

**GENERATION OF A RECOMBINANT HUMAN ADENOSINE KINASE**

BY

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A Thesis  
Submitted to the Faculty of Graduate Studies  
In Partial Fulfillment of the Requirements for the Degree of

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Department of Pharmacology and Therapeutics  
Faculty of Medicine  
University of Manitoba  
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**Generation of a Recombinant Human Adenosine Kinase**

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**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University  
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**of**

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## ABSTRACT

Adenosine is an endogenous neuromodulator that activates cell-surface adenosine receptors to produce anti-inflammatory and anti-convulsant actions, neuroprotection, and anti-nociception. Although adenosine has classically been seen as a retaliatory metabolite that is most active during increased ATP consumption, there is evidence to indicate that adenosine is also active in physiological conditions. Recent research has also suggested that cellular signaling molecules, such as protein kinase C (PKC), may be important in the regulation of endogenous adenosine levels. Our laboratory has previously shown PKC inhibition of the main adenosine-metabolizing enzyme in physiological conditions, adenosine kinase (AK), which catalyzes the phosphorylation of adenosine to produce AMP. Because antibodies to human AK are not available, the purpose of the current project was to create an easily isolated and identified recombinant human AK for use in studying the possible regulation of AK activity by PKC. PCR produced a human adenosine kinase short form (hAKS) cDNA, which was subsequently inserted into a mammalian expression vector that added an Igκ secretion signal to the N-terminus of the recombinant protein, which results in its secretion from the cell, and *c-myc* and hexahistidine tags to the C-terminus of the protein, allowing it to be more easily detected and purified. HEK 293 cells were transfected with the hAKS clone, and RT-PCR, SDS-PAGE, and western analysis were performed to evaluate the success of transfection and expression of the recombinant protein. Sequence analysis of the hAKS clone revealed three point mutations in the hAKS sequence. Cytosolic protein from non-transfected cells and media from transfected cells were evaluated for AK activity. The



results indicate that although transfection appeared to be successful, minimal AK activity was present in transfection samples. The diminished AK activity could be the result of three point mutations in the recombinant AK sequence, or the presence of an N-terminal Igk secretion signal and C-terminal *c-myc* epitope and hexahistidine tag fused to the protein to ease isolation and identification. Future correction of the point mutations, or cloning into a simplified expression vector with fewer tags may restore activity to the recombinant protein, and/or provide information regarding the structure-activity relationship of AK.

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## ABBREVIATIONS

$\mu\text{g}$  -- micrograms

$\mu\text{M}$  -- micromolar

$\mu\text{L}$  -- microlitres

$A_{260}$  -- absorbance at 260 nm

ABC -- ATP-binding cassette

ADA -- adenosine deaminase

ADAC -- adenosine amine congener

ADP -- adenosine diphosphate

AK -- adenosine kinase

AMP -- adenosine monophosphate

ATP -- adenosine triphosphate

BGH -- bovine growth hormone

bp -- base pairs

BSA -- bovine serum albumin

$\text{Ca}^{2+}$  -- free calcium

cAMP -- 3',5'-cyclic adenosine monophosphate

cDNA -- complimentary deoxyribonucleic acid

cGMP -- 3',5'-cyclic guanosine monophosphate

CHA -- N<sup>6</sup>-cyclohexyladenosine

*cib* -- concentrative, inhibitor insensitive, broadly selective nucleoside transporter

*cif* -- concentrative, inhibitor insensitive, formycin B selective nucleoside transporter

CIP – calf intestinal phosphatase

*cit* – concentrative, inhibitor insensitive, thymidine selective nucleoside transporter

IB-MECA – *N*<sup>6</sup>-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide

cN – cytosolic 5'-nucleotidase

CNS – central nervous system

CNT – concentrative nucleoside transporter

CPA – *N*<sup>6</sup>-cyclopentyladenosine

DCF – deoxycoformycin

ddH<sub>2</sub>O – distilled deionized water

DEPC – diethyl pyrocarbonate

dItu – 5'-deoxyiodotubericidin

DMEM – Dulbecco's modified Eagle medium

DNA – deoxyribonucleic acid

dNTPs – deoxynucleotide triphosphates

DPCPX – 1,3-dipropyl-8-cyclopentylxanthine

DTT -- dithiothreitol

*E. coli* – *Escherichia coli*

EDTA – ethylene diamine tetra-acetic acid

EHNA – erythro-9-(2-hydroxy-3-nonyl) adenine

*ei* – equilibrative nucleoside transporter insensitive to NBTI inhibition

eN – ecto 5'-nucleotidase

eN-s – soluble form of ecto 5'-nucleotidase

ENT – equilibrative nucleoside transporter

ERK – extracellular-signal-regulated kinase

*es* – equilibrative nucleoside transporter sensitive to NBTI inhibition

FBS – fetal bovine serum

fmol – femtomoles

$G_i/G_o$  – inhibitory G proteins

$G_s$  – stimulatory G protein

GABA –  $\gamma$ -aminobutyric acid

GI – gastrointestinal

hAKS – human adenosine kinase, short isoform

HEK – human embryonic kidney

HE-NECA – 2-hex-1-ynyl-5'-*N*-ethylcarboxamidoadenosine

hENT – human equilibrative nucleoside transporter

$I_{Cl^-}$  – chloride current

$I_{K^+}$  – potassium current

IgG – immunoglobulin G

Ig $\kappa$  – immunoglobulin  $\kappa$ -chain

IL-6 – interleukin 6

IMP – inosine monophosphate

Itu – iodotubericidin

i.v. – intravenous

Kb – kilo base pairs

$K_i$  – inhibitory constant

$K_M$  – Michaelis Menton constant

kDa – kilodaltons

LB – Luria Bertani

MAP – mitogen-activated protein

MICB – Manitoba Institute of Cell Biology

Mg<sup>2+</sup> – free magnesium

mL – millilitres

mM – millimolar

MOPS – 3-(N-morpholino) propanesulfonic acid

mRNA – messenger ribonucleic acid

MS – multiple sclerosis

MW – molecular weight

NAD<sup>+</sup> – nicotinamide adenine dinucleotide (oxidized form)

NBTI – nitrobenzylthioinosine

NCBI – National Centre for Biotechnology Information

NECA – 5'-N-ethyl-carboxamidoadenosine

ng – nanograms

NGF – neuronal growth factor

NH<sub>2</sub>dAdo – 5'-amino-5'-deoxyadenosine

Ni-NTA – nickel-nitrilotriacetic acid

nM – nanomolar

PBMC – peripheral blood mononuclear cell

PCR – polymerase chain reaction

PKA – protein kinase A

PKC – protein kinase C

PLC – phospholipase C

PMA – phorbol-12-myristate-13-acetate

pmol – picomoles

PSA – prostate specific antigen

REAL – regulator of endogenous adenosine levels

RNA – ribonucleic acid

R-PIA – N<sup>6</sup>-(R-phenylisopropyl)-adenosine

RT-PCR – reverse transcriptase polymerase chain reaction

SAH – S-adenosylhomocysteine

SAHH – S-adenosylhomocysteine hydrolase

SDS – sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – standard error of the mean

TBS – Tris-buffered saline

TTBS – Tween 20 in Tris-buffered saline

TNF $\alpha$  – tumour necrosis factor alpha

U – units

UTR – untranslated regions

UV – ultraviolet

V – volts

V<sub>max</sub> – maximum velocity of permeant influx, or maximum velocity of enzymatic  
reaction



## 1. INTRODUCTION

Adenosine is the purine nucleoside base of adenine triphosphate (ATP). It is a ubiquitous molecule that is important in a number of cellular functions, such as RNA synthesis and energy production, as well as receptor-mediated physiological effects. Adenosine was first described in 1929 as a ubiquitous substance that could cause bradycardia (Drury and Szent-Gyorgi, 1929). Since then, much has been learned of this molecule's metabolic pathway, transport into and out of cells, and receptor signalling pathways. The adenosine system has been a topic of therapeutic interest for decades, and adenosine-based therapies have been investigated for ischaemia (Lasley RD, 1990; Rudolphi et al., 1992; Deckert and Gleiter, 1994; Fredholm, 1997), epilepsy (Zhang et al., 1993; Wiesner et al., 1999), schizophrenia (Ferre et al., 1994; Rimondini et al., 1997), Parkinson's disease (Mally and Stone, 1996; Ongini and Fredholm, 1996; Svenningsson et al., 1999), pain control (Sweeney et al., 1989; Cahill et al., 1995; Reeve and Dickenson, 1995; Sollevi, 1997), asthma (Feoktistov and Biaggioni, 1995; Auchampach et al., 1997), and inflammation (Cronstein, 1994). Currently adenosine is rarely used in therapy, mainly being used for cardiac arrhythmias, but holds potential for the above disorders, as well as others.

### 1.1 Physiological Actions of Adenosine

Adenosine was originally recognized for its effects on the cardiovascular system, namely its ability to produce bradycardia and affect blood flow (Drury and Szent-Gyorgi, 1929; Drury, 1936; Berne, 1963). When it was established that it was involved in the

regulation of blood flow to a number of tissues, including heart (Berne, 1980) and brain (Latini and Pedata, 2001), adenosine was recognized as a ubiquitous biologically active molecule that played an especially important role when energy demand exceeded energy supply. In the past three decades, adenosine has been discovered to have effects on gastrointestinal function, lipolysis, and immune response regulation, in addition to its effects on the cardiovascular system (Fredholm et al., 2001). Adenosine inhibits platelet aggregation (Ledent et al., 1997), and has also been shown to prevent post-ischaemic reperfusion injury due to its anti-inflammatory and anti-thrombotic effects (Lasley RD, 1990; Rudolphi et al., 1992; Jordan et al., 1997). As well, adenosine may mediate the release of allergic mediators from mast cells through its actions on adenosine receptors (Ramkumar et al., 1993; Feoktistov and Biaggioni, 1995; Fozard et al., 1996; Auchampach et al., 1997), and can decrease free radical formation through its interaction with adenosine receptors on neutrophils (Jordan et al., 1997). In the kidney, lack of adenosine increases renin levels and tubuloglomerular feedback is inhibited (Brown et al., 2001; Sun et al., 2001).

In addition to its peripheral effects, adenosine also plays a role in the central nervous system (CNS). It is termed a neuromodulator because it is not a neurotransmitter and does not undergo synaptic release, yet it can enhance or inhibit the effects of neurotransmitters (Latini and Pedata, 2001). Adenosine can accentuate endogenous anti-nociception (Sweeney et al., 1989, 1991) and act as an anti-convulsant (Zhang et al., 1993; Wiesner et al., 1999). It may inhibit motor stimulation to some extent, indicated by the ability of the methylxanthine caffeine, a non-selective adenosine receptor antagonist, to stimulate motor activity. A role for adenosine in the sleep-wake cycle has also been

established (Chagoya de Sanchez, 1995); adenosine levels increase with prolonged wakefulness, and decrease during periods of sleep (Porkka-Heiskanen et al., 1997). Finally, in times of cellular stress (ischaemia, for example), adenosine levels increase due to ATP consumption and inhibition of adenosine metabolism, and the increased extracellular adenosine protects against neuronal damage resulting from excitotoxicity (Schubert et al., 1994).

## 1.2 Adenosine Pathways

The physiological actions of adenosine are mediated by cell-surface adenosine receptors. Adenosine must, therefore, exist in the extracellular space in order to act on these receptors, and so regulation of the amount of adenosine in the extracellular space dictates the physiological actions of adenosine. There are established *classical* and *alternative* cellular pathways of altering extracellular adenosine levels, as well as a *novel* pathway proposed by our laboratory.

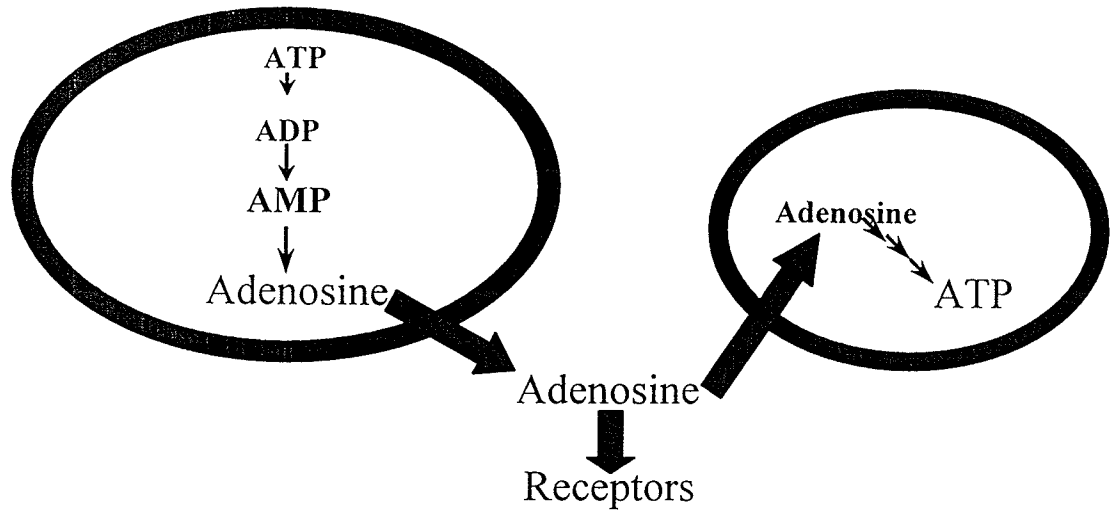
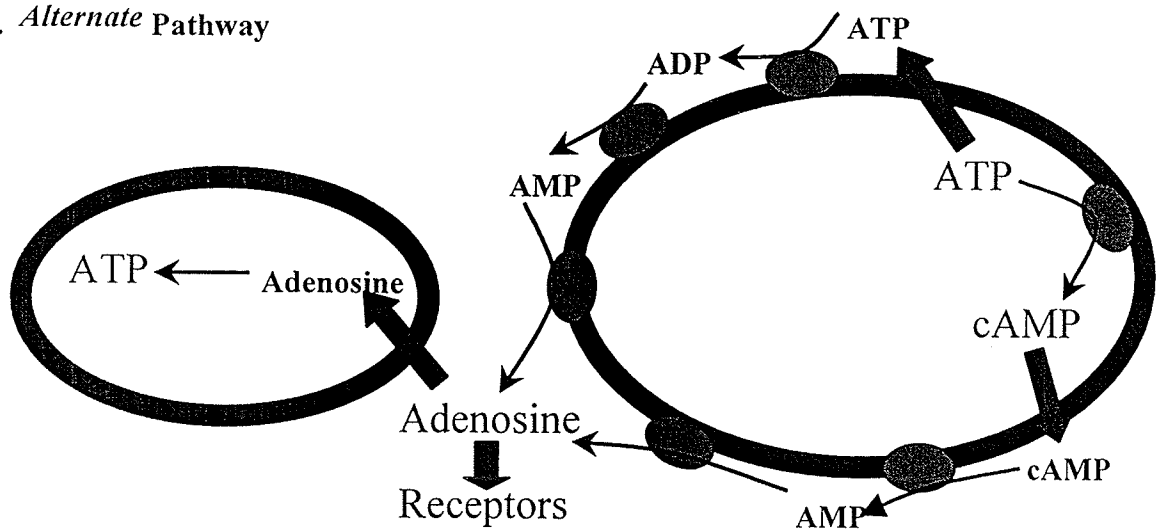
The *classical* view of extracellular adenosine regulation involves regarding adenosine as a “retaliatory metabolite” produced from ATP breakdown in ATP-depleting conditions (Newby, 1984). It is referred to as “retaliatory” due to the fact that adenosine action on adenosine A<sub>1</sub> and A<sub>2</sub> receptors can depress cellular activity and increase blood flow, leading to some preservation and/or replenishment of cellular ATP levels. The *classical* scenario involves extracellular adenosine resulting from intracellular metabolism of ATP to adenosine, subsequent release of adenosine from cells through equilibrative nucleoside transporters, adenosine action on its receptors, adenosine

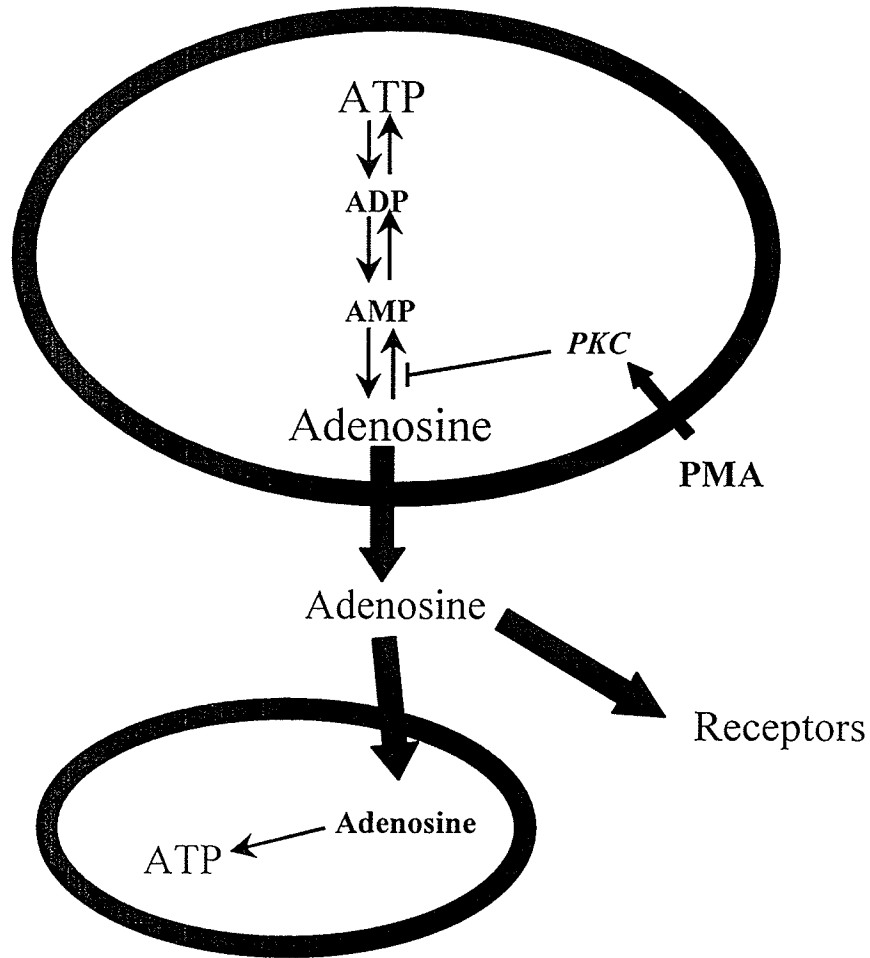
reuptake into cells through nucleoside transporters, and finally, intracellular metabolism of adenosine to replenish ATP stocks. The *classical* pathway is shown in Figure 1A.

The *classical* pathway is widely accepted, but recent evidence has suggested that this view of adenosine as a retaliatory metabolite may not be the only role of adenosine in the body. Although adenosine may be important in neuroprotection during ischaemia and seizures (ATP-depleting conditions), it has also been shown to be active in models representing physiological conditions. The existence of excitatory adenosine A<sub>2A</sub> receptors on striatal and superior collicular neurons suggests that adenosine is not always inhibitory (Cunha et al., 1994; Kurokawa et al., 1996; Ishikawa et al., 1997). As well, the adenosine receptor antagonist caffeine is a recognized stimulant, which suggests that adenosine exerts a tonic inhibitory tone. Finally, studies have shown that adenine nucleotides (ATP; cyclic-AMP or cAMP) can be released from cells and subsequently act on their own receptors (purine P<sub>2</sub> receptors) (Edwards and Gibb, 1993), or be extracellularly metabolized to adenosine (Rosenberg et al., 1994; Dunwiddie et al., 1997), which then acts on its own receptors. To account for the above findings, an *alternate* scenario of the adenosine system has been developed in which adenine nucleotides are released from the cell and metabolized in the interstitium to adenosine, which can then activate cell-surface adenosine receptors. Adenosine can then be taken up by cells through nucleoside transporters and subsequently be metabolized to adenine nucleotides, inosine, or S-adenosylhomocysteine. A schematic of the *alternate* pathway is shown in Figure 1B.

