > ADP > ATP	Adenylate Cyclase
$P2Y_{14}$	UDP-glucose > UDP-galactose	Adenylate Cyclase

Receptor Subtype	Rank Order of Agonist Potency	Signal Transduction
$P2X_1$	2-MeSATP > ATP > α,β-meATP	$I_{Na^+/K^+/Ca^{2+}}$
$P2X_2$	2-MeSATP > ATP, α,β-meATP inactive	I_{Na^+/K^+}
$P2X_3$	2-MeSATP > ATP > α,β-meATP	$I_{Na^+/K^+/Ca^{2+}}$
$P2X_4$	ATP > 2-MeSATP >> α,β-meATP	I_{Na^+/K^+}
$P2X_5$	ATP > 2-MeSATP > ADP	$I_{Na^+/K^+/Ca^{2+}}$
$P2X_6$	ATP > 2-MeSATP > ADP	$I_{Na^+/K^+/Ca^{2+}}$
$P2X_7$	BzATP > ATP > 2-MeSATP > ADP	I_{Na^+/K^+}, pore formation

Abbreviations: 2MeSADP , 2-methylthio-ADP; 2MeSATP, 2-methylthio-ATP; ADP, adenosine 5'-diphosphate; ATP , adenosine 5' - triphosphate; ATPγS, adenosine 5'- triphosphate gamma thiol; BzATP, 2' and 3'-O-(4-benzoylbenzoyl)-ATP; α,β-meATP, α,β-methylene adenosine 5'- triphosphate; UDP, uridine 5'-diphosphate; UTP , uridine 5'- triphosphate; PLC, Phospholipase C; IP_3, inositol triphosphate; I , ion channel current

IV. P2 Receptors in Vascular Cells

The normal arterial wall consists of three layers: the intima, media and adventitia. The single layer of endothelial cells facing the vascular lumen is a very important component of the blood vessel wall in terms of releasing both vasodilators such as nitric oxide (NO) and prostacyclin (PGI_2), and vasoconstrictors like thromboxane A_2 and endothelin. ATP and ADP are components of blood-borne elements, such as platelets and erythrocytes, and can also be released from endothelial cells and smooth muscle cells [10]. The vascular response elicited by these purine nucleotides depends on several factors including the nature of the P2 receptor subtype involved and its location in relation to structural components in the vascular wall [34]. Endothelium-dependent vasorelaxation is due to the release of prostacyclin and NO by the nucleotide-induced activation of $P2Y_2$ as well as $P2Y_1$ receptors [34]. The principal P2Y receptor subtypes that have been functionally characterized in endothelial cells are $P2Y_1$ and $P2Y_2$, but mRNAs for $P2Y_4$ and $P2Y_6$ receptors have also been detected [35]. In smooth muscle cells, however, activation of the $P2Y_2R$ plays a role in vasoconstriction [36,37]. P2 receptor subtypes in different cell types that make up a blood vessel wall can have opposing effects, such as endothelial-mediated vasodilatation or smooth muscle-mediated vasoconstriction [34].

Activation of $P2Y_2$ receptors also causes proliferation and migration of vascular smooth muscle cells [38-40]. $P2Y_1$ receptors are expressed in smooth muscle cells (SMCs) of a number of blood vessel types, and like their endothelial cell (EC) counterparts, mediate vasodilatation [2]. $P2Y_2$ receptors in SMCs mediate the induction of immediate-early and delayed-early cell cycle-dependent genes, consistent with a role for these receptors in vascular proliferation of SMCs [41,42] . A recent study in rat aortic SMCs demonstrated that the $P2Y_2R$ is the predominant functional receptor that responds to ATP and UTP [43]. The $P2Y_6$

receptor is expressed in human cerebral arteries, and mediates vasoconstriction when activated by UDP/UTP [44] similar to results with rat pulmonary and mesenteric arteries [45,46]. Moreover, $P2X_1$ receptor knock-out mice were used to demonstrate that the $P2Y_6$ receptor is the prominent P2 receptor that promotes a contractile response in mesenteric arterial trees [47]. The presence of $P2Y_4$ and $P2Y_6$ receptors has been reported in rat aortic SMCs [41]. Major blood vessels consist of connective tissue and fibroblasts in the outermost layer. A recent study showed that fibroblasts can migrate into the neointima (i.e., the proliferating smooth muscle layer of the subendothelial region of blood vessels), suggesting their possible involvement in the development of vascular diseases such as atherosclerosis and restenosis after angioplasty [48]. Human and rat fibroblasts are known to express $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors [32], suggesting a role for these receptors in fibroblast activation.

P2X receptor subtypes also have been reported in human saphenous vein SMCs, including $P2X_1$, $P2X_2$, $P2X_4$ and $P2X_7$ receptors [49]. Human and rat fibroblasts are known to express $P2X_3$, $P2X_4$, and $P2X_7$ receptors [32].

V. <u>Pharmacology and Signal Transduction of P2Y Receptors</u>

P2Y receptors are found in a wide range of tissue types (Table 2). P2Y receptors serve multiple functions in their host cells, working through two major pathways: 1) activation of intracellular signaling cascades, via the catalytic G protein α subunit; and 2) modulation of membrane ion channel activities, via regulatory G protein βγ subunits ralevic [2]. Also, a third less well understood pathway may involve the physical interaction of P2Y receptors with membrane proteins in their close proximity.

$P2Y_2$ receptors have been reported to couple to G proteins including pertussis toxin-sensitive G_o, pertussis toxin-insensitive $G_{q/11}$, and G_i protein [50-53]. Activated $P2Y_2$

receptors interact with heterotrimeric G proteins and cause GTP/GDP exchange in Gα subunits and dissociation of GTP-Gα from Gβγ subunits. Dissociated GTP-Gα subunits activate phospholipase C (PLC), which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) into the second messengers inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes Ca^{2+} release from endoplasmic reticulum into the cytoplasm. Ca^{2+} and DAG activate a variety of downstream pathways including protein kinase C (PKC) and mitogen-activated protein (MAP) kinases [54,55-57].

The specific downstream signal transduction pathway involved in P2Y receptor activation depends not only on the P2Y receptor subtype but also on the cell type expressing the receptor [2]. In endothelial cells, $P2Y_2$ receptor-mediated calcium mobilization causes phospholipase A_2 (PLA_2) activation, which leads to the release of prostacyclin, and the activation of nitric oxide synthase that leads to the release of NO [53,58]. As with $P2Y_1$ receptors, protein tyrosine phosphorylation and MAP kinase activation seem to be a consequence of $P2Y_2R$ activation in endothelial cells [59,60]. This occurs subsequent to activation of PKC and does not involve IP_3 or elevation of cytosolic Ca^{2+} levels [60]. Stimulation of the $P2Y_2R$ in endothelial cells is known to activate a variety of signaling molecules including the mitogen- and stress-activated protein kinases ERK1/2 (extracellular-signal regulated kinases 1/2), p38, and c-Jun NH_2-terminal kinase, and the small GTPase RhoA [61,62]. More than one subtype of P2Y receptor can regulate responses in endothelial cells, and endothelial cells derived from different vascular beds may express different combinations of receptors [63]. In vascular smooth muscle cells, activation of $P2Y_2$ receptors causes phosphatidylinositol 3-kinase (PI3K)-independent activation of ERK1/2 and proliferation of smooth muscle cells [39].

Src homology domain 3 (SH3) binding sites in the $P2Y_2$ nucleotide receptor ($P2Y_2R$) interact with Src or PI3K and regulate the activities of Src, Pyk2, and growth factor receptors [62]. The integrin binding sequence arginine-glycine-aspartic acid (RGD) in the $P2Y_2R$ interacts with $\alpha_v\beta_3$ integrins and is required for activation of focal adhesion kinase (FAK) and ERK1/2 [52]. The RGD domain in $P2Y_2R$ is required for nucleotide-induced activation of G_o- but not G_q-mediated intracellular calcium mobilization [52]. More recently, Liao Zhongji (unpublished data) has shown that $P2Y_2R$ interaction with α_v integrins is required for it to access and activate G_{12}.

