This is to certify that the

dissertation entitled

A1 Adenosine Receptor Gene: Characterization and Analysis of its Transcription Start Site Heterogeneity

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Physiology

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Date July 22, 1993

A1 ADENOSINE RECEPTOR GENE : CHARACTERIZATION AND ANALYSIS OF ITS TRANSCRIPTION START SITE HETEROGENEITY

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

1993

ABSTRACT

A1 ADENOSINE RECEPTOR GENE: CHARACTERIZATION AND ANALYSIS OF ITS TRANSCRIPTION START SITE HETEROGENEITY

By

Samita Bhattacharya

The primary objective of the research presented in this thesis was to characterize the A1 adenosine receptor gene thereby expanding our understanding of the adenosine receptor-signaling system. Adenosine receptors (AR) are broadly divided into two classes, A1 and A2, based on their differential binding selectivity for a series of adenosine analogs and on their ability to inhibit (A1AR) or stimulate (A2AR) the adenylyl cyclase system. While the A2AR is only known to be coupled to the adenylyl cyclase system, the A1AR has been reported to interact with multiple effector systems. Studies using the A1AR cDNA and the gene will help in understanding the mechanism by which this complicated system is activated, and its ability to interact with multiple effector systems. Recently both A1 and A2 receptors have been cloned and expressed from dog thyroid, rat brain, bovine brain and human brain cDNA libraries. Using a radiolabelled oligonucleotide probe designed from the dog thyroid A1AR clone (RDC7), a partial (2kb) cDNA clone was isolated from a rabbit kidney cDNA library. This clone, designated SB4, lacks the 5'-untranslated region and nucleotides corresponding to those coding for the first 74 amino acids of RDC7. SB4 has 91% identity at the nucleotide level with RDC7 and codes for a protein with 94% identity to the canine A1AR. To obtain the remaining 5'-end of the cDNA, a rabbit genomic library was screened using an 860 base probe, an

*Eco*RI/*Sma*I fragment derived from the 5'-end of SB4 and an A1AR fragment generated by PCR from rat brain mRNA (gift of Dr. M. Lohse). One positive clone was isolated containing a 3.2 kb *Xho*I fragment, which included 2 kb of 5'-flanking region, an exon of 341 nucleotides coding for the first 113 amino acids of the A1AR, followed by an intron. The exon sequence of this clone was 100% identical to SB4. Together the cDNA and the genomic clones provided the entire open reading frame for the rabbit A1AR (RbA1). The cDNA for the rabbit A1AR was further used to isolate and characterize rabbit genomic DNA clones containing the entire coding sequence for the A1AR. Results of restriction analysis and sequencing of genomic clones were consistent with the existence of a single A1AR receptor gene. The complete receptor coding sequence is contained in 2 exons separated by an intron of greater than or equal to 34 kb. Primer extension analysis indicated that transcription of the gene begins at multiple initiation sites, -78, -106, -268 and -322 nucleotides 5' to the ATG translation initiation codon. Neither TATA nor CAAT boxes were found to be present in the 1000 nucleotides that has been sequenced immediately upstream of the translational start site. Examination of the 5'-end sequences of the gene revealed sequences with homology to several transcriptional regulatory sequences. Knowledge of the structure of the receptor gene should facilitate future studies on the structural determinants of the receptor function, the regulation of the receptor expression in various physiological and pathophysiological conditions and lastly the molecular basis of multiple A1AR functions.

To my parents

and

to the memory of my sister Santa.

ACKNOWLEDGMENTS

During the years of my graduate life I have received enormous support from many a people, which resulted in the completion of this work. First and foremost among these is my mentor Dr. William S. Spielman. His guidance allowed me to think independently, develop self-confidence and to acquire scientific insight.

I would like to express my deep appreciation to Dr. David L. Dewitt for introducing me to the field of molecular biology with unsurpassed endurance and care, and for assisting me in developing a critical eye for science. I hope I can some day be as critical a thinker and as compassionate a person as him.

I would like to acknowledge my other committee members, Dr. Seth R. Hootman, Dr. Donald B. Jump and Dr. William L. Smith for their suggestions leading to the improvement of my thesis. I would specially like to thank Dr. William L. Smith for giving me the opportunity to use his laboratory facilities.

Thanks to my friends and colleagues, Dr. Lois J. Arend and Dr. David L. Levier for all their support. My special thanks for Dr. Maria Burnatowska-Hledin for all her intellectual and technical support. Thanks to Linda Shi, Stacey Kramer, Odette Laneuville, Marc Lecomte, Beth Meade, Jim Otto, and Marty Regier for all their help and more so for making me feel a member of their lab. Special thanks for Stacey Kramer for her stimulating discussions and her help with computer programs. Thanks to John Freeman

for all the encouragement. John will always be in my thoughts.

My deep appreciation to Susan Harkema, a good friend in need, for being a pillar of strength and support which enabled me to ride over the hard times of graduate life.

I would like to thank the members in the Department of Physiology, specially Sharon Shaft for her excellent secretarial assistance.

In the end I would like to convey my deepest gratitude to my family, especially my parents, my sisters Tanushree, Jayashree and Santa, my uncle Ganesh Maitra and my aunt Savitri Goswami. Their constant love and encouragement were the foundation of my work.

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I. INTRODUCTION

Adenosine is an ubiquitous purine nucleoside and a byproduct of both extracellular and intracellular metabolism (Collison et al. 1987; Olsson et al. 1990). Due to its short half life (Conway and Cooke 1939), it is likely that adenosine acts in a paracrine or autocrine manner and produces a wide array of physiological effects, including hemodynamic changes, neurotransmitter release, platelet aggregation, lipolysis and renin release and solute transport by the kidneys (McCoy et al. 1993; Lang et al. 1985; Ramkumar et al. 1988; Linden 1991). The actions of adenosine are mediated through G-protein coupled plasma membrane receptors.

Adenosine receptors (AR) are broadly classified as A1 or A2AR, based on their affinities for a variety of adenosine analogs (Daly et al. 1987) and on their ability to inhibit (A1AR) or stimulate (A2AR) the adenylyl cyclase system (Londos et al. 1980). The second messenger systems coupled to A1AR, however, are remarkably diverse, and include adenylyl cyclase, phospholipase C and various ion channels (Linden et al. 1991). This diversity of responses generated by A1AR activation has been interpreted to indicate that subtypes of A1AR may exist, either as products of differential splicing of a multiexon gene or as products of entirely different adenosine receptor genes. A possibility of a single receptor capable of interacting with multiple effector systems, also exists.

Characterization of the A1AR gene or genes is a necessary first step in distinguishing between these possibilities.

The research presented in this dissertation describes the molecular cloning of the cDNA and the characterization of the gene encoding the A1AR from rabbit. The first chapter, the literature review is composed of three sections, which discuss the metabolism of adenosine, the adenosine receptor system and lastly the physiological functions involved with adenosine receptor activation. The projects comprising the thesis are represented as two separate chapters, each comprising of an introduction, methods, results and discussions relevant to each project. The final chapter summarizes the significance of the research.

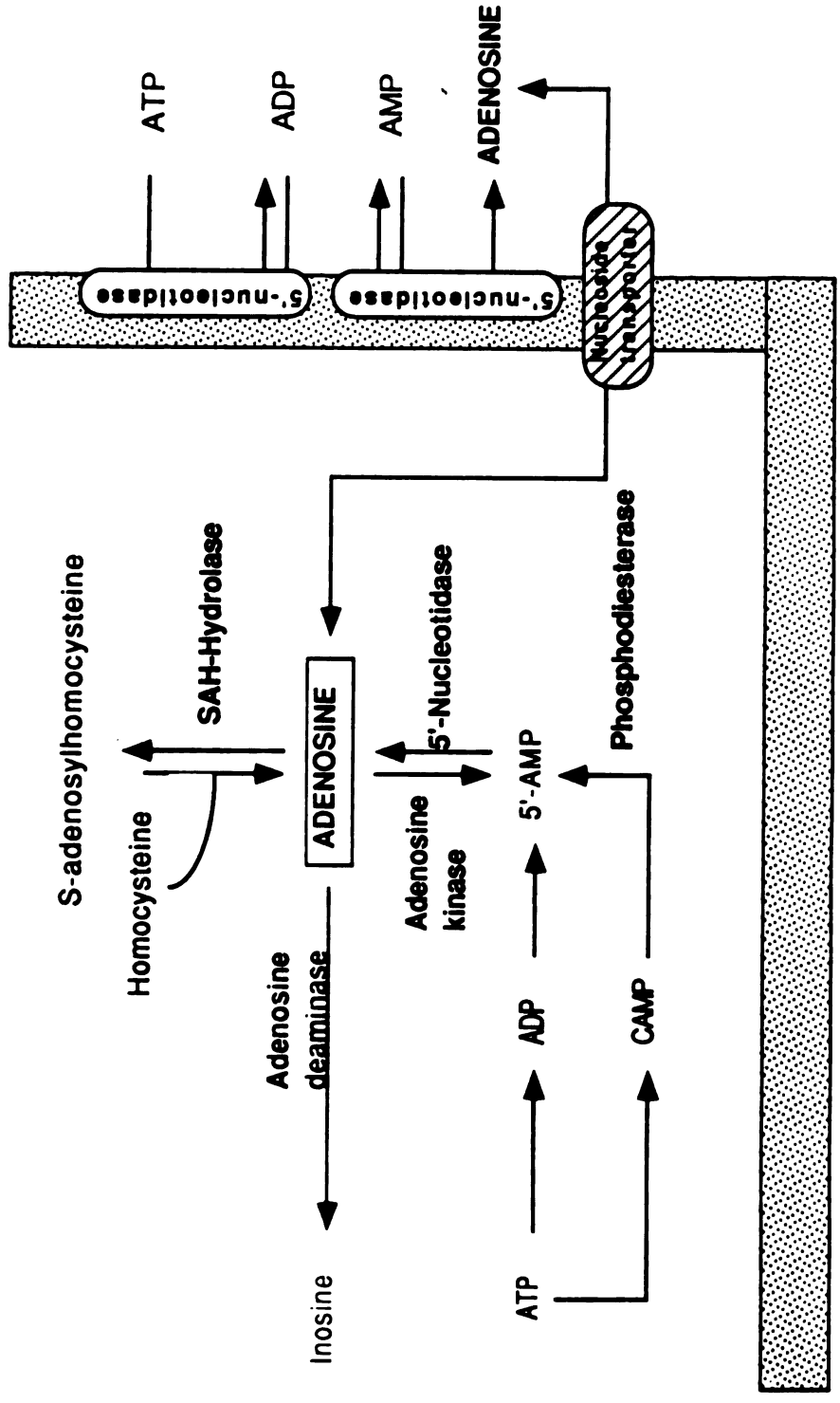
II. LITERATURE REVIEW

A . Adenosine Metabolism Adenosine, the ubiquitous purine nucleoside, is produced intracellularly as well as extracellularly by two distinct metabolic pathways, involving hydrolases. The first pathway involves the enzymatic hydrolysis by 5'-nucleotidase of adenine nucleotides, (Baer et al. 1966 and Pearson et al. 1980) and the second pathway involves the hydrolysis of S-adenosyl homocysteine (SAH) by SAH hydrolase (Schrader 1981). Since the SAH hydroxylase is inhibited by its products, adenosine and inosine, it is postulated to be a minor source of adenosine (Eloranta 1977; Lloyd and Schrader 1987; Schatz et al. 1977). The major source of adenosine is therefore believed to arise from the metabolism of adenine nucleotides (Figure 1).

Adenosine consists of an adenine purine ring and a ribose moiety and is a precursor to, and a metabolite of, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP). Adenosine is also a component of S-adenosyl homocysteine.

Adenosine produced extracellularly or intracellularly, is also degraded by two mechanisms: 1) deamination by adenosine deaminase to inosine or 2) phosphorylation by adenosine kinase to AMP (Plagemann and Wohlhueter 1980). Adenosine deaminase is present both intracellularly as well as in the interstitium (Conway and Cooke 1939; Trams and Lauter 1974), whereas adenosine kinase is an intracellular enzyme. The concentrations of intracellular and extracellular adenosine are maintained by a balance between its rate of production and degradation.

Figure 1 . Adenosine Metabolism : Flow diagram of adenosine sources and sinks.


ATP
ADP
AMP
ADENOSINE
5'-nucleotidase
5'-nucleotidase
Nucleoside transporter
S-adenosylhomocysteine
SAH-Hydrolase
Homocysteine
ADENOSINE
5'-Nucleotidase
Adenosine kinase
5'-AMP
Phosphodiesterase
Adenosine deaminase
ADP
CAMP
Inosine
ATP

The movement of adenosine into and out of the cells is governed by the mechanism of facilitated diffusion, a concentration dependent process. Dipyridamole and related compounds act as inhibitors of this process via the nucleoside transporter. This transporter has been well characterized in red blood cells (Kolassa and Pfleger 1975; Kubler and Bretschneider 1963), aortic endothelial and smooth muscle cells (Pearson et al. 1978), platelets (Sixma et al.1976), and in hepatoma cells (Plagemann 1971; Plagemann and Wohlhueter 1980; Plagemann and Richey 1974). The nucleoside transporter is closely associated with the membrane bound 5'-nucleotidase, thereby suggesting that the production and translocation of adenosine is a single step process (Fox and Kelly 1978).

B . Adenosine receptors

Adenosine consists of a purine ring and a ribose moiety, and is a precursor to, and a metabolite of adenine nucleotides. Under conditions of hypoxia, resulting in a state of decreased cellular energy, the intracellular production of adenosine increases and adenosine is released from the cell, by facilitated diffusion (Olsson and Pearson 1990). Adenosine thus released acts, as described by Newby as "a retaliatory metabolite" (Newby 1987), binding to its plasma membrane receptors and triggering intracellular signaling.

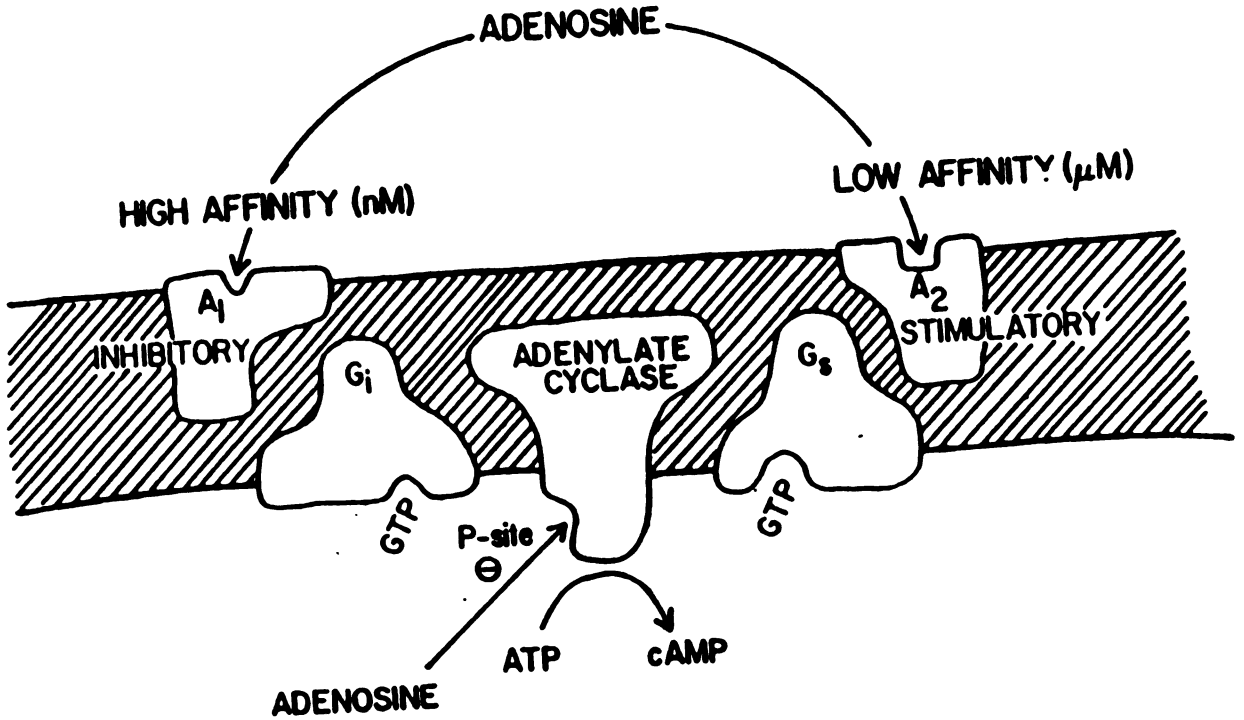
B . 1 . Receptor subtypes

Adenosine receptors are members of the purine receptor family, as originally classified by Burnstock (1978), as P1 or P2 purinergic receptors, depending on their

preference for adenosine or adenine nucleotides, respectively. The xanthine sensitive adenosine receptors, a subclass of P1 purinergic receptors, are further divided into two subtypes, A1 and A2 adenosine receptors. The major criterias used to distinguish between the two adenosine receptors are the binding affinity of the receptors for adenosine and its analogs and their ability to interact with the adenylyl cyclase system. A1AR is a high affinity receptor activated by nanomolar concentrations of agonist which on activation produces inhibition of adenylyl cyclase systems. In comparison A2AR is a low affinity receptor activated by micromolar agonist concentrations and on activation produces increased activity of the adenylyl cyclase. The most commonly used adenosine analogs are, N^6-cyclohexyl adenosine (CHA), R(-)N^6- (2-phenylisopropyl) adenosine (R-PIA), and 5'-N-ethylcarboxamido adenosine (NECA). Agonist binding at the A1AR have a rank order of potency in CHA>R-PIA>NECA, while the potency series for A2AR mediated responses is NECA>R-PIA>CHA. Analogs selective for A1AR typically are substituted at the N^6 positions, whereas analogs substituted at 5' or 2-positions are selective for the A2AR (Daly and Padgett 1992).

Other than A1 and A2AR, there is another intracellular adenosine receptor located on the catalytic subunit of adenylyl cyclase. This site termed the P-site, is responsible for inhibition of adenylyl cyclase. The P-site is activated by millimolor concentrations of adenosine and is not antagonized by methylxanthine, a nonselective antagonist for adenosine receptors. The physiological relevance of the P-site is still in question, since adenosine kinase and adenosine deaminase normally maintain an intracellular concentration of adenosine below the micromolar range. A model for adenosine receptors and their interaction with adenylyl cyclase is presented in Figure 2.

Figure 2 . Model for adenosine receptors and their interaction with adenyl cyclase system. View text for details.



ADENOSINE RECEPTORS

Pharmacological studies suggest that subtypes of A1AR may exist. The order of potency for A1AR in rat adipose tissue is NECA >>> R-PIA, whereas binding characteristics in rat heart and brain indicate similar affinities for NECA and R-PIA (Burns et al. 1987 ; Linden et al. 1985). In various nerves it has also been observed that the potency of NECA to inhibit neurotransmitter release equals R-PIA (Fredholm et al. 1988). The order of A1AR antagonist potency also supports this hypothesis. Gustafsson et al. (1990) have shown that in brain the A1AR specific antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) is much greater in potency in comparison to the xanthine amino cogener (XAC), which has only moderate A1AR selectivity, whereas at the neuromuscular junction XAC is as potent as DPCPX. The functional diversity observed with A1AR stimulation also indicates that subtypes of A1AR may exist. In spite of all these evidence, however the presence of subtypes of A1AR is still not universally accepted. This is due to the fact that other factors such as, 1) differences in the distribution of the generally hydrophobic compounds used to characterize the receptors or 2) differences in the receptor-effector coupling could contribute to these discrepancies. Cloning and expression of the hypothetical subtypes is essential for confirming the existence of A1AR subtypes.