In addition to the established *classical* and *alternate* pathways, our laboratory has proposed a *novel* pathway of adenosine production and action. The *novel* pathway we

propose is similar to the *classical* pathway in that it focuses on intracellular production of adenosine, but differs from the *classical* pathway in that it is relatively independent of cellular ATP levels. Instead, this pathway involves the regulation of adenosine metabolism and transport by signal transduction molecules. It is well-established that in ischaemic heart, the signal transduction molecules protein kinase C (PKC) (Minamino et al., 1995; Kitakaze et al., 1997; Node et al., 1997) and cyclic-GMP (cGMP) (Obata et al., 1998) act to activate ecto-nucleotidase activity and thus increase extracellular adenosine levels, which can contribute to ischaemic preconditioning. A study performed in our laboratory suggested a similar but intracellular action of PKC on the adenosine metabolic pathway (Sinclair et al., 2000). The results indicated that stimulation of PKC activity with phorbol-12-myristate-13-acetate (PMA) can inhibit the activity of adenosine kinase (AK), the enzyme responsible for producing AMP from adenosine. This inhibition of AK activity resulted in increased intracellular adenosine levels, and thus increased extracellular adenosine levels due to efflux through equilibrative nucleoside transporters. As well, PKC stimulation has been shown to increase nucleoside uptake through the hENT1 equilibrative nucleoside transporter (Coe et al., 2002), and it has been suggested that PKC and protein kinase A (PKA) may play opposing roles in regulating ethanol inhibition of adenosine transport (Coe et al., 1996). These findings support a *novel* pathway for adenosine action, in which cell signalling molecules, like PKC, can regulate the activity of enzymes involved in adenosine metabolism, such as the 5'-nucleotidase and adenosine kinase, as well as adenosine transport through equilibrative nucleoside transporters. This *novel* pathway, outlined in

FIGURE 1. Summary of *classical*, *alternate*, and *novel* adenosine pathways.A. *Classical* PathwayB. *Alternate* Pathway

C. *Novel Pathway*

The *classical* (A), *alternate* (B), and *novel* (C) adenosine pathways are shown. The *classical* pathway describes intracellular adenosine production during ATP-depleting conditions, such as ischaemia, followed by release of adenosine into the extracellular space. The *alternate* pathway shows extracellular adenosine production following the release of adenine nucleotides (ATP, cAMP). The *novel* pathway we propose illustrates intracellular adenosine production under the control of cellular signalling molecules, such as PKC. This last pathway suggests adenosine levels are not necessarily dependent on cellular ATP levels, which is in contrast to the *classical* pathway.

Figure 1C, may explain why a number of receptor agonists and cellular signalling molecules can induce or potentiate adenosine release.

### 1.3 Adenosine Receptors

Adenosine produces most of its physiological actions via cell surface receptors. Four distinct adenosine receptors have been characterized to date, including the adenosine A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptors. The adenosine A<sub>1</sub> and A<sub>2a</sub> receptors are well characterized, while less is known about the A<sub>2b</sub> and A<sub>3</sub> receptors.

#### 1.3.1 *The Adenosine A<sub>1</sub> Receptor*

The adenosine A<sub>1</sub> receptor was first cloned from canine tissues in 1989 (Libert et al., 1989), and has since been cloned from a number of other species, including human (Libert et al., 1992; Townsend-Nicholson and Shine, 1992). The recombinant human A<sub>1</sub> receptor is a 36.7 kDa protein consisting of 326 amino acid residues. The sequence is highly conserved (>90%) across species. When adenosine binds to the A<sub>1</sub> receptor, the receptor couples to inhibitory G proteins (G<sub>i</sub>/G<sub>o</sub>), which inhibit the activity of adenylate cyclase and thus decrease cellular levels of the second messenger, cyclic adenosine monophosphate (cAMP) (Fredholm, 1995). Activation of the A<sub>1</sub> receptor also increases K<sup>+</sup> and Cl<sup>-</sup> conductances, which decreases membrane potential, and thus inhibits Ca<sup>2+</sup> uptake and subsequent neurotransmitter release (Fredholm, 1995).

The use of non-selective and selective adenosine A<sub>1</sub> receptor agonists and antagonists has revealed details regarding the distribution of A<sub>1</sub> receptors. The presence of receptor mRNA transcripts in various tissues has also been used to determine and



confirm distribution. In humans, the highest levels of  $A_1$  receptors and mRNA are found in brain (cortex, cerebellum, hippocampus), dorsal horn of the spinal cord, eye, adrenal gland, and atria. Intermediate levels are found in other parts of the brain, skeletal muscle, liver, kidney, adipose tissue, salivary glands, esophagus, colon, antrum, and testis (Fredholm et al., 2001). Based on the distribution data, as well as functional data and confirmation in knockout mice, it is believed that peripheral  $A_1$  adenosine receptors mediate bradycardia, inhibition of lipolysis, reduced glomerular filtration, tubuloglomerular feedback, and ischaemic preconditioning (in the heart) (Fredholm et al., 2001). Adenosine  $A_1$  receptors located in the brain mediate anti-nociception, reduction of sympathetic and parasympathetic activity, neuronal hyperpolarization (thus decreasing neuronal firing), presynaptic inhibition, and ischaemic preconditioning (Fredholm et al., 2001).

### ***1.3.2 The Adenosine $A_{2A}$ Receptor***

As with the adenosine  $A_1$  receptor, the adenosine  $A_{2A}$  receptor was initially cloned from canine tissues (Maenhaut et al., 1990), and has since been cloned from a number of other species, including human (Furlong et al., 1992; Peterfreund et al., 1996). The  $A_{2A}$  receptor is the largest of the adenosine receptors at ~45 kDa and 410 amino acids (Ongini and Fredholm, 1996). It too is highly conserved across species, with over 90% sequence identity across species. Agonism of the  $A_{2A}$  receptor causes it to bind to stimulatory G proteins ( $G_s$ ), which in turn increase adenylylate cyclase activity and thus increase cAMP levels (Fredholm et al., 2001). As well, activation of the  $A_{2A}$  receptor has been shown to affect MAP kinase (Hirano et al., 1996; Sexl et al., 1997) and protein

phosphatase activity (Revan et al., 1996; Svenningsson et al., 2000), independent of changes in cAMP levels.

Receptor protein and receptor mRNA distribution studies have revealed high levels of the  $A_{2A}$  receptor in the spleen, thymus, leukocytes (lymphocytes and granulocytes), blood platelets, striatopallidal GABAergic neurons (caudate-putamen, nucleus accumbens, olfactory tubercle), and the olfactory bulb (Fredholm et al., 2001). Based on mRNA distribution alone, intermediate levels of the  $A_{2A}$  receptor have been found in the vasculature, heart, and lungs. Low levels of the  $A_{2A}$  receptor in other brain regions (hippocampus, cortex) have been confirmed with mRNA and protein distribution studies (Fredholm et al., 2001). The above distribution information suggests that the  $A_{2A}$  receptor is responsible for inhibition of platelet aggregation, inhibition of polymorphonuclear leukocytes, immune function regulation, vasodilation, protection against ischaemic damage, regulation of sensorimotor integration in the basal ganglia, and stimulation of sensory nerve activity (Fredholm et al., 2001). These actions have been confirmed in the  $A_{2A}$  knockout mouse. As well, one type of  $A_{2A}$  knockout mouse has previously displayed anxious behaviour, high blood pressure, and lowered exploratory and pain responses (Ledent et al., 1997), although studies with other  $A_{2A}$  knockouts have not confirmed these findings (Chen et al., 1999; Chen et al., 2000). Such changes in mood, however, may partly explain the physiological effects of caffeine. Finally, there is some evidence to suggest that adenosine  $A_{2A}$  receptors are co-localized with dopamine  $D_2$  receptors in the striatum (Johansson et al., 1997), which may have implications for the pathogenesis and therapy of Parkinson's disease or schizophrenia.

### 1.3.3 *The Adenosine A<sub>2B</sub> Receptor*

The adenosine A<sub>2B</sub> receptor was first distinguished from the A<sub>2A</sub> receptor in 1972 based on differing sensitivities to agonists that increase cellular cAMP levels (Huang et al., 1972). The A<sub>2B</sub> receptor is a 36.4 kDa protein consisting of 332 amino acid residues (Palmer and Stiles, 1995). Similar to the A<sub>2A</sub> receptor, the A<sub>2B</sub> receptor binds adenosine, couples to G<sub>s</sub> proteins, and thus stimulates adenylate cyclase activity, increasing cellular cAMP (Fredholm et al., 2001). However, the binding affinity of the A<sub>2B</sub> receptor for adenosine is much less than that of the A<sub>2A</sub> receptor. The A<sub>2B</sub> receptor has also been suggested to couple to phospholipase C (Pilitsis and Kimelberg, 1998), ERK, and MAP kinase (Feoktistov et al., 1999) pathways independent of effects on cAMP.

Regarding the distribution of the A<sub>2B</sub> receptor, its mRNA is ubiquitous, with high levels found in the gastrointestinal tract, and lower levels found in the lungs and the brain (Fredholm et al., 2001). The receptor mRNA is ubiquitous in the CNS (Dixon et al., 1996; Sebastiao and Ribeiro, 1996) and is localized on astrocytes (Pilitsis and Kimelberg, 1998). Due to its relatively low binding affinity for adenosine, and its ubiquitous distribution, it has been proposed that the A<sub>2B</sub> receptor plays little role in normal physiological conditions. It may be partly responsible for the relaxation of vascular and intestinal smooth muscle, inhibition of monocyte and macrophage function, and stimulation of mast cell mediator release associated with adenosine in some species (Fredholm et al., 2001). The A<sub>2B</sub> receptor is thought to be more important, however, in pathological conditions like ischaemia, when extracellular adenosine levels are elevated due to increased ATP consumption.

### 1.3.4 *The Adenosine A<sub>3</sub> Receptor*

Adenosine A<sub>3</sub> receptor mRNA was initially isolated from RT-PCR of rat testes cDNA that used degenerate oligonucleotide primers directed toward conserved regions of G-protein coupled receptors (Meyerhof et al., 1991). Because the RT-PCR product was most similar to adenosine A<sub>1</sub> and A<sub>2A</sub> receptors (>40% amino acid identity), the receptor was designated a novel adenosine receptor, the A<sub>3</sub> receptor. This receptor has been cloned from human tissues and the cDNA encodes a 318 amino acid, 36.6 kDa protein (Palmer and Stiles, 1995). The A<sub>3</sub> receptor couples to G<sub>i</sub>/G<sub>o</sub> proteins when it binds adenosine, similar to the A<sub>1</sub> receptor, and thus inhibits adenylate cyclase activity and decreases cellular cAMP levels (Abbracchio et al., 1995; Fredholm et al., 2001). Activation of A<sub>3</sub> receptors has also been reported to activate PLC (Abbracchio et al., 1995).

Although knowledge of A<sub>3</sub> receptor distribution and function is still somewhat limited due to lack of effective pharmacological tools, mRNA studies indicate that in most species it is expressed at an intermediate level in the pineal gland, and at low levels in the thyroid, most regions of the brain, adrenal gland, liver, kidney, heart, and intestine (Fredholm et al., 2001). In the human, intermediate levels of A<sub>3</sub> mRNA are found in hippocampus and cerebellum, and low levels are found in the spleen and testis (Fredholm et al., 2001). The low levels of this receptor in the human CNS are ~10 – 30-fold lower than A<sub>1</sub> receptors in the cortex or A<sub>2A</sub> receptors in the striatum (von Lubitz, 1997). The physiological function of the A<sub>3</sub> receptor has not been well established, but it is widely recognized as a regulator of immune function, enhancing mediator release from mast cells (Linden, 1994; Fredholm et al., 2001). It may also play a role in preconditioning in

some species (Fredholm et al., 2001). Both of these functions have been confirmed in the knockout mouse (Salvatore et al., 2000). Due to their low affinity for adenosine, A<sub>3</sub> receptors are thought to play a role in predominantly pathological conditions, such as ischaemia or seizures, when extracellular adenosine levels are increased.

### *1.3.5 Adenosine Receptors and CNS Therapy*

Although it is known to some extent which adenosine receptors mediate what actions of adenosine, this knowledge has not been exploited therapeutically in the CNS. Adenosine and adenosine receptor agonists and antagonists are limited by their blood brain barrier permeability, and adenosine analogues are also limited by their actions in the periphery, which can cause side effects such as lowered heart rate, blood pressure, and body temperature (Guieu et al., 1996; Von Lubitz, 1999). Due to these limitations, current therapeutic research has focused on increasing endogenous adenosine levels as a means of adenosine receptor activation. This can be accomplished by reducing cellular uptake of adenosine either by inhibiting nucleoside transporters, or by inhibiting adenosine metabolism. Both of these strategies can elevate extracellular adenosine concentrations and potentiate adenosine's receptor-mediated effects.

TABLE 1. Summary of adenosine receptor subtypes

Receptor Subtype	A <sub>1</sub>	A <sub>2A</sub>	A <sub>2B</sub>	A <sub>3</sub>
G-protein	G <sub>i</sub> /G <sub>o</sub>	G <sub>s</sub>	G <sub>s</sub>	G <sub>i</sub> /G <sub>o</sub>
Effector system	cAMP; I <sub>K</sub> ; I <sub>Cl</sub>	cAMP; MAP kinase; protein phosphatase	cAMP; PLC; ERK; MAP kinase	cAMP; PLC
Distribution	<i>High</i> : cortex, cerebellum, hippocampus, dorsal horn of spinal cord, eye, adrenal gland, atria <i>Intermediate</i> : rest of brain, skeletal muscle, liver, kidney, adipose tissue, salivary glands, esophagus, colon, antrum, testes	<i>High</i> : spleen, thymus, leukocytes, platelets, striatopallidal GABAergic neurons, olfactory bulb <i>Intermediate</i> : vasculature, heart, lungs <i>Low</i> : rest of brain	<i>Ubiquitous mRNA</i> ; <i>High</i> : GI tract <i>Low</i> : lungs, brain	<i>Intermediate</i> : pineal gland, hippocampus, cerebellum <i>Low</i> : thyroid, rest of brain, liver, kidney, heart, spleen, adrenal gland, intestine, testis
Physiological Actions	<i>Periphery</i> : bradycardia, ischaemic preconditioning in heart, and inhibition of lipolysis, glomerular filtration, tubuloglomerular feedback <i>CNS</i> : anti-nociception, decreased sympathetic and parasympathetic activity, neuronal hyperpolarization, presynaptic inhibition, ischaemic preconditioning	<i>Periphery</i> : vasodilation, inhibition of platelet aggregation, inhibition of leukocytes, protection against ischaemic damage <i>CNS</i> : protection against ischaemic damage, regulation of sensorimotor integration in basal ganglia, sensory nerve activity	Relaxation of vascular and intestinal smooth muscle, inhibition of monocyte and macrophage function, stimulation of mast cell mediator release	Regulator of immune function, mediator release from mast cells, preconditioning
Selective Agonists <sup>a</sup>	CPA; CHA	CGS 21680; HE-NECA	None	CI-IB-MECA
Selective Antagonists <sup>b</sup>	DPCPX; 8-cyclo-pentyltheophylline	SCH 58261; ZM241385	MRS1754; enprofylline	MRE 3008F20; DPCPX

Table 1 summarizes the G-protein and effector system, distribution, physiological actions, and selective agonists and antagonists for each of the adenosine receptor subtypes. Distribution was characterized based on mRNA data, protein data, and/or activity data. Adapted from Fredholm *et al* (Fredholm et al., 2001).

- a. NECA, which was originally thought to be a specific agonist of A<sub>2</sub> receptors, has since been found to have similar affinity for all receptor subtypes.
- b. Adenosine receptor studies were first performed using binding studies with weak non-selective antagonists such as the methylxanthines, including caffeine and theophylline.

## 1.4 Transport Mechanisms Affecting Adenosine Levels

### 1.4.1 *Transport of Adenine Nucleotides*

Extracellular adenosine can increase via direct release of the nucleoside through nucleoside transporters, or via release of adenine nucleotides and subsequent extracellular metabolism of the adenine nucleotides to adenosine. Most evidence suggests ATP is the adenine nucleotide most often released from cells in physiological and pathological circumstances. In the CNS, ATP is packaged in vesicles and undergoes synaptic release from neurons (Zimmermann, 1997; Fields and Stevens, 2000). ATP release from astrocytes and cerebral microvascular endothelial cells is poorly characterized, but may involve increased intracellular  $\text{Ca}^{2+}$ , increased cell volume, and membrane perturbations (Cotrina et al., 2000). Gap junction hemichannels (Cotrina et al., 2000) and ATP-binding cassette (ABC) transporters (Zimmermann, 1996) have been hypothesized to play a role in ATP release from astrocytes and microvascular endothelial cells, and a recent study found that ATP was released from cultured astrocytes in granules, possibly in response to a regulated secretory pathway (Coco et al., 2003). Once in the extracellular space, ATP can act on cell-surface purinergic  $\text{P}_2$  receptors or be metabolized to adenosine, which can then act on adenosine receptors. ATP and other adenine nucleotides are charged molecules, due to the presence of the phosphate groups, and so metabolism to adenosine is usually required for re-entry into cells.

### 1.4.2 *Transport of Adenosine*

Nucleoside transporters are important in the regulation of extracellular adenosine levels in that they control adenosine release into and uptake from the extracellular space,

thus controlling the amount of adenosine available to act on cell-surface adenosine receptors. Their primary function, however, is nucleoside salvage for metabolic processes, such as replication and transcription. Nucleoside transporters are carrier proteins integral to the plasma membrane of cells (Cass et al., 1998). They are divided into equilibrative and concentrative families, and subdivided based on sensitivity to inhibitors and/or selectivity of substrate. All of the nucleoside transporters, however, are permeable to uridine (Cass et al., 1998).

#### 1.4.2.1 Equilibrative Nucleoside Transporters

The equilibrative nucleoside transporters are sodium-independent bi-directional transporters that, as their name suggests, transport nucleosides according to their concentration gradients. These integral membrane proteins are 456 – 457 amino acids in length, and ~36 kDa in size (Cass et al., 1998). They possess eleven transmembrane domains, as well as a large extracellular loop between transmembrane domains 1 and 2, and a large intracellular loop between transmembrane domains 6 and 7 (Cass et al., 1998). Two distinct equilibrative transporters have been characterized and cloned from rat and human tissues: ENT1 (or *es*) and ENT2 (or *ei*).

The ENT1 transporter is sometimes referred to as *es* (*equilibrative-sensitive*) because it is inhibited by nanomolar concentrations of nitrobenzylthioinosine (NBTI) (Pickard et al., 1973). As well, human ENT1 can be inhibited by nanomolar concentrations of dipyridamole, while rat ENT1 requires micromolar concentrations; dilazep is a potent inhibitor in all species studied except rat (Griffith and Jarvis, 1996; Cass et al., 1998). ENT1 transports both purine and pyrimidine nucleosides, and the human form has a  $K_M$  of 20 – 50  $\mu$ M for adenosine (Cass et al., 1998). ENT1 is widely



distributed and exists in many fetal and most adult tissues. In the rat CNS, the mRNA transcript is broadly distributed, and NBTI-binding studies have shown high levels of ENT1 in the striatum, cortex, superior colliculus, substantia nigra, hypothalamus, choroid plexus, and thalamic structures; very low levels are seen in the hippocampus, cerebellum, and white matter (Geiger and Nagy, 1990; Anderson et al., 1999b). ENT1 is present in neurons, astrocytes, and endothelial cells (Cass et al., 1998).