Table 2 : Tissue Distribution of P2Y Receptors
(modified from von Kügelgen et al., 2000 and Burnstock and Knight , 2004)

Subtypes expressed in human tissues	**Tissue distribution**
$P2Y_1$	Platelets, heart, skeletal muscle, neuronal tissue, digestive tract
$P2Y_2$	Lung, heart, skeletal muscle, spleen, kidney, aorta
$P2Y_4$	Placenta, lung, vascular smooth muscle
$P2Y_6$	Lung, heart, aorta, spleen, placenta, thymus, intestine, brain
$P2Y_{11}$	Spleen, intestine, immunocytes
$P2Y_{12}$	Platelets, glial cells
$P2Y_{13}$	Spleen, brain, lymph nodes, bone marrow
$P2Y_{14}$	Placenta, adipose tissue, stomach, intestine, discrete brain regions

VI. Role of P2 Receptors in the Vasculature

Along with mitogenic effects, nucleotides can be involved in vascular inflammation in the following ways:

1) by increasing expression of adhesion and chemoattractant molecules thus facilitating adherence of monocytes to vascular cells [64];
2) by mediating the secretion of pro-inflammatory cytokines [35] by monocytes or macrophages via activation of $P2X_7$ receptors [65] ; and
3) by initiating smooth muscle cell migration [61].

Involvement of nucleotides in the above processes has been demonstrated by *in vitro* and *in vivo* studies in endothelial and smooth muscle cells [61,66]. Nucleotides cause smooth muscle cell migration via an increase in the expression of osteopontin [61]. Nucleotides also increase expression of Monocyte Chemoattractant Protein-1 (MCP-1) in arterial SMCs [41]. Another significant finding is the demonstration that stimulation of P2 receptors can increase release of the cytokines IL-1β, IL-1α, IL-8 and TNF-α [65]. UTP and ATP also can increase expression of the monocyte adherence protein, vascular cell adhesion molecule-1 (VCAM-1) via activation of $P2Y_2$ receptors [64]. Furthermore, ATP and UTP have been shown to induce cell-cell adhesion in a human monocyte/macrophage lineage and neutrophil adherence to human endothelial cell monolayers [66,67]. *In vivo* studies by Seye and colleagues have shown that local UTP delivery to collared rabbit carotid arteries can induce accumulation of macrophages in the intima [68]. Thus, involvement of $P2Y_2$ receptors in leukocyte infiltration and migration suggests that this receptor can be targeted in therapies directed at reducing vascular inflammation and atherosclerotic lesion development [69].

Smooth Muscle Cell Proliferation

P2 receptors are involved in nucleotide-induced vascular smooth muscle cell proliferation, which is a hallmark of vascular diseases such as atherosclerosis and restenosis following angioplasty [42,69]. Acute responses to nucleotides are mediated by both P2X and P2Y receptors, whereas chronic responses are mostly mediated by P2Y receptors [69]. Various cell culture studies have shown that increases in DNA synthesis and the expression of protein markers of cell proliferation occur in vascular smooth muscle cells under the influence of nucleotides [70,71]. Both $P2Y_2$ and $P2Y_4$ receptors have been associated with proliferative responses in vascular smooth muscle cells [72,73]. $P2Y_6$ receptors have also been associated with SMC proliferation in rat aorta [74].

Smooth Muscle Cell Migration

Extracellular nucleotides serve as directional cues for rat aortic SMC migration [61]. Several P2Y receptor subtypes could be involved in nucleotide-mediated SMC migration, although in rat aortic SMCs the effect is thought to be predominantly via the $P2Y_2R$ [37]. Migration in response to UTP involves both $P2Y_2$ and $P2Y_4$ receptors [40]. The migratory capacity of extracellular nucleotides could be mediated by inducing the expression of extracellular matrix (ECM) proteins such as osteopontin, an RGD containing ECM protein [61,68]**.** UTP-induced osteopontin expression involves the transcription factors NFκB and USF-1/USF2 [75]. The role of nucleotides as a chemoattractant is consistent with the concentration range found in pathological vessels [76]. Moreover, findings from previous studies demonstrating the mitogenic activity of extracellular nucleotides for SMCs suggest that nucleotides released from mechanically-

stimulated or damaged cells during the angioplasty process may participate in arterial wall remodeling [69].

Phenotypic Modulation of $P2Y_2R$

Experimental arterial intimal hyperplasia can be evoked by balloon angioplasty, or by the perivascular placement of a silicone collar around an artery [69]**.** An influx of leukocytes precedes the migration and proliferation of vascular SMCs into the intima in both of these models [77]. $P2Y_2R$ mRNA is expressed at low levels in endothelial cells and a few medial smooth muscle cells under normal conditions. In arteries, $P2Y_2R$ expression was increased by balloon angioplasty or collar placement [42,68]. This increase in $P2Y_2R$ expression is also correlated with three phases of neointimal hyperplasia in the collar model [78]**.** The first phase characterized by vascular infiltration of leukocytes begins 2 h after the collar is placed around a rabbit carotid artery [78]. The second phase characterized by medial replication of SMCs begins within 12 h after collar placement [78]. The third phase is characterized by the appearance of sub-endothelial SMCs which begins at day 3 after collar placement [78]**.** The appearance of $P2Y_2R$ mRNA was detected within 3 days of collar placement in SMCs and by day 14 was detected in all intimal and medial smooth muscle cells [68]. In the same experiments, levels of $P2Y_4$ receptor mRNA did not change and $P2Y_6$ receptor mRNA was not detected. Similar observations of $P2Y_2R$ upregulation have been made in the porcine coronary artery stent model [73]. A role for $P2Y_2Rs$ in atherosclerosis is further suggested by the demonstration of $P2Y_2Rs$ in the basilar artery of a rat double-hemorrhage model [79], in coronary arteries of a diabetic dyslipidemic pig model [80] and in human atherosclerotic lesions (Seye and Desgranges, unpublished data).

Pathophysiological Significance of Phenotypic Modulation of P2 Receptors in Smooth Muscle Cells

The shift in the phenotypic status of SMCs from a differentiated contractile phenotype to a synthetic phenotype has been suggested to be related to an increase in the level of P2 receptor expression [37]. In the synthetic phenotype, the mitogenic $P2Y_1$ and $P2Y_2$ receptor transcripts were upregulated, while the contractile $P2X_1$ receptor was completely downregulated and $P2Y_4$ and $P2Y_6$ receptor levels were unchanged [81]. Data indicate that the $P2Y_2R$ is expressed at a high level in medial SMCs of injured rat aorta [42], thus linking it to the poorly differentiated phenotype, a condition similar to that in the post-natal phase of development, where SMCs are confined to the medial layer and are in the immature state [82]. The increased $P2Y_2R$ levels may be required for the modulation of proliferation or vasoreactivity of SMCs by nucleotides [69]. Since both neointimal hyperplasia and vasoconstrictive remodeling are involved in postangioplastic restenosis, these findings suggest that extracellular nucleotides might play a significant role in this process, at least as long as functional endothelial cells, which control intimal thickening and nucleotide-induced vasorelaxation, are not regenerated [69]. SMC $P2Y_2$ receptors are involved in nucleotide-induced constriction of normal arteries [83,84]**.** Long-lasting alterations in vasomotricity after endothelial denudation results in increased sensitivity to vasoconstrictive substances [85,86]. It appears that similar to other receptors of vasoconstrictive factors such as angiotensin II, endothelin, and platelet-derived growth factor (PDGF) that are overexpressed in neointima, nucleotides acting through $P2Y_2$ receptors may play an important role in controlling the vasoactive properties of arteries

under pathophysiological conditions, particularly with chronic constriction at the lesion site that may lead to postangioplastic restenosis [69].

VII. Potential Factors Involved in $P2Y_2$ Receptor Upregulation.

Several factors have been shown to regulate $P2Y_2R$ upregulation. Shear stress induces upregulation of $P2Y_2Rs$ in human blood vessels with intact endothelium [87]. In rat smooth muscle cells, fetal calf serum and growth factors upregulate $P2Y_2R$ mRNA expression by a MAP kinase kinase-dependent pathway [88]. Using quantitative reverse transcription-polymerase chain reaction (RT-PCR), it was shown that phenotypic changes in vascular smooth muscle cells regulate P2 receptor expression [37]. In rat salivary gland cells during short-term culture, a time-dependent increase in $P2Y_2R$ activity occurs that is associated with an increase in the steady-state level of $P2Y_2R$ mRNA, as assessed by RT-PCR [89]. $P2Y_2R$ mRNA upregulation also occurs as an immediate early gene response in T cell differentiation [90], in collared rabbit carotid arteries [68], in response to stab wound injury in astrocytes [91], and in submandibular gland (SMG) cells from the NOD.B10 mouse model of Sjögren's syndrome as compared to wild type mouse SMG cells [92].