Recently Zhou et al. 1992, reported the molecular cloning and characterization of an A3AR (R220) from rat brain. R220 encodes for a protein of 320 amino acids with seven transmembrane domains which possess 50% identity in the amino acid sequences, when compared to the canine A1 and A2AR. R220 was expressed in CHO or COS-7 cells and was observed to bind to the non-selective adenosine agonist [^{3}H]-NECA and to the A1 selective agonist [^{125}I]-APNEA (N^{6}-2-(4-aminophenyl)ethyl adenosine), but not

to the A1 selective antagonist [^{3}H]-DPCPX or to the A2 selective agonist ligand [^{3}H]CCS21000. Competitive binding studies using [^{125}I]-APNEA demonstrated that the binding could be inhibited by adenosine ligands with a potency order of R-PIA = NECA > S-PIA but not by the A1AR specific antagonist DPCPX. Although functional studies indicated that R220 inhibited forskolin stimulated cAMP accumulation through a pertussis toxin sensitive G-protein, a phenomenon linked to A1AR activation, pharmacological studies suggest that R220 could be an A3 receptor.

B . 2 . Receptor structure

Photoaffinity crosslinking of [^{125}I]APNEA into rat cerebral cortex membranes, followed by isolation of cross-linked proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Koltz et al. 1985; Lohse et al. 1986; Stiles et al. 1985) identified a protein of molecular weight 36,000 which displayed all of the pharmacological properties of A1AR. Purification of A1AR through chromatographic methods also supported this interpretation. Further studies of A1AR structure indicated that the protein is glycosylated. Deglycosylation of A1AR from rat brain and adipose cells results in a protein of molecular weight of 32,000 (Klotz and Lohse 1986; Stiles 1986). The functional role for this glycosylation remains to be determined. Similar photoaffinity labelling of A2AR by [^{125}I] PAPA-APEC, a selective A2AR agonist, demonstrated that the molecular weight of A2AR is 45,000.

The structures of the adenosine receptors has been further established by molecular cloning. Recently a number of A1 and A2 adenosine receptors have been cloned, sequenced, and expressed from different species (Maenhaut et al. 1990; Libert et

al. 1991; Mahan et al. 1991; Olah et al. 1992 and Bhattacharya et al. 1993). Both the A1 and the A2 receptors show a high degree of homology between species (91-92%). These receptors are small relative to other receptors of the G protein-coupled receptor superfamily, the A1AR being 36,000 and the A2AR being 45000 in molecular weight, as indicated by photoaffinity labelling.

Like other G-protein linked receptors, the A1 and A2AR have seven transmembrane domains (Mahan et al. 1991). Comparing the AR with several other G-protein coupled receptors however reveal some unique characteristics, which include: 1) a relatively low molecular weight; 2) one or two consensus glycosylation sites on the second extracellular loop rather than on the amino terminus; and 3) the presence of a relatively short third intracellular loop (34 amino acids) in the A1AR, compared with other receptors that inhibit adenylyl cyclase.

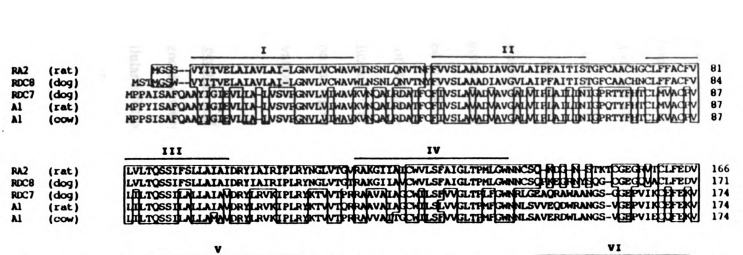
B . 3 . Molecular cloning of adenosine receptors:

Although much is understood regarding adenosine and its effects on a variety of physiological systems, only recently have the primary structures of the adenosine receptors been determined by molecular cloning. Libert et al. (1989) were the first to clone the adenosine receptors, and used oligonucleotides designed on the basis of conserved regions of other genes encoding the superfamily of G-protein linked receptor proteins. Sets of degenerate oligonucleotides from putative transmembrane regions (TM3 & TM6), were used as primers in the polymerase chain reaction (PCR) to amplify cDNA fragments of human thyroid tissue. These PCR products were subsequently used as probes to screen a dog thyroid cDNA library to obtain full length clones. Five clones

were isolated and identified as receptors : 1) RDC1, identical to a recently cloned human vasoactive intestinal peptide receptor (Shreedharan et al. 1991); 2) RDC4, thought to be a 5-HT receptor (Libert et al. 1989); 3) RDC5, the dog analog of the α-adrenergic receptor; and two orphan clones, namely 4) RDC7 and 5) RDC8. Based on ligand binding, tissue distribution and second messenger coupling RDC8 was subsequently identified as A2AR (Maenhaut et al. 1990). Tissue distribution has been an useful criteria for identifying orphan receptors, a classical example of which is the characterization of rat brain cannabinoid receptor (Matsuda et al. 1990). The 51% identity of RDC7 to RDC8 in its nucleotide sequence made RDC7 a possible candidate for A1AR cDNA clone. Subsequently ligand binding and second messenger coupling experiments confirmed that RDC7 codes for A1AR (Libert et al. 1991).

cDNA clones encoding the A1AR from rat brain (Mahan et al. 1991; Reppert et al. 1991), bovine brain (Olah et al. 1992), human brain (Libert et al. 1992) and from rabbit kidney (Bhattacharya et al. 1993) were subsequently isolated using probes derived from RDC7. The amino acid sequence of adenosine receptors are aligned and compared in Figure 3. The two adenosine A2 receptors cloned from rat and canine cDNA libraries not only share 97% identity in their amino acid sequences, but the rat A2 receptor also shares 65%, 64% and 63% amino acid identities with those of rat A1, canine A1 and bovine A1 receptors. Thus not only are A2 and A1 receptors highly homologous within species, but there is also a high degree of homology between the two receptors in their amino acid sequences. The maximum homologies between the A2 and the A1 receptors are in the transmembrane domains, which in G-protein coupled receptors is the region thought to be involved in ligand binding; the major differences in the A1 and A2

Figure 3. **The alignment and comparisons of the predicted amino acid sequences of the adenosine receptors isolated from different species.** The figure shows the comparison of the A2 and A1 adenosine receptors isolated from rat, canine and bovine cDNA libraries. Amino acids identical to the rat A2 are boxed. The putative seven transmembrane domains are overlined.


I
II
III
IV
V
VI
VII
RA2 (rat)
RDC8 (dog)
RDC7 (dog)
A1 (rat)
A1 (cow)

sequences third intracellular loop and in the C-terminal, which are thought to be involved in G-protein coupling.

As mentioned before, Zhou et al. (1992), have reported the molecular cloning and characterization of an adenosine receptor from rat brain (R220) which possesses an unique binding characteristics and which they therefore refer to as A3AR. R220 encodes for a protein of 320 amino acids, with seven transmembrane domains. When compared with RDC7 and RDC8, R220 is only 50% identical. R220 when expressed in CHO and COS-7 cells, was observed to bind to the nonselective adenosine agonist NECA and the A1 selective agonist APNEA, but not to the A1 selective antagonist DPCPX or to the A2 selective agonist ligand CCS21000. Competitive binding studies using [^{125}I]-APNEA demonstrated that the binding could be inhibited by adenosine ligands with a rank order of potency of R-PIA = NECA > S-PIA, but not by the antagonist DPCPX. Functional studies indicated that the protein R220 inhibited the forskolin stimulated cAMP accumulation through a pertussis toxin sensitive G-protein, a phenomenon observed with A1AR activation.

B . 4 . <u>Receptor - effector coupling :</u>

Adenosine receptors belong to the class of hormone receptors that are coupled to the intracellular effector systems via guanine nucleotide binding proteins. The low affinity A2AR is coupled to activation of adenylyl cyclase, whereas the high affinity A1 receptor inhibits its activity. Both A1 and A2 receptors are coupled to adenylyl cyclase through the guanine nucleotide binding protein Gs and Gi, respectively (Spielman and Arend 1991). The second messenger systems coupled to A1AR, however are remarkably diverse

(Table I), and includes adenylyl cyclase, phospholipase C and various ion channels (Linden 1991). This diversity of responses generated by A1AR activation has been interpreted to indicate that subtypes of A1AR may exist, either as products of differential splicing of a multiexon gene or as products of entirely different adenosine receptor genes. A third possibility is that a single receptor exists which is capable of interacting with multiple effector systems.

B . 5 . Adenosine receptor regulation :

Regulation of receptor function protects the cell from either over or under-activation of its effector systems. It is generally accepted that prolonged exposure to an agonist leads to desensitization of the receptor which may be receptor specific (homologous) or generalized (heterologous) for receptors coupled to similar second messenger system. The alternate situation, exposure to antagonists, often results in enhancement of subsequent responses to agonists. Although desensitization of the adenosine receptor has been observed in various systems, the molecular mechanisms responsible for desensitization are yet to be determined. Generally it is believed that desensitization can result from reduction of the size of the receptor population by sequestration, by regulation of receptor transcription and or translation rates, from alteration in expression of G-proteins, or from covalent modification such as phosphorylation leading to reduced function of the receptor (Hausdroff et al. 1990).

Homologous desensitization of the A2AR has been observed in rat brain striatum (Porter et al. 1988), rat vascular smooth muscle cells (Anand-Shrivastava et al. 1989), hamster DDT_1-MF-2 cells, a cell line derived from the vas deferens smooth muscle

Table I . Responses Coupled To A1 Adenosine receptor :

RESPONSE	TISSUES
Inhibition of adenylyl cyclase	RCCT-28A cells Heart Pituitary Adipose
Activation of Kach channels	Atria
Inactivation of Ca++ channels	Mouse sensory neurons
Increased inositol phosphate production	RCCT-28A cells cortex FRTL-5 thyroid cells
Inhibition of inositol phosphate production	Guinea pig cortex GH3 pituitary cells
Decreased chloride transport	RCCT-28A cells Shark rectal glands

(Ramkumar et al. 1991), rat kidney fibroblasts (Newman and Levitzki 1983) and in LLCPK1 cells, a cell line developed from porcine kidney (Levier and Spielman, 1992). The desensitization of A2AR in all these systems, appears to be rapid, within a period of 24 hrs.

Desensitization studies of A1AR have been carried out using adipocytes (Hoffman et al. 1986; Parsons and Stiles 1987; Green 1987; Longabaugh et al. 1989; Green et al. 1990; Green et al. 1992), DDT_1-MF-2 cells (Ramkumar et al. 1991), rabbit kidney (Arend and Spielman 1992) and embryonic chicken heart (Shryock et al. 1989). Desensitization in all these systems, unlike the A2AR was observed to have a slower time of onset. In DDT_1-MF-2 cells, it was observed that prolonged exposure to the A1 agonist resulted in decreased numbers of A1AR. Phosphorylation, sequestration, and uncoupling of the A1AR appeared to account only in part for the decrease in the A1AR number. These results suggest that the receptor may be regulated at the transcriptional and or translational level. A1 adenosine receptor numbers are also observed to increase in response to stimulation by glucocorticoids in DDT_1-MF-2 cells (Gerwins et al.1991) and thyroid hormones (Rapiejko et al 1987). With the molecular cloning of adenosine receptor cDNA and the gene, it is now possible to elucidate the molecular mechanisms underlying regulation of the adenosine receptors.

C . <u>Physiological effects of adenosine receptor activation:</u>

The physiological role of adenosine has been recognized for more than 60 years. Drury and Szent-Gyorgi, in 1929 first demonstrated a role for adenosine in the regulation of the cardiovascular system and vascular muscle tone. The effects of adenosine,

however, are not confined solely to the cardiovascular system, but are manifested in the nervous system, renal system, pulmonary system, gastrointestinal and immune systems (Table II). The physiological actions of adenosine are initiated by binding of adenosine to its plasma membrane G-protein coupled receptors and the subsequent change in intracellular signaling mechanisms. The actions of adenosine on different systems are summarized below :

1. **Cardiovascular system :** Adenosine has negative chronotropic, dromotropic and inotropic effects (Olsson and Pearson et al. 1990) in the heart. Drury and Szent Gyorgi (1929) were the first to report, a transient decrease in heart rate following adenosine infusion. Further investigations revealed that adenosine acts directly on the SA node, AV nodes and Bundle of His in experimental animals (Belardinelli et al. 1980). The effects of adenosine are blocked by methylxanthines and increased by inhibitors of adenosine uptake such as dipyridamole (Belardinelli et al. 1981; Burne et al. 1963). The negative inotropic effect of adenosine may be mediated by the activation of K-channels through A1AR (Kurachi et al. 1986; Belardinelli et al. 1987). In ventricular tissues it appears that adenosine acts as an inhibitor of adrenergic stimulation (Dobson et al. 1987).

In vascular beds, adenosine acts as a potent vasodilator (Li and Fredholm 1983) except in the renal vascular system (Macias et al. 1983) and in the placenta (Olsson et al. 1990). The vasodilatory effect of adenosine is probably mediated via activation of A2AR on endothelial cells and releasing the endothelium derived relaxing factor (Olsson and Bunger 1987).

Table II. Functional Effects Of Adenosine Receptor Activation :

TISSUE	EFFECT	RECEPTOR
Nervous System		
peripheral	↓ transmitter release	A 1
central	↓ neuronal firing	A 1
Heart	anti-adrenergic	A 1
Smooth Muscle		
vascular	relaxation	A 2
trachea	relaxation	A 2
taenia coli	relaxation	A 2
Platelets	anti-aggregatory	A 2
Fats cells	anti-lipolytic	A 1
Masts cells	degranulation	?
Lymphocytes	immunosuppression	?
Kidneys		
afferent art.	contraction	A 1
efferent art.	relaxation	A 2
JGA, renin	inhibition	A 1
release	stimulation	A 2
erythropoietin	inhibition	A 1
	stimulation	A 2
adren. trans.	presynaptic inhibition	A 1
collecting	cAMP (↑ H2O perm. ?)	A 2
tubule	cAMP (↓ H2O perm. ?)	A 1
thick limb	cAMP (↑ J Na)	A 2
	cAMP (↓ J Na)	A 1

2. Nervous system : The role of adenosine as a modulator of neurologic function was first suggested by Drury and Szentz-Gyorgi in 1929. Subsequently adenosine was reported to inhibit the release of neurotransmitters, including norepinephrine, dopamine, serotonin, acetylcholine, GABA, aspartate, and glutamate (Stone 1981; Spignoli et al. 1984; Ebstein et al. 1982). This inhibition appears to be independent of calcium entry into neurons.

In 1954 Feldberg and Sherwood were the first to demonstrate the cessation of spontaneous motor activity in cats upon intraventricular injection of adenosine. This led to further investigation and now adenosine and its analogs have been found to produce sedative actions. Adenosine in high doses also has been shown to protect against seizure (Dragunow et al. 1985).

3. Renal system : The diverse effects of adenosine are best illustrated in the renal system. In the kidney, adenosine has been reported to produce a variety of responses, including hemodynamic changes, renin release and solute transport (Spielman and Arend 1991; Churchill and Churchill 1988; Osswald 1983; Spielman and Thompson 1982; Yagil et al. 1990; Dillingham and Anderson 1985). In contrast to peripheral vascular beds, adenosine infusion into the renal artery results in vasoconstriction (Osswald et al. 1978) which in turn decreases glomerular filtration rate (GFR). The mechanism underlying the adenosine induced decrease in GFR, appears to be due to a decrease in glomerular hydrostatic pressure resulting from preglomerular A1AR-mediated vasoconstriction and a more slowly developing postglomerular A2AR-mediated vasodilation (Rossi et al. 1988; Rossi et al. 1987). Since an increase in distal nephron

perfusion rate produces an afferent arteriolar vasoconstriction and a resultant decrease in single nephron GFR, it can be interpreted to serve as a mechanism to limit large increases in fluid and solute delivery and consequent alterations in tubule and excretory functions (Briggs and Schnermann 1990). This whole phenomenon has been termed tubulo-glomerular feedback (TFG). Studies have shown that the specific A1AR antagonist DPCPX inhibits TGF when administered either into the tubule lumen or into the peritubular capillary circulation (Schnermann et al. 1990).

In addition to hemodynamic changes, adenosine infusion also produces a biphasic renin release. At low adenosine concentrations A1 receptors are activated inhibiting renin release, whereas at high adenosine concentrations which activates A2 receptors, renin release is augmented (Osswald et al 1978).

Adenosine also has been shown to regulate erythropoietin production (Ueno et al. 1988) and inhibition of neurotransmitter release (Hedqvist et al. 1976) in the kidney. In exhypoxic polycythemic mice, activation of A2AR produces stimulation of radioiron incorporation into red blood cells and A1AR activation is shown to inhibit this process. Moreover, stimulation of A2AR enhances erythropoietin production in renal carcinoma cells whereas A1AR stimulation inhibits erythropoietin production (Ueno et al. 1987).

In the kidney, adenosine acts at prejunctional A1AR to inhibit the release of norepinephrine from sympathetic neurons. However, it has also been reported that adenosine increases the sensitivity of the kidney to norepinephrine through a post-junctional mechanism (Hedquist et al. 1976).

Decrease in urine flow and solute excretion also has been reported to result from elevated levels of intrarenal adenosine (Yagil et al. 1990; Miyamoto et al. 1988). A direct

tubular action of adenosine has been reported in rabbit cortical collecting tubule (Dillingham and Anderson 1985;), canine medullary thick ascending limb and cortical thick ascending limb (Anand-Shrivastava et al. 1986).

4. **Pulmonary system :** Adenosine causes bronchoconstriction in asthmatic lung which is antagonized by the adenosine receptor antagonist, theophylline (Cushley et al. 1984). Theophylline also has been demonstrated to inhibit the action of adenosine to potentiate the antigen-induced histamine release from guinea pig lung (Welton et al. 1976).

5. **Gastrointestinal system :** In rats, the adenosine receptor antagonist, theophylline, augments the production of stress induced gastric ulcers (Geiger and Glavin 1985; Watt et al. 1987). Adenosine analogs have been shown to induce gastric lesions (Ushijina et al. 1985). It has also been shown that in pancreas, caffiene induces pancreatic secretion during fasting and theophylline induces the release of amylase from isolated pancreatic acinar cells (Korman et al. 1980).

6. **Immune system :** Inherited deficiency of adenosine deaminase, the enzyme that catalyzes the breakdown of adenosine to inosine, which leads to elevated levels of adenosine, has long been known to have detrimental effects on the development of the immune system. Among the major functions of adenosine in the immune system, are lectin stimulated proliferation of lymphocytes, inhibition of interleukin-2 production by T-lymphocytes (Averill et al. 1985), modification of T-lymphocyte effector functions,

inhibition or facilitation of cytokine production, and suppression of natural killer activity of neutrophils (Cronstein et al. 1985).

7. Platelet : The anti-aggregatory effect of adenosine on platelets has long been recognized (Born 1964). This effect is blocked by the methylxanthines and enhanced by dipyridamole, the inhibitor of adenosine uptake.

III. MOLECULAR CLONING OF A1 ADENOSINE RECEPTOR cDNA FROM RABBIT

A . INTRODUCTION

Adenosine receptors are members of the purine receptor family, broadly classified as P1 (adenosine) or P2 (ATP, ADP, or AMP) purinergic receptors (Brunstock 1978). The P1 receptors have been further subdivided into A1 and A2 subtypes based on their affinities for a variety of adenosine analogs (Daly et al. 1987) and on their ability to inhibit (A1) or stimulate (A2) adenylyl cyclase (Londos et al. 1980). The second messenger systems coupled to the A1 adenosine receptor, however, are remarkably diverse, and include adenylyl cyclase, phospholipase C and various ion channels (Linden, 1991). This diversity of responses generated by A1AR activation has been interpreted to indicate that subtypes of the A1AR may exist, either as products of differential splicing of a multiexon gene or as products of entirely different adenosine receptor genes. However, the possibility of a single receptor capable of interacting with multiple effector systems also exists.