The ENT2 transporter is differentiated from ENT1 in that it has low sensitivity to inhibition by NBTI, dipyridamole, dilazep, and propentofylline; micromolar concentrations of each compound are needed to inhibit transport through ENT2. For this reason, it was previously named *ei* (*equilibrative-insensitive to NBTI*) (Cass et al., 1998). ENT2 transports both purines and pyrimidines, like ENT1. Adenosine has a  $K_M$  of 30 – 70  $\mu\text{M}$  for transport by ENT2 (Griffith and Jarvis, 1996), but transport of guanosine by ENT2 is 10-fold lower than it is by ENT1 (Hammond, 1992; Ward et al., 2000), making ENT2 more selective for adenosine. Uridine is transported with a  $K_M$  of 70 – 280  $\mu\text{M}$  (Griffith and Jarvis, 1996), which is similar to its  $K_M$  for the ENT1 transporter. Less is known about the distribution of ENT2, as selective inhibitors are not available for binding studies. In human fetal and rat CNS preparations, ENT2 activity has been demonstrated in neurons (synaptosomes) and astrocytes (Lee and Jarvis, 1988; Gu et al., 1996). In rat brain, ENT2 mRNA is found predominantly in neurons in the hippocampus, dentate gyrus, cerebellum, cerebral cortex, and striatum (Anderson et al., 1999a). The ENT2 mRNA transcript is widely distributed in human and rat brain (Anderson et al., 1999a), but regional differences in expression of the ENT2 protein are unknown.

#### 1.4.2.2 Concentrative Nucleoside Transporters

Concentrative nucleoside transporters transport nucleosides against their concentration gradient in a sodium-dependent manner. Because adenosine concentrations are higher within the cell than they are in the extracellular space, an adenosine gradient exists from the cytosol to the extracellular space; concentrative nucleoside transporters usually transport adenosine against this gradient, into cells. In general, concentrative nucleoside transporters are 648 – 658 amino acid proteins consisting of 13 – 14 transmembrane domains (Cass et al., 1998). There are six functionally distinct sodium-dependent nucleoside transporters that are differentiated based on permeant selectivity, NBTI sensitivity, and sodium/nucleoside stoichiometry. However, little is known about each transporter in terms of function and distribution.

The CNT1, or N2, transporter is one of three concentrative nucleoside transporters that have been cloned (Huang et al., 1994). It is sometimes referred to as *cit* (*concentrative, insensitive to NBTI, thymidine selective*). The human CNT1 clone is a 648 amino acid, 71 kDa protein with an estimated 13 transmembrane domains (Huang et al., 1994). CNT1 is pyrimidine selective, but adenosine is also a permeant. Its  $K_m$  for adenosine is 15 – 26  $\mu\text{M}$ , which is similar to its  $K_M$  for uridine (21 – 37  $\mu\text{M}$ ) (Cass et al., 1998); however, adenosine permeability is poor compared to uridine, with respective  $V_{\text{max}}/K_M$  ratios of 0.003 and 0.57 (Yao et al., 1996). In human and rat, CNT1 activity has been demonstrated primarily in kidney and intestines (Griffith and Jarvis, 1996), in rat brain, CNT1 mRNA is widely distributed (Anderson et al., 1996b).

The CNT2, or N1, transporter is the only characterized sodium-dependent nucleoside transporter that prefers adenosine as a permeant (Cass et al., 1998). The

human CNT2 clone is a 658 amino acid protein containing 14 transmembrane domains, as well as a number of consensus sites for phosphorylation by protein kinases. CNT2 transports predominantly purine nucleosides, including adenosine, guanosine, and formycin B (an analog of inosine). Thus, previously it was called *cif* (*concentrative, insensitive to NBTI, formycin B selective*). *In vitro* studies have indicated its  $K_m$  for adenosine is 1 – 14  $\mu\text{M}$  (Griffith and Jarvis, 1996; Cass et al., 1998). CNT2 transports purines into the cell unless the sodium gradient is disturbed (Borgland and Parkinson, 1997). In human, CNT2 mRNA is found in liver, kidney, intestine, heart, skeletal muscle, pancreas, placenta, lung, and brain (Griffith and Jarvis, 1996; Cass et al., 1998).

The third of the cloned sodium-dependent nucleoside transporters is CNT3, or N3 (Ritzel et al., 2001). It was previously called *cib* (*concentrative, insensitive to NBTI, broadly selective*). CNT3 is permeable to both purines and pyrimidines. Distribution studies have shown the transporter to be present in leukemic blast cells, choroid plexus, and intestine (Cass et al., 1998). Little else is known about this transporter.

The remaining three sodium-dependent nucleoside transporters have not been well characterized. N4 appears to be similar to CNT1, but it is inhibited by guanosine (Griffith and Jarvis, 1996). It was originally isolated from renal brush border cells. N5 was isolated from human leukemic cells; it seems to transport guanosine selectively, and is sensitive to inhibition by NBTI (Griffith and Jarvis, 1996). Finally, the N6 transporter is quite similar to N5 except it allows adenosine permeation (Griffith and Jarvis, 1996). Future cloning of these last three sodium-dependent transporters will be needed in order to establish their individual roles.

## 1.5 Adenosine Metabolism

The intracellular and extracellular adenosine metabolic pathways dictate the amount of adenosine available to cell-surface adenosine receptors. Adenosine is formed and degraded intracellularly, and can then be released through nucleoside transporters into the extracellular space. Extracellular dephosphorylation of adenine nucleotides by an ecto-ATPase and, subsequently, the ecto 5'-nucleotidase (eN), as well as the deamination of adenosine by low levels of extracellular adenosine deaminase can also influence extracellular adenosine levels.

### 1.5.1 5'-Nucleotidase

The majority of adenosine is formed from adenine nucleotides via dephosphorylation by 5'-nucleotidase (EC 3.1.3.5). To date, three isoforms of this enzyme have been characterized: ecto 5'-nucleotidase (eN), cytosolic 5'-nucleotidase I (cN-I), and cytosolic 5'-nucleotidase II (cN-II) (Zimmermann, 1992). There is also some data to suggest that soluble forms of eN exist in the cytosol; these are referred to eN-s (Zimmermann, 1996). All of the above isoforms predominantly hydrolyse phosphate groups from 5'-monophosphates, but are also capable of metabolizing nucleotides (eg. ADP, ATP) and complex nucleotides like NAD to varying extents (Zimmermann, 1996).

#### 1.5.1.1 Ecto 5'-Nucleotidase (eN)

The ecto-5'-nucleotidase (eN; EC 3.1.3.5) is responsible for the extracellular dephosphorylation of AMP to adenosine and free phosphate. Dephosphorylation via eN is a transport-independent mechanism for increasing extracellular adenosine. This mechanism has been well characterized, mostly in heart. A glycosylphosphatidylinositol

at the C-terminus keeps eN anchored in the cell membrane; cleavage of this moiety produces a soluble form of eN (eN-s) found in cytosol, vesicles, and synaptic spaces (Zimmermann, 1992, 1996). eN-s is responsible for approximately 30% of total 5'-nucleotidase activity in CNS (Zimmermann, 1996). eN is present in most non-neuronal cells in rat brain *in vivo* (Kreutzberg et al., 1978; Snyder et al., 1983), but only in hippocampal neurons (Zimmermann, 1996, 2000). *In vitro* data suggest, however, eN presence in astrocytic and neuronal cultures (Stefanovic et al., 1976; Snyder et al., 1983; Meghji et al., 1989). In rat CNS, eN activity is highest in hippocampus and cerebellum, and lower in cortex, basal ganglia, and superior colliculus (Zimmermann, 1996, 2000).

eN has a native molecular weight of 125 – 150 kDa (Zimmermann, 1992). The enzyme consists of two identical subunits that are 62 – 74 kDa and 574 – 577 amino acids each (Misumi et al., 1990a; Misumi et al., 1990b). The  $K_M$  of eN for AMP and IMP is 1 – 50  $\mu$ M (Zimmermann, 1992). This enzyme requires zinc for activity, and is inhibited by micromolar levels of ATP and ADP. Interestingly, eN is inhibited by concentrations of methylxanthines that antagonize adenosine receptors, which may complicate the interpretation of the mechanism by which methylxanthines produce their physiological effects (Fredholm et al., 1978; Heyliger et al., 1981). However, it has been noted that inhibition of eN does not decrease extracellular adenosine significantly, so eN's contribution to methylxanthine effects may be minimal.

#### 1.5.1.2 Cytosolic 5'-Nucleotidase I (cN-I)

In addition to the ecto 5'-nucleotidase, there are two intracellular 5'-nucleotidases, cytosolic 5'-nucleotidase I and II (cN-I and cN-II, respectively). The cN-I isoform preferentially metabolizes intracellular AMP, while the cN-II isoform prefers

intracellular IMP as a substrate (Zimmermann, 1992). The cN-I isoform is of more direct relevance to intracellular adenosine levels.

cN-I (EC 3.1.3.5) dephosphorylates AMP to produce adenosine and free phosphate, with a  $K_M$  for AMP of 2-8 mM (Zimmermann, 1992). Despite the belief that cN-I is of primary relevance in adenosine formation, little is known about the function or distribution of this enzyme. It has been suggested that the rise in intracellular adenosine levels following ATP depletion is due to increased activity of cN-I on AMP. The adenosine produced can then be transported out of the cells in order to produce its physiological effects through interaction with adenosine receptors.

Most research regarding cN-I has been performed on cN-I from cardiac tissues. It was originally purified from pigeon heart in 1988 by Newby (Newby, 1988), and later from rabbit (Yamazaki et al., 1991), rodent (Skladanowski et al., 1996), canine (Darvish and Metting, 1993), and human (Oka et al., 1994; Skladanowski et al., 1996; Hunsucker et al., 2001) hearts. The isolated protein run on SDS-PAGE is 40 kDa, but the native protein appears to be a ~150 kDa tetramer (Skladanowski and Newby, 1990). Northern hybridization of pigeon and human tissues has revealed high levels of cN-I mRNA in cardiac tissues and skeletal muscle, low levels in total brain, and no detectable mRNA in kidney, blood vessel, and liver (Sala-Newby et al., 1999; Hunsucker et al., 2001). This variable distribution further confuses the understanding of the role that cN-I plays in the adenosine metabolic pathway.

cN-I has been cloned from pigeon heart (Sala-Newby et al., 1999) and human heart (Hunsucker et al., 2001). The human clone of cN-I is a 358 amino acid protein of 39.5 kDa. It has no sequence homology with eN or cN-II. cN-I activity can be increased

by high concentrations of intracellular ADP ( $>10 \mu\text{M}$ ) due to an increase in affinity for AMP, and due to an increase in the  $V_{\text{max}}$  of the reaction (Itoh et al., 1986). This increase in AMP affinity allows AMP to be metabolized to adenosine at concentrations  $<1 \text{ mM}$ . As well, free magnesium is required for cN-I activity; at  $1 \text{ mM}$ ,  $\text{Mg}^{2+}$  can increase cN-I activity (Yamazaki et al., 1991), while at  $10 \text{ mM}$ ,  $\text{Mg}^{2+}$  inhibits cN-I activity (Skladanowski and Newby, 1990). Finally, the optimum pH range for cN-I is  $6.8 - 7.8$ , and below  $6.8$ , activity of the isolated protein is completely abolished (Plagemann et al., 1988; Skladanowski and Newby, 1990; Yamazaki et al., 1991; Darvish and Metting, 1993).

Due to the limited distribution of cN-I, the role of cN-I in intracellular adenosine production is unclear. Transfection of cells with the cloned cN-I produces adenosine release during ATP depletion (Sala-Newby et al., 2000). As well, cellular levels of AMP, ADP, and  $\text{Mg}^{2+}$  are increased in hypoxic, ATP-depleting conditions, which would increase the activity of cN-I, if present. To date, no selective cell-permeable cN-I inhibitors are available to clarify the role of cN-I in intracellular adenosine production.

### ***1.5.2 S-Adenosylhomocysteine Hydrolase (SAHH)***

Although dephosphorylation of adenine nucleotides by 5'-nucleotidases is the predominant mechanism of adenosine formation, another enzyme exists that is capable of adenosine production. S-adenosylhomocysteine hydrolase (SAHH; EC 3.3.1.1) catalyzes the reversible cleavage of S-adenosylhomocysteine into adenosine and homocysteine. In heart, SAHH accounts for one third of adenosine production under normoxic conditions and almost none in hypoxic conditions (Lloyd et al., 1988; Deussen et al., 1989), but in

the brain, SAHH does not seem to be important in adenosine production (Latini and Pedata, 2001). The reverse reaction, in which adenosine and homocysteine are combined to create S-adenosylhomocysteine, is also catalyzed by SAHH, and seems to be preferred by the enzyme (Briske-Anderson and Duerre, 1982; Doskeland and Ueland, 1982). This reverse reaction may be more relevant in that it plays a role in the regulation of heart (Borst et al., 1992), liver (Doskeland and Ueland, 1982), and kidney (Kloor et al., 1996) adenosine levels. In the CNS, however, neither reaction appears to be important in regulating adenosine levels (Latini and Pedata, 2001). This is because although SAHH is widely distributed in the CNS and its affinity for adenosine is comparable to that of the main metabolic enzyme, adenosine kinase, low intracellular levels of homocysteine limit this method of adenosine metabolism. This pathway could, however, be important in CNS conditions where homocysteine levels are elevated, such as alcoholism (Bleich et al., 2000b; Bleich et al., 2000a) or vitamin B<sub>12</sub> deficiency (Penix, 1998), in which case adenosine would be required by SAHH to form SAH.

### *1.5.3 Adenosine Deaminase (ADA)*

Adenosine is predominantly metabolized by adenosine deaminase and adenosine kinase. Adenosine deaminase (ADA; EC 3.5.4.4) catalyzes the irreversible deamination of adenosine (or deoxyadenosine), producing ammonia and inosine (or deoxyinosine). There are a number of types of ADA, but the free nucleoside ADA is of most relevance to adenosine levels.

Although ADA is ubiquitous in mammalian tissues, tissue ADA activity is quite variable. High ADA activity has been reported in thymus, spleen, and gastrointestinal



tract; lower activity is reported in kidney, lung, and CNS (van der Weyden and Kelley, 1976). Within the CNS, ADA activity is greatest in hypothalamus and olfactory bulb, while the lowest activity is seen in hippocampus and striatum (Geiger and Nagy, 1986). As well, cellular distribution differences may exist, since astrocytic ADA activity is ~10-fold higher than neuronal ADA activity (Ceballos et al., 1994).

Although ADA exists primarily as an intracellular enzyme, there is an extracellular form, named ecto-ADA. This isoform has been documented in fibroblasts, blood, and endothelial cells (Franco et al., 1998). Most research has been performed in immune cells, as ecto-ADA binds CD26 to maintain its extracellular orientation (Franco et al., 1998; Cristalli et al., 2001). In the immune system it is thought to be important in cell signalling and immune system development. It is unclear what function ecto-ADA may have in the CNS, but it may be involved in CNS development and neuronal activity (through effects on adenosine levels) (Franco et al., 1997). As well, ecto-ADA has been reported to bind to and increase the affinity of adenosine  $A_1$  receptors (Ciruela et al., 1996; Saura et al., 1996; Saura et al., 1998).

There are two main cytosolic forms of the free-nucleoside ADA: ADA<sub>1</sub> and ADA<sub>2</sub>. ADA<sub>1</sub> is the main active isoform, since ADA<sub>2</sub> activity is only prevalent in human serum and plasma (Cristalli et al., 2001). ADA<sub>1</sub> exists as either a 363 amino acid, 33 kDa monomeric enzyme, or a 280 kDa dimer-combining protein complex (Cristalli et al., 2001). The ADA<sub>1</sub> isoform is a low-affinity, high-capacity enzyme with a  $K_M$  for adenosine of ~45  $\mu$ M (Wiginton et al., 1981). As well, ADA activity is highest between pH 7.0 and 7.4 (van der Weyden and Kelley, 1976). The activity of ADA is subject to endogenous inhibition by its product, inosine ( $K_i = 60 - 153 \mu$ M), as well as

hypoxanthine ( $K_i = 410 \mu\text{M}$ ) and guanosine ( $K_i = 190 \mu\text{M}$ ) (Fox and Kelley, 1978; Centelles et al., 1988). Besides pH and inhibition by endogenous purines, ADA lacks endogenous regulation and is instead seen as a “housekeeping” enzyme. Because of their relative  $K_M$  values for adenosine, it has been hypothesized that in a cell possessing both adenosine kinase and ADA activity, adenosine kinase activity will be of most relevance in physiological conditions, while ADA activity will be more relevant in pathophysiological conditions in which adenosine levels are elevated. Therapeutically, ADA inhibitors have been investigated to maintain elevated adenosine levels in certain CNS pathophysiological conditions, such as ischaemia (Phillis and O'Regan, 1989; Lin and Phillis, 1992; Gidday et al., 1995) or seizure (Zhang et al., 1993), although none are in current clinical use.

#### ***1.5.4 Adenosine Kinase (AK)***

Adenosine kinase (AK; EC 2.7.1.20) is the major metabolic enzyme for adenosine in physiological conditions. AK catalyzes the phosphorylation of adenosine (and related nucleosides) to produce AMP (or related 5'-monophosphate nucleotides). This reaction is an important step in replenishing ATP stocks in the cell, and is also important for metabolizing adenosine to control its cellular and extracellular levels.

AK has been shown to be a ubiquitous enzyme, occurring in all mammalian cell types and tissues investigated, and in the majority of eukaryotic species studied. AK has not been detected in prokaryotes, which may be due to their ability to synthesize purines *de novo*. Levels of AK vary depending on cell type and location. In rats, the highest levels of AK mRNA, protein, and activity have been demonstrated in liver and kidney,

with lower levels in spleen, lung, brain, heart, and skeletal muscle (Sakowicz et al., 2001). In Rhesus monkeys, AK activity was highest in liver, intermediate in kidney, lung, and erythrocytes, and low in brain, heart, and skeletal muscle (Krenitsky et al., 1974). In human autopsy samples, roughly equivalent levels of AK activity were seen in liver, kidney, pancreas, and brain cortex, and lower levels were seen in lung (Snyder and Lukey, 1982). However, there is some discrepancy between AK activity and mRNA studies in human, which show low mRNA levels but high activity in brain, and intermediate mRNA levels but low activity in skeletal muscle (Snyder and Lukey, 1982). In the rat CNS, the highest level of AK activity is in the olfactory bulb and cerebellum, and the lowest activity was reported in the septum and posterior hypothalamus (Geiger et al., 1997). There is no such AK activity data, unfortunately, for regions of the human CNS.

#### 1.5.4.1 Adenosine Kinase Structure

AK lacks similarity in structure to other nucleoside kinases. The majority of nucleoside kinases are dimers with subunits of 26 – 31 kDa which favour 2'-deoxynucleosides as substrates, and which contain the common kinase 1a, 2, and 3a nucleotide-binding motifs (Spychala et al., 1996). Cloning of AK has revealed it to be distinct from other nucleoside kinases, as it lacks the “classical” ATP-binding motif (an N-terminal P loop), acts as a monomer, and possesses a relative specificity for adenosine as a substrate (Spychala et al., 1996; McNally et al., 1997). It appears that AK shares more sequence similarity with microbial ribokinases and fructokinases, and its overall X-ray crystal structure is similar to that of *E. coli* ribokinase (Matthews et al., 1998). Thus AK belongs to the PfkB family of carbohydrate kinases.

Cloning of the human AK gene revealed two distinct isoforms of the enzyme which differ only at the N-terminus of the proteins; the larger isoform possesses an extra 16 amino acids at its N-terminus (McNally et al., 1997). Low sequence similarity exists in the 5' UTR and initial 5' coding region of the cDNAs, but the sequences are 100% identical following bases 105 and 113 of the short and long cDNA, respectively. It is thought that the two isoforms of the enzyme are produced from two distinct mRNA transcripts, which are a result of differential splicing of a common gene.

AK isolated from a number of species has been shown to be a monomer of 38 – 54 kDa (Latini and Pedata, 2001). The human AK isoform a, or “hAK-short”, is the form of the enzyme that appears most similar to the AK isolated and studied from most species and tissues. hAK-short is a 38.7 kDa protein consisting of 345 amino acids (Spychala et al., 1996; McNally et al., 1997). The human AK isoform b, or “hAK-long”, was only distinguished as a separate form in 1997 following cloning studies by McNalley *et al.* hAK-long is an approximately 40.5 kDa protein that consists of 361 amino acids (McNally et al., 1997). Aside from the N-terminal difference, the two AK isoforms share structural features.