Among the prime candidates for factors that can cause upregulation of human $P2Y_2R$ expression are cytokines, growth factors and shear stress. Positioning a silicone collar around the carotid artery of rabbits induces vascular injury possibly due to hindrance of transmural blood flow, leading to retention of cytokines/growth factors within the segment enclosed by the collar [77].

Cytokines and Growth Factors

Previous studies have reported that cytokines such as tumor necrosis factor-α (TNF-α) in combination with interleukin 1-β (IL-1β), and IL-1β in combination with interferon-γ, induce $P2Y_2R$ upregulation in rat SMCs and increase mitogenic responses to nucleotides [81]. In SMCs and other cell types, upregulation of $P2Y_2Rs$ has been associated with stimulation of PKC, cyclooxygenase, and MAP kinases [88,93].

TNF-α released from macrophages and T-lymphocytes, injured human coronary artery endothelial cells and activated SMCs is one of the most important promoters of inflammation [94]. TNF-α induces activation of major signaling pathways involved in the activation of endothelial cells (i.e., the IκB kinase/nuclear factor κB [IKK/NF-κB] pathway) [94]. It is possible that NF-κB elements in the $P2Y_2R$ 5'-flanking region may be involved in the transcriptional regulation of $P2Y_2R$ expression.

Laminar and Shear Stress

Certain flow-sensitive proteins are regulated by the transcription factor Sp1. For example, Sp1 is involved in regulatory mechanisms of flow-sensitive proteins, such as endothelial Toll-like receptor 2 in human coronary artery endothelial cells [95] . It has also been shown that fluid shear stress-induced transcriptional activation of the VEGF receptor-2 gene and Flk-1/KDR (fetal liver kinase-1) requires Sp1-dependent DNA binding [96,97]. The presence of Sp1 and EGR1 (early growth response-1) binding sites immediately upstream and close to the putative transcription start sites of the $P2Y_2R$ gene indicates that these sites could potentially be involved in the regulation of $P2Y_2R$ transcription (Figure 2).

Vascular stress conditions including ischemia/oxidative stress, vascular flow, and mechanical stretch are known to cause increased release of nucleotides [98,99]. Shear stress affects endothelial structure and function, both *in vivo* and *in vitro*, and is implicated as a contributing factor in the development of cardiovascular diseases [100,101]. Endothelial cells, because of their unique localization at the blood/blood vessel wall interface, are constantly exposed to fluid mechanical forces, such as shear stress derived from flowing blood. A shear stress response element in the PDGF A-chain promoter was described that contains a binding site for the transcription factors EGR1/Sp1 [102].

Other Inflammatory Mediators

Lipopolysaccharide (LPS) has been shown to cause vascular endothelial growth factor (VEGF) upregulation via activation of Sp-1 [103]. LPS has been shown to be involved in the upregulation of $P2Y_2$Rs in rat SMCs [104].

Phenotypic Changes

In the synthetic phenotype of SMCs, mitogenic $P2Y_1$ and $P2Y_2$ transcripts were upregulated by 342- and 8-fold, respectively, while the contractile $P2X_1$ receptor was completely downregulated and expression levels of $P2Y_4$ and $P2Y_6$ receptors were unchanged [37]. This plasticity of P2 receptor expression may be important in the transition from the contractile to the synthetic SMC phenotype.

Thus, many factors at the site of injury could cause upregulation of $P2Y_2$Rs. By studying the $P2Y_2$R promoter, we aim to find the cis and trans acting factors involved in $P2Y_2$R expression. Furthermore, we hope to delineate the signaling pathway involved in $P2Y_2$R upregulation.

VIII. Eukaryotic Promoters And Prediction of Regulatory Elements

Eukaryotic promoters are DNA sequences that regulate gene expression at the level of transcription. These promoters have a complex block-modular structure and contain numerous short functional elements, i.e., transcription factor binding sites [105]. The distal elements have no uniform location, are dispersed in the 5'-flanking region up to ~ 1 kb upstream of the transcription start site (TSS), and are involved in specific transcriptional regulation of gene expression (e.g., tissue- and cell-specific regulation, etc.) [106]. The proximal elements, which encompass the TSS, are called core promoters and are involved in formation of the basal transcription complex [106].

Core Promoters

The narrow region around the TSS has been considered to be important for understanding promoter functionality [107] and this region is termed the "core promoter". Core promoters are also considered to control basal transcription [105]. The core promoter region [105,107-109] can be defined as the minimal continuous segment of DNA sufficient for accurate initiation and directionality of transcription. However, the definition is ambiguous regarding the length of the region covered by the core promoter. In most cases, promoter elements considered important for the functionality of the core promoter region can be spread over the segment -60 to +50 bp relative to the TSS [110], and thus it makes sense to consider this segment as representing the core promoter. An often overlooked component of the core promoter is its active participation in the regulation of eukaryotic gene expression. The core promoter is actually the ultimate target of all transcription factors and co-regulators that control the transcriptional activity of every gene [109].

The common elements belonging to the basal transcription complex are:

1) A TATA box with consensus sequence TATA(A/T)A(A/T), the first eukaryotic core promoter motif to be identified [111,112].
2) Inr with the consensus sequence of $YYA_{+1}N(T/A)YY$ [113,114] or $TCA_{+1}(G/T)T(C/T)$ in Drosophila [115-117].
3) A subset of TATA boxes possesses an upstream sequence termed the BRE, which is a recognition site for the binding of TFIIB [118]. The consensus sequence for the BRE is (G/C)(G/C)(G/A)CGCC, where the 3'-C of the BRE is immediately followed by the 5'-T of the TATA box.
4) DPE or downstream promoter element is a recognition site for TFIID and is conserved from *Drosophila* to humans [109].

Most promoters contain a combination of one or two of these basic core promoter elements and all of these elements are known to interact with each other. Transcription initiation begins with the formation of a basal transcription complex in the promoter region several dozen bp long located around the TSS [105]. In turn, assembly of the basal transcription complex at TATA-containing promoters starts with the recognition of TATA boxes by the TATA-binding protein (TBP) [105]. On average, the sequence -300 to -50 bp of the TSS positively contributes to core promoter activity [105]. Interestingly, a large comprehensive study of promoter function showed the presence of putative negative elements around -1000 to -500 bp upstream of the TSS for 55% of genes tested [119].

IX. Cloning of the $P2Y_2$ Receptor and Mapping of the Gene

$P2Y_2$ receptors ($P2Y_2Rs$) were previously known as P_{2U} receptors, [73,120-122]. The human $P2Y_2R$ gene has been mapped to 11q13.5-14.1 on chromosome 11 [123]. G protein-coupled receptor genes for the β-adrenergic receptor, angiotensin receptor-like-1 and muscarinic cholinergic receptor-1 have also been localized to chromosome 11 [124].

CHAPTER 2 : CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF THE $P2Y_2$ RECEPTOR GENE PROMOTER

I. Introduction

Atherosclerosis is a multi-factorial disease involving endothelial dysfunction and is thought to be initiated by vascular injury. Extracellular nucleotides that are released from a variety of arterial and blood cells can bind to the $P2Y_2$ receptor ($P2Y_2R$) and modulate proliferation and migration of smooth muscle cells, which is known to be involved in intimal hyperplasia that accompanies atherosclerosis and post-angioplasty restenosis. A direct pathological role is reinforced by recent evidence showing that activation of the $P2Y_2R$ mediates intimal hyperplasia [68]. Moreover, the $P2Y_2R$ is upregulated in vascular smooth muscle cells and endothelial cells in response to tissue injury [42]. These findings also suggest that the $P2Y_2R$ is a potential target for the pharmacological control of the progression of atherosclerosis and post-angioplasty restenosis. However, despite the potential role of the $P2Y_2R$ in the atherogenic process, the mechanisms governing $P2Y_2R$ upregulation remain unknown.