Our laboratory has long been interested in adenosine receptors, its structure, function and signalling in the rabbit kidney. In RCCT-28A cells, a cell culture line developed from the rabbit cortical collecting tubule, adenosine receptors were shown to be coupled to adenylyl cyclase via guanine nucleotide binding proteins (Arend et al. 1987). The A2AR, which is coupled to the stimulatory guanine nucleotide protein,

activates the cyclase system, whereas the A1AR, which is coupled to the inhibitory guanine nucleotide binding protein (G_i), inhibits the cyclase system. It also has been observed that stimulation of the A1AR in RCCT-28A cells not only produces an inhibition of the cyclase but also a stimulation of the phospholipase C activity (Arend et al. 1989). It is not clear whether there is one A1AR which is capable of interacting with more than one effector system or whether there are subtypes of A1AR which have the same ligand binding domains but different effector coupling domains. Molecular cloning and expression of the A1AR will help to resolve this controversial area.

This report describes the cloning of an A1AR from rabbit. The deduced amino acid sequence of this receptor and its comparison with other cloned A1AR is presented in this chapter. This data show that the rabbit A1AR displays a high degree of similarity with the dog thyroid, rat brain, bovine brain and the human brain A1ARs (Libert et al., 1991; Mahan et al., 1991; Reppert et al., 1991; Olah et al., 1992; Libert et al., 1992).

B . MATERIALS AND METHODS

MATERIALS: [γ-^{32}P]ATP (specific activity of 6000 Ci/mmol), [^{35}S]ATP (specific activity of 1200 Ci/mmol) and [α-^{32}P]dCTP (specific activity of 800 Ci/mmol) were obtained from DuPont-New England Nuclear; restriction enzymes, T4 DNA ligase and Random primer labelling kit were obtained from Boehringer-Manheim or BRL; λ-ZAPII cDNA library was from Stratagene; λ-EMBL3 SP6/T7 rabbit genomic library containing *Sau*3AI partial digest of rabbit kidney DNA was from Clontech; nitrocellulose filters (pore size 0.45 µm) were from Schleicher and Schuell; agarose (type I) was

obtained from Sigma; oligonucleotides used for library screening and for sequencing were synthesized by the Macromolecular Structure, Synthesis, and Sequencing Facility Laboratory at Michigan State University and the Sequencing Laboratory at SmithKline Beecham Pharmaceuticals; DNA sequencing kits were obtained from United States Biochemicals.

METHODS:

Designing an oligonucleotide probe: The nucleotide sequence of the canine A1AR protein RDC7 (Libert et al. 1991 was deduced with the help of a computer program "Backtranslate" distributed by the Genetics Computer Group, Wisconsin. Each amino acid is coded by a set of three nucleotides called a codon. However most of the amino acids can be coded by more than one codon, which is referred to as degeneracy of codon. It has been observed that in case of degenerate codons, each species has a preference for a particular codon over others. The Backtranslate program statistically compares a group of different messages from a particular species, and generates a nucleotide sequence for a protein containing the best fit codon for that species. The nucleotide sequence of RDC7 thus obtained from this Backtranslate program was used to generate a 60-mer oligonucleotide probe, termed WSS1, in order to screen the rabbit kidney cDNA library.

The 60 mer oligonucleotide designated, WSS1 (5'-GAAGAAGTTGAAGTAIA-CCATGTACTCCATGGAGATIACCTTCTCAAACTCACACTTGAT-3') was synthesized based on the region comprising a part of the second extracellular loop and fifth

transmembrane domain (166-186 amino acid) of RDC7, the dog thyroid A1AR (Libert et al. 1991). WSS1 was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and the labelled probe was first used for Northern blotting.

Northern Blot : Poly-(A)$^+$ RNA was isolated by a two step process from kidney and brain tissues of white male New Zealand rabbit and also from RCCT-28A cells, a cell culture line developed from rabbit cortical collecting tubule (Arend et al. 1989). Total cellular RNA was first isolated by extraction with guanidinium isothyocyanate and cesium chloride separation, followed by poly-(A)$^+$ mRNA isolation using oligo-d(T) cellulose column (Sambrook et al. 1989). The mRNA isolated (10 µg/lane) was electrophoresed on a 1% agarose-formaldehyde gel and blotted overnight onto nitrocellulose, immobilized by baking at 80°C for 1 hour and hybridized to labelled WSS1. Hybridization conditions were 20% formamide, 5X SSPE (0.75M NaCl, 0.05M NaH_2PO_4, 0.005M EDTA, pH 7.4), 5X Denhardt's solution (a stock solution of 50X Denhardt's containing 5 gm Ficoll Type 400 (Pharmacia), 5 gm Polyvinylpyrrolidone and 5 gm Bovine Serum Albumin Fraction V was used to prepare the working solution), 0.1% sodium dodecyl sulphate, 0.25 mg/ml of salmon sperm DNA, with 2X10^6 cpm/ml γ-^{32}P labelled probe, for overnight at 42°C. The filters were washed with 6X SSC (0.9M NaCl, 0.09M Sodium Citrate, pH 7.0) containing 0.1% SDS for 15 min. at room temperature, followed by 15 min wash at 55°C and exposed for autoradiography. WSS1 was shown to hybridize to a message length of 2.4 kb, the reported size for canine A1AR.

cDNA library screening : End labelled WSS1 was then used to screen a rabbit kidney cDNA library (λ-ZAPII cDNA library cloned in the *Eco*RI cloning site). Duplicate nitrocellulose filters which were used for phage lifts were hybridized with 20% formamide, 5X SSPE, 5X Denhardt's solution, 0.1% sodium dodecyl sulphate, 0.25 mg/ml of salmon sperm DNA, with $2X10^6$ cpm/ml of the γ-^{32}P labelled oligonucleotide, WSS1, for overnight at 42°C. The filters were washed with 6X SSC/0.1% SDS for 15 min at room temperature, followed by a 15 min wash at 55°C and exposed for autoradiography. One positive clone designated SB4, was isolated, purified and sequenced.

DNA sequencing : The insert isolated from the positive clone was ligated in M13mp19 in both orientations and sequencing was performed on both strands using [^{35}S]dATP, as per the manufacture's instruction, using Sequenase ver. 2.0 kit (U. S. Biochemical). Sequencing data indicated that SB4 was the rabbit analog of RDC7 but was missing the first 74 amino acid of RDC7. In order to get a full length clone I further screened a rabbit genomic library.

Genomic library screening : A rabbit genomic library (a λ-EMBL3 library cloned in the *Bam*HI restriction site) was purchased from Clontech. The library was screened with an 870 nucleotide fragment from the 5'-end of SB4 (an *Eco*RI and a *Sma*I fragment; SB4E/S), as per the cDNA library screening protocol, with the exception that the 1) probe was labeled with [α-^{32}P]dCTP using a random primer labeling kit (Boehringer-Manheim); 2) the hybridization solution contained 50% formamide and 3) the washes following hybridization were more stringent (2X SSC containing 0.1% SDS

for 15 min at room temperature followed by 0.2X SSC containing 0.1% SDS for 15 min at 65°C). Eight positive clones were isolated and plaque purified. Duplicate nitrocellulose filters used for plaque lifts from the eight clones were hybridized to two sets of probes: a) the 5'-end *Eco*RI/*Sma*I fragment of SB4 (SB4E/S) and b) a PCR fragment generated from rat brain mRNA, using oligonucleotides (21 mer) designed from RDC7, which included the first 300 nucleotide of the 5'-end starting from the translation start site (kindly provided by Dr. Lohse, Genzentram/MPI for Biochemistry, Martinsried, FRG). This PCR fragment therefore contained the nucleotides that were missing in SB4. One genomic clone, named clone-1, gave a positive signal with both the probes and was therefore further characterized.

Southern blot : Phage DNA isolated from Clone-1, was digested with *Xho*I and electrophoretically separated on a 1% agarose gel containing 0.5 µg/ml of ethidium bromide, blotted overnight onto nitrocellulose and immobilized by baking at 80°C for 1 hour. Southern blot analysis was performed using labelled SB4E/S fragment and the PCR fragment as probes. A 3.2 kb *Xho*I fragment of clone-1 was found to hybridize to both SB4E/S and the PCR probe. Sequencing data indicated that this 3.2 kb *Xho*I fragment contained approximately 2 kb of 5'-flanking region, an exon of 341 nucleotides followed by an intron.

PCR and ligation of genomic clone-1 and SB4 to generate a full length cDNA encoding the A1AR from rabbit: The genomic clone-1 and SB4 were ligated using the polymerase chain reaction (PCR) to generate a full length cDNA encoding the A1AR

from rabbit (Figure 10). A pair of reverse and complementary primers were synthesized from the exon-1 and intron-1 of genomic clone-1 as follows :

PRIMER	SEQUENCE	PRODUCT SIZE
7033 (intron-1)	$^{5'}$GGCACTGCCAGGCTCGTGTTCCT$^{3'}$	571 nucleotide
7198 (exon-1)	$^{5'}$CGGAATTCTGCTGATGTGCCCAGCTG$^{3'}$	

Primer 7198, contains a restriction site for *Eco*RI, as underlined and is 30 bp upstream of the translational start site. For PCR, a mixture including 5 pmol primers, 2 U Taq DNA polymerase (Perkin Elmer), 1.25 mM each of dATP, dCTP, dGTP and dTTP was used. The total volume was brought to 100 µl with 1 X PCR buffer (80 mM Tris-HCl, pH 8.9; 20 mM $(NH_4)_2SO_4$; 5 mM $MgCl_2$) and 50 ng of the 3.2 kb *Xho*I fragment as template. The PCR cycle included denaturation at 95°C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min, for 25 cycles. After PCR, one-tenth of the sample was electrophoresed on a 1% agarose gel to verify the size of the products. The PCR product was digested with *Eco*RI and *Sac*I to obtain a product of 310 nucleotides, starting 30 bp upstream of the translational start site and subcloned into pUC19 (pUC19+5'ORF) for sequencing and further verification. SB4 and pUC19+5'ORF were digested with *Sac*I and *Pst*I and religated to generate the full open reading frame for the rabbit A1AR.

C. RESULTS

(a) Isolation of an A1AR-encoding cDNA

An oligonucleotide probe, WSS1 was synthesized based on the nucleotide sequence of RDC7, the canine A1AR (amino acid 166-186; Figure 4). On Northern blot analysis of poly(A)$^+$ mRNA isolated from rabbit cortical collecting tubule cells (RCCT-28A), rabbit kidney and rabbit brain, end labeled WSS1 hybridized to a 2.4 kb mRNA, the reported size of mRNA for canine A1AR (Figure 5). Therefore WSS1 was used as a probe to screen a rabbit kidney λ-ZAPII cDNA library, cloned in the *Eco*RI cloning site. A single positive clone containing a 3 kb insert was isolated. The cDNA insert of the clone was comprised of two *Eco*RI fragments 2 kb and 1 kb in length. To verify whether both of the fragments originated from the same mRNA, a Northern blot analysis of rabbit kidney mRNA was performed with the two individual *Eco*RI fragments. The 2 kb and the 1 kb fragments hybridized to two different sized mRNAs indicating that they were probably derived from different messages (data not shown). This may be due to an artifact in the cDNA library two different messages were ligated bluntly resulting in a single insert. Since the 2 kb fragment (designated SB4) hybridized to a 2.4 kb mRNA, the reported size of mRNAs for canine A1AR, we sequenced SB4 in both orientations. Comparison of amino acid sequence obtained from the largest open reading frame to that of the canine receptor protein sequence (RDC7) indicated 94% identity. SB4 was however, found to lack 5'-UTR sequences and nucleotides corresponding to the first 74 amino acids of RDC7 (Figure 6). Moreover, no poly (A) tail was observed at the 3'-end of the clone, although oligo dT primers were used to construct this library.

Figure 4 . Flow diagram depicting the strategy involved in screening the cDNA library. RDC7, the canine A1AR, with its putative seven transmembrane domains is shown to the right. The shaded amino acids indicate the region from which WSS1 was designed.

RDC7 Amino acid sequence

Backtranslate

RDC7 Nucleotide sequence

Oligonucleotide probe

Northern blot

Screen rabbit kidney cDNA library

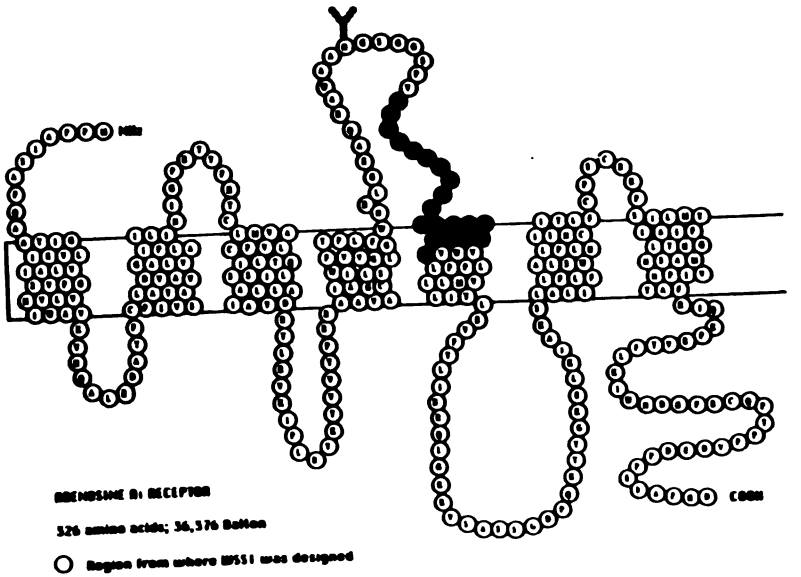


Figure 5 . **Northern blot analysis of A1AR mRNA.** Poly(A)$^+$ mRNA (10 µg/lane) was run on an agarose/formaldehyde gel, transferred to nitrocellulose and probed with ^{32}P-labelled oligonucleotide, WSS1. Tissues analyzed were rabbit kidney and brain and RCCT-28A cells, a cell culture line developed from rabbit cortical collecting tubule. Position of the RNA molecular weight markers are shown to the right.

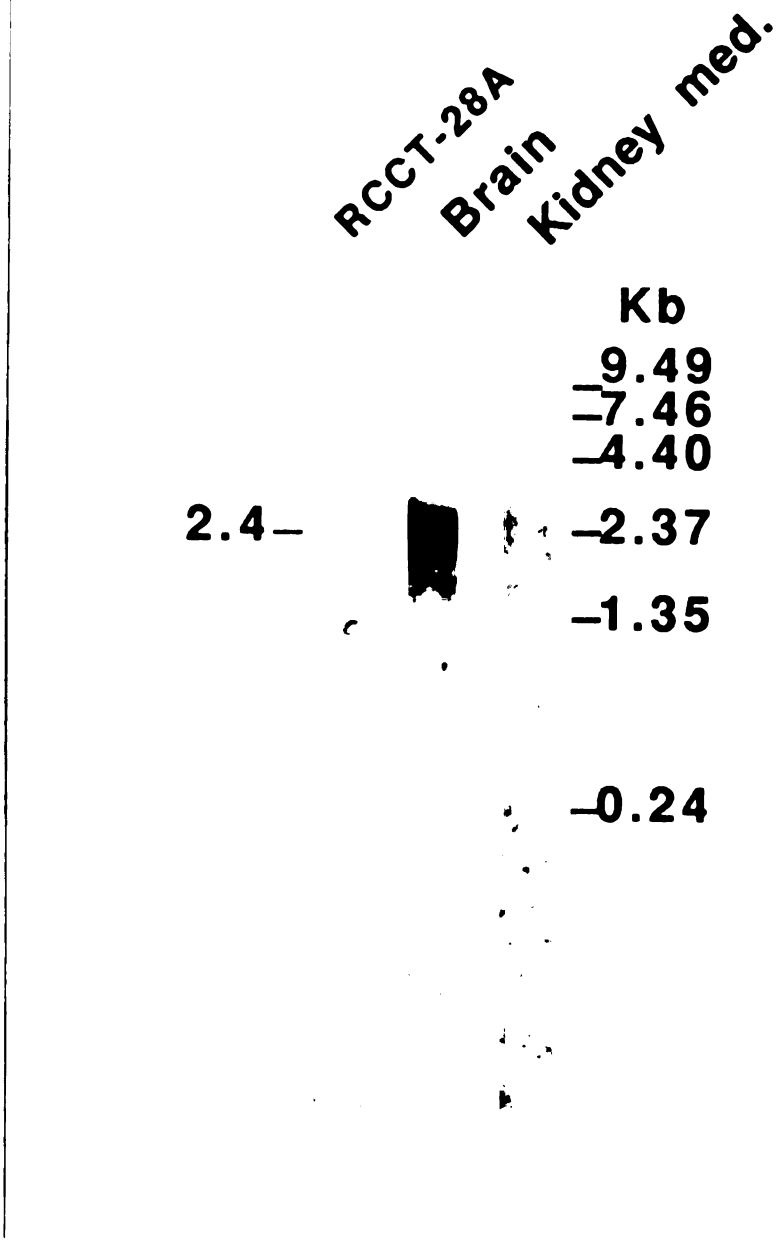

RCCT-28A
Brain
Kidney med.
Kb
9.49
7.46
4.40
2.4
2.37
1.35
0.24

Figure 6 . **Comparison of the amino acid sequence of RDC7, the canine A1AR, with SB4.** Vertical lines indicate amino acids that are identical and dots indicate the differences in nucleotide numbers in a set of codon for a particular amino acid.

```
RDC7        75 TYFHTCLMVACPVLILTQSSILALLAIAVDRYLRVKIPLRYKTVVTPRRA 124
               |||||||||||||||||||||||||||||||||||||||||.|||||||
SB4          1 TYFHTCLMVACPVLILTQSSILALLAIAVDRYLRVKIPLRYKAVVTPRRA 50

RDC7       125 AVAIAGCWILSFVVGLTPLFGWNRLGEAQRAWAANGSGGEPVIKCEFEKV 174
               ||||||||||:||||||:||||.| |.|||||||||.||||||||||||
SB4         51 AVAIAGCWILSLVVGLTPMFGWNNLREVQRAWAANGSVGEPVIKCEFEKV 100

RDC7       175 ISMEYMVYFNFFVWVLPPLLLMVLIYLEVFYLIRRQLGKKVSASSGDPQK 224
               ||||||||||||||||||||||||||||||||||:||.|||||||:|
SB4        101 ISMEYMVYFNFFVWVLPPLLLMVLIYLEVFYLIRRQLSKKASASSGDPHK 150

RDC7       225 YYGKELKIAKSLALILFLFALSWLPLHILNCITLFCPSCRKPSILMYIAI 274
               ||||||||||||||||||||||||||||:|||||||.|||||:|.||
ŞB4        151 YYGKELKIAKSLALILFLFALSWLPLHILNCVTLFCPSCQKPSILVYTAI 200

RDC7       275 FLTHGNSAMNPIVYAFRIQKFRVTFLKIWNDHFRCQPTPP..VDEDPPEE 322
               ||||||||||||||||:||||||||||||||||.|.|:  .||| |||
SB4        201 FLTHGNSAMNPIVYAFRIHKFRVTFLKIWNDHFRCRPAPAGDGDEDLPEE 250

RDC7       323 APHD 326
                |:|
SB4        251 KPND 254
```