X-ray crystallography studies show the hAK-short protein is composed of alpha-helices and beta-sheets folded into a large domain and a small domain, with the active site present on the large domain and the smaller domain acting as a flap overtop of the active site (Matthews et al., 1998). There is a binding site for the ribose moiety of adenosine, which is similar to the ribose-binding site in *E. coli* ribokinase (Sigrell et al., 1998). A similar binding site for the ATP (or other NTP, or adenosine) exists, and is similar to the ADP site of *E. coli* ribokinase. Finally, there is at least one magnesium-

binding site between the adenosine and ATP-binding sites of the enzyme (Matthews et al., 1998).

#### 1.5.4.2 Adenosine Kinase Activity

AK is the predominant adenosine-metabolizing enzyme in physiological conditions, as basal cellular adenosine levels approximate the  $K_M$  for AK. The reaction catalyzed by AK (adenosine + MgATP  $\rightarrow$  AMP + Mg-ADP) has been documented to proceed in an ordered bi-bi mechanism (Henderson et al., 1972; Palella et al., 1980; Richard et al., 1980; Rotllan and Miras Portugal, 1985; Hawkins and Bagnara, 1987; Mimouni et al., 1994; Spychala et al., 1996); MgATP<sup>2-</sup> binds first, allowing adenosine to bind to its site, and AMP and MgADP are subsequently released. A ping-pong bi-bi mechanism has also been proposed (Chang et al., 1983), with the creation of a phosphorylenzyme after the dissociation of MgADP, but there is no evidence to date to support this hypothesis. As well, the positioning of adenosine and MgATP<sup>2-</sup> binding sites so close to one another would facilitate direct transfer of the phosphate group to adenosine from MgATP<sup>2-</sup>, without the necessity of a phosphorylenzyme intermediate (Matthews et al., 1998).

AK is considered a high-affinity, low-capacity enzyme (as opposed to ADA, which is a low-affinity, high-capacity enzyme). AK has been reported to have a  $K_M$  for adenosine ranging from 0.2 – 20  $\mu$ M, depending on species and tissue conditions (Chang et al., 1980), although another study comparing recombinant AK with AK purified from human heart, human lymphoblasts, and pig liver reported  $K_M$  values of 40 – 75 nM (Spychala et al., 1996). The  $V_{max}$  of the reaction ranges from 0.3 to 2.2  $\mu$ mol/min/mg protein (Miller and Adamczyk, 1979; Chang et al., 1980). AK is also subject to substrate

inhibition (Palella et al., 1980; Fisher and Newsholme, 1984); adenosine concentrations of 2.5  $\mu\text{M}$  or more (when adenosine's  $K_M$  is 0.4  $\mu\text{M}$ ) inhibited enzyme activity in human placenta (Palella et al., 1980; Fisher and Newsholme, 1984). It is believed that in the optimal concentration range, adenosine binds to the high affinity adenosine site and the ATP of  $\text{MgATP}^{2-}$  binds to the lower affinity adenosine site; at higher concentrations, adenosine binds to both sites, thus preventing proper binding of the  $\text{MgATP}^{2-}$  and inhibiting the reaction (Geiger et al., 1997; Matthews et al., 1998).

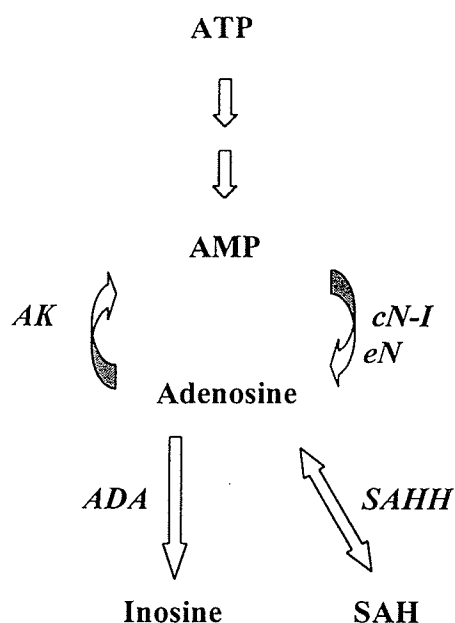
A number of endogenous factors are capable of affecting AK activity, which may explain the wide range of  $K_M$  and  $V_{\max}$  values. Most important among these factors is cellular/cytosolic pH. Optimal pH for AK activity is between 6.2 and 6.8, since the pK of the  $\gamma$ -phosphate of ATP is 6.5, meaning that as pH decreases from physiological pH, more  $\text{ATP}^{4-}$  is available to combine with  $\text{Mg}^{2+}$  to create the  $\text{MgATP}^{2-}$  needed to initiate the ordered bi-bi reaction (Palella et al., 1980). As well, as pH decreases, so does the  $K_M$  of AK for adenosine, and the capacity of adenosine for substrate inhibition of AK (Maj et al., 2000). At pH 5 – 6, AK is active and seems to be resistant to inhibition by  $\text{Mg}^{2+}$ , ATP, or adenosine; if pH increases above 7.5, AK activity decreases rapidly.

In addition to pH, certain endogenous substances can alter AK activity. As mentioned, sufficient free  $\text{Mg}^{2+}$  and  $\text{ATP}^{4-}$  are needed to form  $\text{MgATP}^{2-}$  and thus initiate the reaction. The ideal ratio of  $\text{Mg}^{2+}/\text{ATP}$  is 0.5 – 1. If the ratio is greater than 1, free  $\text{Mg}^{2+}$  can potently inhibit AK activity (Lindberg et al., 1967; Miller et al., 1979; Chang et al., 1980; Palella et al., 1980). (This may occur in ATP-depleting conditions like hypoxia and ischaemia.) As well, pentavalent ions have been shown to be required for AK activity in a number of species; inorganic phosphate has been shown to decrease the  $K_M$

and increase the  $K_i$  of adenosine for AK, and to increase the  $V_{max}$  of the reaction if abnormally high concentrations are present (Maj et al., 2000, 2002). As mentioned above, excessive adenosine can produce substrate inhibition; AMP and MgADP can also produce product inhibition (Palella et al., 1980; Mimouni et al., 1994). AMP is a competitive inhibitor of AK activity with a  $K_i$  of 140  $\mu\text{M}$ , and it is also a non-competitive inhibitor with a  $K_i$  of 500  $\mu\text{M}$ . ADP is a non-competitive inhibitor with a  $K_i$  of 11 – 500  $\mu\text{M}$ . Finally, as detailed in section 1.2 (Adenosine Pathways), stimulation of the cellular signalling molecule PKC by PMA inhibits the ability of AK to phosphorylate adenosine (Sinclair et al., 2000), suggesting a role for endogenous regulation of AK activity *via* cell signalling pathways.

It is obvious from the number of endogenous factors controlling AK function that the activity of this enzyme is complex and highly regulated. This could be due to the numerous cellular roles of adenosine, from RNA synthesis and nucleotide production to receptor activation leading to second messenger signalling. In some cell types, it has also been shown that there is a seemingly futile cycling of adenosine to AMP to adenosine again, which is controlled by AK and cN-I (Bontemps et al., 1983), as shown in Figure 2. This could be important in moment-to-moment regulation of adenosine levels. Because of this cycling, and also simply because AK controls adenosine levels in physiological conditions, inhibition of AK is being pursued for its therapeutic potential. Inhibition of AK could quickly increase intracellular adenosine levels (assuming adenosine levels are not already elevated), leading to increased release of adenosine *via* equilibrative nucleoside transporters into the extracellular space, where it could act on cell-surface receptors to produce beneficial actions. This will be discussed in the following section.

**FIGURE 2. Adenosine metabolism.**



A general schematic of the main players in the adenosine metabolic pathway is shown. Adenosine kinase (AK) is thought to be the most important metabolic enzyme in basal conditions, whereas adenosine deaminase (ADA) is thought to be more relevant in conditions in which adenosine levels are elevated, such as ischaemia. The cycle of AK and cN-I activities has been shown to be extremely active (in cardiac tissue), which may provide an endogenous mechanism for increasing adenosine levels quickly in times of need. Abbreviations are as follows: AK, adenosine kinase; cN-I, cytosolic 5'-nucleotidase; eN, ecto-5'-nucleotidase; ADA, adenosine deaminase; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase.



## 1.6 Therapeutic Potential of Adenosine

Due to wide distributions of adenosine receptors, transporters, and related metabolic enzymes, adenosine is an attractive therapeutic target for a number of conditions. However, despite its therapeutic potential, the development of adenosine-related therapies has been slow. Currently adenosine itself is sold as Adenocard<sup>TM</sup> (i.v.) for acute treatment of paroxysmal supraventricular tachycardia, and as Adenoscan<sup>TM</sup> (i.v.) for thallium cardiac imaging when evaluating coronary artery disease in patients unable to exercise (Fredholm et al., 2001). However, adenosine therapy has not yet been applied to CNS pathologies in the clinical setting.

The adenosine system is being investigated for a number of therapeutic possibilities. Outside of the CNS, the majority of studies are investigating the adenosine system for use in cardiovascular conditions (ischaemia), asthma (inflammatory component), and lymphoma. Within the CNS, the therapeutic potential of adenosine lies in its role as a neuromodulator, neuroprotectant, anti-nociceptive, and anti-inflammatory agent. Adenosine therapy holds promise in areas such as ischaemia, epilepsy, pain management, and Parkinson's disease. Most CNS therapies are thus focussed on receptor activation or inhibition with synthetic compounds or adenosine itself, subsequent to increasing extracellular adenosine levels. This can be accomplished through the use of receptor agonists and antagonists, inhibitors of nucleoside transport, or inhibitors of AK and ADA metabolism of adenosine.

As well, a number of established clinical agents whose mechanisms of action were previously poorly understood are now being found to work partly, or entirely, through the adenosine pathway. These agents include theophylline (used in asthma),

methotrexate (Cronstein et al., 1995) and sulfasalazine (Gadangi et al., 1996) (anti-inflammatories), dipyridamole and dilazep (anti-thrombotic), morphine (Sweeney et al., 1989; Cahill et al., 1995) (anti-nociception), nitric oxide donors (Fallahi et al., 1996; Rosenberg et al., 2000), antipsychotics, caffeine, and ethanol (Dar, 1990). Thus, an understanding of the adenosine system is important not only for the development of new adenosine-based therapies, but also for understanding the mechanisms behind the therapeutic effects of compounds in current use.

### *1.6.1 Adenosine Receptor Therapy*

The use of adenosine receptor agonists and antagonists has been widely investigated, but very few compounds have progressed to clinical trials. In the 1970s, adenosine receptor agonists were investigated predominantly as antihypertensives (Fredholm et al., 2001), but currently such agonists and antagonists are being investigated for a wide range of therapies.

Outside of the CNS, agonists and antagonists of adenosine receptors are being investigated for a variety of disorders. Adenosine A<sub>3</sub> receptor agonists have been shown to inhibit lymphoma cell proliferation; stimulation of A<sub>1</sub> and A<sub>3</sub> receptors has also resulted in increased production of granulocyte-colony-stimulating factor, which is chemoprotective (Fredholm et al., 2001). In asthma, adenosine acting on A<sub>2B</sub> and A<sub>3</sub> receptors has been shown to induce bronchoconstriction due to the release of allergic mediators from mast cells. The non-selective adenosine receptor antagonist, theophylline, has been used as an anti-asthmatic agent, and although the mechanism of action is as of yet unknown, it has been suggested that it may produce its effects through

the blockade of adenosine receptors, since a related methylxanthine, enprofylline, selectively blocks the A<sub>2B</sub> receptor at therapeutic concentrations (Fredholm et al., 2001). In cardiac ischaemia, A<sub>2A</sub> receptor activation by CGS 21680 has been shown to protect tissues from reperfusion injury by inhibiting neutrophil accumulation and thus reducing inflammation; stimulation of the A<sub>3</sub> receptor may have similar effects. As well, the anti-inflammatory effects of adenosine receptor agonism can produce ischaemic preconditioning, which can protect the heart from further ischaemic insult (Fredholm et al., 2001). The anti-inflammatory agents methotrexate and sulfasalazine have been shown to produce their effects through elevation of extracellular adenosine levels and subsequent activation of A<sub>2</sub> receptors on inflammatory cells (Cronstein et al., 1995; Gadangi et al., 1996); the elevation of adenosine by these agents has been shown to involve the dephosphorylation of adenine nucleotides by ecto-5'-nucleotidase (Morabito et al., 1998). Finally, the A<sub>1</sub> receptor agonist GR79236 is being investigated for insulin resistance in type II diabetes mellitus (Heseltine et al., 1995), as well as primary headache (Fredholm et al., 2001; Honey et al., 2002).

Within the CNS, adenosine agonists and antagonists have been investigated widely for use in ischaemia, anti-nociception, epilepsy, Parkinson's disease, and schizophrenia, and may also have potential as anti-inflammatories in haemorrhage and multiple sclerosis. A<sub>1</sub> agonism in focal and global ischaemia models (rats, gerbils, mice, pigs) can decrease mortality, neuronal loss (due to hyperpolarization of cells and decreased release of excitatory amino acids), and behavioural dysfunction when the agonist is administered pre- or post-ischaemia (Bischofberger et al., 1997). The selective agonist adenosine amine congener (ADAC) has been shown to be particularly effective in

this respect, as it reduces mortality and neuronal damage at a lower dose (75 – 100 µg/kg) than other A<sub>1</sub> agonists, and does not produce the usual cardiovascular side effects, namely bradycardia (Von Lubitz et al., 1996). Although A<sub>1</sub> receptor agonists seem to be most important in neuroprotection in cerebral ischaemia, agonism of the A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptors may also have benefits in ischaemia. Stimulation of A<sub>2A</sub> and A<sub>2B</sub> receptors can increase cerebral blood flow (due to vasodilation and inhibition of platelet activity), and thus may provide neuroprotection (Rudolphi et al., 1992); however, A<sub>2A</sub> receptor agonism can also cause excitatory actions, such as the potentiation of ischaemia-induced glutamate and aspartate release (O'Regan et al., 1992), which may make this an undesirable therapeutic alternative. Stimulation of A<sub>2</sub> receptors may also enhance re-growth and differentiation of neurons post-ischaemia (Neary et al., 1996), and stimulation of A<sub>2B</sub> receptors may also release neuroprotective substances from astrocytes, such as interleukin-6 (Schwaninger et al., 1997), which protects against glutamate toxicity and stimulates differentiation, and possibly neuronal growth factor (NGF), neurotrophin-3, and pleiotrophins (Neary et al., 1996). Finally, A<sub>3</sub> receptor stimulation may suppress tumour necrosis factor alpha (TNFα) release from human macrophages (Sajjadi et al., 1996) and cultured rat microglia (Schubert et al., 1997), suggesting a possible protective role for A<sub>3</sub> receptor stimulation in cerebral ischaemia, although this hypothesis is still poorly understood.

Adenosine analogues have also been investigated for pain response modulation. Both intrathecally- and systemically-administered adenosine analogues produce anti-nociception in tactile, pressure, and heat models of acute pain (Sollevi, 1997). The anti-nociceptive effect is due to activation of the A<sub>1</sub>, and not the A<sub>2</sub>, receptors present in the

spinal cord, since the A<sub>1</sub> agonist CPA was more effective in producing anti-nociception than the A<sub>2A</sub> agonist CGS 21680 in a rat model (Reeve and Dickenson, 1995). As well, methylxanthines have been shown to decrease morphine- and  $\beta$ -endorphin-mediated anti-nociception, and opioid receptor activation releases adenosine, suggesting that adenosine contributes to the anti-nociceptive effects of opioids (Sweeney et al., 1987). Finally, the anti-inflammatory effects of A<sub>1</sub> receptor activation could also act to indirectly decrease pain associated with the inflammatory response.

Adenosine is a therapeutic target in epilepsy because during convulsions, neurons use ATP quickly, producing increased levels of endogenous adenosine, which then can act as an anti-convulsant. Enhancement of endogenous adenosine in rat prepiriform cortex decreased susceptibility to seizures (Zhang et al., 1993), likely through A<sub>1</sub> receptor activation, as A<sub>1</sub> receptor agonists also inhibited seizures in mice (von Lubitz et al., 1994) and suppressed status epilepticus in rats, while A<sub>2</sub> receptor activation produced no anticonvulsant effect (Young and Dragunow, 1994). However, A<sub>1</sub> receptor stimulation causes sedation, which is a drawback when considering A<sub>1</sub> receptor agonists as therapeutic compounds. In addition to A<sub>1</sub> receptor agonism, A<sub>3</sub> receptor activation may have anticonvulsant properties, as chronic IB-MECA protected mice against chemically-induced seizures (von Lubitz et al., 1995).

In Parkinson's disease and schizophrenia, adenosine antagonists or agonists, respectively, are attractive therapeutically based on the co-localization of adenosine A<sub>2A</sub> receptors and dopamine D<sub>2</sub> receptors in the striatum, as well as the known effects of methylxanthines (namely motor stimulation) (Fredholm et al., 2001). The co-localization was determined following the realization in the mid-1970s that caffeine mimicked or

potentiated the motor effects of dopamine receptor agonists (Fuxe and Ungerstedt, 1974; Fuxe et al., 1975; Fredholm and al., 1976). Antagonism of  $A_{2A}$  receptors in a primate model of Parkinson's disease was found to be synergistic with  $D_2$  receptor agonism (Kanda et al., 2000), and there has been no evidence to indicate that tolerance develops to the motor-stimulating effects of selective  $A_{2A}$  antagonists (Fredholm et al., 2001). Theophylline has been shown to improve the anti-Parkinsonian effects of L-dopa (Mally and Stone, 1994), further supporting the use of adenosine antagonists in Parkinson's disease therapy. In addition to the implications for Parkinson's disease, the co-localization of  $A_{2A}$  receptors and dopamine  $D_2$  receptors in the striatum suggest a role for adenosine-based therapy in psychotic disorders like schizophrenia. Classical anti-psychotic drugs (haloperidol, phenothiazines) produce their effects through antagonism of dopamine  $D_2$  receptors; chronic blockade of  $D_2$  receptors, usually in the dorsal striatopallidal system, can lead to tardive dyskinesias (Ferre, 1994). Because  $D_2$  receptors are co-localized with the  $A_{2A}$  receptors predominantly in the ventral striatopallidal system,  $A_{2A}$  receptor agonists may be able to be used as anti-psychotics without producing extrapyramidal side effects like tardive dyskinesia.

Finally, the role of adenosine agonism is being investigated for anti-inflammatory effects in haemorrhage and multiple sclerosis (MS). Activation of striatal  $A_{2A}$  receptors with the selective agonist CGS 21680 in a rat model of intracerebral haemorrhage resulted in decreased  $TNF\alpha$  mRNA expression, parenchymal neutrophil infiltration, and cell death (Mayne et al., 2001). Adenosine levels and  $A_1$  receptor protein levels have been shown to be significantly decreased in the plasma and peripheral blood mononuclear cells (PBMCs), respectively, taken from MS patients as compared to those taken from

control subjects (Mayne et al., 1999). As well, cytokines like  $\text{TNF}\alpha$  and interleukin-6 (IL-6) have been implicated in MS;  $A_1$  receptor activation by the selective agonist R-phenylisopropyl-adenosine (R-PIA) was shown to modulate  $\text{TNF}\alpha$  and IL-6 production in PBMCs from control subjects and MS patients, respectively. The effects of R-PIA were blocked by the  $A_1$  receptor antagonist DPCPX (Mayne et al., 1999). A subsequent study indicated that  $A_1$  receptor mRNA and protein expression were diminished in brain and PBMCs from MS patients as compared to healthy controls and patients with other neurological diseases (Johnston et al., 2001). Thus, adenosine may play a role in the pathogenesis of MS *via* decreased  $A_1$  receptor levels and thus increased inflammation.