In this study, we have initiated attempts to investigate the human $P2Y_2R$ gene promoter by cloning its 5'-flanking region into pGL3, a luciferase reporter vector, followed by transfection in human coronary artery endothelial cells and SH-SY5Y neuroblastoma cells. A serial deletion analysis of a 2071 bp 5'-flanking region in SH-SY5Y neuroblastoma cells showed that interspersed positive and negative response elements might be involved in the regulation of the $P2Y_2R$ gene. Moreover, the deletion of a 175 bp region including exon1 and a 41 bp immediate upstream region completely

abolished promoter function showing that the core promoter is located in this region. These results indicate that the $P2Y_2R$ gene promoter is a GC rich promoter with multiple transcription start sites and uses an array of positive and negative response elements in the regulation of gene expression. Furthermore, other experiments indicate that the cytokine IL-1β may be involved in downregulation of $P2Y_2R$ expression in human coronary artery endothelial cells. Further studies are needed to identify specific cis and trans acting factors involved in the regulation of $P2Y_2R$ gene expression. This study will potentially lead to the identification of novel pathways involved in the regulation of $P2Y_2R$ gene expression, information that might be useful to suppress neointimal hyperplasia in atherosclerosis and restenosis after angioplasty.

II. Materials and Methods:

Vectors

pGL3-basic vector and SV40 promoter pGL3 vector were obtained from Promega (Madison, WI, USA). CMV-pGL3, which has the CMV promoter cloned in the basic pGL3 vector, was a gift from David Pintel (Department of Microbiology and Molecular Immunology, University of Missouri-Columbia, USA).

RNA Isolation

PolyA$^+$ RNA was isolated from total RNA of human coronary artery endothelial cells (HCAECs). The Oligotex kit from Qiagen (Valencia, CA, USA) was used for PolyA$^+$ mRNA isolation and total RNA was isolated using the RNeasy Midi kit from Qiagen, according to the manufacturer's instructions. Total RNA obtained from 1 confluent T-75 flask of HCAECs was around 7-15 μg. The concentration and purity was determined by measuring absorption at 260 nm and 280 nm. A 260/280 ratio of 1.8 – 2.0 was considered to indicate relatively pure RNA. Five μg of total RNA or 0.1 μg of PolyA$^+$ RNA was used for the primer extension reaction.

Primer Extension Reaction

Single-stranded 43-mer anti-sense oligonucleotide (10 pmol) (5'-CTCTCGCCACTGCGCTGCGCTTCTCCTCTCAGGGTGCCGTCGC-3'),

(Tm=75.3ºC) corresponding to exon1 in the P2Y$_2$R gene sequence was designed and chemically synthesized, end-labeled using polynucleotide kinase from Promega (Madison, WI, USA) and [γ-^{32}P]-adenosine 5`-triphosphate from Perkin Elmer (Boston, MA, USA)**;** EasyTides® adenosine 5`-triphosphate, 250 μCi (9.25 MBq), specific activity: 3000 Ci (111 TBq)/mMole). Labeled primers (1 pmol) were used for

hybridization with 5 μg of polyA$^+$ RNA. The primer was annealed with the mRNA at 5 ºC below it's melting temperature (Tm) for 20 min. After annealing, the reaction was maintained at room temperature for 10 min. AMV reverse transcriptase was added for 30 min at 41 ºC to yield the corresponding cDNA. Sample Buffer from the Promega kit was added and AMV reverse transcriptase was inactivated by incubating all samples at 90 ºC for 10 min. The products were then analyzed on a 8% (w/v) polyacrylamide denaturing gel. Then, 1.2 kb kanamycin-positive mRNA from the Promega kit was used with a control primer as a positive control. The negative control included diethylpyrocarbonate-(DEPC-) treated water instead of mRNA in the reaction.

PCR Amplification of the Putative $P2Y_2R$ Gene Promoter Region from Genomic DNA

Using genomic DNA obtained from Promega as template, the 5'-flanking region of the $P2Y_2R$ gene was amplified. The primers designed were Mlu-2071 (5-CGGACGCGTGGCAGGAGGACTGCTTGGACTCAGC-3') and Xho-134 (5'-CCGCTCGAGCTCTCGCCACTGCGCTGCGC-3') and were PAGE purified by IDT (Coralville, IA, USA), which amplified a 2071 bp 5'-flanking region including the region encoding the first exon of $P2Y_2R$ as reported for the mRNA variants 1 and 2 with accession IDs of NM_176072 and NM_002564, respectively (NCBI). Primers Mlu-4620 and Xho-134 were used to amplify a 5380 bp fragment, the longest variant with respect to the 5'-end. The primers selected were between 24-30 nucleotides long with an annealing temperature of 68-72 ^oC. The following PCR parameters were used in preliminary studies to yield a product on a 0.8% (w/v) agarose gel stained with ethidium bromide: pre-

amplification denaturation at 94 ^{o}C for 1 min, followed by 35-40 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 66 ^{o}C for 1 min and elongation at 68 ^{o}C for 6 min.

Construction of the Initial Promoter Reporter Construct

The above generated fragments were digested using MluI and XhoI and ligated to the pGL3 vector digested with the same enzymes. The construct obtained was labeled as pNJ2071, transformed into JM109 competent cells and positive clones were identified by restriction enzyme digestion and confirmed by sequencing using RVprimer3 (5′-CTAGCAAAATAGGCTGTCCC-3′) and GLprimer2 (5′-CTTTATGTTTTTGGCGTCTTCCA-3′) (Promega, Madison, WI). The pGL3 vector has multiple cloning sites just upstream and downstream of the inserted promoter in the following order: KpnI, SacI, MluI, NheI, SmaI, XhoI, Bgl II, and Hind III.

Serial Deletion of the Promoter from the 5' End

To delineate the boundaries of the minimal functional promoter and regulatory elements within the 5'-flanking region of the $P2Y_2R$ gene, a series of 5' serial deletions of the initial promoter-reporter (luciferase) construct were made using the Erase-a-base system from Promega (Madison, WI, USA) based on the method of Henikoff [125] and were transiently expressed in HCAECs and the luciferase activities of the reporter construct were measured. The pNJ2071 construct contains a 2071 bp upstream region cloned at the MluI and XhoI sites. This construct was first digested using Mlu I and Kpn I. This digestion provided a linear construct of approximately 6960 bp that has a 5'-overhang at the Mlu I end of the construct and has a 3'-overhang at the Kpn I end. Exonuclease III was used to digest the 5'-overhang using the Erase-a-base kit from

Promega. A time-dependent reaction at 37 ^{o}C was used to obtain a linear construct that had deletions of various sizes as shown in Figure 4.

Transient Transfections

SH-SY5Y neuroblastoma cells were obtained from ATCC, and cultured using Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 10% FBS , L-glutamate and non-essential amino-acids. SH-SY5Y neuroblastoma cells were plated in 24 well plates for 24 h in antibiotic-free medium. SH-SY5Y cells were seeded in 24 well plates and transfected at 70-80% confluence. Transient transfections were performed using Lipofectamine 2000 with 1.92 pmoles of each of the 6 serially deleted constructs (Table 3) and pNJ-1937/-41. To compensate for the different amounts of DNA arising due to size differences of the various deletion constructs, equal concentrations of the plasmid DNA were used by adding non-interfering DNA (pBLUESCRIPT SK-) with each of the serial deletions. Renilla luciferase driven by thymidine kinase (pHRLTK) from Promega (Madison, WI, USA) was used as an internal control and co-transfected with each of the deletion constructs.

Human coronary artery endothelial cells were obtained from Cambrex and were maintained in EGM-2 medium. Transfection was done by electroporation using Nucleofector Reagent from Amaxa Biosystem (Gaithersburg, MD, USA). The cells were first dispersed by trypsinization, as described in the manual for HCAEC Nucleofector Reagent (VPB-1001) then collected and resuspended in Nucleofector Reagent. At least 5 μg of DNA were used for transfection of 5 X 10^5 cells.

Plasmids for transfection were prepared and purified using a Maxiprep kit purchased from Macherey-Nagel (Easton, PA, USA).