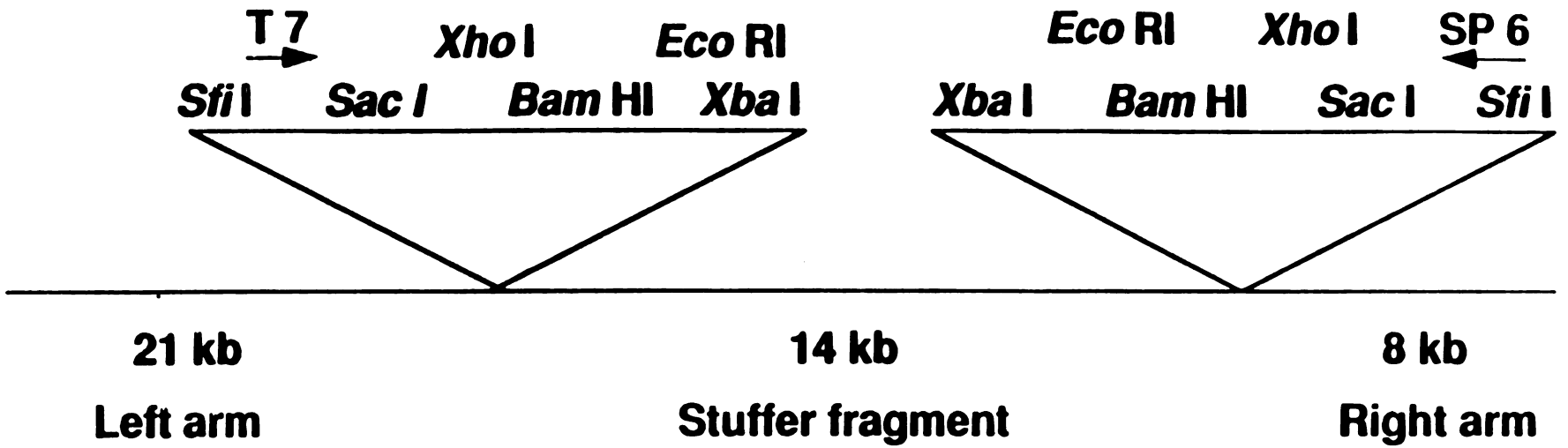
(b) Isolation of genomic A1AR clones

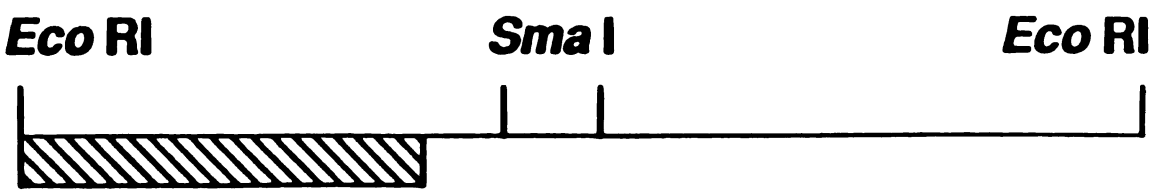
To obtain a clone encoding the N-terminal sequence of the rabbit A1AR we screened a rabbit genomic library (Clontech) with an *Eco*RI/*Sma*I fragment derived from the 5'-end of SB4. A schematic of the λ-EMBL3 vector of the genomic library with its cloning site and the probes used to screen the library is depicted in Figure 7. Eight positive clones were identified and rescreened with a PCR fragment from the 5'-end of the rat brain A1AR generated using oligonucleotide primers designed from RDC7. This PCR fragment included the first 300 nucleotide of the 5'-end of the rat brain A1AR begining with the translation initiation codon (kindly provided by Dr. Martin Lohse, Genzentram/MPI for Biochemistry, Martinsried, FRG). Only one of the eight clones, designated clone-1, hybridized to this PCR fragment. Clone-1 was rescreened, purified and its DNA was extracted (Sambrook et al. 1989). Southern blot analysis of the PCR positive clone revealed a 3.2 kb *Xho*I fragment which hybridized to both the SB4 probe and the PCR fragment (Figure 8). Sequence analysis demonstrated that the 3.2 kb *Xho*I fragment contained the 5'-UTR, an exon of 341 nucleotides followed by an intron with a consensus exon/intron splice site. The exon sequence was 100% identical to SB4 at the region of overlap (Figure 9) and the deduced amino acid sequence was 98% identical to that of the canine A1AR (RDC7). Together, the cDNA and the genomic clone provided the entire open reading frame for the rabbit A1AR.

(c) The amino acid sequence of rabbit A1AR and its comparison to other A1ARs

A part of the 5'-open reading frame was amplified from the 3.2 kb *Xho*I fragment using PCR with primers made from exon-1 with an *Eco*RI site at the 5'-end, 30 bp

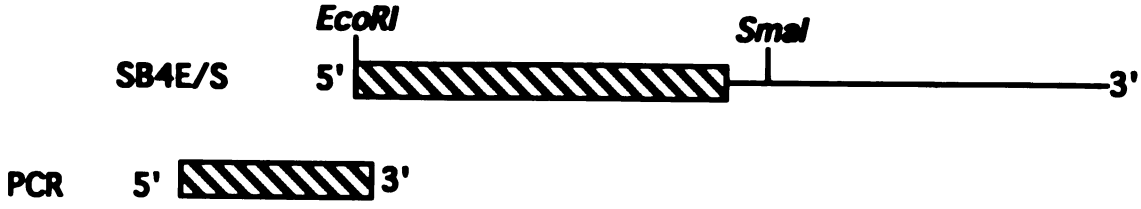
Figure 7 . Schematics of the λ-EMBL3 cloning site of the genomic library vector and the probes used to screen the library.


T 7
Xho I
Eco RI
Sfi I
Sac I
Bam HI
Xba I
Eco RI
Xho I
SP 6
Xba I
Bam HI
Sac I
Sfi I
21 kb
Left arm
14 kb
Stuffer fragment
8 kb
Right arm


Eco RI
Sma I
Eco RI
SB 4

PCR

Figure 8 . **Southern blot analysis of the genomic clone-1.** DNA isolated from genomic clone-1 was digested with the restriction enzyme *Xho*I, run on agarose gel, transferred to nitrocellulose and probed with random labelled SB4(E/S) and the PCR fragment. Positions of the λ-*Hind*III digest, molecular weight markers are shown to the left.


EcoRI
SmaI
SB4E/S
5'
3'
PCR
5'
3'

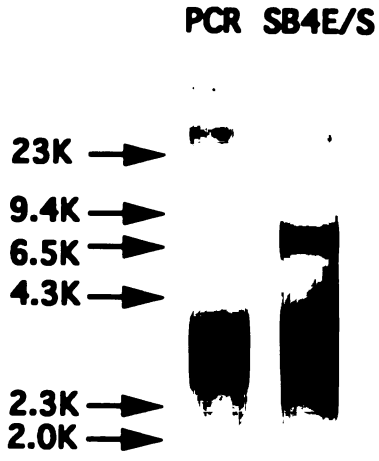
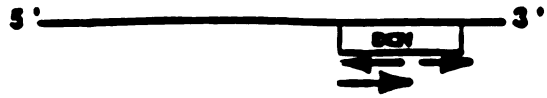

PCR SB4E/S
23K
9.4K
6.5K
4.3K
2.3K
2.0K

Figure 9 . **Sequence obtained from the 3.2 kb *Xho*I fragment and its comparison with SB4.** The top figure illustrates the sequencing map of the 3.2 kb *Xho*I fragment from genomic clone-1. Arrows indicate the length and direction of sequence obtained from each primers. The middle figure shows the nucleotide sequence obtained from the 3.2 kb *Xho*I fragment, including the first exon and the exon/intron border. The 5' UTR and the intron sequence is shown in small letters. The figure below shows the nucleotide sequence compared to SB4.




```
  1   ttctgctgat gtgcccagcc tgtgcccgcc ATGCCGCCCT CCATCTCGGC CTTCCAGGCC
 61   GCCTACATTG GCATCGAGGT GCTCATCGCG CTGGTCTCGG TGCCAGGGAA CGTGCTGGTG
121   ATCTGGGCCG TGAAGGTGAA CCAGGCACTG CGGGACGCCA CCTTCTGCTT CATCGTGTCG
181   CTGGCAGTGG CTGACGTGGC CGTGGGCGCC CTGGTCATCC CGCTGGCCAT CCTCATCAAC
241   ATCGGCCCCG AGACCTACTT CCACACCTGC CTCATGGTGG CCTGTCCTGT CCTCATCCTC
301   ACCCAGAGCT CCATCCTGGC CCTGCTGGCC ATCGCCGTGG ACCGCTACCT CCGCGTCAAG
361   ATTCCTCTCC Ggtgagtcca ---------- (INTRON 1) ----
```

```
Clone-1    252 GACCTACTTCCACACCTGCCTCATGGTGGCCTGTCCTGTCCTCATCCTCA 301
               ||||||||||||||||||||||||||||||||||||||||||||||||||
SB4          8 GACCTACTTCCACACCTGCCTCATGGTGGCCTGTCCTGTCCTCATCCTCA 57

Clone-1    302 CCCAGAGCTCCATCCTGGCCCTGCTGGCCATCGCCGTGGACCGCTACCTC 351
               ||||||||||||||||||||||||||||||||||||||||||||||||||
SB4         58 CCCAGAGCTCCATCCTGGCCCTGCTGGCCATCGCCGTGGACCGCTACCTC 107

Clone-1    352 CGCGTCAAGATTCCTCTCCGgtgagtcca
               ||||||||||||||||||||
SB4        108 CGCGTCAAGATTCCTCTCCG..........
```

upstream from the translation start site and a reverse and complementary primer from intron-1 (Figure 10). The 571 nucleotide product thus obtained, after size verification on an agarose gel, was digested with *Eco*RI and *Sac*I to obtain a fragment of 310 bp in length. This *Eco*RI and *Sac*I fragment from PCR was ligated into pUC19 (pUC+5'ORF), and sequenced. Both genomic clone-1 and SB4 possess an unique *Sac*I site at the 5'-end and therefore SB4 was digested with *Sac*I and *Pst*I, generating a 1487 bp long fragment containing the nucleotides coding for a part of the open reading frame and a part of the 3'-UTR. This fragment was then ligated to the *Sac*I/*Pst*I site into the vector containing the PCR product (pUC+5'ORF; Figure 10). The vector containing the entire open reading frame of the rabbit A1AR (pUC+RBA1) was sequenced from both ends by the forward and reverse primers made from pUC19 for further verification. The nucleotide and the amino acid sequence obtained from the rabbit A1AR is shown in Figure 11.

The rabbit A1AR was compared to other A1ARs as shown in Figure 12. This comparison demonstrated that: (1) there is a high degree of interspecies homology among the A1ARs (2) each contain seven putative transmembrane spanning domains characteristic of G-protein linked receptors, (3) the rabbit A1AR contains two additional amino acid residues, (4) each receptor contains serine residues in the third intracellular loop, which may be a potential site for phosphorylation involved in regulation of receptor function and (5) all the A1AR cloned so far with the exception of canine A1AR contain two consensus glycosylation sites. Canine A1AR has only one consensus glycosylation site. The five A1ARs cloned to date are 92% identical in their transmembrane region I, II and V, and in the first intracellular loop. Histidine 278 and 251 in bovine A1AR has been identified as an important residue in both agonist and antagonist binding (Olah et

Figure 10. Schematic representation of the strategy involved in generating a full length clone encoding the rabbit A1AR.

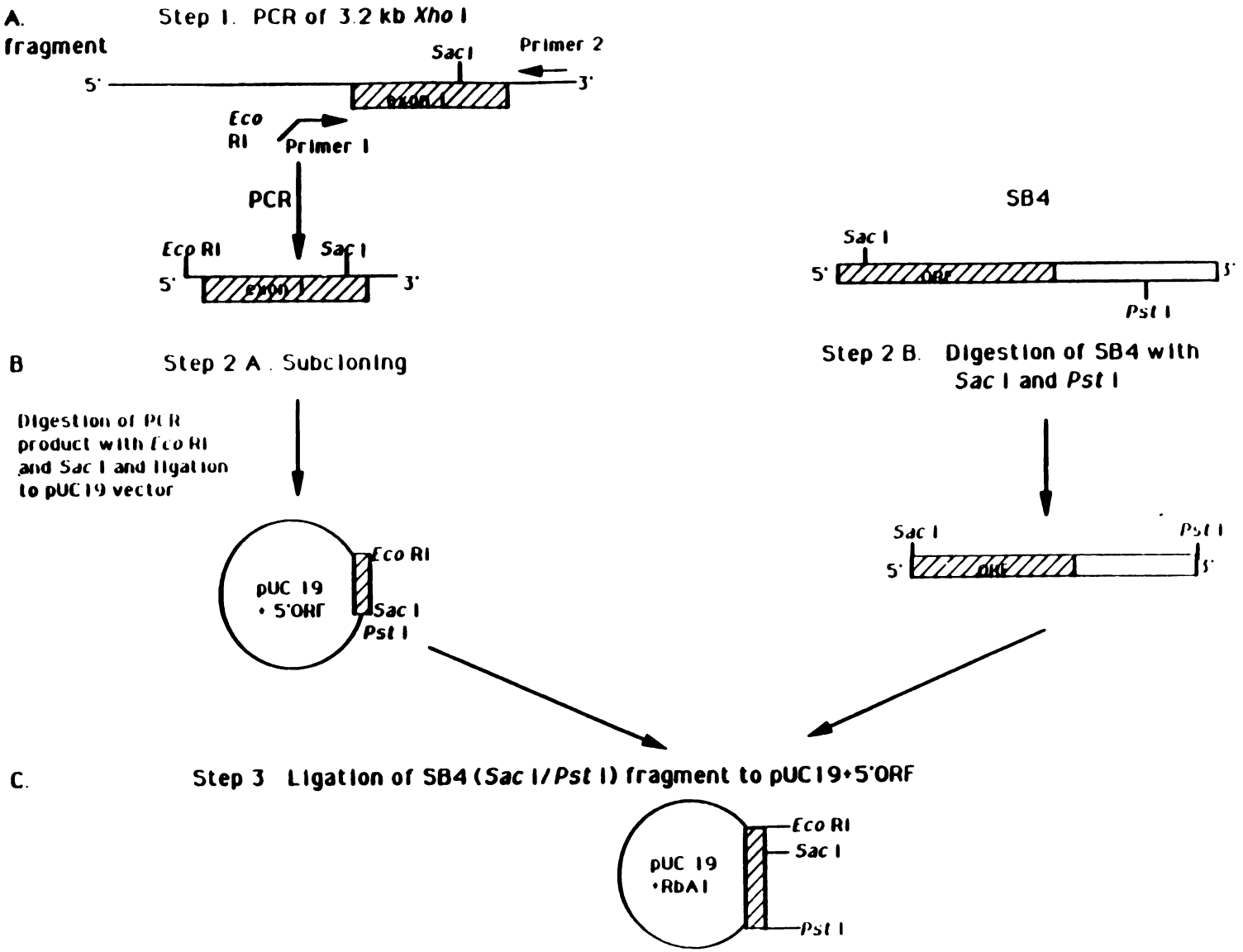
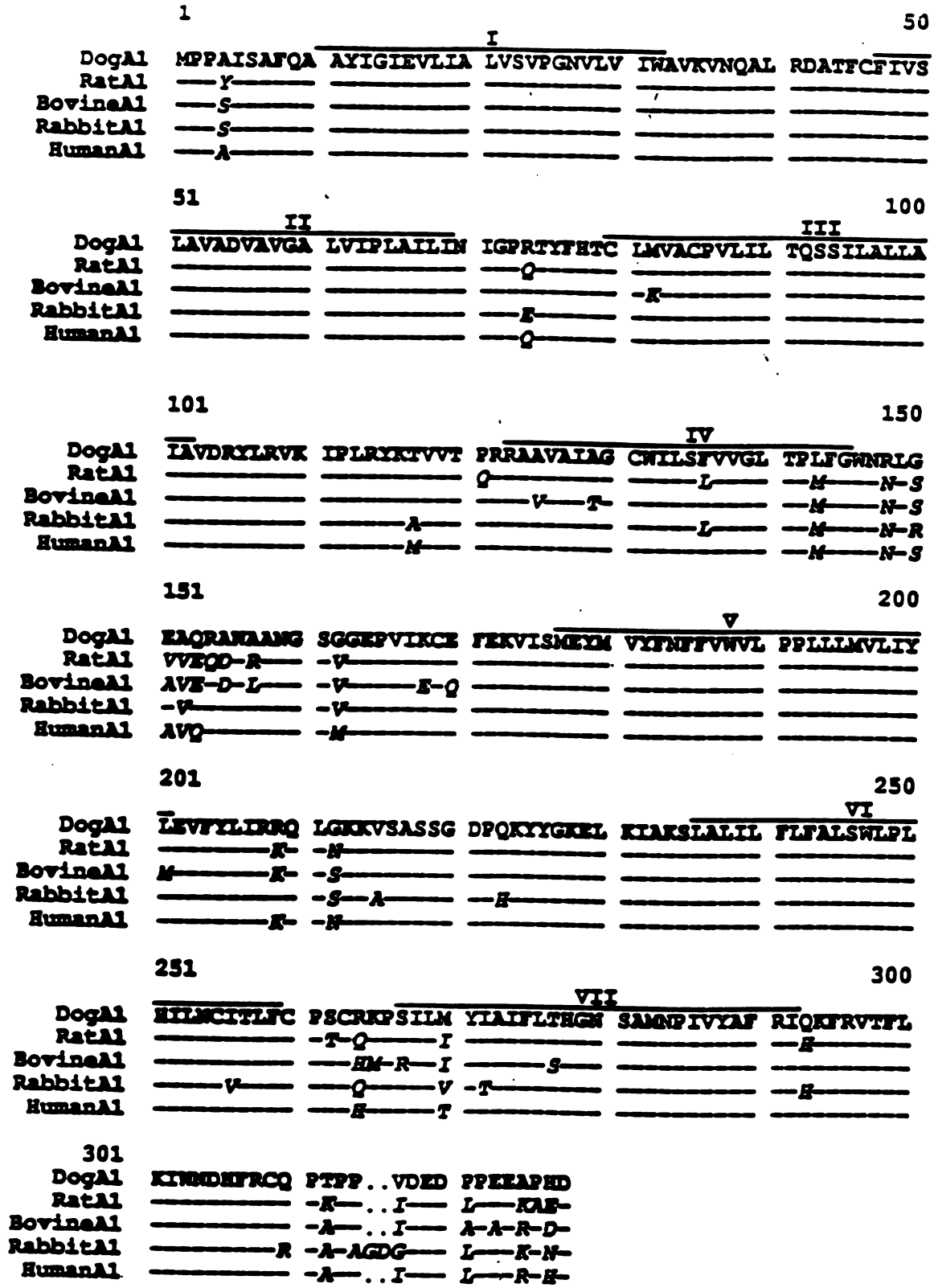

A.
Step 1. PCR of 3.2 kb Xho I fragment
Sac I
Primer 2
5'
3'
Eco RI
Primer 1
PCR
Eco RI
Sac I
5'
3'
SB4
Sac I
5'
3'
Pst I
B
Step 2 A. Subcloning
Digestion of PCR product with Eco RI and Sac I and ligation to pUC19 vector
pUC 19 + 5'ORF
Eco RI
Sac I
Pst I
Step 2 B. Digestion of SB4 with Sac I and Pst I
Sac I
Pst I
5'
3'
C.
Step 3 Ligation of SB4 (Sac I/Pst I) fragment to pUC19+5'ORF
pUC 19 +RbAI
Eco RI
Sac I
Pst I