Despite the many potential therapeutic uses for adenosine receptor agonists and antagonists, development of such compounds is subject to a number of limitations. For CNS therapeutics, peripherally administered agonists and antagonists must be able to cross the blood-brain barrier, which has been a major setback for a number of compounds. As well,  $A_1$  receptor agonism is associated with hypothermia (Miller and Hsu, 1992), which has been suggested to be the true cause of adenosine-related neuronal protection in ischaemic models, and not more direct protective decreases in neuronal activity brought on by  $A_1$  receptor agonism.  $A_1$  receptor agonists have also been shown to be neuroprotective upon acute administration, but neurotoxic with chronic administration (Jacobson et al., 1996). Finally, due to the widespread distribution of adenosine receptors in the body, peripherally administered compounds for CNS disorders can cause side effects in the periphery, such as bradycardia and hypotension (Guieu et al., 1996; Von Lubitz, 1999), and bronchoconstriction in asthmatics (Cushley et al., 1983), depending on which receptor subtypes are activated by the doses administered.

### 1.6.2 REAL Therapeutic Agents

Although some selective adenosine receptor agonists and antagonists possess therapeutic potential, current research is largely focused on regulators of endogenous adenosine levels, or REAL agents (Geiger et al., 1997). This class of agents includes nucleoside transport inhibitors, to prevent adenosine reuptake into cells, and inhibitors of the metabolic enzymes AK and ADA, to decrease intracellular adenosine metabolism, thus increasing adenosine levels within cells, and subsequently increasing the amount of adenosine released from cells. It is thought that REAL agents could increase extracellular adenosine levels in a more tissue-specific manner, and thus decrease side effects associated with adenosine receptor agonists and antagonists. For example, in the case of cerebral ischaemia, an adenosine transport inhibitor could inhibit adenosine uptake from the extracellular space into cells, thus prolonging the duration of increased levels of adenosine and its action on adenosine receptors in the affected area. Because adenosine levels should only be elevated in the ischaemic area, the inhibitor should have little effect on other areas of the body which possess normal physiological adenosine concentrations.

#### 1.6.2.1 Inhibition of Adenosine Transport

Currently, the non-selective adenosine transport inhibitor, dipyridamole (Persantine<sup>TM</sup>) is used clinically as an anti-thrombotic agent, since adenosine is able to inhibit platelet aggregation *via* stimulation of A<sub>2A</sub> receptors. As well, the antiplatelet (Dawicki et al., 1985, 1986) and vasodilating (Saito et al., 1982; Zhang et al., 1991) effects of dilazep are thought to be due at least in part to adenosine uptake inhibition; nanomolar concentrations of dilazep inhibit the ENT1 transporter in most species, and



micromolar concentrations are able to inhibit the ENT2 transporter (Griffith and Jarvis, 1996; Cass et al., 1998). Adenosine transport inhibitors could also be useful in CNS conditions like cerebral ischaemia or seizure. Inhibition of adenosine transport has potential to be tissue-specific, as it would potentiate adenosine levels that have been raised due to decreased ATP synthesis and increased ATP consumption. As well, the increased adenosine levels would act on all available receptor subtypes, depending on their affinity for adenosine, which may be of some added benefit.

One REAL agent that has been studied is propentofylline, which has been demonstrated to be an inhibitor of adenosine uptake via three specific nucleoside transporter subtypes (ENT1, ENT2, CNT2) in cultured cells (Parkinson et al., 1993). It is thought that it is through the inhibition of the above transporter subtypes that propentofylline is able to increase endogenous extracellular adenosine levels (Andine et al., 1990), and provide neuroprotection in a number of animal stroke models (Parkinson et al., 1994). Propentofylline has also been shown to improve glucose utilization in human stroke patients (Huber et al., 1993). In addition to its effects on transport, however, propentofylline can also block adenosine receptors at high concentrations, inhibit cAMP phosphodiesterase, and increase the release of nerve growth factor in brain (Parkinson et al., 1994). Therefore, it remains unclear as to whether the neuroprotective effects of propentofylline are entirely due to its inhibition of adenosine transport.

Another REAL transport inhibitor that has been studied to some extent is nitrobenzylthioinosine (NBTI), which selectively inhibits ENT1/*es* at nanomolar concentrations (Cass et al., 1998). NBTI has been shown to decrease locomotor activity and seizures in mice, as well as decrease nociception (Geiger and Fyda, 1991). In cats,

NBTI was able to enhance the sleep-promoting effects of adenosine (Porkka-Heiskanen et al., 1997), and in pig global ischaemia, NBTI injected directly into cerebral cortex improved perfusion following an ischaemic insult (Gidday et al., 1996). However, NBTI has poor solubility in aqueous solutions, and crosses the blood-brain barrier poorly, despite its lipophilicity (Anderson et al., 1996a).

In addition to the inhibition of adenosine transport, there is some evidence to suggest that adenosine transport is affected in opioid-induced anti-nociception and ethanol-induced motor incoordination. Morphine treatment of rats was found to cause adenosine release from primary afferent nerve terminals in the dorsal horn of the spinal cord (Sweeney et al., 1989; Cahill et al., 1995); the release is believed to be mediated by the mu and delta opioid receptors (Cahill et al., 1995, 1996). The increase in extracellular adenosine may then act on adenosine A<sub>1</sub> receptors to enhance opioid-induced anti-nociception. In 1984, Proctor and Dunwiddie hypothesized that the CNS depressant effects of ethanol may be mediated by adenosine (Proctor and Dunwiddie, 1984). Since then, cross-tolerance between ethanol and adenosine has been noted in brain (Dar et al., 1994), and ethanol has been observed to potentiate the effects of adenosine by inhibiting its uptake (Nagy et al., 1990) and enhancing its release (Clark and Dar, 1989). As well, intracerebellar microinfusion of the A<sub>1</sub> receptor agonist CHA was found to accentuate ethanol-induced motor incoordination, and the antagonist DPCPX was found to antagonize the motor effects of ethanol (Dar, 1996). The exact mechanism for ethanol's effects on extracellular adenosine levels is still somewhat unclear, but it appears to involve alteration of adenosine transport.

Currently there are few other widely studied adenosine transport inhibitors. Transport inhibitors like propentofylline and NBTI hold significant therapeutic value, if the limitations of administration (solubility, blood-brain barrier permeability) and selectivity are overcome.

#### 1.6.2.2 Adenosine Deaminase Inhibitors

An additional means of increasing endogenous adenosine levels is through the use of inhibitors of adenosine metabolism as REAL agents. Inhibitors of ADA are thought to be more relevant in pathophysiological conditions when adenosine concentrations can reach the micromolar range, which approximates the  $K_M$  of ADA for adenosine (~45  $\mu\text{M}$ ), thus making ADA the predominant metabolic enzyme in such conditions.

Inhibitors of AK are thought to have relevance in physiological conditions, because basal adenosine levels (20 – 200 nM) are closer to the range of the  $K_M$  of AK for adenosine (40 – 75 nM), making AK the predominant metabolic enzyme in normal physiological conditions.

ADA inhibitors have been studied in the periphery for a number of conditions. ADA inhibitors have been studied for their use in viral infection and some lymphoproliferative disorders (Cristalli et al., 2001). They can potentiate antileukemic and antiviral nucleosides, since ADA inhibitors prevent the deamination (and inactivation) of such compounds. The ADA inhibitor deoxycoformycin (DCF) has been used clinically to treat hairy-cell leukemia (Klohs and Kraker, 1992). ADA inhibitors have recently been shown to attenuate myocardial ischaemic injury and improve recovery of contractility and metabolism post-ischaemia, since they enhance adenosine effects and

preserve ATP levels (Cristalli et al., 2001). As well, inhibition of ecto-ADA may be useful in hypertension, since adenosine acting on  $A_{2A}$  receptors decreases blood pressure.

ADA inhibitor use in the CNS is currently under investigation for ischaemia, excessive neuroexcitation, seizure activity, and pain control. Under normal physiological conditions, the commonly used ADA inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) produced no effect on endogenous adenosine levels or neuronal activity (Pak et al., 1994). However, in ischaemic conditions, the ADA inhibitor deoxycoformycin (DCF) increased adenosine release and recovery of nucleotides (Phillis and O'Regan, 1996), and when given before the ischaemic insult, DCF decreased neuronal damage in gerbil models (Phillis and O'Regan, 1989). As well, when given after the ischaemic insult, DCF decreased neuronal injury in newborn rats (Gidday et al., 1995). However, in some rat and gerbil models of ischaemia, DCF was not shown to be neuroprotective (Phillis, 1990; Delaney et al., 1993), and there is a surprising lack of effect of ADA inhibitors in models of pain and seizure (Zhang et al., 1993; Geiger et al., 1997). EHNA and DCF do not induce anti-nociception when administered alone and do not enhance opioid-induced anti-nociception (Geiger et al., 1997). When administered with exogenous adenosine, DCF was able to potentiate the anticonvulsant effects of adenosine, but when administered alone, DCF showed little or no ability to protect against bicuculline methiodide-induced seizures (Zhang et al., 1993).

### 1.6.2.3 Adenosine Kinase Inhibitors

AK inhibitors appear to hold much more potential than ADA inhibitors in terms of protection from insult in physiological conditions, since AK is the predominant adenosine-metabolizing enzyme in physiological conditions. AK inhibition is seen as a

means of increasing adenosine levels in an event- and site-specific manner, thus providing the beneficial effects of adenosine where they are needed, while minimizing the undesirable side effects seen with systemically administered adenosine and adenosine agonists. The most common AK inhibitors are 5'-iodotubericidin (Itu) and 5'-amino-5'-deoxyadenosine (NH<sub>2</sub>dAdo), both of which are effective at nanomolar concentrations (Geiger et al., 1997). Most AK inhibitors currently being investigated are analogues of these two compounds with similar potency to the parent compounds, but with increased bioavailability and cellular permeability, as well as a decreased likelihood of being metabolized by AK or other enzymes (Kowaluk et al., 1998).

AK inhibition has been investigated in inflammation, pain modulation, seizure activity, and ischaemia. AK inhibitors have been proven to reduce inflammation *in vivo* (Kowaluk et al., 1998; Kowaluk and Jarvis, 2000). These REAL agents have also been reported to produce analgesia *in vivo* when administered peripherally or spinally. Intrathecal NH<sub>2</sub>dAdo was shown to enhance opioid-induced anti-nociception in mice (Keil and DeLander, 1992). In general, AK inhibitors have been effective in treating acute nociceptive pain, chemically-induced pain, and neuropathic pain; these anti-nociceptive effects were able to be blocked by adenosine receptor antagonists like theophylline, indicating elevated endogenous adenosine was the mechanism by which the anti-nociception was produced (Kowaluk et al., 1998; Kowaluk and Jarvis, 2000).

AK inhibitors are also being investigated for the treatment of seizures, and to prevent damage resulting from ischaemia. Because adenosine has been shown to be neuroprotective in cases of ischaemic and seizure-induced damage, it is clear why REAL agents like AK inhibitors are being pursued therapeutically. Injection of Itu into the rat

preperiform cortex protected against damage caused by bicuculline methiodide-induced seizures; systemically administered 5'-deoxyiodotubericidin (dItu) protected against pentylenetetrazol-induced seizures (Zhang et al., 1993). Centrally-acting adenosine receptor antagonists were able to block the neuroprotective effects of AK inhibitors in this and other studies (Zhang et al., 1993; Wiesner et al., 1999; Ugarkar et al., 2000b; Ugarkar et al., 2000a). In the case of ischaemia, AK inhibitors would perhaps not be thought to be neuroprotective, since the increased endogenous adenosine levels resulting from increased ATP consumption and decreased ATP synthesis should inhibit AK by substrate inhibition. ADA metabolism would be hypothesized to be more important in such conditions. However, AK inhibitors have been shown to decrease neuronal infarct size, and neurological deficits following ischaemic insults, in a number of cerebral ischaemia models (Miller et al., 1996; Tatlisumak et al., 1998). For example, systemic dItu protected rats against damage caused by transient focal ischaemia (Jiang et al., 1997). It is still unclear why AK inhibition is effective in ischaemia models, but the success of these REAL agents has led to their being investigated in clinical trials. Finally, there is evidence to indicate that the effects of nitric oxide donors on the nervous system are caused by nitric oxide-induced adenosine release (Fallahi et al., 1996), and it has been suggested that this release of adenosine is a result of adenosine kinase inhibition (Rosenberg et al., 2000). Nitric oxide-induced increases in extracellular adenosine levels may be important in the regulation of behavioural states.

Due to the extensive therapeutic potential of adenosine and adenosine analogues, it is important to first elucidate the metabolic and cellular pathways and systems that

govern endogenous adenosine levels. This information can then be used to develop therapeutically-relevant adenosine receptor agonists and antagonists, as well as REAL agents. Such information is necessary for developing adenosine-based CNS therapy in particular, as few compounds to date are able to cross the blood-brain barrier and produce the desirable effects in the CNS.

## 2. OBJECTIVE

The purpose of this project was to create a recombinant human adenosine kinase isoform a (hAK-short; hAKS) for future use in adenosine metabolic pathway studies. A recombinant hAKS would especially assist the study of the metabolic regulation of endogenous adenosine levels by cellular signalling molecules like PKC.



### 3. MATERIALS AND METHODS

#### 3.1 Creation of the pSecTag2 B/AKS Vector

All materials were purchased from Invitrogen/Gibco Life Technologies (Burlington, Ontario) unless otherwise noted.

##### 3.1.1 PCR

PCR was performed in a 100  $\mu$ L reaction containing 100 ng template (the Invitrogen GeneStorm<sup>®</sup> pcDNA3.1/GS ADK vector), 400 nM primers (University of Calgary DNA Services, Calgary, Alberta), 200  $\mu$ M dNTPs, 4 mM MgSO<sub>4</sub>, ThermoPol Reaction Buffer (New England Biolabs, Pickering, Ontario), and 1 U Deep Vent<sub>R</sub><sup>®</sup> DNA Polymerase (New England Biolabs). Deep Vent<sub>R</sub><sup>®</sup> DNA Polymerase is a thermostable DNA polymerase isolated from *Pyrococcus* sp. GB-D which possesses a 3'-5' exonuclease ability, resulting in higher fidelity synthesis of cDNA (Cariello et al., 1991). A high fidelity enzyme was chosen due to the size of the desired PCR product (~1 Kb), which could be prone to mismatches with a non-proofreading enzyme.

PCR cycling was performed using a Perkin Elmer DNA Thermal Cycler (Perkin Elmer, Woodbridge, Ontario). Following initial denaturation (94°C, 3 min), amplification was performed for 25 cycles consisting of denaturation (94°C, 30 s) and a combined annealing and elongation step (68°C, 3 min). A final elongation step of 68°C for 7 min was done to ensure completion of cDNA products. The primer sequences used for amplification of the coding region of the short form of AK were: 5'-CCCAAGCTT ACGTCAGTCAGAGAAAATATTC-3' (forward, with a Hind III restriction site at 5'

end) and 5'-CCGCTCGAGTGTGGAAGTCTGGCTTCTCAG-3' (reverse, with Xho I restriction site at 5' end). The target sequence for AKS in the pcDNA3.1/GS vector was 1037 bp.

Products were analyzed by 1% agarose gel electrophoresis. Gels contained ethidium bromide and were visualized and photographed under UV light.

### **3.1.2 Zero Blunt® TOPO® Cloning of AKS PCR Product**

The AKS PCR product was cloned into the Invitrogen pCR®-Blunt II-TOPO® vector (see Appendix for vector map) using the Zero Blunt® TOPO® PCR Cloning kit, in order to ensure the production of overhanging ends on the AKS cDNA for subsequent ligation into pSecTag2B expression vector. The pCR®-Blunt II-TOPO® vector is pre-linearized and topoisomerase-activated to shorten ligation time and to improve cloning efficiency.

A 6 µL TOPO cloning reaction containing 3 µL PCR product, 10 ng pCR®-Blunt II-TOPO® vector, 240 mM NaCl, 12 mM MgCl<sub>2</sub>, 10% glycerol, 10 mM Tris-HCl (pH 7.4), 0.2 mM EDTA, 0.2 mM DTT, 0.02% Triton X-100, 20 µg/mL BSA, and phenol red, was incubated for 10 min at room temperature. The reaction was then added to 100 µL One Shot *Escherichia coli* and incubated on ice for 15 min. Cells were heat-shocked at 42°C for 30 s, and then 250 µL of SOC medium was added on ice. Cells were allowed to recover at 37°C for one hour with shaking at 225 rpm. All shaking was performed at 225 rpm, unless otherwise noted. Transformants were selected by plating on Luria-Bertani (LB) agar containing 50 µg/mL kanamycin and overnight incubation at 37°C. Colonies present on the plates the following day are expected to contain vector with AKS

insert, as the pCR<sup>®</sup>-Blunt II-TOPO<sup>®</sup> vector without an insert contains an undisturbed lethal *E. coli lacZ $\alpha$ -ccdB* fusion gene which codes for the toxic topoisomerase poison, CcdB.

Transformants were grown overnight at 37°C in LB broth containing 100 µg/mL ampicillin with shaking. The following day, plasmid DNA was isolated using the Gibco Concert<sup>®</sup> Rapid Plasmid Miniprep System. Briefly, bacteria were pelleted by centrifugation for 2 min at 16 000 x g in an Eppendorf Centrifuge 5415C microcentrifuge (Brinkmann Instruments Incorporated, Mississauga, Ontario) and resuspended in cell suspension buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 20 mg/mL RNase A). Cell lysis solution (200 mM NaOH, 1% SDS (w/v)) was added, incubated at room temperature for 5 min, and then a neutralization solution (acetate and guanidine HCl) was added. The mixture was centrifuged at 16 000 x g for 10 min and supernatant was poured into spin cartridges. The spin cartridge apparatus was centrifuged at 16 000 x g for 1 min, flow-through was discarded, and the cartridge was washed with wash buffer (proprietary solution of NaCl, EDTA, and Tris-HCl, pH 8.0). Plasmid DNA was eluted with TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and stored at 4°C until restriction endonuclease digestion.

Plasmid DNA was digested at 37°C for 90 – 150 min with Hind III and Xho I (1 U each) or EcoR I (2 U) in 20 µL reactions in order to evaluate PCR success and TOPO Cloning. Restriction digests were run on 1% agarose gels containing ethidium bromide and then visualized and photographed using UV trans-illuminescence.

### 3.1.3 Cloning of AKS into the Invitrogen pSecTag2 B Vector

The pSecTag2 B vector was purchased from Invitrogen. The vector features Zeocin<sup>TM</sup> and ampicillin resistance, an Igk leader sequence (translated onto the N-terminus of the recombinant protein), a region containing a variety of restriction sites for insertion of desired gene sequence, a *c-myc* coding region (translated onto the C-terminus of the recombinant protein), a polyhistidine coding sequence (translated onto the C-terminus of the recombinant protein), and T7 promoter and BGH reverse priming sequences, which were used for sequencing the vector. The Appendix contains a map of the pSecTag2 vector, as well as the sequence of the pSecTag2 B vector specifically, around the area for insertion of the PCR product. PCR primers for AKS had mutated start and stop codons in order to allow the Igk leader sequence, *c-myc* epitope, and polyhistidine tag to be translated as part of the recombinant protein.

The pSecTag2 B vector was digested with Hind III and Xho I (1 U each) for 120 min in order to produce overhangs for ligation of the AKS insert into the vector. The pSecTag2 B plasmid was then dephosphorylated with 1 U calf intestinal phosphatase (CIP) at 37°C for 60 min prior to ligation with the insert.

The AKS gene sequence was removed from the pCR<sup>®</sup>-Blunt II-TOPO<sup>®</sup> vector by restriction endonuclease digestion with Hind III and Xho I (1 U each) for 90 min. This produced the overhang ends for ligation into pSecTag2 B. The AKS insert was gel-purified on a 1% agarose gel and incubated with the digested, dephosphorylated pSecTag2 B at 14°C in the presence of T4 DNA ligase (1 U) overnight at ratios of 1:1 and 1:2 (vector:insert). Chemically competent DH5 $\alpha$  *E. coli* were then transformed with the ligation mixtures for 30 min on ice, followed by a 45 s heat-shock at 42°C, and 2 min

on ice. LB broth was added and cultures were allowed to recover for 1 hour at 37°C with shaking. Transformants were selected by plating on LB agar containing 100 µg/mL ampicillin incubated at 37°C overnight.