Table 3 : Concentrations of plasmid DNA used in transfection of SHSY-5Y neuroblastoma cells

Promoter Construct	Plasmid Size (bp)	pmol	Amount. of DNA (μg)	Filler (μg)	pHRGTK (μg) (0.192 pmol)
pNJ-1937/+134	6889	0.384	1.72	0	0.12
pNJ-1678/+134	6630	0.384	1.66	0.06	0.12
pNJ-1320/+134	6272	0.384	1.564	0.156	0.12
pNJ-1086/+134	6038	0.384	1.504	0.216	0.12
pNJ-692/+134	5644	0.384	1.406	0.314	0.12
pNJ-266/+134	5218	0.384	1.3	0.42	0.12
pNJ-235/+134	5187	0.384	1.292	0.428	0.12
pNJ-1937/-41	6714	0.384	1.67	0.05	0.12
pGL3basic	4818	0.384	1.2	0.52	0.12
SV40pGL3	5010	0.384	1.248	0.472	0.12
CMVpGL3	5611	0.384	1.397	0.329	0.12

Dual Luciferase Reporter Assay System

The various promoter reporter constructs obtained from deletions and mutations as described above were transfected into human SH-SY5Y neuroblastoma cells using Lipofectamine 2000. The concentrations of each maxiprep DNA (Nucleobond Plasmid Maxi Kit-Macherey Nagel) (Easton, PA, USA) were estimated using 1:10 dilution and calculated using the Nanodrop method (NanoDrop® ND-1000 Spectrophotometer) (Wilmington, DE, USA). After 24 h, cells were washed with phosphate buffered saline and lysed with passive lysis buffer as described in the dual luciferase reporter system manual (E1910) from Promega (Madison, WI, USA). Ten µl of the lysate supernatant were analysed for luciferase (LUC) reporter activity on a single tube luminometer (Turner Biosystems, Sunnyvale, CA, USA) using the dual luciferase reporter system. The transfection efficacy was normalized by a luciferase internal control vector pHRL-TK from Promega (Madison, WI, USA). A minimum of triplicate experiments were carried out. A ratio of 2:1 of experimental vector:internal control vector was used for each of these experiments. For the cytokine stimulation experiments, after 16-24 h, cells were treated with cytokines for another 24 h, washed with phosphate buffered saline and lysed with passive lysis buffer. The luciferase activity for each sample was calculated as a ratio of experimental divided by the renilla luciferase activity. These values were then used to calculated the Percentage relative response ratio (RRR) as follows. The positive control used was SV40-pGL3 and negative control used was basic-pGL3.

$$\text{Percentage RRR} = \frac{(\text{experimental sample ratio}) - (\text{negative control ratio})}{(\text{positive control ratio}) - (\text{negative control ratio})} \times 100$$

III. Results and Discussion:

1) Genomic Organization of the $P2Y_2R$ Gene

The three reference sequences (Refseqs) for *$P2Y_2R$* mRNA that are shown below were obtained from cDNA libraries from human renal cell carcinoma, airway epithelium and adult blood leukocytes as indicated in Figure 1. The diagrammatic representation below shows the exon-intron structure of *$P2Y_2R$* as derived by schematic computational analysis [126]. The similarity in the color indicates the similarity in the sequence between the three mRNA transcripts. The *$P2Y_2R$* is proposed to encode at least three mRNA variants each of which have three exonic and two intronic regions. The coding sequence is intronless and the three variants differ in the 5'-untranslated region (UTR).

Figure 1: $P2Y_2R$ gene organization

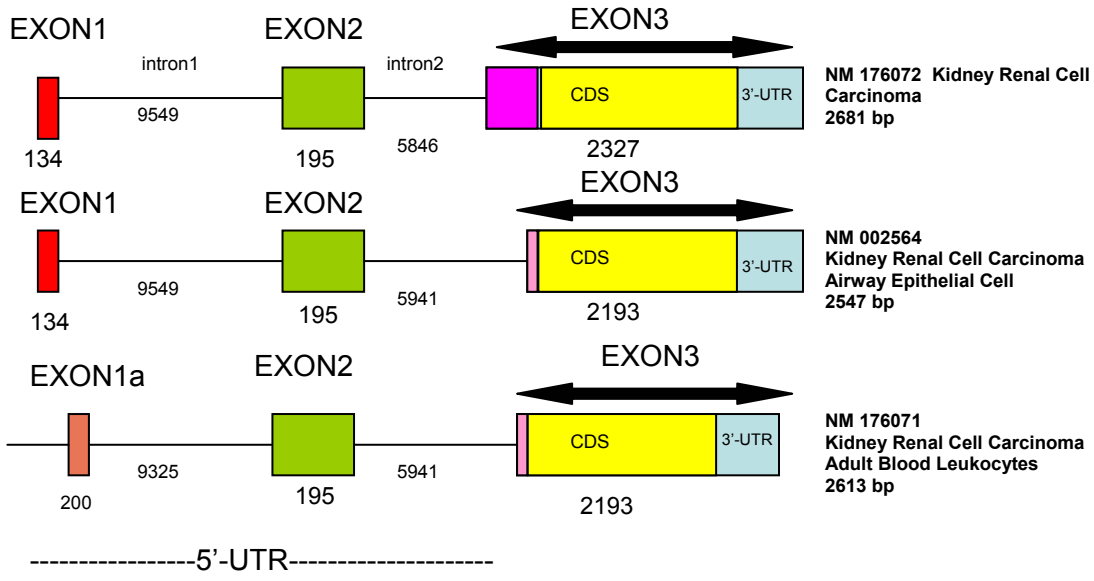

Figure 1: $P2Y_2R$ gene structure and organization

$P2Y_2R$ transcripts from the NCBI (National Center for Biotechnology Information) Database – Refseqs. Color scheme is modified such that the regions with similar sequences have the same color.

2a) Cloning of the $P2Y_2R$ Gene 5'-upstream Sequence

The 2.071 kb upstream of the ATG start codon was cloned into the pGL3 Basic luciferase reporter plasmid to generate the plasmid pNJ-2071/+134 and the sequence was analyzed by the Proscan computer program [127]. A consensus TATA box was not found in the 2.071 kb region analyzed. Furthermore, in the same sequence, a CpG island was predicted when CpG island-finding software (CpG Finder, Softberry) (Softberry Inc., Mount Kisco, NY, USA) was used. The CpG island was 274 bp long in a region spanning exon 1 of these transcripts with a P(CpG)/exp score of 0.895 (threshold is 0.6). This score represents the Min gc_ratio = P(CpG)/(expected)P(CpG) and is defined as the minimal ratio of the observed to expected frequency of CpG dinucleotide in the island The percentage of CG content was found to be 75.9%. This information is consistent with vertebrate genomes that contain CpG islands in regions about 1-2 kilobases in length where the dinucleotide CpG is present at the expected frequency and in an unmethylated form [128]. The location of these CpG islands is almost always coincident with the 5'-end of genes, often overlapping the first exon. [129] . It is estimated that 56% of all human genes are associated with CpG islands [128]. The unmethylated status in some cases is dependent on binding of the transcription factor Sp1 [130]. We also found putative binding sites for Sp1 within the same region (Figure 2). The region being analyzed is thus a likely candidate for analysis of the $P2Y_2R$ gene promoter.

Reference website for the software used:

http://softberry.com/berry.phtml?topic=cpgfinder&group=programs&subgroup=promoter

Results presented as raw output below:

Search parameters:

Length of the CpG island: >= 100

% GC: 50.0

P(CpG)/exp: 0.600

Output is as follows:

Found: 1 CpG Island

Start: 1

end: 32

chain: +

CpG: 13

% CG: 75.0

CG/GC: 0.812

P(CpG)/exp: 0.973(0.98)

P(CpG): 0.131

Length of the CpG island : 274

2b) Promoters Predicted by the Promoter Predicting Programs (PPPs) Identified the Region for Experimental Verification

Promoter Predicting Programs (PPPs) are important *in silico* tools for guiding experimental biologists. Once the approximate putative regions for promoters have been detected using PPPs, reporter gene assays based on a series of deletion mutants can be used to further narrow down the DNA regions that play the most important role in the promoter activities [131]. The information on transcription factor binding sites predicted by the commonly available and non-commercial softwares (TESS, University of

Pennsylvania; Matinspector 2.0, Genomatix Software GmbH, Germany) was assimilated and the most relevant sites are presented in Figure 2.