Figure 11 . The nucleotide sequence of the rabbit A1AR clones and its deduced amino acid sequence. The oligonucleotide probe WSS1 derived from the canine A1AR (Libert et al. 1991) was used to isolate a 2-kb rabbit A1AR cDNA (SB4) comprised of nucleotide 260-2215. The 5'-end of SB4 and a PCR fragment derived from the 5'-end of the rat A1AR were used to isolate a genomic clone (clone-1), which contained the 5'-UTR, an exon of 341 nucleotide and a part of the first intron of the rabbit A1AR gene. The region of overlap between exon-1 of genomic clone-1 and SB4 is shown in bold (nucleotide 260-378). The putative mRNA splice site is underlined. The 5'-end and the 3'-end UTRs are shown in lower case whereas capital letters indicate the open reading frame. The stop codon is shown as an asterisk. The sequence is registered with the GeneBank Database under accession No. L01700.

```
   1  cgtgccgttc tgctgatgtg ccccagcctgt gccccgccATG CCGCCCTCCA TCTCGGCCTT   60
                                              M   P  P  S  I   S  A  F    8

  61  CCAGGCCGCC TACATTGGCA TCGAGGTGCT CATCGCGCTG GTCTCGGTGC CAGGGAACGT  120
   9  Q  A  A   Y  I  G  I   E  V  L   I  A  L   V  S  V  P   G  N  V   28

 121  GCTGGTGATC TGGGCCGTGA AGGTGAACCA GGCACTGCGG GACGCCACCT TCTGCTTCAT  180
  29  L  V  I   W  A  V  K   V  N  Q   A  L  R   D  A  T  F   C  F  I   48

 181  CGTGTCGCTG GCAGTGGCTG ACGTGGCCGT GGGCGCCCTG GTCATCCCGC TGGCCATCCT  240
  49  V  S  L   A  V  A  D   V  A  V   G  A  L   V  I  P  L   A  I  L   68

 241  CATCAACATC GGCCCCGAGA CCTACTTCCA CACCTGCCTC ATGGTGGCCT GTCCTGTCCT  300
  69  I  N  I   G  P  E  T   Y  F  H   T  C  L   M  V  A  C   P  V  L   88

 301  CATCCTCACC CAGAGCTCCA TCCTGGCCCT GCTGGCCATC GCGGTGGACC GCTACCTCGG  360
  89  I  L  T   Q  S  S  I   L  A  L   L  A  I   A  V  D  R   Y  L  R   108

 361  CGTCAAGATT CCTCTCCGGT ACAAGGCAGT GGTGACGCCG CGCAGGGCGG CGGTAGCCAT  420
 109  V  K  I   P  L  R  Y   K  A  V   V  T  P   R  R  A  A   V  A  I   128

 421  CGCCGGCTGC TGGATCCTCT CGCTCGTGGT GGGCCTGACG CCCATGTTCG GCTGGAACAA  480
 129  A  G  C   W  I  L  S   L  V  V   G  L  T   P  M  F  G   W  N  N   148

 481  CCTGCGGGAG GTGCAGCGGG CCTGGGCGGC CAACGGCAGC GTCGGGGAGC CGGTGATCAA  540
 149  L  R  E   V  Q  R  A   W  A  A   N  G  S   V  G  E  P   V  I  K   168

 541  GTGCGAGTTC GAGAAGGTCA TCAGCATGGA GTACATGGTG TACTTCAACT TCTTCGTGTG  600
 169  C  E  F   E  K  V  I   S  M  E   Y  M  V   Y  F  N  F   F  V  W   188

 601  GGTGCTGCCC CCGCTACTGC TCATGGTCCT CATCTACCTG GAGGTCTTCT ACCTGATCCG  660
 189  V  L  P   P  L  L  L   M  V  L   I  Y  L   E  V  F  Y   L  I  R   208

 661  CCGGCAGCTC AGCAAGAAGG CGTCGGCCTC CTCCGGAGAC CCGCACAAGT ACTACGGCAA  720
 209  R  Q  L   S  K  K  A   S  A  S   S  G  D   P  H  K  Y   Y  G  K   228

 721  GGAGCTGAAG ATCGCCAAGT CGCTGGCCCT CATCCTCTTC CTATTCGCCC TCAGCTGGCT  780
 229  E  L  K   I  A  K  S   L  A  L   I  L  F   L  F  A  L   S  W  L   248

 781  GCCTCTGCAC ATCCTGAACT GTGTCACCCT CTTCTGCCCA TCCTGCCAGA AGCCCAGCAT  840
 249  P  L  H   I  L  N  C   V  T  L   F  C  P   S  C  Q  K   P  S  I   268

 841  CCTCGTCTAC ACCGCCATCT TCCTCACGCA CGGCAACTCG GCCATGAACC CCATCGTCTA  900
 269  L  V  Y   T  A  I  F   L  T  H   G  N  S   A  M  N  P   I  V  Y   288

 901  CGCCTTCCGC ATCCACAAGT TCCGGGTCAC CTTCCTCAAG ATCTGGAACG ACCACTTCCG  960
 289  A  F  R   I  H  K  F   R  V  T   F  L  K   I  W  N  D   H  F  R   308

 961  CTGCCGGCCC GCACCCGCCG GCGACGGCGA CGAGGACCTC CCGGAAGAGA AGCCCAACGA 1020
 309  C  R  P   A  P  A  G   D  G  D   E  D  L   P  E  E  K   P  N  D   328

1021  CTAGgcgctg ccctctgctc ttccagccca gccggtcctc ccctcccctc cgccccaagg 1080
                •                                                      348

1081  ggcccctttcc caaaggagtg cacccctgca ccccgggggct cggagtgggg agggaacccg 1140
1141  cgccccaggg cgcgaggagc gtcttgagcc cacccacctg cctgaccacc ccaggagtga 1200
1201  gcagggcccc cgttcaaggg gtgggtgggc cccgggggagg ctgggcgtgc agagggggccg 1260
1261  tgtgggcgga tgaggctgcc tggacttctg cgccaggcag gggtctgctt gttgggggtgc 1320
1321  tggtgatgca gaagacagga tgctctccgg gagggagggg agcagcacgt ggggaggggg 1380
1381  tgtcttggcc ttgctttctc tgcgcagcag aaggggctca gaagcagcta agggacagga 1440
1441  ctcaaggagc ctccatcccc acctccaagg actctgccca gggccgtgcc aggtgccagg 1500
1501  cggccgacte aggatcgcac tttggagagg cagaaagagt gggtccaaaa atcatttctg 1560
1561  acctgggctt ttattctgca ccagcccttg gactgtgggc agggggtggag tggtgggtgc 1620
1621  tggcctcaca cagccaccag gcggcagcac tgagcccctcc ttgcccctgca cctcgctggg 1680
1681  cggcgcctgg ctgtgccagc cggatggcac cgtgtatact gcccaatgga gggggaaccc 1740
1741  aaccatgcca acatgctgcc tgccagcccc ccccaggaag acgaagaggg gaagggtccc 1800
1801  tgcaggcttg gtcggtgctc accggggcac ctgcgggggtg ggaagtggcg ggcaggagtc 1860
1861  tcaatcacct gggcccacca cagtttaata gaaggcaatc gcaaaaacat acagcaagct 1920
1921  ctagccatta ccattctcct aggtatctat tttacctac ttcaagcatc ggagtattac 1980
1981  gaaacatctt ttactatttc agatggagtc tacggatcaa cattcttcat agccacagga 2040
2041  tttcacggtc ttcacgtcat cattgggtcc acttttctta cggtctgcct tctacgacaa 2100
2101  tttaactttc actttacatc gaaccaccac tttggttttg aagcagccgc atgatactga 2160
2161  cactttgtag atgtagtctg actattccta tatgtatcaa tctattgatg agggt       2215
```

Figure 12 . Comparison of the deduced amino acid sequences of canine, rat, bovine, rabbit and human A1AR. Identical amino acids are represented by dashes and the dissimilar amino acids are in italics. Solid lines represent putative transmembrane domains.


1
50
I
DogAl MPPAISAFQA AYIGIEVLIA LVSVPGNVLV IWAVKVNQAL RDATFCFIVS
RatAl —Y— — — — — — — — — —
BovineAl —S— — — — — — — — — —
RabbitAl —S— — — — — — — — — —
HumanAl —A— — — — — — — — — —
51
100
II
III
DogAl LAVADVAVGA LVIPLAILIN IGPRTYFHTC LMVACPVLIL TQSSILALLA
RatAl — — — — — — — — —Q— — — — — — — — — —
BovineAl — — — — — — — — — — — — — — — —K— — — — — —
RabbitAl — — — — — — — — —E— — — — — — — — — —
HumanAl — — — — — — — — —Q— — — — — — — — — —
101
150
IV
DogAl IAVDRYLRVK IPLRYKTVVT PRRAAVAIAG CWILSFVVGL TPLFGWNRLG
RatAl — — — — — — — — — — — — — — —Q— — — — — —L— — — —M— — —N-S
BovineAl — — — — — — — — — — — — —V— —T— — — — — — — —M— — —N-S
RabbitAl — — — — — — — —A— — — — — — — — — — — —L— — — —M— — —N-R
HumanAl — — — — — — — —M— — — — — — — — — — — — — — —M— — —N-S
151
200
V
DogAl EAQRAMAANG SGGEPVIKCE FEKVISMEYM VYFNFFVWVL PPLLLMVLIY
RatAl VVEQD-R— —V— —
BovineAl AVE-D-L— —V— — —E-Q — — — — — — — — — — — — — — — —
RabbitAl —V— — — — — —V— — — — — — — — — — — — — — — — — —
HumanAl AVQ— — — — — —M— — — — — — — — — — — — — — — — — —
201
250
VI
DogAl LEVFYLIRRQ LGKKVSASSG DPQKYYGKEL KIAKSLALIL FLFALSWLPL
RatAl — — — — — —K— —N— — — — — — — — — — — — — — — — — —
BovineAl M— — — — — —K— —S— — — — — — — — — — — — — — — — —
RabbitAl — — — — — — — —S—A— — — —H— — — — — — — — — — — —
HumanAl — — — — — — — —K— —N— — — — — — — — — — — — — — —
251
300
VII
DogAl HILNCITLFC PSCRKPSILM YIAIFLTHGN SAMNPIVYAF RIQKFRVTFL
RatAl — — — — — — — —T-Q— — — — —I — — — — — — — — — —H— — — —
BovineAl — — — — — — — —HM-R— —I — — — — —S— — — — — — — — —
RabbitAl — — — —V— — — —Q— — — —V —T— — — — — — — — — —H— — — —
HumanAl — — — — — — — —H— — — — —T — — — — — — — — — — — —
301
DogAl KIMMDHFRCQ PTPP..VDED PPEEAPHD
RatAl — — — — — — — — —K— —..I— — — L— — —KAE-
BovineAl — — — — — — — — —A— —..I— — — A-A-R-D-
RabbitAl — — — — — — — — —R —A-AGDG— — — L— — —K-N-
HumanAl — — — — — — — — —A— —..I— — — L— — —R-E-

al. 1992). Mutation of His-278 to Leu-278 decreased both agonist and antagonist binding by 90%. When His-251 was mutated to Leu-251 antagonist affinity and the number of receptors recognized by the antagonist decreased, however the binding affinity of agonist remained the same although the number of receptors detected by an agonist was reduced. These His residues are conserved among all of the A1AR isolated so far. The major differences between the various A1ARs are in the C-termini, in the transmembrane domain IV and VII, and in the extracellular loop II and III.

D. DISCUSSION

A partial cDNA encoding an A1AR which lacks nucleotides corresponding to those coding for the first 74 amino acids of the canine A1AR was isolated from a rabbit kidney cDNA library. The remaining 5'-end was obtained from a rabbit genomic library clone which contained the 5'-flanking region, the first exon, and part of the first intron. Together, the cDNA and the genomic clone provided the entire open reading frame encoding the rabbit A1AR. This conclusion was based on a comparison of the open reading frame obtained from the rabbit clone to other cloned A1ARs.

The deduced amino acid sequence of the rabbit A1AR was shown to possess a significant homology to A1ARs from canine, rat, bovine and human. The encoded proteins are all identical in molecular mass with 326 amino acids except for the rabbit A1AR which has 328 amino acids. The five A1ARs cloned to date are 92% identical in their overall amino acid structure. The rabbit A1AR differs from the canine, rat, bovine and human receptors by 26, 26, 33, and 23 amino acid substitutions, respectively. It is

generally believed that the transmembrane domains of the G-protein coupled receptors are involved in ligand binding (Strader et al. 1989; O'Dowd et al. 1989) and therefore are more conserved within species. Comparisons of the putative transmembrane domains of the A1ARs isolated from different species revealed the presence of only 6, 4, 11 and 4 amino acid differences between the canine, rat, bovine and human A1ARs. There are 100% identity in the transmembrane domains I and II while the most diverse are domains IV and VII.

The recent cloning and purification of the A1AR and A2AR and the development of selective radioligands will help to understand the structure, function and regulation of the adenosine receptor systems. Although the physiological effects of adenosine have been well characterized for more than 60 years, only recently have the receptors been cloned which will help us to understand the system at the biochemical and molecular biological levels.

IV. CHARACTERIZATION OF THE A1 ADENOSINE RECEPTOR GENE AND ANALYSIS OF TRANSCRIPTION START SITE HETEROGENEITY

A . INTRODUCTION

Although a physiological role of adenosine have been observed since the last 70 years, (Drury and Szent-Gyorgi 1929; and Burne 1963) it is only recently that the cDNA for the adenosine receptors have been cloned thereby allowing for investigations at the molecular level. Recently, we and others have reported the cloning, sequence analysis and expression of the cDNA encoding both the A2 and the A1 adenosine receptors (Libert et al.1989; Maenhaut et al. 1990; Reppert et al. 1991; Libert et al. 1992; Olah et al. 1992; Bhattacharya et al. 1993). Analysis of both A1 and A2 receptors amino acid sequence and function indicated the proteins belong to the superfamily of the G-protein coupled receptors. This superfamily consists of a diverse group of receptors in terms of ligand binding and functional characteristics, yet they all appear to share common structural features (i.e. seven transmembrane domains) and mechanisms of signal transduction (i.e. activation of specific G-proteins). In addition to the adenosine receptors, this receptor superfamily includes the opsins (Nathans and Hogness et al. 1983), the adrenergic receptors (Lefkowitz and Caron 1988), the muscarinic receptors (Kubo et al. 1986; Peralta et al. 1987; Bonner et al.1987; Bonner et al 1988; Shapiro et al. 1988), dopamine receptors (Grandy et al. 1989; Sokoloff et al. 1990; Van Tol et al. 1991), thyroid

stimulating hormone receptors (Parmentier et al. 1989), leutinizing hormone receptors (Loosfelt et al. 1989; McFarland et al. 1989), endothelin receptor (Hosoda et al. 1992), tachykinin receptors (Hershey et al. 1991; Takahashi et al. 1992), the secretin receptor (Ishihara et al. 1991), the cannabinoid receptor (Gerard et al. 1991), the serotonin 1c receptor (Julius et al. 1988), thromboxane A2 receptor (Hirata et al. 1991), the antidiuretic hormone receptor (Birnbaumer et al. 1992; Alain et al. 1992; Lolait et al. 1992), the oxytoxin receptor (Kimura et al. 1992), the product of the oncogene *mas* (Young et al. 1986; Jackson et al. 1988), the yeast mating factor receptors (Burkholder et al. 1985; Nakayama et al. 1985), and a dictyostelium cAMP receptor (Klien et al. 1988).

A distinguishing feature that all G-protein coupled receptors have in common is their genomic structure. Most of the receptor genes that belong to this superfamily have been found to be intronless, although there are some exceptions including the dopamine D2, D3 and D4 receptors (Grandy et al. 1988; Sokoloff et al. 1990; Van Tol et al. 1991), the opsins (Nathans and Hogness et al. 1984), the tachykinins (Hershey et al. 1991; Takahashi et al. 1992), the leutinizing hormone receptors (Tsai-Morris et al. 1993) and the endothelin receptor (Hosoda et al. 1992). In the present work I have cloned and characterized the gene encoding the A1AR which belongs to the intron containing G-protein linked receptor gene family.

Adenosine receptors are found in a wide variety of cell types and tissues and are known to be regulated by agonist-induced desensitization (Parsons et al. 1987; Longabaugh et al. 1989), antagonist-induced sensitization (Ramkumar et al. 1988), thyroid hormone (Rapiejko and Malbon 1987) and by glucocorticoids (Gerwins and

Fredholm 1991). Little is known about the biochemical mechanisms underlying the regulation of this receptor system. To begin to investigate the regulation of the receptors, I have cloned and characterized the 5'-flanking region of the gene encoding the rabbit A1AR using the A1AR cDNA from rabbit as a probe (Bhattacharya et al. 1993).

B . MATERIALS AND METHODS

MATERIALS :

Reagents were obtained as follows : [γ-^{32}P]ATP (specific activity, 6000 Ci/mmol), [α-^{32}P]dCTP (specific activity, 800 Ci/mmol), and [^{35}S]dATP (specific activity, 1200 Ci/mmol) were from DuPont-NewEngland Nuclear; restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, and a random primer DNA labelling kit, were from Boehringer-Manheim; RNAse inhibitor was from Promega; an avian myeloblastosis virus reverse transcriptase was from GIBCO; λ-EMBL3 SP6/T7 rabbit genomic library containing *Sau*3AI partial digest of rabbit kidney DNA was from Clontech; nitrocellulose filters (pore size 0.45 µm) were from Schleicher & Schuell; agarose (type I) was from Sigma; Bluescript KS(+) was from Stratagene; a Taq dye primer cycle sequencing kit was from Applied Biosystems; DNA sequencing Kit was from United States Biochemicals; oligonucleotides were synthesized by the Macromolecular Structure, Synthesis, and Sequencing Facility Laboratory at Michigan State University and by the Sequencing Laboratory at SmithKline Beecham Pharmaceuticals, Philadelphia.

METHODS :

Analysis of the gene copy number : Rabbit kidney genomic DNA (Clontech), was digested with restriction enzymes *Bam*HI and *Pst*I, electrophoresed on 1% agarose gels, transferred to nitrocellulose and immobilized by baking for 1 hour at 80°C. A 3'-*Hae*II fragment of 658 bp derived from 3'-untranslated region of SB4 was labelled with [α-^{32}P]dCTP (800 Ci/mmol) by random priming and hybridized to genomic DNA blots in 20% formamide, 6X SSPE (0.75 M NaCl, 0.05 M NaH_2PO_4, 0.005 M EDTA, pH 7.4), 5X Denhardt's solution, 0.1% Sodium Dodecyl Sulphate, 0.2 mg/ml Salmon Sperm DNA at 42°C for overnight. DNA blots were washed in 2X SSC (0.3 M NaCl, 0.03 M Sodium Citrate, pH7.0) containing 0.1% SDS for 15 min at room temperature followed by a 15 min wash with 0.2X SSC containing 0.1% SDS at 55°C.

Selection and analysis of genomic clones : As mentioned in chapter III a rabbit genomic library was screened with a gel-purified *Eco*RI-*Sma*I fragment containing the coding sequence of the previously cloned cDNA encoding the rabbit A1AR, SB4 (Bhattacharya et al. 1993). The cDNA probe was randomly labelled with [α-^{32}P]dCTP (800 Ci/mmol) by random primer synthesis and approximately 2X 10^6 recombinants were screened at moderate stringency (20% formamide at 42°C for overnight). Eight genomic clones were isolated from the library which were further purified and amplified. As mentioned in chapter III, one genomic clone named as clone-1, contained the 5'-UTR, exon-1 and a part of intron-1. In order to isolate the rest of the gene, phage DNA from the remaining 7 genomic clones were isolated and characterized by restriction

endonuclease mapping and Southern Blot analysis, using the cDNA encoding the rabbit A1AR, the primers originating from different regions of the A1AR cDNA isolated from rabbit and also the cDNA for the canine A2AR as probes. The A2AR was used as a probe to distinguish and eliminate clones that would produce stronger signals with A2AR probes in comparison to A1AR probes. The pattern of hybridization of the restriction digests of these genomic clones to the different A1AR cDNA fragments, primers originating from the cDNA, and the canine A2AR cDNA, facilitated in identifying three overlapping genomic clones namely, clone-4, 5 and 7, containing a 2.3 kb *Xho*I restriction fragment which contained the remaining part of the gene encoding the A1AR cDNA. Clone-7 was used as a representative of the three clones for characterization.