The following day, LB broth containing 100 µg/mL ampicillin was inoculated with selected transformants, and cultures were grown overnight at 37°C with shaking. The next day, plasmid DNA was isolated using the Gibco Concert<sup>®</sup> Rapid Plasmid Miniprep System as previously described. Restriction endonuclease digestions with either Hind III and Xho I (1 U each, 90 min), or EcoR I and Bgl II (1 U each, 150 min), were performed on the plasmid DNA to determine proper insertion of the AKS cDNA. Restriction digests were run on 1% agarose gels containing ethidium bromide and were visualized and photographed with UV trans-illumination.

In order to produce enough plasmid DNA for sequencing and subsequent transfection, 500 mL of LB broth was inoculated with pSecTag2 B/AKS transformants and was grown overnight at 37°C with shaking. The bacteria were then pelleted in a Sorvall<sup>®</sup> RC-5B Refrigerated Superspeed Centrifuge for 15 min at 4300 x g and 4°C (Kendro, Asheville, NC). Plasmid DNA was isolated using the QIAGEN<sup>®</sup> Plasmid Maxi Kit (QIAGEN Incorporated, Mississauga, Ontario). Briefly, cells were resuspended in a cell suspension solution (50 mM Tris·Cl, pH 8.0, 10 mM EDTA, 100 µg/mL RNase A) and were lysed by addition of a cell lysis solution (200 mM NaOH, 1% SDS (w/v)). A neutralization solution (3.0 M potassium acetate, pH 5.5) was added and cultures were centrifuged at 20 000 x g for 30 min at 4°C. Supernatant was applied to an equilibrated QIAGEN-tip 500 column, washed twice with provided wash buffer (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol (v/v)), and eluted with provided elution buffer (1.25 M

NaCl, 50 mM Tris·Cl, pH 8.5, 15% isopropanol (v/v)). The plasmid DNA in the eluate was precipitated with isopropanol and pelleted at 20 000 x g for 30 min at 4°C. The pellet was washed twice with cold 70% ethanol and resuspended in nuclease-free water.

To further ensure proper insertion of the AKS insert into the pSecTag2 B plasmid, and to evaluate success of PCR cloning of AKS, 200 ng plasmid DNA was combined with 10 pmol primer (T7 promoter primer for vector/insert junction at 5' end AKS, sense strand: 5'-TAATACGACTCACT ATAGGG-3'; or BGH reverse primer for vector/insert junction at 5'end AKS, antisense strand: 5'-TAGAAGGCACAGTCGAGG-3'; primers were synthesized by University of Calgary DNA Services) and sent to the Manitoba Institute of Cell Biology (MICB) for sequencing. In order to sequence the middle of the AKS gene in the plasmid, primers were designed to anneal with portions of the AKS sequence, one near the 5'end, sense strand (bases 18 – 37 of AKS sequence; 5'-TGGCT CAGTGGATGATTCAA-3'), and one near the 5'end, antisense strand (bases 607 – 626 of AKS sequence; 5'-TCTCCCTTGGGTGAAGATCA-3'). 200 ng plasmid DNA was combined with 10 pmol primer (synthesized by Invitrogen Custom Primers) and sent to MICB for sequencing.

## **3.2 Transfection of HEK 293 Cells with pSecTag2 B/AKS**

### **3.2.1 Culture of HEK 293 Cells**

All cell culture materials were purchased from Gibco Life Technologies (Burlington, Ontario) unless otherwise noted. HEK 293 cells were a kind gift from the laboratory of Dr. D. S. Sitar (University of Manitoba, Winnipeg, Manitoba). Cells were maintained in low glucose Dulbecco's modified Eagle medium (DMEM) containing 10%

fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL fungizone) in a humidified 37°C incubator aerated with 95% air/5% CO<sub>2</sub>. Two days prior to transfection, media was removed from cells and cells were washed with citrate saline (134 mM sodium chloride; 15 mM sodium citrate; chemicals were purchased from Fisher Scientific, Whitby, Ontario), detached with trypsin-EDTA (0.25% trypsin, 1 mM EDTA), and subcultured into fresh DMEM (10% FBS, 1% antibiotic/antimycotic) in 6-well plates (3 x 10<sup>5</sup> cells/well) or into T-75 flasks (2.5 x 10<sup>6</sup> cells/flask). This cell density allowed cells to be 40 – 70% confluent on the day of transfection.

### ***3.2.2 Transfection of HEK 293 Cells with pSecTag2 B/AKS in 6-Well Plates***

The initial transfection of HEK 293 cells with the pSecTag2 B/AKS vector was performed using Mirus *TransIT*<sup>®</sup>-293 Transfection Reagent (Mirus Corporation, Madison, WI). This reagent employs cationic liposomes and is optimized for simple and reproducible transfection of HEK 293 cells with minimal cell toxicity. On the day of transfection, two solutions containing 9 µL of the *TransIT*<sup>®</sup>-293 Transfection Reagent and 600 µL low-glucose, serum-free DMEM were prepared. The mixtures were incubated for 15 min at room temperature, and then 3 µg sterile plasmid DNA (pSecTag2 B/AKS or pSecTag2/PSA; diluted to 0.1 µg/µL prior to use) was added to each of the liposome/medium mixtures. (The pSecTag2/PSA plasmid was included with the Invitrogen pSecTag2 kit as a control plasmid, and it contains the sequence for prostate specific antigen.) The mixtures were incubated 15 min at room temperature in order to allow liposome-DNA complexes to form.

Meanwhile, the media was removed from the HEK 293 cells in the 6-well plate and was replaced with 2 mL fresh low-glucose DMEM (10% FBS, 1% antibiotic/antimycotic) per well. Following liposome-DNA complex formation, 213  $\mu$ L liposome-DNA-media mixture was added drop-wise to each well and the plate was rocked gently to distribute the complexes evenly over the cells. Three wells of HEK 293 cells were transfected with pSecTag2 B/AKS, and three were transfected with pSecTag2/PSA. Cells were returned to the 37°C, 95% air/5%CO<sub>2</sub> incubator and transfection was allowed to proceed for approximately 48 hours, following which the media was removed from each well into sterile microcentrifuge tubes. (The media was expected to contain the recombinant AKS, since the Igk leader sequence translated onto the N-terminus of the protein allows the protein to be secreted from the cell; the leader sequence is detached as the protein moves through the cell membrane.) Media samples were stored at 4°C until western analysis and adenosine kinase function assays could be performed.

### ***3.2.3 Transfection of HEK 293 Cells with pSecTag2 B/AKS in T-75 Flasks***

A subsequent transfection was performed in T-75 cell culture flasks in order to acquire more media containing the secreted recombinant hAK-short for western and activity assay purposes. The transfection proceeded as above, except a single transfection solution was prepared for each of two flasks, one for transfection with the pSecTag2 B/AKS vector and one for the pSecTag2/PSA control vector. Each transfection solution contained 24  $\mu$ L of the *TransIT*<sup>®</sup>-293 Transfection Reagent and 1.6 mL low-glucose, serum-free DMEM. Following the 15 minute incubation at room temperature, 8  $\mu$ g of the appropriate plasmid DNA was added to the liposome/media mixture and DNA-



liposome complexes were allowed to form for 15 min at room temperature. The transfection then proceeded as in the 6-well plates, except cells were washed first with serum-free media, and then covered with 15 mL fresh, serum-free media. A portion of the media collected from each transfection was concentrated using Centricon<sup>®</sup> YM-10 Centrifugal Filter Units (Millipore Canada Limited, Nepean, Ontario) in order to increase the concentration of the recombinant protein in the media.

### 3.3 Western Analysis of Transfected HEK 293 Media Samples

All materials were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise noted. Twenty-one microlitres of each media sample (~3 µg protein) was added to 7 µL 4X sample buffer (0.125 M Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate, 0.2% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) and samples were denatured by heating in a boiling water bath for 5 min. Samples from the initial transfection were loaded onto a 14% polyacrylamide gel and electrophoresed at 170 V for 1 hour. Samples from the subsequent flask transfection were loaded onto a 10% gel and electrophoresed at 80 V until samples entered the resolution gel, and then at 200 V for 45 min. A BenchMark Prestained Protein Ladder (GibcoBRL Life Technologies, Burlington, Ontario) and a c-myc positive protein lysate from the laboratory of Dr. R. Shiu at the University of Manitoba (as a positive control) were also loaded and electrophoresed on the SDS-PAGE of the initial transfection media samples; a MultiMark<sup>®</sup> Multi-Colored Standard (Invitrogen Corporation, Burlington, Ontario) and a Positope<sup>™</sup> control protein (Invitrogen Corporation, Burlington, Ontario) were loaded and electrophoresed on the SDS-PAGE of the T-75 flask transfection media samples. (The

Positope<sup>TM</sup> control protein contains a *c-myc* epitope, in addition to other commonly used antibody-binding epitopes, and was used to evaluate primary antibody binding.) Following electrophoresis, samples were transferred from the gel to Boehringer-Mannheim PVDF membrane (Roche Applied Science, Laval, Quebec) using a Bio-Rad Trans-Blot<sup>®</sup> SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Mississauga, Ontario). Transfer proceeded 50 min at 15 V. Following transfer, the membrane was temporarily stained with Ponceau S dye, and the gel with Coomassie Brilliant Blue to evaluate the efficiency of transfer. Ponceau S dye was then removed from the membrane by successive washes in ddH<sub>2</sub>O, and the membrane was blocked in 2% skim milk made in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5; chemicals were purchased from Fisher Scientific, Whitby, Ontario) for 1.5 h. The membrane was then incubated at 4°C overnight in Sigma anti-myc 9E10 antibody diluted 1/5000 in 1% skim milk, or Roche anti-myc 9E10 antibody (Roche Applied Science, Laval, Quebec). The membrane was then washed for 3 x 10 min in TTBS (0.1% Tween 20 in TBS) and incubated 30 – 60 min at room temperature in Sigma peroxidase-conjugated goat anti-mouse IgG. The blot was subsequently washed 3 x 10 min in TTBS, and was visualized with Boehringer Mannheim Chemiluminescence Substrate (Roche Applied Science, Laval, Quebec). Blots were placed in cassettes with Konica Medical Film (Konica Canada Incorporated, Mississauga, Ontario) and the chemiluminescence reaction was allowed to develop the film for 30 s to 10 min. Film was then developed in an AFP Imaging MINI-MED/90 X-ray Film Processor (AFP Imaging Corporation, Elmsford, NY) and a digital photograph was taken. Digital

photographs were also taken of the Coomassie-stained gel for the westerns of the second transfection, since problems were encountered with antibody binding to the blot.

### 3.4 Nickel Chromatography of Transfected Media Samples

All In order to purify any recombinant protein existing in the media, transfected media samples were applied to QIAGEN<sup>®</sup> Ni-NTA Spin Columns (QIAGEN Incorporated, Mississauga, Ontario). Ni-NTA spin columns contain a nickel-nitrilotriacetic acid (NTA) resin that binds hexahistidine tags present on proteins when a sample is passed through the resin during centrifugation.

All chemicals were purchased from Fisher Scientific (Whitby, Ontario) unless otherwise noted. Initially, media samples were dialyzed at 4°C overnight in a buffer containing 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, and 10 mM imidazole. This was done in order to remove sodium bicarbonate and amino acids (glycine, glutamate) from the media sample, since these agents are not compatible with the Ni-NTA resin, and to introduce a low concentration of imidazole to inhibit non-specific binding of proteins to the resin. The next day, Ni-NTA Spin Columns were equilibrated by applying 600 µL of the dialysis buffer mentioned above to the columns and centrifuging for 2 min at 700 x g in an IEC Micromax microcentrifuge; the flow-through was discarded. Following column equilibration, 600 µL of each dialysed transfected media sample was applied to each Ni-NTA Spin Column. Columns were then centrifuged for 2 min at 700 x g, following which the flow-through was discarded. Samples bound to the Ni-NTA resin were then washed twice with 600 µL of wash buffer containing 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, and 20 mM imidazole, pH 8.0; for each wash, wash buffer was applied to the spin

columns and columns were centrifuged at 700 x g for 2 min. Wash eluates were set aside for SDS-PAGE analysis. Potential hexahistidine-tagged proteins bound to the Ni-NTA resin were then eluted twice; each elution was performed by applying 150  $\mu$ L of elution buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 250 mM imidazole, pH 8.0) to each Ni-NTA Spin Column and centrifugation of spin columns for 2 min at 700 x g. Eluates were collected and eluates and wash samples were analyzed by SDS-PAGE (as performed for western analysis in section 3.3) with Coomassie Brilliant Blue (Sigma Chemical Company, St. Louis, MO) staining.

### **3.5 RT-PCR of Transfected HEK 293 mRNA**

All materials were purchased from Invitrogen Corporation (Burlington, Ontario) unless otherwise noted.

#### ***3.5.1 RNA Isolation***

All labware (microcentrifuge tubes, pipet tips, syringes, needles) was sterile to prevent contamination of isolated RNA samples. Water used in the RNA isolation was treated overnight with DEPC (Sigma Chemical Company, St. Louis, MO) and autoclaved the next day.

Non-transfected, pSecTag2 B/AKS-transfected, and pSecTag2/PSA-transfected HEK 293 cells from the second transfection were detached from the flasks following the collection of media, pelleted by centrifugation, and stored at  $-20^\circ\text{C}$  until RNA could be isolated using the Invitrogen S.N.A.P.<sup>TM</sup> Total RNA Isolation Kit. Cells were homogenized in lysis buffer (2.5 mM Tris, 0.25 mM EDTA, 5.25 M guanidine HCl, 1.5%

Triton X-100) and centrifuged at 16 000 x g in an IEC Micromax microcentrifuge to pellet cellular debris. The supernatant was transferred to a fresh microcentrifuge tube, isopropanol was added, and the mixture was placed in a spin column. The spin column apparatus was centrifuged at 16 000 x g for 1 min, at which point the filtrate was discarded. The column was washed twice with 100 mM NaCl in 75% ethanol, and the nucleic acids were eluted from the column with RNase-free water. RNase-free DNase was added to the eluate with appropriate buffer (4 mM Tris, pH 8.0; 600  $\mu$ M MgCl<sub>2</sub>; 200  $\mu$ M CaCl<sub>2</sub>) and incubated at 37°C for 10 min in order to remove DNA from the RNA sample. Binding buffer (7M guanidine-HCl; 2% Triton X-100) was added to the solution, followed by isopropanol, and the mixture was passed through another spin column by centrifugation at 16 000 x g for 1 min. The column was washed twice and then the RNA was eluted with 10 mM Tris, pH 8.0. The concentration of isolated RNA was determined by measuring the A<sub>260</sub>, and using the equation:

$$[\text{RNA}] = (A_{260})(0.04 \mu\text{g}/\mu\text{L})(\text{dilution factor})$$

Isolated RNA was stored at -20°C until RT-PCR was performed.

### 3.5.2 RT-PCR

The RT step was performed using the Invitrogen ThermoScript™ RT-PCR System Kit. For each RT reaction, 3  $\mu$ g total RNA, oligo(dT)<sub>20</sub> primer (5  $\mu$ M final concentration), and DEPC-treated water were combined in a thin-walled PCR tube to a total volume of 10  $\mu$ L. The solution was heated to 65°C for 5 min to denature RNA and

then placed on ice. The remaining components for the RT reaction were added (250 mM Tris acetate, pH 8.4, 375 mM potassium acetate, 40 mM magnesium acetate, 5 mM DTT, 40 U RNaseOUT™, 1 mM dNTPs, 15 U ThermoScript™ RT) to a final volume of 20 µL. The reaction was incubated at 57°C for 60 min, and then heated to 85°C for 5 min to terminate the reaction. *E. coli* RNase H (2 U) was added to each reaction and reactions were incubated at 37°C for 20 min. This was done in order to digest the majority of the RNA, leaving only the first-strand cDNAs in the reaction tubes. An aliquot of each completed RT reaction was used immediately for PCR, and the remaining reactions were placed at -20°C for storage.

PCR was performed using Invitrogen Platinum *Taq* DNA Polymerase (as part of the ThermoScript RT System). For each PCR reaction, 5 µL of the cDNA produced in the RT reaction was used. For a final reaction volume of 50 µL, the following components were added to each PCR reaction (final concentrations are indicated): 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.82 mM MgCl<sub>2</sub>, 200 nM each dNTP, 200 nM each primer (forward and reverse), 2 U Platinum *Taq* DNA Polymerase, DEPC-treated water. A drop of mineral oil was placed overtop of each reaction in order to prevent condensation. Primer sequences were as follows: 5'-GTACTGCTGCTCTGGGTTCC-3' (AKS and PSA forward; the primer anneals to part of the Igk leader sequence), 5'-TCCAACAAATGCATCTCCAG-3' (AKS reverse; the primer anneals near the 3' end of the AK short sequence), 5'-GGTGTCTTGATCCACTTCC-3' (PSA reverse; the primer anneals near the 3' end of the PSA sequence).

Reactions were placed in a MJ Research PTC-100™ Programmable Thermal Controller (Fisher Scientific, Whitby, Ontario) and heated for 2 min at 94°C in order to

denature the template cDNA and to activate the polymerase. Initial denaturation was followed by 28 cycles of 94°C for 30 s (denaturation), 55°C for 30 s (annealing), and 72°C for 1 min (extension). A final elongation step of 72°C for 7 min was performed to finish extending incomplete cDNAs. PCR products were analyzed by agarose gel electrophoresis (1% agarose gel) and visualized with ethidium bromide staining. A Kodak Electrophoresis Documentation and Analysis System (Fisher Scientific, Whitby, Ontario) camera was used to capture an image of the gel using Kodak digital science dS 1D v2.0.2 software (Fisher Scientific, Whitby, Ontario). The remaining portions of the PCR reactions were stored at -20°C.

### **3.6 Assay for Recombinant Adenosine Kinase Activity**

Bovine serum albumin was purchased from GibcoBRL Life Technologies (Burlington, Ontario). The adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was purchased from Research Biochemicals International (Natick, MA). Glycerol was purchased from Boehringer Mannheim (Roche Applied Science, Laval, Quebec). 2-Mercaptoethanol and ATP were purchased from Sigma Chemical Company (St. Louis, MO). MgCl<sub>2</sub>, KCl, NH<sub>4</sub>COOH, and HCl were purchased from Fisher Scientific (Whitby, Ontario). [<sup>3</sup>H]Adenosine was purchased from NEN Life Sciences (Mississauga, Ontario). Ethanol was purchased from Commercial Alcohols Incorporated (Toronto, Ontario).

For a positive control, human promonocytic U937 cells (for 6-well plate transfection) or non-transfected HEK 293 cells (for flask transfection) were lysed by homogenization in cold 50 mM Tris-HCl, pH 7.4 (Fisher Scientific, Whitby, Ontario) and

cytosolic protein was obtained by centrifugation in a Fisher Scientific Micro-Centrifuge Model 235V (Fisher Scientific, Whitby, Ontario) at 16 000 x g for 20 minutes at 4°C. Supernatant was removed into a fresh microcentrifuge tube and stored at 4°C until use.

Prior to the AK activity assay, the concentration of protein in transfection media samples and the U937 and HEK 293 cytosolic extracts was measured using the Bradford protein assay (Bradford, 1976). Bradford reagent was purchased from BioRad Laboratories (Mississauga, Ontario). Protein measurements were conducted in 96-well plates, and readings were taken with a Molecular Devices E<sub>max</sub> Precision Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA).