Figure 2: Putative binding sites in the immediate 5'-flanking region of the $P2Y_2R$ gene

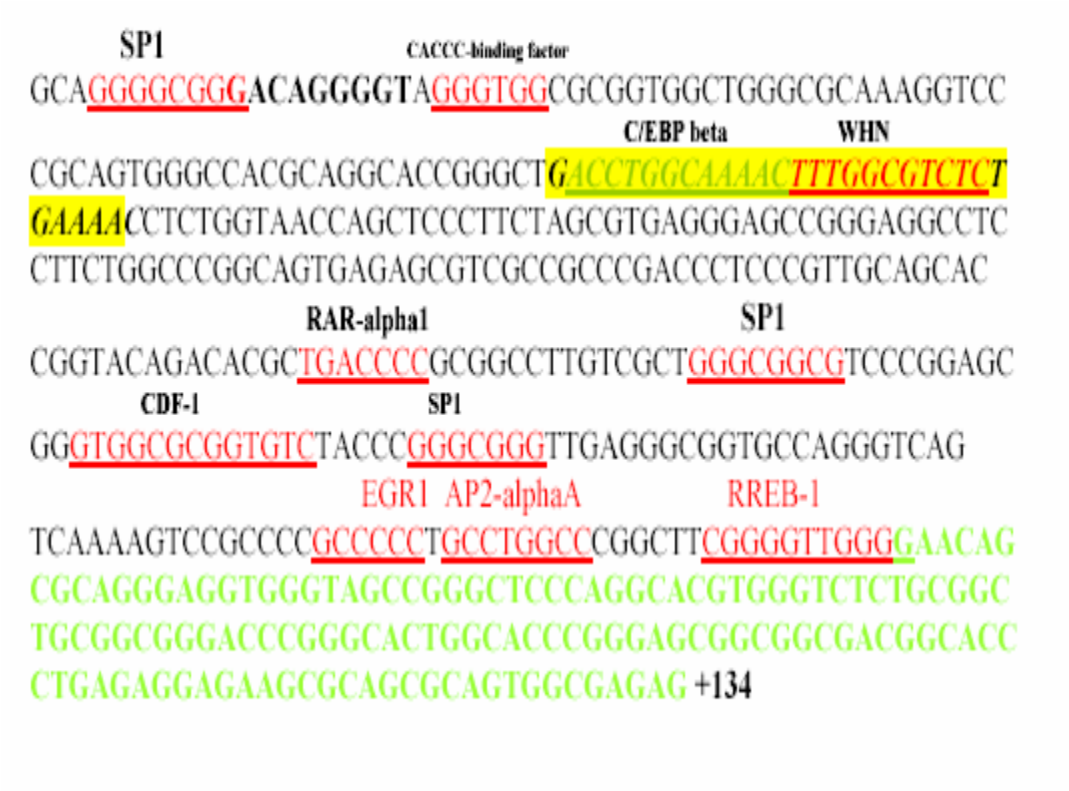

Figure 2: Putative binding sites in the immediate 5'-flanking region of the $P2Y_2R$ gene: Putative Transcription factor binding sites are indicated by underlined sequences. The green region represents 134 bp of exon 1 and the region in yellow represents a 31 bp sequence deleted in pNJ-235/+134.

3) <u>Determination of Transcription Start Site(s) by Primer Extension Analysis</u>

We used primer extension analysis to identify the probable transcription initiation site(s) in the $P2Y_2R$ gene with mRNA extracted from HCAECs. The single-stranded 43-mer oligonucleotide used was highly specific for $P2Y_2R$ mRNA exon 1. The expected cDNA product size was 134 bp if the transcription start site for $P2Y_2R$ mRNA conformed to mRNA transcripts arrived at for $P2Y_2R$ mRNA by *in silico* analysis [126], as shown in Figure 1. The positive control was a 1.2 kb kanamycin-positive *in vitro* product that was expected to give a product size of 84 bp. The chief cDNA products obtained by primer extension were smaller (57 bp and 84 bp) and larger (200 bp) than predicted (Figure 3). More than one transcription start site has been known to be associated with TATAless promoters [132]. However, formation of products due to secondary structures in mRNA in the primer extension reaction cannot be ruled out. Further experiments such as 5'-RACE (Rapid Amplification of cDNA ends) analysis and RNAse protection assays need to be done to confirm the transcription start sites.

Figure 3: Determination of transcription start site(s) by primer extension analysis.

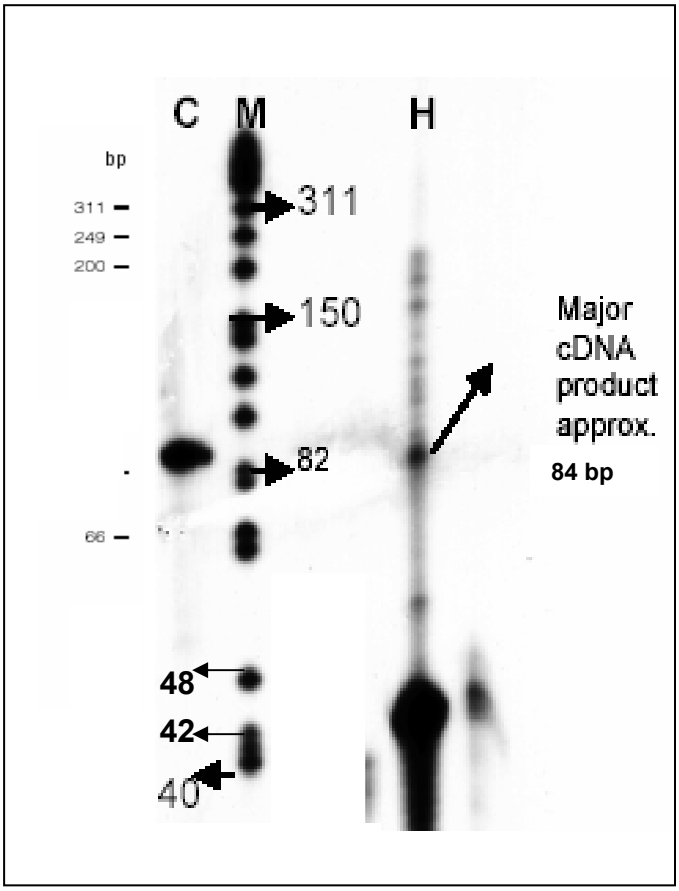

Figure 3. Determination of transcription start site(s) by primer extension analysis. Primer extension analysis revealed one major transcription start site in extracts of mRNA from human coronary artery endothelial cells (H) and some weak intensity products of sizes from 100-230 bp. A positive control (C) from the Promega kit was included that produced a band at 84 bp. Hinf I digested markers (M) were used as standards to compare the sizes of the products**.**

4) <u>Deletion Analysis of the Promoter from the 5'-end</u>

In initial studies, we tested the pNJ-1937/+134 construct for promoter activity in cell lines previously reported to show endogenous $P2Y_2R$ expression, such as HCAECs and human salivary gland (HSG) cells. To define the mechanisms controlling transcriptional regulation of *$P2Y_2R$* in HCAECs, a series of deletion constructs (Figure 4) were generated and transiently transfected in SH-SY5Y neuroblastoma cells.

Deletion mutant pNJ-1678/+134 exhibited a drastic decrease in reporter activity as shown by a decrease in percentage of relative response ratio (RRR) (percentage of RRR is calculated as relative increases for pGL3 expressed as a percentage of SV40 promoter-driven luciferase activity as described in 'methods' section) by 119% (Figure 4). Deletion mutant pNJ-1320/+134 exhibited slightly greater reporter activity (Figure 4). Deletion mutant pNJ-1086/+134 showed luciferase activity similar to the pNJ-1678/+134 mutant (Figure 4). The pNJ-692/+134 mutant exhibited some reporter activity, similar to the pNJ-1320/+134 mutant (Figure 5). Thus, the 5'-flanking region showed interspersed positive and negative regulatory elements. Furthermore, the pNJ-266/+134 mutant showed similar reporter activity to pNJ-1937/+134, which was further dramatically increased for deletion mutant pNJ-235/+134 (Figure 4). Analysis of this 31 bp sequence showed putative binding sites for C/EBPβ (CCAAT enhancer binding protein), WHN (winged helix nude), E2F, and MyT1 (myelin transcription factor 1) zinc finger transcription factors known to act as repressors of transcription activity of other genes [133-135]. Moreover, the analysis of luciferase reporter activity for the deletion mutant pNJ-1937/-41 revealed a complete loss of activity, especially when compared to the full-

length construct pNJ-1937/+134 and pNJ-235/+134 (Figure 4). Analysis of this region revealed multiple putative sites for Sp1 and a putative site for EGR1.