The DNA restriction fragments of clone-7, containing exons and flanking regions of the gene were subcloned into the M13mp19 or pUC19 vectors and sequenced. Sequencing was performed by the dideoxy method (Sanger et al. 1977) with Sequanase version 2.0 (United States Biochemical) using either double stranded pUC 19 or single stranded M13mp19. Sequencing data indicated that genomic clone-7 contained a part of intron-1 and exon-2. Since a polyadenylation signal was not obtained from the 2.3 kb fragment of genomic clone-7, direct sequencing of the phage DNA from clone-7 was performed using the TaqDyedeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems) until a polyadenylation signal was obtained.

A restriction map of the A1AR gene was obtained from Southern blot analysis using the T7 and SP6 primers as well as primers that were synthesized corresponding to the 5' and 3'-ends of the A1AR cDNA. These oligonucleotide primers were end labeled with [γ-^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase and were used as probes

for Southern hybridizations of restriction digests of genomic clones.

The size of intron-1 was determined by restriction digest and Southern blotting of the genomic DNA isolated from rabbit (Clontech) using fragments from two ends of intron-1. Digestion of genomic DNA was carried out for 24 hours with 10 µg of DNA and 100 U of the restriction enzyme *Eco*RI, blotted onto nitrocellulose, baked at 80°C and hybridized for 24 hrs using cDNA and genomic probes labeled randomly with [α-^{32}P]dCTP (800 Ci/mmol).

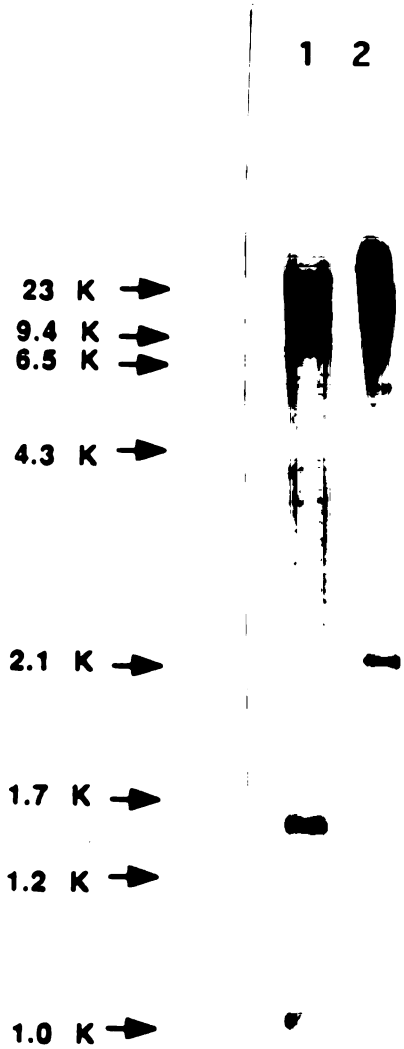
Primer Extension Analysis : RNA extracted from rabbit brain (Sambrook et al. 1989) was used to isolate poly(A)$^+$ RNA by chromatography on oligo(dT) cellulose (Pharmacia) and used for primer extension analysis. Two 30 mer oligonucleotides, WSS23, 5'-CTCGATGCCAATGTAGGCGGCCTGGAAGGC-3' complementary to base pairs 19 to 48 of the rabbit A1AR gene; and WSS30 5'-AGCCCTTCCTCTG-CCTAGGTCCCCTCCCTT-3', complementary to the base pairs -206 to -177 of the A1AR gene, starting from the translation start site, were used in primer extension. These oligonucleotides were end labelled at the 5'-ends with T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol) to a specific activity of 2X 10^8 cpm/µg. Labeled primer (2X 10^6 cpm) was combined with 10 µg of poly(A)$^+$ mRNA in 30 µl of 40 mM Pipes pH 6.4, containing 1 mM EDTA pH 8.0, 0.4 M NaCl, and 80 % formamide. Samples were denatured for 10 min at 85°C and then hybridized for overnight at 30°C. The resultant oligonucleotide:mRNA hybrids were precipitated with ethanol and resuspended in 20 µl of 25 mM Tris-Cl, pH 8.3, containing 10 mM dithiothreitol, 37.5 mM KCl, 1.5 mM $MgCl_2$, 40 U RNasein, and 1 mM each of dATP, dCTP, dGTP and dTTP. This was

subjected to reverse transcription by addition of M-MLV reverse transcriptase (200 U) and incubating at 50°C for 60 min following extraction with phenol:chloroform (1:1) and ethanol precipitation. The pellet was resuspended in 80% formamide sample buffer. The products were denatured by heating to 95°C and then loaded onto a 5% polyacrylamide gel containing 7 M urea along with sequencing reactions using the 3.2 kb *Xho*I fragment as the template.

C . RESULTS

Gene copy number determination: To determine the organization and the structure of the rabbit A1AR gene, Southern blot analysis of the chromosomal DNA isolated from rabbit kidney was performed. As shown in Figure 13, a single hybridization band was obtained on using a probe from the 3'-end of the rabbit A1AR cDNA, SB4 (*Hae*II fragment, 658 bp; Figure 17). Digestion of the genomic DNA with *Bam*HI or *Pst*I gave rise to a single positive band (2.1 kb for *Bam*HI and 1.5 kb for *Pst*I) that correlated with the genomic restriction map (Figure 17). A smear of radioactivity was however, observed at the upper portion of the blot, which may indicate the presence of other undigested restriction fragment or may be due to the presence of multiple copies of the gene. Therefore this preliminary observation may indicate the presence of a single copy of the A1AR gene and the absence of pseudogenes in the rabbit genome, but for further verification of this data rabbit genomic DNA needs to be digested with other restriction enzymes and probed with different regions of the cDNA or the gene.

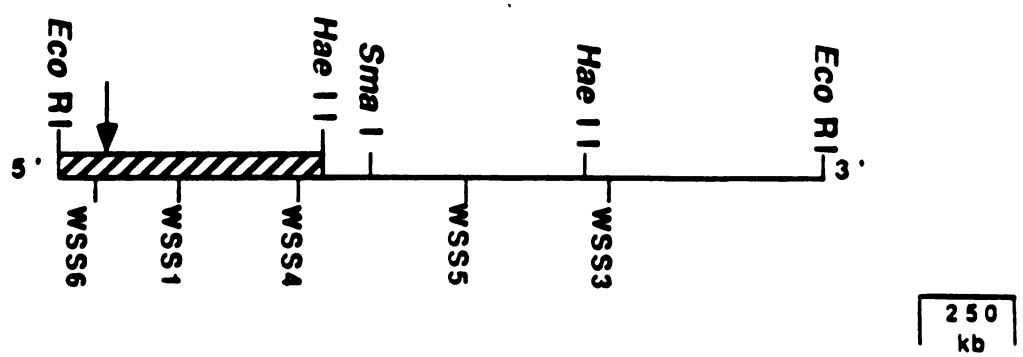
Figure 13 . Analysis of the rabbit A1AR gene by Southern blot hybridization. High molecular weight rabbit DNA (10 µg/lane) digested with *Pst*I and *Bam*HI restriction enzymes (lane 1 and 2 respectively), size fractionated by electrophoresis on 1% agarose gel, transferred to nitrocellulose, immobilized and probed with rabbit A1AR cDNA (658 bp *Hae*II fragment from 3'-UTR), as described under "Materials and Methods". Sizes of the molecular weight markers are indicated on the left.


1
2
23 K
9.4 K
6.5 K
4.3 K
2.1 K
1.7 K
1.2 K
1.0 K

Isolation and characterization of genomic clones encoding rabbit A1AR: A rabbit cDNA clone encoding the A1AR (SB4) has previously been isolated and characterized (Bhattacharya et al. 1993). With an 860 bp *Eco*RI/*Sma*I fragment of SB4 we screened a rabbit genomic library, a λ-EMBL3 library (Figure 7) and isolated eight positive clones: clone-1, clone-2, clone-4, clone-5, clone-6, clone-7, clone-2.1 and clone-2.2. As mentioned before clone-1 was found to contain the 5' flanking region, exon-1 and part of intron-1. So in order to investigate which of the other seven clones contained the rest of the gene encoding the A1AR, we decided to characterize the remaining genomic clones. The phage DNA isolated from the genomic clones were digested with *Xho*I and Southern blot analysis was carried out using the probes depicted in Figure 14. The A2AR has not been cloned from rabbit, but data suggests that A2AR cloned so far from different species show a high degree of identity in their amino acid sequences (Figure 3) within species and also to the A1AR sequence cloned from different species. We therefore used RDC8, the canine A2AR, as a probe to isolate and eliminate the clones that would give stronger signals with RDC8 in comparison to SB4.

Results of Southern blot analysis are shown in Figure 15 and 16 and is summarized in Table III. The results indicated as follows: 1) clone-2 and 6 hybridized strongly to the canine A2AR cDNA probe as opposed to the rabbit A1AR cDNA, and therefore may contain the gene encoding the A2AR; 2) clone-2.1 and 2.2 are identical clones that hybridize more strongly to the rabbit A1AR cDNA probe but did not hybridize to all the other primers made from the cDNA, and hybridized less strongly to A2AR cDNA. A 2.2 *Sac*I restriction fragment from clone 2.1 which hybridized to most of the cDNA probes was sequenced. When this sequence was compared to the SB4

Figure 14 . The different probes used for characterization of the genomic clones. 1) *Eco*RI and *Sma*I fragment from the 5'-end and *Eco*RI and *Hae*II fragment from the 3'-end of the rabbit cDNA, SB4; the primers designed from different regions of SB4, namely, WSS6, WSS1, WSS4, WSS5 and WSS3; and the canine A2AR cDNA, RDC8. The arrow denotes the position of the intron-1 with respect to the cDNA sequence.



SB4

1. SB4, 5'-end *Eco* R I/*Sma* I fragment
2. SB4, 3'-end *Eco* R I/*Hae* II fragment
3. WSS6, 17 mer, position 80
4. WSS1, 60 mer, position 286
5. WSS4, 17 mer, position 642
6. WSS5, 17 mer, position 1124
7. WSS3, 17 mer, position 1465
8. RDC8, the canine A2 adenosine receptor cDNA.

Figure 15 . Southern blot analysis of eight genomic clones. The phage DNA isolated from each clone was digested with *Xho*I, size fractionated on 1% agarose gel, transferred to nitrocellulose, immobilized and hybridized with different probes. Blots A-D are from the same gel, each containing *Xho*I digest of the eight genomic clones. The clones are designated on the top of each lanes. The radiolabelled probes used for high stringency hybridization were the following region of the cDNA SB4: blot (A) 5'-end *Eco*RI and *Sma*I fragment; (B) 3'-end *Eco*RI and *Hae*II fragment (C) WSS1 and (D) WSS3. Shown to the left are the sizes in kb of the molecular weight markers.

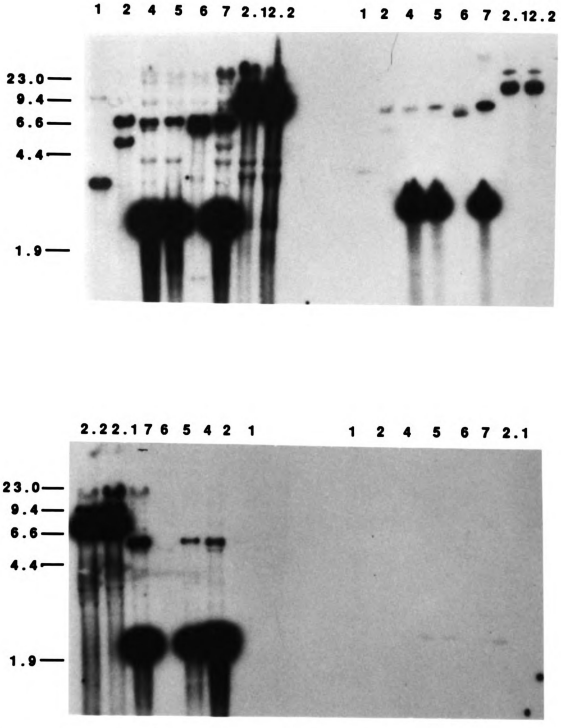

A.
B.
1 2 4 5 6 7 2.1 2.2
1 2 4 5 6 7 2.1 2.2
23.0
9.4
6.6
4.4
1.9
2.2 2.1 7 6 5 4 2 1
1 2 4 5 6 7 2.1
23.0
9.4
6.6
4.4
1.9

Figure 16 . Southern blot analysis of the genomic clones. The genomic clones are designated on top of each lanes. The radiolabelled probes used for each high stringency hybridization were (A) WSS4, (B) WSS5 and (C) RDC8, the canine A2AR. Shown to the left are the sizes in kb of the high molecular weight markers.

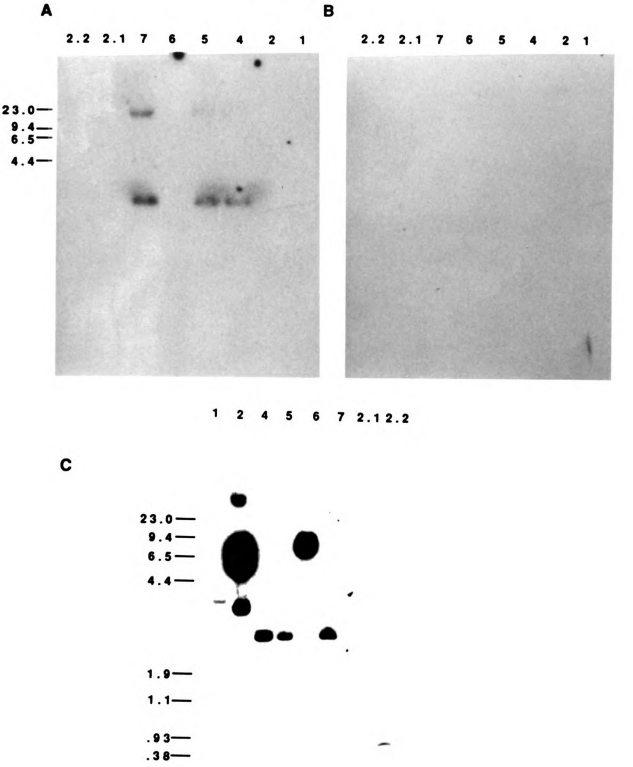
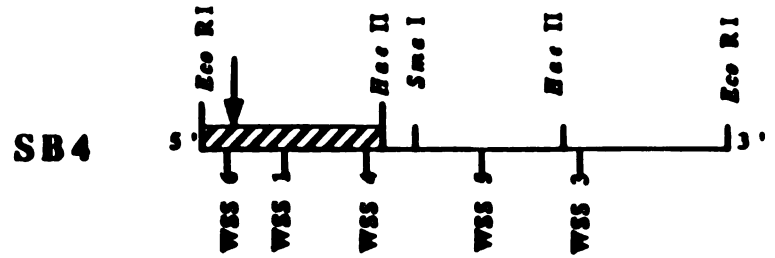

A
2.2 2.1 7 6 5 4 2 1
23.0
9.4
6.5
4.4
B
2.2 2.1 7 6 5 4 2 1
1 2 4 5 6 7 2.1 2.2
C
23.0
9.4
6.5
4.4
1.9
1.1
.93
.38

Table III **Southern blot analysis of the genomic clones.** The genomic clones were digested with restriction enzyme *Xho*I, size fractionated on an 1% agarose gel, transferred to nitrocellulose and probed with different regions of the A1AR cDNA (SB4) and the canine A2AR cDNA (RDC8) (depicted on the top pannel). The arrow depicts the position of intron-1 with respect to the cDNA. The table shows the probe used for hybridization and the results obtained. (+) denotes positive signal, (-) denotes negative signals and (++) denotes very strong positive signal.



GENOMIC CLONES		PROBES USED IN CHARACTERIZING THE GENOMIC CLONES							
Name	Fragment XhoI in kb	SB4 5'-E/S	SB4 3"-E/S	WSS1	WSS4	WSS5	WSS3	WSS6	RDC8
1	3 kb	++	-	-	-	-	-	++	+
2	6.5 kb	+	-	-	-	-	-	-	++
	5.4 kb	+	-	-	-	-	-	++	++
	3.1 kb	-	-	-	-	-	-	-	++
4	2.3 kb	++	++	++	+	-	+	-	+
5	2.3 kb	++	++	++	+	-	+	-	+
6	6.5 kb	+	-	-	-	-	-	-	++
	6.0 kb	+	-	-	-	-	-	++	++
7	2.3 kb	++	++	++	+	-	+	-	+
2.1	10 kb	++	++	++	-	-	-	-	+
2.2	10 kb	++	++	++	-	-	-	-	+

sequence, it matched 100% to a part of the third intracellular loop after which it completely diverged (data not shown). The genomic library was made with a partial digest of *Sau*3AI restriction enzyme and a *Sau*3AI restriction site was observed at the point of divergence. The data therefore indicated that they could encode for different adenosine receptor clone or may result due to an artifact of the genomic library; 3) a 2.3 kb *Xho*I fragment from three genomic clones, namely clone-4, 5, and 7 hybridized very strongly to both the 5'-end (*Eco*RI/*Sma*I) and 3'-end (*Eco*RI/*Hae*II) fragments and to all the sequencing primers except for WSS6, the primer upstream of intron-1, suggesting that clone-4, 5, and 7 are overlapping clones containing a major portion of the A1AR gene starting from a part of the first intron. Clone-7 was chosen arbitrarily as a representative for all three clones for characterization of the A1AR gene. The 2.3 kb *Xho*I fragment from clone-7 was subcloned and sequenced. Sequencing data indicated that genomic clone-1 and 7 contained the entire coding region of the gene in two exons, exon-1 present in clone-1 and exon-2 in clone-7.

Structure of the rabbit A1AR gene: Southern blot analysis and comparison of the sequence of the rabbit A1AR gene with that of the cDNA (Bhattacharya et al. 1993) established the restriction map of the A1AR gene (Figure 17). The size of intron-1 was deduced from Southern blot analysis. Genomic DNA from rabbit was digested with *Eco*RI and hybridized to the 3'-end of genomic clone-1 (the 3.2 kb *Xho*I fragment) and a 658 bp *Hae*II fragment from the 3'-untranslated region of the gene, present in genomic clone-7. The restriction enzyme *Eco*RI was selected based on the restriction map which indicated the absence of a restriction site for *Eco*RI in the intron segment present in the two genomic clones, clone-1 and 7. (Figure 17). As shown in Figure 18 both the probes

Figure 17 . Schematic representation of the rabbit A1AR gene and cDNA. (A), the restriction map of the two genomic clones, clone-1 and 7. There is no overlap between the two genomic clones; B, *Bam*HI; E, *Eco*RI; H, *Hae*II; P, *Pst*I; S, *Sfi*I; X, *Xho*I. (B), the schematic of the A1AR gene. The closed boxes represent the exons. The restriction sites described in A are represented by vertical lines. (C), the structure of the rabbit A1AR cDNA. The region encoding the membrane spanning domain are represented by closed boxes and are numbered. The region encoding the non-membrane spanning domain are represented by open boxes. The translation start site (ATG) and the termination point (GAC)are indicated.