On the day of the assay, an aliquot (80 µL) of each sample was denatured by heating to 85°C for 10 min and then placed on ice until use. To begin the assay, 20 µL of either sample or denatured sample was added to 80 µL of reaction mixture (final concentrations: 0.1% bovine serum albumin, 0.5 µM EHNA, 5% glycerol, 1.6 mM MgCl<sub>2</sub>, 50 mM 2-mercaptoethanol, 1.2 mM ATP, 50 mM KCl, and 20 µM [<sup>3</sup>H]adenosine) in microcentrifuge tubes, in triplicate, and reactions were incubated at 37°C for 2 h. Reactions were then terminated by heating to 85°C for 10 min. Twenty microlitres of each reaction were then spotted onto DE81 ion exchange filters (Fisher Scientific, Whitby, Ontario) and spots were allowed to dry. (DE81 ion exchange filters bind phosphorylated compounds and so should bind the [<sup>3</sup>H]AMP created by a successful AK reaction.) The filters were then placed in scintillation vials and were washed twice with 5 mL 1 mM NH<sub>4</sub>COOH, twice with 5 mL distilled deionized water, and twice with 5 mL 100% ethanol. Each wash lasted 5 min. Following the final ethanol wash, 250 µL of 0.2 M HCl and 250 µL of 0.8 M KCl were added to each tube. Tubes were vortexed



briefly, incubated at room temperature for 10 min, vortexed briefly again, and 4.5 mL scintillation fluid (Beckman Coulter Canada Incorporated, Mississauga, Ontario) was added to each tube. After vortexing to mix thoroughly, radioactivity was determined by scintillation counting in a Beckman LS 6000TA counter (Beckman Coulter Canada Incorporated, Mississauga, Ontario) for 5 min/sample.

Activity of AK was expressed as fmol [ $^3\text{H}$ ]AMP formed/mg protein. Results were paired (denatured sample and non-denatured sample) and analyzed with one-tailed paired t-tests. Data analysis and graphing was performed using GraphPad PRISM<sup>®</sup> Version 3.00 software (GraphPad Software Incorporated, San Diego, CA).

## 4. RESULTS

### 4.1 Creation of pSecTag2 B/AKS Vector

PCR was performed with the Invitrogen GeneStorm<sup>®</sup> pcDNA3.1/GS ADK vector as a template in order to produce a short-form human adenosine kinase cDNA for molecular cloning purposes. Restriction sites for Hind III and Xho I restriction endonucleases were designed into the PCR primers in order to assist subsequent ligation. Successful PCR produced a linear cDNA of ~1050 bp; a product of 1039 bp was expected for the short isoform of human adenosine kinase. The PCR product was restriction digested with EcoR I in order to further clarify whether the cDNA was that of the short form of hAK. Following a 2 hour digest at 37°C, gel electrophoresis revealed two bands of ~1050 bp (undigested PCR product) and ~850 bp, which approximated the theoretical digestion band of 836 bp. A band corresponding to the 203 bp fragment expected from the digestion was not seen, but may have migrated off the gel.

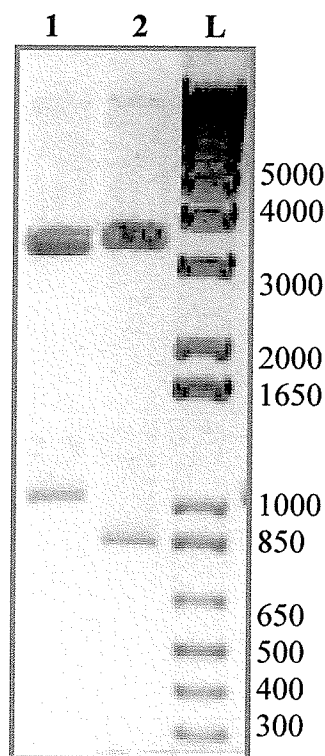
Following the synthesis of the hAKS cDNA, TOPO cloning of hAKS was performed in order to more easily digest the hAKS insert ends with Hind III and Xho I to produce the sticky ends required for ligation into the mammalian expression vector, pSecTag2 B. The pCR<sup>®</sup>-Blunt II-TOPO<sup>®</sup> vector map is shown in The Appendix, with restriction sites and insertion site indicated. Insertion of hAKS into the TOPO vector was evaluated by two methods. First, TOP 10 *E. coli* successfully transformed with a successful ligation mixture will grow on LB agar plates containing ampicillin, whereas unsuccessful transformants will not grow (due to lack of ampicillin resistance), and bacteria transformed with unsuccessful ligation mixtures will not grow (due to a lethal gene surrounding the insertion site). The fact that colonies were present on the LB agar

ampicillin plates suggests that a fragment was inserted into the TOPO vector. Secondly, in order to ensure hAKS insertion (and not insertion of an incomplete PCR product), plasmid DNA was isolated from cultures of transformed bacterial colonies, and restriction digestions were performed on the isolated DNA. A Hind III/Xho I digest of the plasmid DNA is predicted to produce fragments of 3833 bp, 1039 bp, 60 bp, and 38 bp. (These last two fragments were not expected to be seen due to their small size; they are a result of two sets of Hind III and Xho I restriction sites, one set in the vector near the insertion site, and one set at the ends of the hAKS insert. hAKS restriction sites are indicated in Figure 5 below; vector restriction sites are presented in The Appendix). Agarose gel electrophoresis revealed bands of ~3500 bp and ~1050 bp, as shown in Figure 3. Because the pCR<sup>®</sup>-Blunt II-TOPO<sup>®</sup> vector is designed with EcoR I sites flanking the insertion site, a second digest with EcoR I was performed, with expected fragments of 3915 bp, 848 or 842 bp (depending on orientation of PCR product insertion), and 209 or 214 bp. A band of 1039 bp (the size of the hAKS insert) was not expected because the hAKS gene sequence also contains one EcoR I restriction site. Gel electrophoresis indicated DNA fragments of ~3700 bp and ~850 bp (Figure 3). It is possible that the ~210 bp fragment ran off the gel. The digestion results, in addition to the survival of *E. coli* on LB agar containing ampicillin, strongly suggest hAKS was successfully inserted into the TOPO vector.

In order to excise the hAKS cDNA with sticky ends, the TOPO/AK vector was digested with Hind III and Xho I. The hAKS cDNA was purified by agarose gel electrophoresis. The pSecTag2 B expression vector was also digested with Hind III and Xho I to produce complimentary sticky ends for ligation, and then was dephosphorylated

prior to ligation. Ligation was performed overnight, and the following day DH5 $\alpha$  *E. coli* were transformed with the ligation mixtures. Plasmid DNA was isolated from successful transformants and restriction digestions were performed to determine whether hAKS had inserted into pSecTag2 B, and if so, whether it had inserted in the proper orientation for transcription. A Hind III/Xho I digest gave ~5100 bp and ~1050 bp bands, and a suggestion of a band >5100 bp (due to the thickness of the 5200 bp band). These results correlate well with the expected digestion fragments of 5092 bp and 1039 bp, and the size of the undigested vector (6131 bp). An EcoR I/Bgl II digest was expected to give DNA fragments of 4939 bp and 1192 bp, and gel electrophoresis indicated that the sizes of the actual DNA fragments (~5000 bp, ~1200 bp, and a band >5100 bp) approximated those of the expected fragments and undigested vector. The gel electrophoresis results for both digests are shown in Figure 4. hAKS restriction sites for Hind III, Xho I, and EcoR I are indicated in Figure 5 (sequencing results); vector restriction sites for Hind III and Xho I are indicated also in The Appendix.

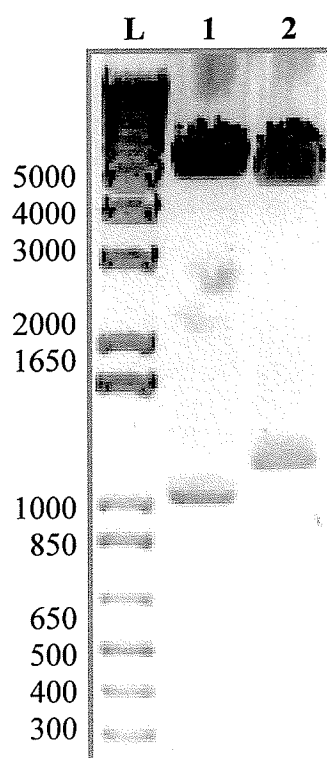
To determine whether the hAKS insert contained any errors (insertions, deletions, mismatches) at the junctions with pSecTag2 B, or within the hAK sequence, pSecTag2 B/AKS plasmid DNA samples were sent for sequencing at the Manitoba Institute of Cell Biology. The combined sequencing results are shown in Figure 5, and the translated sequence is shown in Figure 6. The junctions between the vector and insert were free of errors; however, the internal hAK sequence contained 4 single-base variations from the Genbank sequence, 3 of which were determined to lead to amino acid changes, shown in Figure 6. Two mismatches were determined to be due to differences between the PCR template (Invitrogen GeneStorm<sup>®</sup> pcDNA3.1/GS ADK vector) and the Genbank

**FIGURE 3. Restriction digestions of TOPO/AKS plasmid DNA.**

The TOPO/AKS plasmid was digested with Hind III/Xho I (1) or EcoR I (2) in order to determine whether the hAKS PCR product had been inserted into the TOPO vector.

Numbers on the right side of the figure are base pair indications for the 1 Kb Plus ladder (L). For the Hind III/Xho I digest, fragments of 1039 bp and 3519 bp were expected.

For the EcoR I digest, fragments of 3931 bp, 848/842 bp (depending on orientation of insert), and 209/214 bp (depending on orientation) were expected. The results suggest that the hAKS PCR product was successfully inserted into the TOPO vector.

**FIGURE 4. Restriction digestions of pSecTag2 B/AKS vector.**

The pSecTag2 B/AKS plasmid DNA was digested with Hind III/Xho I (1) or EcoR I/Bgl II (2). Numbers on the left side indicate the base pair sizes of the 1 Kb Plus ladder (L). The Hind III/Xho I digest was expected to produce DNA fragments of 5092 bp and 1039 bp. The EcoR I/Bgl II digest was expected to produce DNA fragments of 4939 bp and 1192 bp. The results indicate that the hAKS sequence was indeed inserted into the pSecTag2 B vector.

FIGURE 5. Combined sequencing results for pSecTag2 B/AKS.

<sup>1</sup> ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGT  
 Ig<sub>K</sub> leader sequence (secretion signal)

<sup>53</sup> CCACTGGTGACGCGGCCAGCCGGCCAGGCGCGCGCCGTACGA<sup>A</sup>AGCTTA  
 Hind III site  
 5' primer for AKS

<sup>104</sup> CGTCAGTCAGAGAAAATATTCTCTTTGGAATGGGAAATCCTCTGCTTG  
 5' primer for AKS (cont'd)

<sup>152</sup> ACATCTCTGCTGTAGTGGACAAAGATTCCTTGATAAGTATTCTCTGAA  
<sup>201</sup> ACCAAATGACCAAATCTTGGCTGAAGACAAACACAAGGAAGTCTTGA  
<sup>249</sup> TGAAGTTGTGAAAAAATTCAAAGTCGAATATCATGCTGGTGGCTCTAC  
 EcoR I site

<sup>297</sup> CCA<sup>A</sup>GAATTCAATTAAGTGGCTCAGTGGATGATTCAACAGCCACACAA  
<sup>345</sup> AGCAGCAACATTTTTTGGATGCATTGGGATAGATAAATTTGGGGAGAT

<sup>393</sup> CCTGAAGAGAAAAGCTGCTGAAGCCCATGTGGATGCTCATTGCTACGA  
<sup>441</sup> GCAGAATGAGCAGCCAACAGGAAGTGTGCTGCATGCATCACTGGTG  
<sup>488</sup> ACAACAGGTCCCTCATAGCTAATCTTGCTGCTGCCAATTGTTATAAAAA  
<sup>537</sup> GAAAAACATCTTGATCTGGAGAAAAACTGGATGTTGGTAGAAAAAGC  
 CAC

<sup>585</sup> AAGAGTTTGTATATAGCAGGCTTTTTTCTTACAGTTTCCCAGAGTCA  
<sup>634</sup> GTATTAAGGTGGCTCACCATGCTTCTGAAAACAACAGGATTTTCACT  
 C

<sup>682</sup> TTGAATCTATCTGCACCGTTTATTAGTCAGTTCTACAAGGAATCATTGA  
 T

<sup>731</sup> TGAAAGTTATGCCTTATGTTGATATACCTTTTGGAAATGAGACAGAAG  
<sup>779</sup> CTGCCACTTTTGCTAGAGAGCAAGGCTTTGAGACTAAAGACATTAAG  
<sup>827</sup> AGATAGCCAAAAAGACACAAGCCCTGCCAAAGATGAACTCAAAGAGG  
<sup>874</sup> CAGCGAATCGTCATCTTCACCCAAGGGAGAGATGACACTATAATGGCT  
<sup>922</sup> ACAGAAAGTGAAGTCACTGCTTTTGCTGTCTTGGATCAAGACCAGAAA  
<sup>970</sup> GAAATTATTGATACCAATGGAGCTGGAGATGCATTTGTTGGAGGTTTT  
<sup>1018</sup> CTGTCTCAACTGGTCTCTGACAAGCCTCTGACTGAATGTATCCGTGCT  
<sup>1066</sup> GGCCACTATGCAGCAAGCATCATAATTAGACGGACTGGCTGCACCTTT  
 Xho I site

<sup>1113</sup> CCTGAGAAGCCAGACTTCCACA<sup>C</sup>TGAGGAGGGCCCGAACAAAAACTCA  
 3' primer for AKS (complimentary sequence) *c-myc* epitope

<sup>1164</sup> TCTCAGAAGAGGATCTGAATAGCGCCGTGACCATCATCATCATCATT  
*c-myc* epitope (cont'd) poly His

<sup>1215</sup> GA

Above are the combined sequencing results for the sense strand of pSecTag2 B/AKS from the transcriptional start codon (ATG) to the transcriptional stop codon (TGA). The PCR primer sequences for hAKS, Ig<sub>K</sub> leader sequence, *c-myc* epitope, and hexahistidine

tag are underlined and labelled. The Hind III and Xho I restriction sites used for ligation of the hAKS insert into the pSecTag2 B vector are italicized and labelled as well. The EcoR I restriction site used in restriction digestions to confirm insertion of hAKS is indicated with italics as well. The four mismatch mistakes are indicated in the middle of the hAKS insert sequence, with the correct bases indicated above in italics. Two of the mistakes ( $C^{617}A^{618}C^{619} \rightarrow ACA$ ;  $T^{58} \rightarrow C$ ) are due to differences between the PCR template sequence (Invitrogen GeneStorm<sup>®</sup> pcDNA3.1/GS ADK vector) and the Genbank sequence (accession NP\_001114; GI: 4501943); both mismatches lead to amino acid changes. The remaining two mismatches ( $A^{434} \rightarrow G$ ;  $C^{708} \rightarrow T$ ) are due to PCR errors; only one of these mistakes leads to an amino acid change ( $A^{434} \rightarrow G$ ).



FIGURE 6. Translated recombinant hAKS amino acid sequence.

<sup>1</sup> ATGGAGACAGACACACTCCTGCTATGGGTA<sup>19</sup>CTGCTGCTCTGGGTTCCAGGTTCCACT  
M E T D T L L L W V L L L W V P G S T<sup>19</sup>  
<sup>58</sup> GGTGACGCGGCCAGCCGCGCCAGGCGCGCGCCGTACGAAGCTTACGTCAGTC  
G D A A Q P A R R A R R T K L T S V<sup>37</sup>  
<sup>112</sup> AGAGAAAATATTCTCTTTGGAATGGGAAATCCTCTGCTTGACATCTCTGCT  
R E N I L F G M G N P L L D I S A<sup>54</sup>  
<sup>163</sup> GTAGTGGACAAAGATTTCTTGATAAGTATTCTCTGAAACCAAATGACCAA  
V V D K D F L D K Y S L K P N D Q<sup>71</sup>  
<sup>215</sup> ATCTTGGCTGAAGACAAACACAAGGAACTGTTTGATGAACTTGTGAAAAAA  
I L A E D K H K E L F D E L V K K<sup>88</sup>  
<sup>266</sup> TTCAAAGTCGAATATCATGCTGGTGGCTCTACCCAGAATTCAATTAAGTG  
F K V E Y H A G G S T Q N S I K V<sup>105</sup>  
<sup>317</sup> GCTCAGTGGATGATTCAACAGCCACACAAAGCAGCAACATTTTTTGGATGC  
A Q W M I Q Q P H K A A T F F G C<sup>123</sup>  
<sup>368</sup> ATTGGGATAGATAAATTTGGGGAGATCCTGAAGAGAAAAGCTGCTGAAGCC  
I G I D K F G E I L K R K A A E A<sup>140</sup>  
<sup>419</sup> CATGTGGATGCTCATTGCTACGAGCAGAATGAGCAGCCAACAGGAACTTGT  
H V D A H C Y E Q N E Q P T G T C<sup>157</sup>  
<sup>470</sup> GCTGCATGCATCACTGGTGACAACAGGTCCCTCATAGCTAATCTTGCTGCT  
A A C I T G D N R S L I A N L A A<sup>174</sup>  
<sup>521</sup> GCCAATTGTTATAAAAAGGAAAAACATCTTGATCTGGAGAAAAACTGGATG  
A N C Y K K E K H L D L E K N W M<sup>191</sup>  
<sup>572</sup> TTGGTAGAAAAAGCAAGAGTTTGTATATAGCAGGCTTTTTTCTTACAGTT  
L V E K A R V C Y I A G F F L T V<sup>208</sup>  
<sup>623</sup> TCCCCAGAGTCAGTATTAAGGTGGCTCACCATGCTTCTGAAAACAACAGG  
S P E S V L K V A H H A S E N N R<sup>225</sup>  
<sup>674</sup> ATTTTCACTTTGAATCTATCTGCACCGTTTATTAGTCAGTTCTACAAGGAA  
I F T L N L S A P F I S Q F Y K E<sup>242</sup>  
<sup>725</sup> TCATTGATGAAAGTTATGCCTTATGTTGATATACCTTTTGGAAATGAGACA  
S L M K V M P Y V D I P F G N E T<sup>259</sup>  
<sup>776</sup> GAAGCTGCCACTTTTGCTAGAGAGCAAGGCTTTGAGACTAAAGACATTA  
E A A T F A R E Q G F E T K D I K<sup>276</sup>  
<sup>827</sup> GAGATAGCCAAAAAGACACAAGCCCTGCCAAAGATGAACTCAAAGAGGCAG  
E I A K K T Q A L P K M N S K R Q<sup>293</sup>  
<sup>878</sup> CGAATCGTCATCTTCACCCAAGGGAGAGATGACACTATAATGGCTACAGAA  
R I V I F T Q G R D D T I M A T E<sup>310</sup>  
<sup>929</sup> AGTGAAGTCACTGCTTTTGGCTGCTTGGATCAAGACCAGAAAGAAATTATT  
S E V T A F A V L D Q D Q K E I I<sup>327</sup>  
<sup>980</sup> GATACCAATGGAGCTGGAGATGCATTTGTTGGAGGTTTTCTGTCTCAACTG  
D T N G A G D A F V G G F L S Q L<sup>344</sup>  
<sup>1031</sup> GTCTCTGACAAGCCTCTGACTGAATGTATCCGTGCTGGCCACTATGCAGCA  
V S D K P L T E C I R A G H Y A A<sup>361</sup>  
<sup>1082</sup> AGCATCATAATTAGACGGACTGGCTGCACCTTTCCTGAGAAGCCAGACTTC  
S I I I R R T G C T F P E K P D F<sup>378</sup>  
<sup>1133</sup> CACACTCGAGGAGGGCCCGAACAAAACTCATCTCAGAAGAGGATCTGAATAGC  
H T R G G P E Q K L I S E E D L N S<sup>396</sup>  
<sup>1187</sup> GCCGTCGACCATCATCATCATCATTTGA  
A V D H H H H H H<sup>405</sup>stop

Above is the amino acid sequence for the recombinant hAKS, translated from the combined pSecTag2 B/hAKS sequencing results. Amino acid changes are indicated in bold and underlined (the Ser203 → Ser nonsense change is also shown). The pSecTag2 B/hAKS sequence is annotated as in Figure 5.

sequence. The other two mismatches were determined to be due to PCR errors. The differences are summarized in Table 2.

#### 4.2 Cellular Expression of pSecTag2 B/AKS

HEK 293 cells were transfected with the pSecTag2 B/AKS plasmid because these cells have 75 – 80% transfection efficiency with the optimized Mirus *TransIT*<sup>®</sup> Transfection Reagent. Media was removed from cells at 48 h to be analyzed by western blotting, and for subsequent use in AK activity assays. Cells from the flask transfection were then washed with sodium citrate, trypsinized, pelleted by centrifugation, and stored at –20°C until RNA could be isolated for RT-PCR analysis.