We analyzed the similarity of the putative C/EBPβ binding sequence to the consensus binding sequence of C/EBP [136], and according to TESS this sequence was graded as a good match. C/EBPβ belongs to a family of transcription factors composed of at least five distinct members known to be associated with the regulation of cell growth and differentiation [136]. It is thought that C/EBPβ has a possible role in the brain, since it is known that C/EBPβ mRNA is widely expressed in adult mouse brain [136]. Constitutive expression of C/EBPβ is particularly high in liver, intestine, lung, adipose tissue, spleen, kidney and myelomonocytic cells [136]. C/EBPβ protein is widely distributed in the brain of post-natal rats when the dendritic arbor of neurons develops [137]. C/EBPβ overexpression significantly increases programmed cell death, probably through a mechanism involving the cell cycle-dependent proteins p53 and p21 [136]. C/EBPβ might play an important role in the regulation of neuronal differentiation and cell death [138]. We postulate that C/EBPβ is one of the factors involved in the repression of $P2Y_2R$ gene expression, as shown by the increase in luciferase activity with deletion mutant pNJ-235/+134 (Figure 4). The role of the $P2Y_2R$ in the nervous system is less well understood, but it has been shown that $P2Y_2Rs$ can couple to neuronal ion channels [139], can activate nerve growth factor/TrkA signaling [140], and can modulate pain responses [141]. It has been suggested that $P2Y_2Rs$ and their associated signaling pathways may be important factors regulating astrogliosis in brain disorders [142].

Combining the data from the primer extension analysis and the deletion analysis, it can be concluded that the region between -41 to +134 plays an important role in the

initiation of $P2Y_2R$ transcription. This region might represent the core promoter of the $P2Y_2R$ gene and may be responsible for conferring basal transcriptional activity. This can be concluded because pNJ-1937/-41 has all the regulatory elements that are present in other constructs except the region that has been deleted from -41 to +134. Furthermore, the $P2Y_2R$ flanking region shows interesting putative cis-regulatory elements that should be mutated further to determine their involvement in the transcriptional regulation of $P2Y_2R$ expression. More conclusive information on transcription factors involved in $P2Y_2R$ gene expression can be obtained by site-directed mutagenesis of specific transcription factor binding sites, as shown in Table 4. Furthermore, gel mobility shift assays and chromatin immunoprecipitation assays can be carried out with wild type and mutated constructs to confirm the involvement of these putative transcription factor binding sites.

Figure 4: Luciferase activity of the promoter deletion constructs

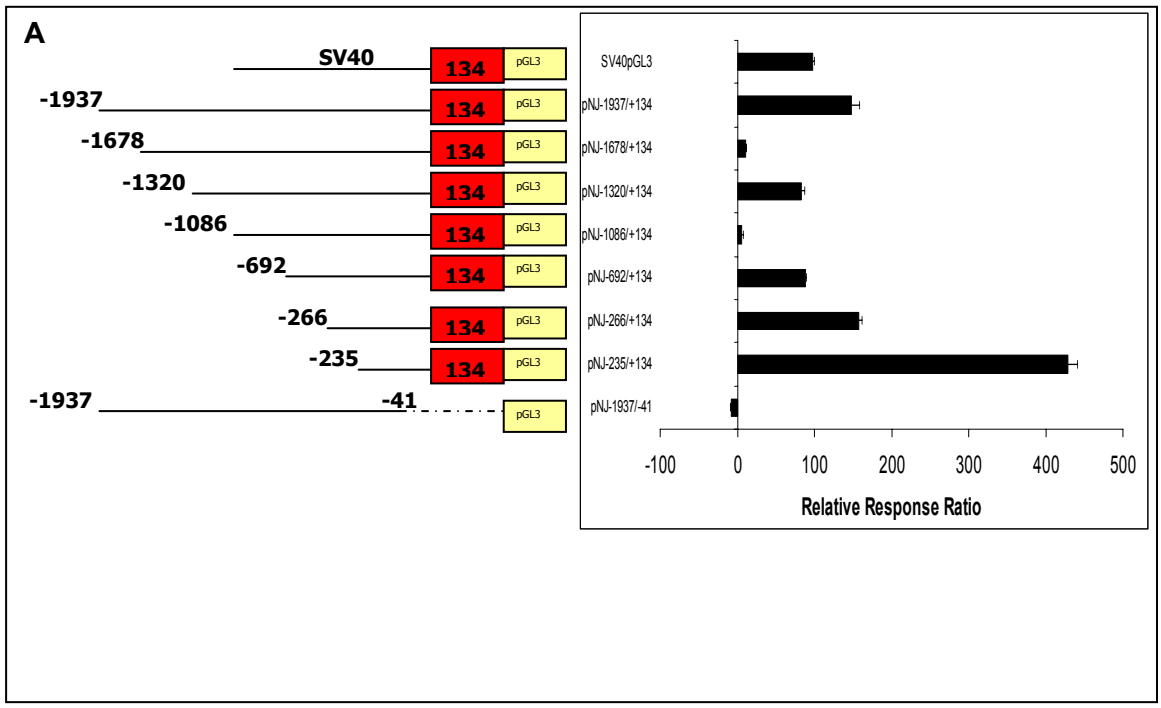

Figure 4. Luciferase activity of the promoter deletion constructs: A- Serial deletions of the pNJ2071 (pNJ-1937/+134) construct. B- Luciferase activities of the deletion plasmids transfected into SH-SY5Y cells. Values corrected to the promoterless plasmid reporter activity are expressed as a percentage of control SV40 promoter-driven reporter activity and represent the mean ±S.E. of results from at least three experiments.

5) Effect of IL-1β on the Luciferase Reporter Activity Driven by the 2071 Bp 5'-flanking Region

In rat smooth muscle cells, it is known that interleukin (IL)-1ß induces a time- and dose-dependent upregulation of $P2Y_2$ receptor mRNA, which was dramatically enhanced when combined with interferon-γ or tumor necrosis factor-α [104]. We wanted to see if IL-1-1β can induce upregulation of the promoter driven reporter activity in human coronary artery endothelial cells. To analyze this effect, human coronary artery endothelial cells were transfected with the full-length construct pNJ2071 followed by stimulation with IL-1β at 50 ng/ml for 24 h, and luciferase activities were determined as shown in Figure 5. This indicates that at this particular IL-1β concentration and at this particular time interval, IL-1β decreased promoter activity by 20%. It is possible that certain repressors in the 5'-flanking region of $P2Y_2R$ could be involved in the IL-1β-induced decrease in promoter activity. Similar experiments in SH-SY5Y cells also indicated either no response or a slight decrease in luciferase activity induced by IL-1β. Future experiments should include transfection of human coronary artery endothelial cells by several serial deletion mutants to test the effects of IL-1β and the effect of a concentration dose response of IL-1β. These experiments may indicate the elements involved in IL-1β induced downregulation of $P2Y_2R$ promoter activity. It is possible that in human coronary artery endothelial cells IL-1β has an opposite effect, but this needs to be further confirmed using other quantitative approaches at the endogenous mRNA level. IL-1β is known to be involved in the repression of the transcription of cartilage-derived retinoic acid-sensitive protein (CD-RAP) via C/EBPβ [133] in rat chondrocytes. Interestingly, the

deletion analysis has indicated that there might be a functional C/EBPβ binding element in the *P2Y$_2$R* putative promoter region (Figures 2 and 4).

Figure 5: Effect of IL-1β on luciferase reporter activity for the 2071 bp 5'-flanking region of *P2Y$_2$R* expressed in human coronary artery endothelial cells.