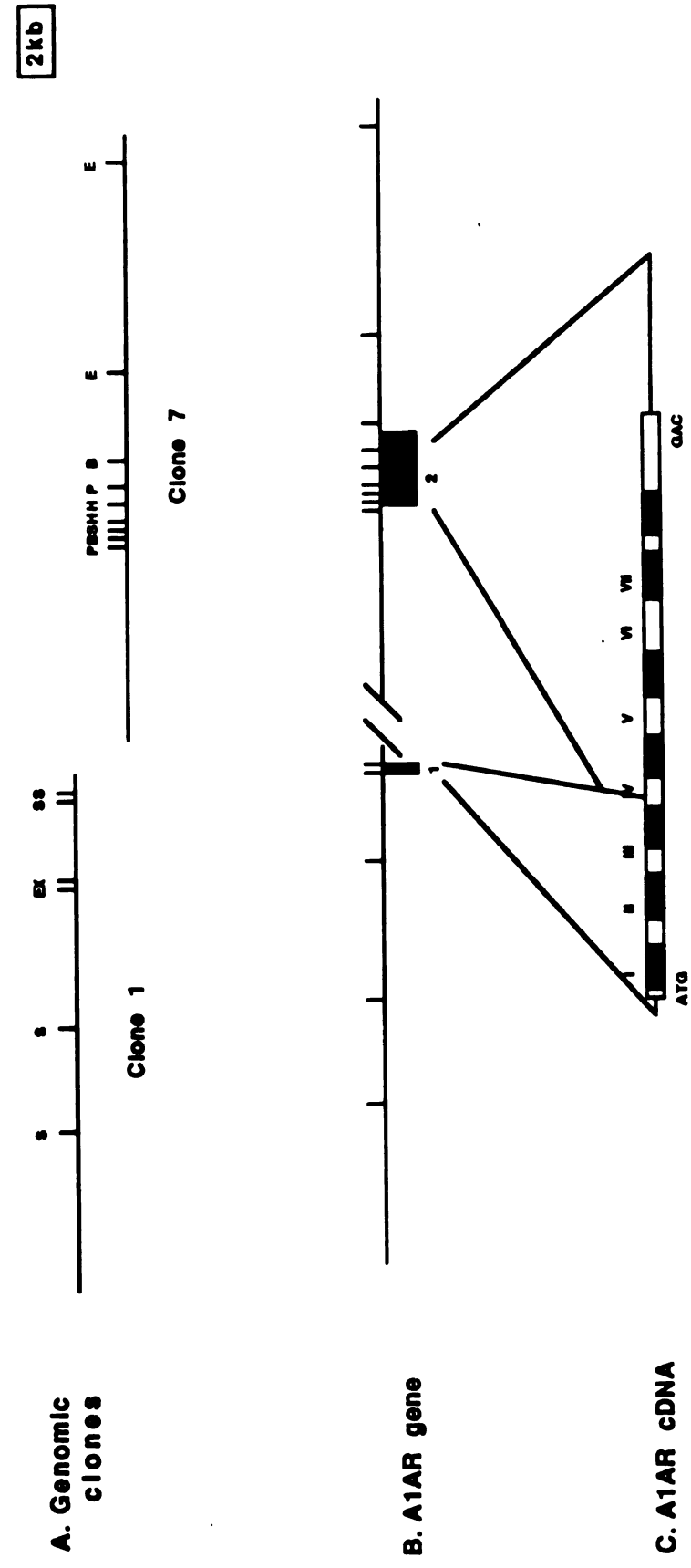
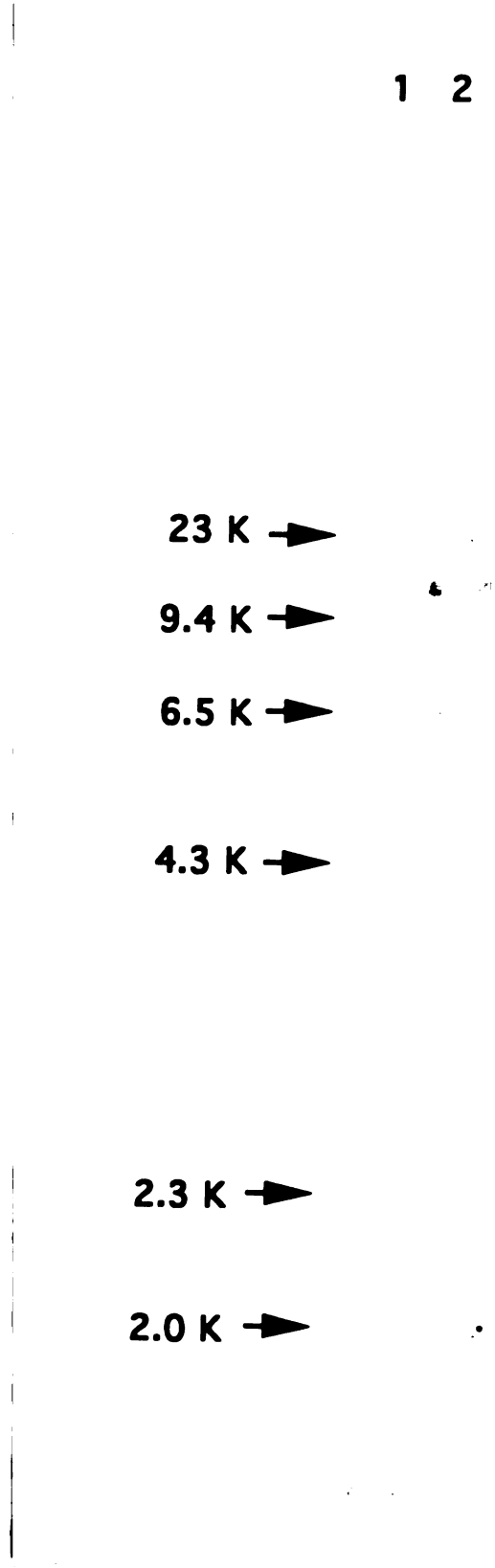

2kb
A. Genomic clones
Clone 1
Clone 7
B. A1AR gene
C. A1AR cDNA
ATG
TAC

Figure 18 . Southern blot hybridization analysis to determine the size of intron 1 of A1AR gene. 10 µg of rabbit genomic DNA digested with *Eco*RI were probed with the 5'-end of intron-1 (lane2) and the 3'-end of exon-2 (lane 1). Sizes in kb of the molecular weight markers are indicated on the left.


1 2
23 K
9.4 K
6.5 K
4.3 K
2.3 K
2.0 K

hybridized to fragments of different size. The 3.2 kb *Xho*I fragment hybridized to a 14 kb fragment and the 658 *Hae*II fragment hybridized to a 25 kb *Eco*RI fragment. Therefore by comparing to the restriction map (Figure 17) and by simple addition and substraction of the intron size obtained from the two genomic clones, namely clone-1 and 7, the size of intron-1 was calculated to be either equal to or greater than 34 kb. For better approximation of the intron size, the rabbit genomic DNA needs to be digested with restriction enzymes that recognizes eight bases, as *Not*I or *Sfi*I and probed with restriction fragments originating from 5' and 3'-ends of the intron-1, in order to obtain a single hybridization band with both the probes. Other G-protein linked receptor genes also have been known to contain introns of great length whose exact sizes are not determined. Some examples includes the human endothelin-A receptor (intron-2, >16 kb; intron-3 >11 kb;; Hosoda et al. 1992) and the Substance P receptor (intron-1 > 15 kb; intron-2 >23 kb; Hershey et al. 1991). As shown in Figure 19, the A1AR gene is composed of two exons and the splice site agrees with consensus donar/acceptor (GT/AG) splice site sequence (Breathnach and Chambon 1981).

Analysis of the 3'-flanking sequence of the rabbit A1AR gene: A potential polyadenylation sequence in the 3'-end of the gene was observed on sequencing the genomic clone-7. This feature has been reported to represent conserved areas for transcription termination and 3'-end processing (Birnstiel et al. 1985). However, on comparing the sequence obtained from the 3'-end of the gene to the cDNA revealed that the 3'-end of the gene does not match with the cDNA sequence. This region is shown in bold in Figure 19. Since polyadenylation signal was obtained at the 3'-end of the gene,

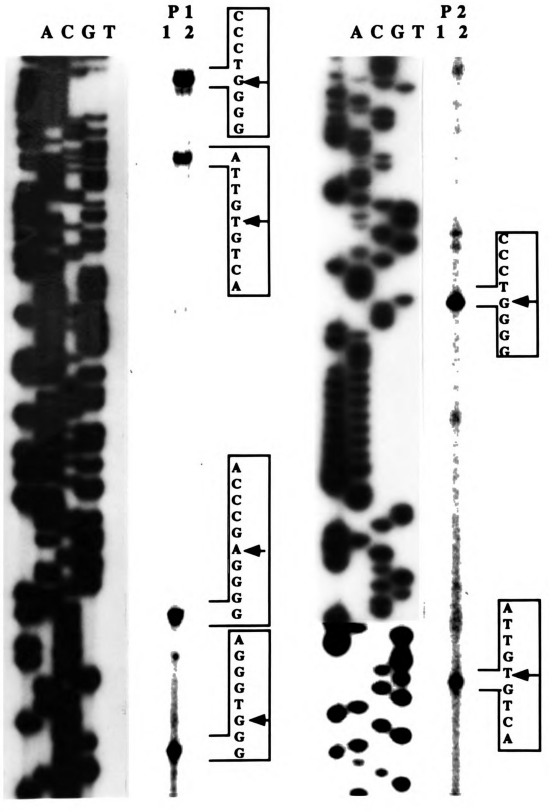
Figure 19 . The nucleotide and the amino acid sequence of the rabbit A1AR gene. Exon sequences are indicated in uppercase. The 3'-flanking region that do not match with the cDNA are indicated in bold. The polyadenylation signal is underlined.

```
   1  TTCTGCTGAT GTGCCCAGCC TGTGCTCGCC ATGCCGCCCT CCATCTCGGC CTTCCAGGCC GCCTACATTG
   1                                 M  P  P  S   I  S  A   F  Q  A   A  Y  I  G

  70  GCATCGAGGT GCTCATCGCG CTGGTCTCGG TGCCAGGGAA CGTGCTGGTG ATCTGGGCCG TGAAGGTGAA
  15    I  E  V   L  I  A   L  V  S  V   P  G  N   V  L  V   I  W  A  V   K  V  N

 140  CCAGGCACTG CGGGACGCCA CCTTCTGCTT CATCGTGTCG CTGGCAGTGG CTGACGTGGC CGTGGGCGCC
  38    Q  A  L   R  D  A   T  F  C  F   I  V  S   L  A  V  A   D  V  A   V  G  A

 210  CTGGTCATCC CGCTGGCCAT CCTCATCAAC ATCGGCCCCG AGACCTACTT CCACACCTGC CTCATGGTGG
  61    L  V  I   P  L  A   I  L  I  N   I  G  P   E  T  Y  F   H  T  C   L  M  V  A

 280  CCTGTCCTGT CCTCATCCTC ACCCAGAGCT CCATCCTGGC CCTGCTGGCC ATCGCCGTGG ACCGCTACCT
  85    C  P  V   L  I  L   T  Q  S  S   I  L  A   L  L  A   I  A  V  D   R  Y  L

 350  CCGCGTCAAG ATTCCTCTCC Ggtgagtcca ---------- (INTRON 1) ----gccccg cagGTACAAG
 108    R  V  K   I  P  L  R                                                Y  K

 379  GCAGTGGTGA CGCCGCGCAG GGCGGCGGTA GCCATCGCCG GCTGCTGGAT CCTCTCGCTC GTGGTGGGCC
 117  A  V  V  T   P  R  R   A  A  V   A  I  A  G   C  W  I   L  S  L   V  V  G  L

 449  TGACGCCCAT GTTCGGCTGG AACAACCTGC GGGAGGTGCA GCGGGCCTGG GCGGCCAACG GCAGCGTCGG
 141    T  P  M   F  G  W   N  N  L  R   E  V  Q   R  A  W   A  A  N  G   S  V  G

 519  GGAGCCGGTG ATCAAGTGCG AGTTCGAGAA GGTCATCAGC ATGGAGTACA TGGTGTACTT CAACTTCTTC
 164    E  P  V   I  K  C  E   F  E  K   V  I  S   M  E  Y  M   V  Y  F   N  F  F

 589  GTGTGGGTGC TGCCCCCGCT ACTGCTCATG GTCCTCATCT ACCTGGAGGT CTTCTACCTG ATCCGCCGGC
 187  V  W  V  L   P  P  L   L  L  M   V  L  I  Y   L  E  V   F  Y  L   I  R  R  Q

 659  AGCTCAGCAA GAAGGCGTCG GCCTCCTCCG GAGACCCGCA CAAGTACTAC GGCAAGGAGC TGAAGATCGC
 211    L  S  K   K  A  S   A  S  S  G   D  P  H   K  Y  Y   G  K  E  L   K  I  A

 729  CAAGTCGCTG GCCCTCATCC TCTTCCTATT CGCCCTCAGC TGGCTGCCTC TGCACATCCT GAACTGTGTC
 234    K  S  L   A  L  I  L   F  L  F   A  L  S   W  L  P  L   H  I  L   N  C  V

 799  ACCCTCTTCT GCCCATCCTG CCAGAAGCCC AGCATCCTCG TCTACACCGC CATCTTCCTC ACGCACGGCA
 257  T  L  F  C   P  S  C   Q  K  P   S  I  L  V   Y  T  A   I  F  L   T  H  G  N

 869  ACTCGGCCAT GAACCCCATC GTCTACGCCT TCCGCATCCA CAAGTTCCGG GTCACCTTCC TCAAGATCTG
 281    S  A  M   N  P  I   V  Y  A  F   R  I  H   K  F  R   V  T  F  L   K  I  W

 939  GAACGACCAC TTCCGCTGCC GGCCCGCACC CGCCGGCGAC GGCGACGAGG ACCTCCCGGA AGAGAAGCCC
 304    N  D  H   F  R  C  R   P  A  P   A  G  D   G  D  E  D   L  P  E   E  K  P

1009  AACGACTAGG CGCTGCCCTC TGCTCTTCCA GCCCAGCCGG TCCTCCCCTC CCCTCCGCCC CAAGGGGCCC
 327  N  D  *
1079  CTTCCCAAAG GAGTGCACCC CTGCACCCCG GGGCTCGGAG TGGGGAGGGA ACCCGCGCCC CAGGGCGCGA
1149  GGAGCGTCTT GAGCCCACCC ACCTGCCTGA CCACCCCAGG AGTGAGCAGG GCCCCCGTTC AAGGGGTGGG
1219  TGGGCCCCGG GGAGGCTGGG CGTGCAGAGG GGCCGTGTGG GCGGATGAGG CTGCCTGGAC TTCTGCGCCA
1289  GGCAGGGGTC TGCTTGTTGG GGTGCTGGTG ATGCAGAAGA CAGGATGCTC TCCGGGAGGG AGGGGAGCAG
1359  CACGTGGGGA GGGGGTGTCT TGGCCTTGCT TTCTCTGCGC AGCAGAAGGG GCTCAGAAGC AGCTAAGGGA
1429  CAGGACTCAA GGAGCCTCCA TCCCCACCTC CAAGGACTCT GCCCAGGGCC GTGCCAGGTG CCAGGCGGCC
1499  GACTCAGGAT CGCACTTTGG AGAGGCAGAA AGAGTGGGTC CAAAAATCAT TTCTGACCTG GGCTTTTATT
1569  CTGCACCAGC CCTTGGACTG TGGGCAGGGG TGGAGTGGTG GGTGCTGGCC TCACACAGCC ACCAGGCGGC
1639  AGCACTGAGC CCTCCTTGCC CTGCACCTCG CTGGGCGGCG CCTGGCTGTG CCAGCCGGAT GGCACCGTGT
1709  ATACTGCCCA ATGGAGGGGG AACCCAACCA TGCCAACATG CTGCCTGCCA GCCCCCCCCA GGAAGACGAA
1779  GAGGGGAAGG GTCCCTGCAG GCTTGGTCGG TGCTCACCGG GGCACCTGCG GGGTGGGAAG TGGCGGGCAA
1849  GGACTGGCCG GAGACCGCTC TCGAGGAAGG TCCACTGGCC CTTAGATGCC CAAAGCTCAG GCTGGGGCTT
1919  TGGGACGCTC TTGACAGATG GACTCCAGGC ATTTAGGGAT CTGGGAAAGG CCTGGCCTGC AGCCTCTGTG
1989  CACAGGGCCT GCTGGAAGGG GTTCTAGACC CTTCTGTTGG AGGCGGGTGT GAGGCCATGA GGCTAATAAA
2170  AGACCGGACC CTGGCGGAGA TCAT
```

it is highly unlikely that there is any artifact in the genomic clone. On the other hand, as reported before no polyadenylation tail was observed at the 3'-end of the cDNA although the library from which the cDNA clone was isolated was designed by reverse transcribing the mRNA using oligo dT primers. Therefore it is likely to be an artifact of the cDNA library.

Analysis of the transcription start site of the rabbit A1AR gene: The transcription initiation site of the A1AR gene was mapped by primer extensions using two primers WSS-23 (PE-1, 19 to 48) and WSS-30 (PE-2, -206 to -117) (Figure 21). In primer extension with the rabbit brain poly(A)$^+$ RNA, WSS-23 revealed four signals at -78, -106, -268 and -322 and WSS-30 revealed two strong signals at -268 and -322 (Figure 20). No bands were observed in the tRNA control lanes. WSS-23 produced a single large extended product and a smaller product (Figure 20). Since the primer WSS-30 produced a single extended product, this probably reflects an incomplete extension by primer WSS-23. RNase protection assay was used to confirm the location of the transcriptional start site determined by primer extension using a cRNA produced by *in vitro* transcription of a plasmid constructed by subcloning the region containing the 5'-end of the A1AR gene from the genomic phage clone-1 into the plasmid Bluescript KS(+) using T7 RNA polymerase. Poly(A)$^+$RNA from rabbit brain was used to hybridize to the cRNA, tRNA and yeast RNA. Yeast RNA and tRNA were used as negative controls. RNase protection assay with the cRNA produced protected fragments that were not only different from the extended products but were also present in the negative control lanes. This reflects that either the cRNA had secondary structure and therefore

Figure 20 . Primer extension analysis of the transcription start site for the rabbit A1AR. Primer extension using oligonucleotides WSS23 (PE-1, 19 to 48) and WSS30 (PE-2, -206 to -177). The end labelled primers were hybridized to 10 µg of poly(A)$^+$ RNA from the rabbit brain and extended with reverse transcriptase (lane 2). 10 µg of tRNA was used as negative control (lane 1). The primer extended products were analyzed on a 5% polyacrylamide denaturing gel and their sizes determined by comparison with the dideoxy-termination products from sequencing reactions run in adjacent lanes, of the rabbit A1AR gene using the same primer.

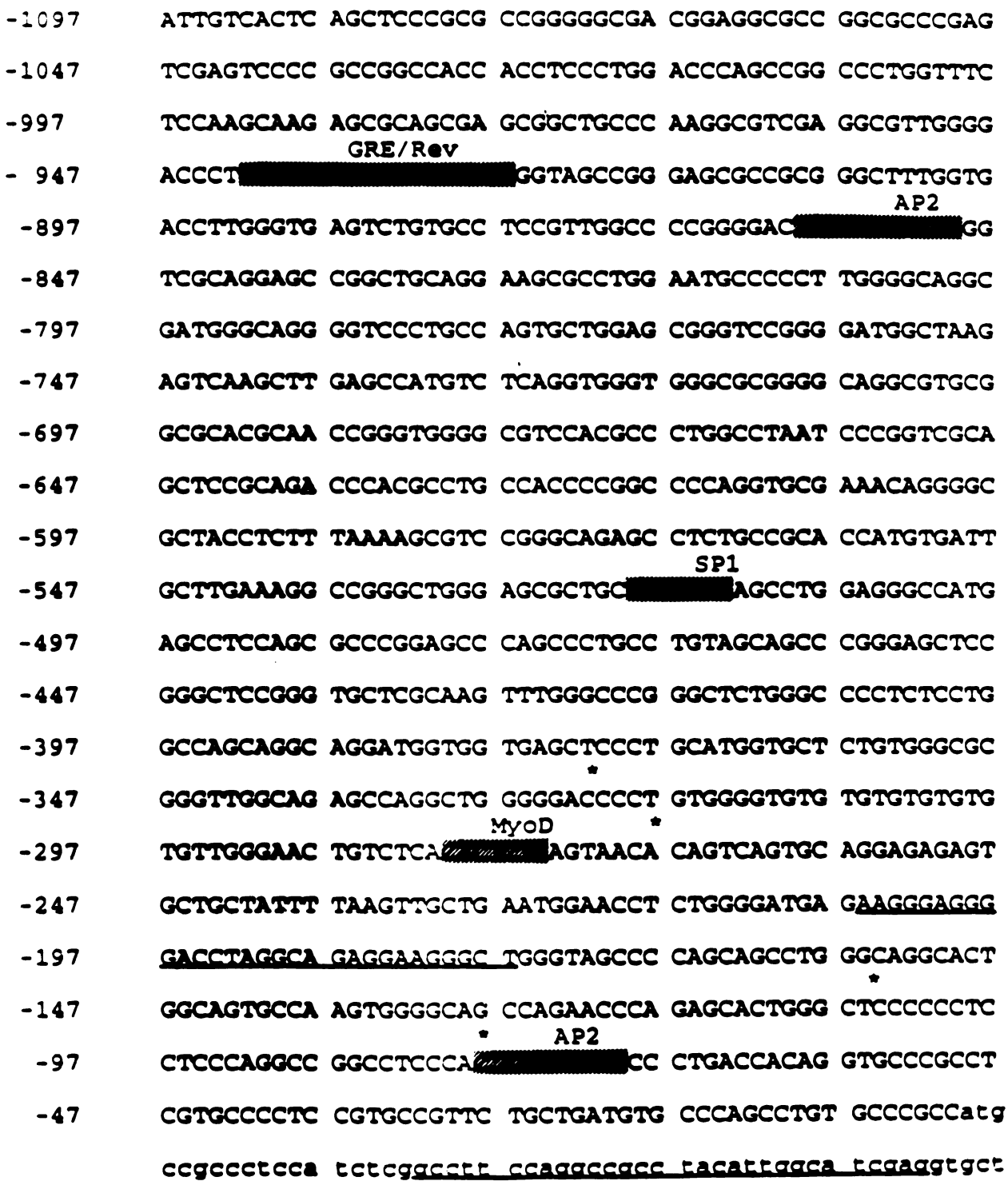

A C G T
P1
1 2
C C C T G G G G
A T T G T G T C A
A C C C G A G G G G
A G G G T G G G
P2
A C G T 1 2
C C C T G G G G
A T T G T G T C A

was protected or the polymerase was transcribing nonspecifically from the opposite strand.

When the transcription start site for other G-protein linked receptors were looked for, it was observed that transcription from most G-protein receptor genes originated from a specific point. Some of the examples include the human endothelin-A receptor, the transcription start site of which determined by primer extensions is 502 bp upstream of the translation start site (Hosoda et al. 1992); the bovine rodopsin has a transcription start site at 96 bp upstream of the methionine initiation codon (Nathans and Hogness 1983); and the transcription initiation site of the substance P receptor as identified by solution hybridization-nuclease protection assay, is at 576 bp upstream from the translation start methionine (Hershey et al. 1991). For LH receptor, however, primer extension studies indicate the presence of multiple transcriptional initiation sites (Tsai-Morris et al. 1991). A1AR transcripts as determined by primer extension appears to be heterologous and four sites were identified.

Characterization of the 5'-end of the rabbit A1AR gene: The sequence of approximately 1000 bp of the 5'-flanking region upstream of the translation start site was determined and is presented in Figure 21. As shown in the figure, the 5'-flanking region of the rabbit A1AR gene was observed to have a high G+C content (67.5%). A TATA or a CAAT box sequence, which determines the specificity of mRNA synthesis initiation by RNA polymerase II, was not found in the 5'-flanking region of the A1AR gene (Breathnach and chambon 1981; McKnight and Tijan 1986). Therefore the heterologous transcription initiation observed on primer extension analysis may have resulted from a

Figure 21 . Nucleotide sequence of the 5'-flanking region of the rabbit A1AR gene. The position of the oligonucleotide used for primer extensions are underlined. The transcription start site as indicated by the primer WSS23 and WSS30 (underlined) are marked by an astericks. The putative regulatory sequences are marked. Numbering begins with the translation initiation codon.


-1097 ATTGTCACTC AGCTCCCGCG CCGGGGGCGA CGGAGGCGCC GGCGCCCGAG
-1047 TCGAGTCCCC GCCGGCCACC ACCTCCCTGG ACCCAGCCGG CCCTGGTTTC
-997 TCCAAGCAAG AGCGCAGCGA GCGGCTGCCC AAGGCGTCGA GGCGTTGGGG
GRE/Rev
- 947 ACCCT GGTAGCCGG GAGCGCCGCG GGCTTTGGTG
AP2
-897 ACCTTGGGTG AGTCTGTGCC TCCGTTGGCC CCGGGGAC GG
-847 TCGCAGGAGC CGGCTGCAGG AAGCGCCTGG AATGCCCCCT TGGGGCAGGC
-797 GATGGGCAGG GGTCCCTGCC AGTGCTGGAG CGGGTCCGGG GATGGCTAAG
-747 AGTCAAGCTT GAGCCATGTC TCAGGTGGGT GGGCGCGGGG CAGGCGTGCG
-697 GCGCACGCAA CCGGGTGGGG CGTCCACGCC CTGGCCTAAT CCCGGTCGCA
-647 GCTCCGCAGA CCCACGCCTG CCACCCCGGC CCCAGGTGCG AAACAGGGGC
-597 GCTACCTCTT TAAAAGCGTC CGGGCAGAGC CTCTGCCGCA CCATGTGATT
SP1
-547 GCTTGAAAGG CCGGGCTGGG AGCGCTGC AGCCTG GAGGGCCATG
-497 AGCCTCCAGC GCCCGGAGCC CAGCCCTGCC TGTAGCAGCC CGGGAGCTCC
-447 GGGCTCCGGG TGCTCGCAAG TTTGGGCCCG GGCTCTGGGC CCCTCTCCTG
-397 GCCAGCAGGC AGGATGGTGG TGAGCTCCCT GCATGGTGCT CTGTGGGCGC
-347 GGGTTGGCAG AGCCAGGCTG GGGGACCCCT GTGGGGTGTG TGTGTGTGTG
MyoD
-297 TGTTGGGAAC TGTCTCA AGTAACA CAGTCAGTGC AGGAGAGAGT
-247 GCTGCTATTT TAAGTTGCTG AATGGAACCT CTGGGGATGA GAAGGGAGGG
-197 GACCTAGGCA GAGGAAGGGC TGGGTAGCCC CAGCAGCCTG GGCAGGCACT
-147 GGCAGTGCCA AGTGGGGCAG CCAGAACCCA GAGCACTGGG CTCCCCCCTC
AP2
-97 CTCCCAGGCC GGCCTCCCA CC CTGACCACAG GTGCCCGCCT
-47 CGTGCCCCTC CGTGCCGTTC TGCTGATGTG CCCAGCCTGT GCCCGCCatg
ccgccctcca tctcggccct ccagggcggc tacattggca tcgaggtgct

lack of a well defined TATA or a CAAT box motif. However, there are a few G + C-rich regions that contains a GC box homologous sequence, GGGCGG in its core (5 out of 6 base pair matches with the consensus sequence). The GC box sequence which binds to the transcription factor SP1, when found in multiple copies can direct transcription initiation from a TATA less promoter (Smale et al. 1990; Miwa et al. 1987). In human endothelin-A receptor the 5'-flanking region lacks a typical TATA box but contain a potential SP-1 binding site which is thought to be responsible for initiation of transcription (Hosoda et al. 1992).

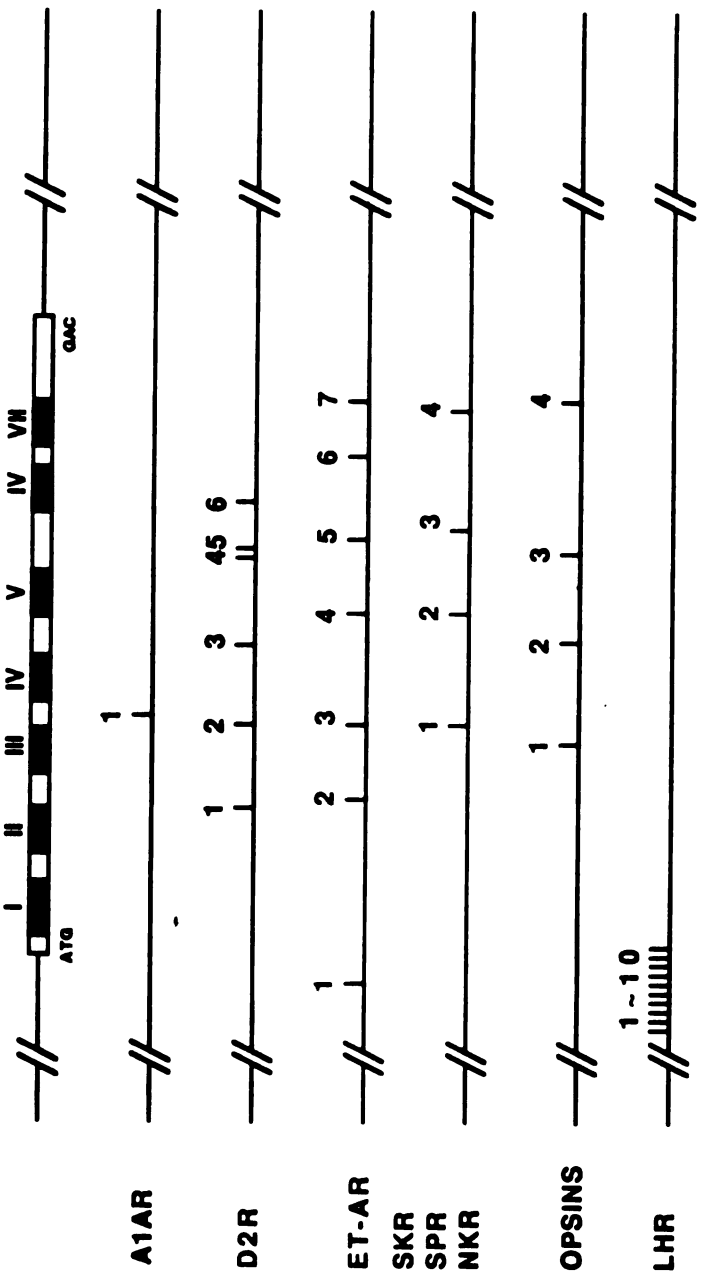
The 5'-flanking region of the gene also contained several sequences with homology to other known transcriptional regulatory elements. Consensus, potential *cis-acting* elements for MyoD (TCTCACACCTGAGTAA; Lassar et al. 1989) is present at positions -285 to -270. Sequences with homology to the AP-2 consensus regulatory binding site [(T/C)C(G/C)CC(C/A)(G/C)(G/C)(G/C)] are also located at the positions -78 to -70 and -859 to -850 (Imagawa et al. 1987). A consensus sequence for binding of the transcription factor SP1 (GGGCGG) is located at position -519 to -514 (Li et al. 1991). There are however presence of reverse SP-1 sequences at positions -1040 to -1035 and at -54 to -49. When the 5'-flanking region was searched for hormone responsive element consensus sequences, a sequence in reverse orientation to the glucocorticoid responsive element (GGGGCAGAACAGCTCC; Lerner et al. 1980) was found between position -942 to -927.

D . DISCUSSION

In the present study the organization, structure and the copy number of the rabbit A1AR gene have been clarified. Genomic clones containing a rabbit A1AR gene have been isolated using cDNA as a probe. The present study also demonstrates that the rabbit genome may contain a single copy of the A1AR gene, and that the gene contains only one intron in the coding region (Figure 17).

The genes for most of the G-protein coupled receptors lack introns within their coding regions (Kobilka et al. 1987), which has greatly facilitated isolation of many receptor genes (Bonner et al. 1987). Subsequent studies have however revealed the presence of introns in the coding region of certain G-protein coupled receptor genes. This include the genes for the dopamine D2, D3 and D4 receptors (Grandy et al. 1988; Sokoloff et al. 1990; Van Tol et al. 1991), the tachykinins (Hershey et al. 1991; Takahashi et al. 1992), the opsins (Nathans and Hogness et al. 1984), the leutinizing hormone receptors (Tsai-Morris et al. 1993) and the endothelin receptor (Hosoda et al. 1992). The A1AR gene elucidated in the present study belongs to the family of intron containing G-protein linked receptor genes. The exon/intron splice sites of the G-protein coupled receptor genes are compared in Figure 22. Comparison data shows that A1AR gene is unique from the rest of the G-protein linked receptor gene, in its exon/intron arrangement, since it has only one intron that separates the coding region. The genes for the ET-AR, SKR, SPR, NKR, D2R, and D4R all have an intron at the same location immediately after the third membrane spanning domain. This finding may suggest that the genes for these receptors may have originated from the same ancestral intron-

Figure 22 . The locations of the exon/intron splice sites of G protein-linked receptors are compared. The exon/intron splice site are compared between the cDNAs for the A1AR, dopamine D2 receptor (D2R), endothelin receptor (ET-AR), substance K receptor (SKR), substance P receptor (SPR), neuromedin K (NKR), opsins, and luteinizing hormone receptor (LHR). The seven transmembrane spanning A1AR is shown schematically in the first line. The closed boxes represent the membrane spanning domains. The linear lines represents the cDNAs for G protein-linked receptors. The vertical lines indicates the exon/intron splice sites and are numbered.


I
II
III
IV
V
IV
VII
ATG
OAC
A1AR
1
D2R
1 2 3 45 6
ET-AR
1 2 3 4 5 6 7
SKR
1 2 3 4
SPR
NKR
OPSINS
1 2 3 4
LHR
1~10

containing gene whereas the gene encoding the A1AR may belong to a different gene family.

Multiple initiation sites for rabbit A1AR transcription were observed on primer extension analysis. Sequence analysis of the 5'-end demonstrated the absence of a classical TATA box, which plays a role in directing the start of transcription to a specific site. The absence of well defined TATA or CAAT boxes is consistent with the heterogeneity of the transcription initiation sites. It would be interesting to determine whether or not any stimulation could induce transcription predominantly from one of the four initiation sites.

The 5'-flanking region of the rabbit A1AR gene was sequenced and all sequence compilation, alignment and searches for the putative regulatory elements were performed using the Wisconsin Genetics Computer Group Programs. A sequence reverse in orientation to the glucocorticoid responsive element was found in the 5'-flanking region. Glucocorticoids have been shown to upregulate the A1AR (Gerwins and Fredholm 1991). Glucocorticoids affect gene expression via activation of the glucocorticoid receptor, which in turn, interacts with glucocorticoid responsive sequences to increase or decrease the transcription of the hormone responsive gene. It is therefore tempting to speculate that glucocorticoids might be involved in the modulation of A1AR gene expression. To determine if this mechanism is physiologically relevant, transfection assays with plasmids containing the appropriate flanking sequences and reporter genes will be required.

Other possible transcription factor binding site consensus sequences include the SP1 binding site, an AP2 binding site and MyoD binding site. Particularly interesting is the presence of the alternating purine and the pyrimidine nucleotides which are reported

to have the potential to form Z-DNA (Nordheim and Rich 1983). It is noted that variation in the length of the repeating units among individuals causes genetic polymorphism (Weber and May 1989). Therefore the CA repeat turned out to be an abundant source of genetic markers. The sequence $(GT)_{18}$ in the A1AR gene could be used as a polymorphic DNA markers to determine whether or not the A1AR gene is linked to any genetic diseases.

In, summary, several putative regulatory sequences have been identified in a rabbit A1AR gene. To determine the significance of these sequences will require further *in vivo* analysis of their enhancer activities.

V . SUMMARY AND CONCLUSIONS

The research presented in this dissertation provided the following results:

1. The deduced amino acid sequence of rabbit A1AR shows significant homology to A1ARs cloned from canine, rat, bovine and human.
2. Rabbit A1AR belongs to a family of intron-containing G-protein linked receptor genes.
3. Preliminary studies indicate that the rabbit genome may contains a single copy of A1AR gene.
4. The transcription start site for the rabbit A1AR gene as analyzed by primer extension, appears to be heterogenous.
5. Several putative regulatory elements are present in the 5'-flanking region of the gene.

The deduced amino acid sequence of rabbit A1AR was shown to possess a significant homology to A1ARs from canine, rat, bovine and human. The encoded proteins are all identical in molecular mass with 326 amino acid except for rabbit A1AR which has 328 amino acid. The five A1ARs cloned so far are 92% identical in their overall amino acid structure. The identity is more pronounced in the transmembrane domains, which in G-protein coupled receptors are known to be involved in ligand binding.

The cloned A1AR from rabbit, will help to evaluate the structure, function and

regulation of the A1 adenosine receptor system at the biochemical and molecular biological levels. The expression of the A1AR clone in a cell system, will help to resolve the controversial issue of whether or not a single receptor is capable of interacting with multiple effector systems.

The gene encoding for the rabbit A1AR elucidated in the present study belongs to the family of intron-containing G-protein linked receptor genes. Comparison of A1AR gene structure to other intron-containing G-protein linked receptor gene revealed that the exon/intron arrangement of the A1AR gene is unique from the rest, since it possesses only one intron that separates the coding region. The genes for the endothelin receptor, substance K receptor, substance P receptor, and dopamine receptor have multiple introns and they all have an intron at the same location immediately after the third membrane spanning domain. This may indicate that the genes for these receptors may have originated from the same ancestral intron-containing gene, whereas the gene encoding the A1AR may belong to a different family.

Introns are intervening sequences that are spliced out when heteronuclear RNA is processed to mRNA. Occasionally errors arises due to splicing which result in splice variants which may be similar in structure but different in function. One such example is the dopamine D2 receptor. The two splice variants of the D2 dopamine receptor varies in 81 nucleotides, and they have the same pharmacological character. It is however not known if they have any differences in function. The gene structure for A1AR will help to classify if subtypes of A1AR may arise due to differential splicing of the same gene.

The present study also indicated that the rabbit genome contains a single copy of the A1AR gene which is a necessary first step for studies concerning A1AR gene

regulation and expression.

Analysis of the transcription start point by primer extension has indicated the heterogeneity of its transcription. Sequence analysis of the 5'-end demonstrated the absence of a classical TATA box, which has the role of directing the start of transcription to a specific site. The absence of well defined TATA box is consistent with the heterogeneity of the transcription initiation sites. It would be interesting to determine whether or not any stimulation could induce predominant transcription from one of the four initiation sites.

The 5'-flanking region of the rabbit A1AR gene was sequenced and all sequence compilation, alignment and searches for the putative regulatory elements were performed using the Wisconsin Genetics Computer Group Programs. A sequence reverse to the glucocorticoid responsive element was found in the 5'-flanking region. Other possible transcription factor binding site consensus sequences include, SP1 binding site, AP2 binding site and MyoD binding site. In summary, several putative regulatory sequences have been identified in a rabbit A1AR gene. To determine the significance of these sequences will require further *in vivo* analysis of their enhancer activity.

Cloning and characterization of the A1AR gene is therefore the first necessary step in evaluating the A1AR function and regulation at the biochemical and molecular level.

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VI . LITERATURE CITED

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