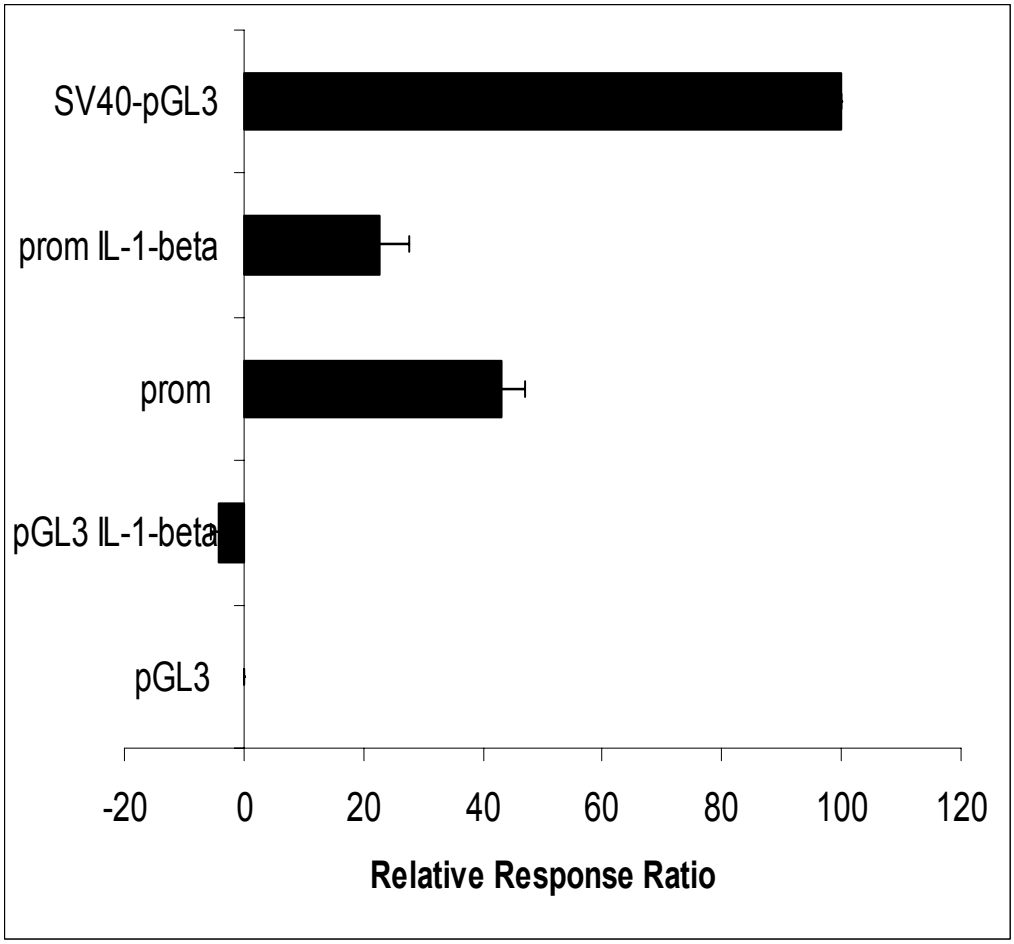

Figure 5. Effect of IL-1β on luciferase reporter activity for the 2071 bp 5'-flanking region of $P2Y_2R$ expressed in human coronary artery endothelial cells. Stimulation of HCAEC transfectants with 50 ng/ml IL-1β for 24 h showed that the 5'-flanking region of $P2Y_2R$ (prom) exhibits a slight negative regulation in response to IL-1β.

IV. Summary

Several cis-regulatory elements are present in the 5'-flanking region of $P2Y_2R$ that was analyzed. Deletion analysis indicates that certain elements and/or interaction between elements present between 235 bp upstream and 2071 bp upstream of the putative transcription initiation site might be important for repression of $P2Y_2R$ gene expression in SH-SY5Y cells. In particular, the sequence between deletion 5 and deletion 6 (pNJ-235/+134) showed the presence of putative binding sites for C/EBP and WHN, which have been shown to be involved in repression of other genes [133,143]. The data from the primer extension analysis indicates the presence of multiple transcription initiation sites in the immediate (within approximately 60 - 70 bp) $P2Y_2R$ 5'-flanking region as well as some strong initiation sites within exon1. Moreover, we found that the ability of this putative promoter region to drive luciferase reporter gene expression was completely lost when the region between -46 and +134 bp was deleted. These results indicate the presence of transcription initiation sites in the region between -46 to +134 bp. Furthermore, stimulation experiments indicated that IL-1β might be involved in downregulation of $P2Y_2R$ activity in human coronary artery endothelial cells. Taken together, these findings suggest the presence of a GC rich promoter for $P2Y_2R$ that utilizes an array of interspersed positive and negative regulatory elements that might be involved in sequence-specific and signal-specific stimulation of $P2Y_2R$ gene expression.

Table 4 : Putative transcription factor binding sites in the 5'-flanking region of $P2Y_2R$ gene
(as present in the respective construct) [144,145]

pNJ -235/+134

Family/matrix	Further Information	Opt.	Position	Str.	Core sim.	Matrix sim.	Sequence (red: ci-value > 60 capitals: core sequence)
V$MAZF/MAZR.01	MYC-associated zinc finger protein related transcription factor	0.88	-26/-38	(-)	1.000	0.916	ggggggcGGGGcgg
V$RARF/RAR.02	Retinoic acid receptor, member of nuclear receptors, DR5 site	0.70	-28/-56	(+)	0.960	0.702	ccagGGTCagtcaaaagtccgcccc gccc
V$SP1F/SP1.01	Stimulating protein 1, ubiquitous zinc finger transcription factor	0.88	-29/-43	(-)	1.000	0.913	ggcgGGGCggacttt
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation	0.95	-101/-111	(-)	1.000	0.950	gggACGCcgcc
V$SP1F/SP1.02	Stimulating protein 1, ubiquitous zinc finger transcription factor	0.85	-102/-116	(+)	1.000	0.914	cgctGGGCggcgtcc
V$MYBL/VMYB.02	v-Myb	0.90	-152/-164	(-)	1.000	0.958	tgcAACGggaggg
V$SP1F/SP1.02	Stimulating protein 1, ubiquitous zinc finger transcription factor	0.85	-163/-177	(-)	1.000	0.863	ggtcGGGCggcgacg
V$WHZF/WHN.01	Winged helix protein, involved in hair	0.95	-172/-182	(-)	1.000	0.985	gcgACGCtctc

	keratinization and thymus epithelium differentiation						

pNJ -235/+134 to pNJ-266/+134

V$E2FF/E2F.02	E2F, involved in cell cycle regulation, interacts with Rb p107 protein	0.84	-251/-261	(-)	1.000	0.886	agagacgcCAAAgtttt
V$WHZF/WHN.01	Winged helix protein, involved in hair keratinization and thymus epithelium differentiation	0.95	-252/-262	(-)	1.000	0.953	gagACGCcaaa
V$MYT1/MYT1.02	MyT1 zinc finger transcription factor involved in primary neurogenesis	0.88	-258/-270	(-)	1.000	0.993	ccaAAGTtttgcc

APPENDIX

Future Work and Directions

Preliminary deletion analysis studies with HCAEC transfectants indicated very different results than obtained with SH-SY5Y cell transfectants. These results need to be confirmed by repeated analysis. These results suggest that negative regulation is the hallmark for $P2Y_2R$ expression using transient transfection approaches. This is consistent with Dr. Seye's earlier work showing that the $P2Y_2R$ is normally expressed at low levels in rabbit carotid arteries [68].

Futhermore, to precisely define the regions of the promoter necessary for $P2Y_2R$ basal expression in HCAECs, serial site-directed mutagenesis of the core promoter should be performed. Mutant promoter constructs can be created in pGL3 basic vector, each containing 10 consecutive mutated bases, and expressed in HCAECs and other cell types for assay of luciferase activity.

The results obtained by the luciferase assays for the deletion constructs transfected in SH-SY5Y cells should be confirmed using real time PCR or competitive RT-PCR assays.

We have already generated some constructs to begin ribonuclease protection assays for the confirmation of the transcription start site(s) of $P2Y_2R$ that were indicated by the primer extension analysis.

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