Western analysis of media from pSecTag2 B/AKS-transfected and pSecTag2/PSA-transfected cells was performed to determine whether recombinant protein (AKS or the positive transfection control, PSA) was produced by transfected HEK 293 cells in the initial 6-well plate transfection. Media samples were analyzed because the Igκ leader sequence contained in the pSecTag2 vector and translated onto the N-terminus of the recombinant proteins allows the proteins to be secreted from the cells into the extracellular media. (The Igκ signalling fragment is removed as the protein passes through the membrane.) Western blot analysis revealed bands at ~35 kDa and ~47 kDa for the recombinant PSA and AKS, respectively, as shown in Figure 7. The recombinant PSA was expected to run at 33 kDa. The short isoform of human AK is a 39 kDa protein, but with the *c-myc* and hexahistidine tags, it was expected to run at ~42 kDa. The recombinant AK may have run higher, however, due to unexpected post-translational modifications to the protein, or due to changes in secondary and tertiary

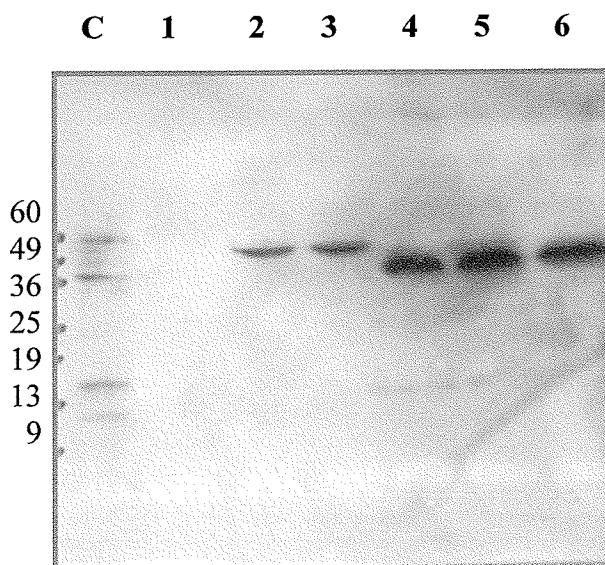
TABLE 2. Mismatch errors in pSecTag2 B/AKS sequence.

Genbank Codon	pSecTag2 B/AKS Codon	Cause of Mismatch	Amino Acid Change
TAC	TGC	PCR error	Tyr112 → Cys
CAC	ACA	GeneStorm® sequence difference	His173 → Thr
AGC	AGT	PCR error	None (Ser203 → Ser)
CTT	CCT	GeneStorm® sequence difference	Leu220 → Pro

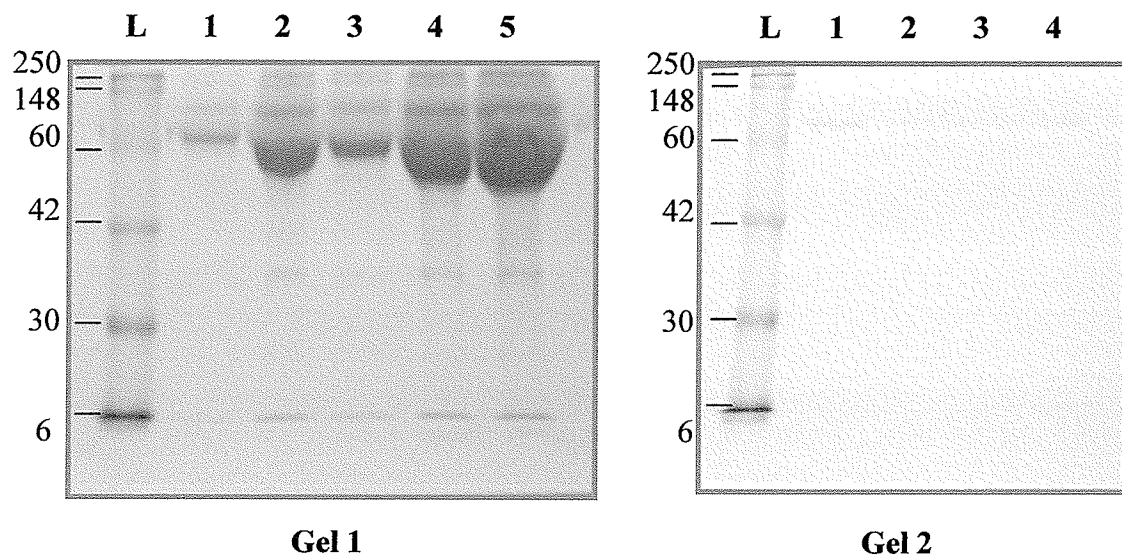
The above table summarizes the mismatch mistakes in the pSecTag2 B/AKS sequence, their causes, and the resultant amino acid changes. Amino acids are numbered according to their positions in the native hAKS. The implications of the amino acid changes are reviewed in the Discussion section in terms of AK structure and activity.

structure due to the mismatches present in the cDNA. However, SDS in the sample buffer and gel should denature the protein and negate such a problem. It could also be possible that the Igk leader sequence was not cleaved from the protein as it passed through the plasma membrane; with the leader sequence still attached, the recombinant protein has a predicted size of 45.3 kDa. Predicted molecular weights of the recombinant AKS were calculated with the use of the online Statistical Analysis of Protein Sequences program of Dr. S. Karlin at Stanford University.

Western analysis was performed on the subsequent flask transfection as described in the Materials and Methods section. However, minimal or no antibody binding was seen upon development of the films. Both Sigma and Roche anti-myc 9E10 primary antibodies were used, in case the Sigma antibody had become unstable, but antibody binding was not obtained after a number of analyses. SDS-PAGE analyses of media samples, and nickel column-purified media from the pSecTag2 B/AKS transfection, were performed and stained with Coomassie Brilliant Blue in order to examine whether proteins of the expected sizes for recombinant hAKS and PSA were present. Both SDS-PAGE analyses indicated a lack of recombinant hAKS or PSA in the transfection media and a lack of recombinant hAKS in the nickel-purified sample. The results are shown in Figure 8. However, Coomassie Brilliant Blue staining of SDS-PAGE is not a sensitive protein detection method, and so a small amount of recombinant protein could have been present in the media samples but was not detectable by this method. As well, if such a small amount of recombinant protein was present, purification by nickel column chromatography may not have increased the concentration of recombinant protein enough to allow it to be detectable by SDS-PAGE/Coomassie Brilliant Blue staining. In order to

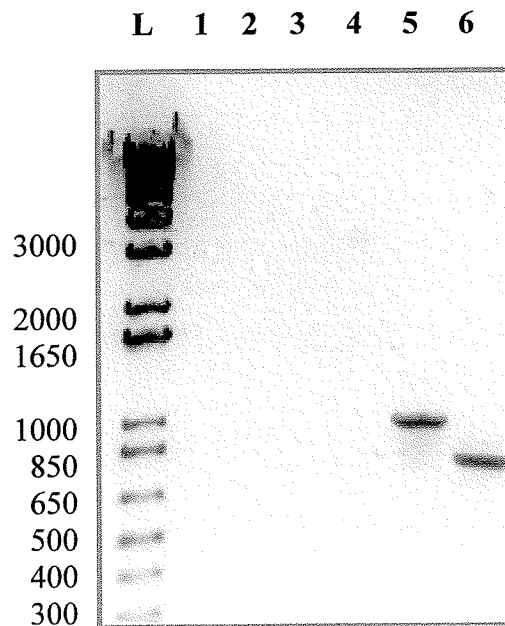
**FIGURE 7. Western analysis of initial transfection media samples.**

The results of the western analysis performed on the media samples taken from the initial transfection are shown. Numbers on the left indicate molecular weight expressed in kDa (each size corresponds to a blue dot where bands of the ladder were). A sample containing a protein possessing a c-myc epitope was obtained from the laboratory of Dr. R. Shiu at the University of Manitoba and run as a positive control (a band of 60 kDa was expected; the control appears to be somewhat degraded). Lanes 1, 2, and 3 are media samples taken from the three wells of the six well plate transfected with pSecTag2 B/AKS. The recombinant hAKS has a theoretical molecular weight of ~40 kDa. The positive samples (2 and 3) may have run higher due to unknown post-translational modifications. Lanes 4, 5, and 6 are media samples taken from the three wells transfected with pSecTag2/PSA. The secreted recombinant PSA was expected to be ~33 kDa, but may be larger if glycosylated. These results indicate that the initial transfection in 6-well plates was successful, and that hAKS has lower electrophoretic mobility than expected.

**FIGURE 8. Protein gels of media samples from second transfection.**

The results of SDS-PAGE followed by Coomassie Brilliant Blue staining are shown.

Molecular weights (in kDa) are indicated on the left of each gel. In **Gel 1** the samples are as follows: molecular weight marker (**L**); pSecTag2 B/AKS transfection media (**1**); concentrated pSecTag2 B/AKS transfection media (**2**); pSecTag2/PSA transfection media (**3**); concentrated pSecTag2/PSA transfection media (**4**); media from non-transfected cells, containing FBS (**5**). The protein present in the transfected media samples appears to be remnant FBS, since the bands are of similar size and relative intensity as those in the non-transfected cell media sample. No hAKS or PSA protein bands are apparent. **Gel 2** shows the results of attempted purification of recombinant hAKS from the media sample using nickel column chromatography. Molecular weights are indicated by the marker in the first lane (**L**). Each of two washes of the column (**1** and **2**) are shown, as well as each of two elution steps (**3** and **4**). Only one band is apparent in the first wash (**1**) at approximately 60 kDa, which could be a small fraction of FBS that was detained on the column. The results suggest no recombinant protein in media samples.

**FIGURE 9. RT-PCR analysis of RNA isolated from transfected HEK 293 cells.**

RT-PCR was performed on RNA isolated from non-transfected, pSecTag2 B/AKS-transfected, and pSecTag2/PSA-transfected cells. The numbers on the left indicate the sizes of the 1 Kb Plus ladder (L) bands in base pairs. Lanes 1 and 2 are controls with each set of primers (those for pSecTag2 B/AKS or those for pSecTag2/PSA, respectively) and no template. Lanes 3 and 4 are RT-PCR of non-transfected HEK 293 RNA with pSecTag2 B/AKS and pSecTag2/PSA primers, respectively, which were performed in order to ensure the primers were specific for the recombinant genes. Lane 5 shows the RT-PCR of RNA isolated from pSecTag2 B/AKS-transfected HEK 293 cells; a PCR product of 983 bp was expected. Lane 6 shows the RT-PCR of RNA isolated from pSecTag2/PSA-transfected HEK 293 cells; a PCR product of 745 bp was expected. The results support successful transfection of HEK 293 cells with the recombinant DNAs.



investigate whether the transfection was successful, RT-PCR analysis was performed on RNA isolated from transfected and non-transfected cells. The results are shown in Figure 9. RT-PCR analysis of cellular RNA from non-transfected cells, pSecTag2 B/AKS-transfected cells, and pSecTag2/PSA-transfected cells was performed to evaluate transcription of plasmid DNA to mRNA. RT-PCR of non-transfected cells was expected to produce no bands, RT-PCR of pSecTag2 B/AKS-transfected cells was expected to produce a cDNA of 983 bp, and RT-PCR of pSecTag2/PSA-transfected cells was expected to produce a cDNA of 745 bp. RT-PCR of RNA from the above cell types revealed no DNA fragment in the non-transfected RNA sample, and DNA fragments of ~1000 bp for the pSecTag2 B/AKS-transfected cells, and ~800 bp for the pSecTag2/PSA-transfected cells. Thus, RT-PCR analysis suggests successful transfection and expression of the pSecTag2 B/AKS and pSecTag2/PSA plasmids in the T-75 flask transfection. It is still unclear as to why western analyses and SDS-PAGE were not successful for media samples from the second transfection. It is possible that for some reason the proteins were not secreted, but western analyses of the initial 6-well plate transfection indicated that the leader sequence was effective in secreting the proteins from the cell.

#### **4.3 Determination of Activity of Recombinant AKS**

Media samples from pSecTag2 B/AKS-transfected cells were used in AK activity assays in order to determine whether the recombinant AKS was functional, that is, able to phosphorylate [<sup>3</sup>H]adenosine to produce [<sup>3</sup>H]AMP. Assays with the media collected from the initial transfection were allowed to proceed overnight at 37°C in order to accumulate enough [<sup>3</sup>H]AMP for scintillation counting purposes. Assays with the media

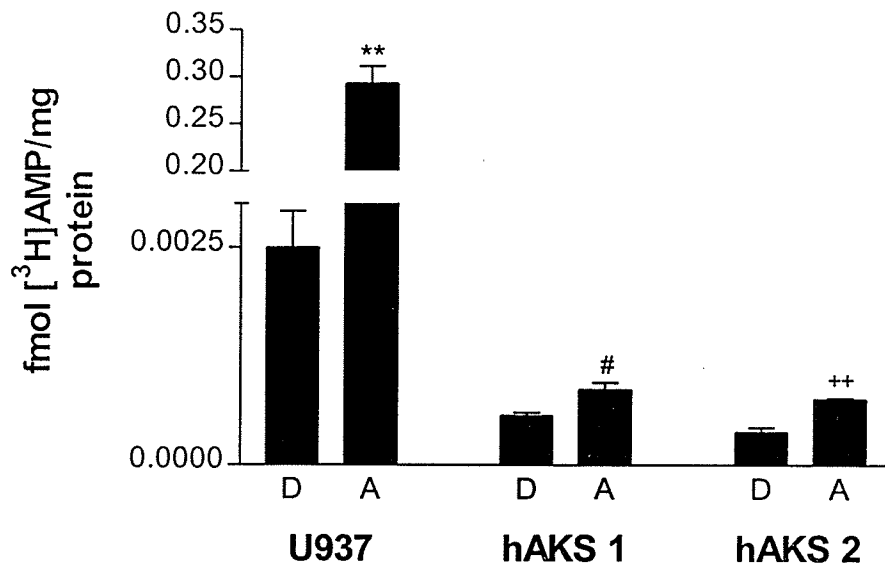
collected from the T-75 flask transfection were allowed to proceed only two hours at 37°C, as it was decided that two hours was sufficient for accumulating enough [<sup>3</sup>H]AMP for counting purposes.

AK activity in media samples from pSecTag2 B/AKS-transfected cells, cytosolic protein from human promonocytic U937 cells (assays with initial transfection media), and non-transfected HEK 293 cells (T-75 flask transfection media), was compared to AK activity in denatured samples using paired t-tests. The U937 or HEK 293 cytosolic protein was assayed as a positive control for the assays. Denatured samples were negative controls.

Results indicated significant AK activity in media samples from either transfection, although activity was minimal compared to the positive control cytosolic protein samples. The amount of radioactivity present in denatured AK media reactions was approximately three times higher than the amount of radioactivity in denatured cytosolic protein reactions and so an AK inhibitor, 5'-iodotubericidin (Itu), was added to the reaction mixture in order to determine if there was residual AK activity in denatured samples. The addition of Itu, however, did not alter the amount of radioactivity in denatured AK media reactions (results are not shown). The results from the AK assays are shown in Figure 10.

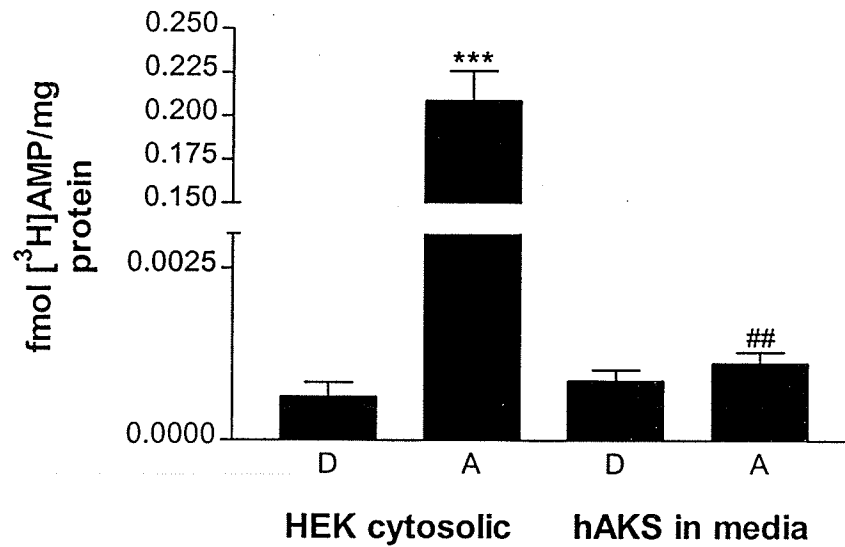
FIGURE 10. AK activity in media from pSecTag2 B/AKS-transfected HEK 293.

A.



B.

D = denatured sample  
A = active sample



The results of a single 18 h AK assay with the 6-well plate transfection media (**A**) and four 2 h AK assays with the flask transfection media (**B**) are shown. Assays were incubated at 37°C and performed in triplicate. Data are expressed in fmol [<sup>3</sup>H]AMP formed/mg protein. Error bars indicate standard error of the mean (SEM). In both graphs data was paired (denatured and active protein) and analyzed by one-tailed paired t-tests. In **A** each active sample (U937 cytosolic protein, **U937**; media from one well of pSecTag2 B/hAKS-transfected HEK 293 cells, **hAKS 1**; media from a second well of pSecTag2 B/hAKS-transfected HEK 293 cells, **hAKS 2**) had significantly more activity than their denatured counterpart; \*\*, ++ = p<0.01; # = p<0.05. In **B** each active sample (cytosolic protein from non-transfected HEK 293 cells, **HEK cytosol**; media from pSecTag2 B/hAKS-transfected HEK 293 cells, **hAKS in media**) was also significantly more active than its denatured counterpart; \*\* = p<0.001; ## = p<0.01. It should be noted that the media sample in **B** may contain no recombinant hAKS, as western analysis revealed no recombinant protein in the media samples, nickel column purification eluted no recombinant protein (as determined by SDS-PAGE), and SDS-PAGE of media samples suggested the protein present in transfection samples was residual FBS. Our laboratory has previously observed minimal AK activity in FBS, likely due to contamination during production. Thus, the AK activity observed in **B** could be due to a contaminating phosphorylating enzyme in residual FBS in the media and not to the recombinant hAKS itself. Because the transfection in **A** was performed in the presence of FBS, that activity seen could also be due to a contaminating enzyme in the FBS, even though western analysis supports the presence of a recombinant hAKS in the media.

## 5. DISCUSSION

The purpose of this project was to create a recombinant human adenosine kinase, short form (hAKS) for future use in adenosine research in our laboratory. A recombinant hAKS would better enable the elucidation of the adenosine metabolic pathway, the effects of inhibitors upon it, and the cellular factors which may play a role in the regulation of this pathway. It was especially hoped to be useful in determining the nature of the endogenous regulation of AK by cell signalling molecules, like PKC. A recombinant hAKS was produced; unfortunately, no activity was determined to be present in media samples from transfected cells. The minimal activity in media samples from the second transfection could be attributed to an apparent lack of recombinant hAKS in the samples, although the presence/absence of AK in the media remains unclear; an additional transfection is underway, following which both extracellular media and cytosolic protein will be examined in western analyses and AK activity assays for the presence of recombinant protein. The media samples from the initial transfection did, however, contain recombinant hAKS, as seen in western analysis, but also possessed diminished activity. It is unclear whether the activity present was due to recombinant AK or a contaminating phosphorylating enzyme in the FBS in the media. If the decreased enzyme activity is that of the recombinant AK, it could be due to a number of features of the recombinant protein, including the possible inefficient removal of the N-terminal Igk secretion signal upon passage through the cellular membrane into the extracellular media, the presence of a *c-myc* epitope and hexahistidine tag at the C-terminus, and single base changes in the DNA sequence leading to amino acid changes in the protein.

The pSecTag2 B vector was chosen for cloning hAKS because it improves the detection, isolation, and purification of the resultant recombinant protein. The Ig $\kappa$  leader sequence, a protein fragment of 21 amino acids, is attached to the N-terminus of the recombinant protein in order to direct the completely synthesized protein to the cellular membrane for secretion; the secretion signal is then removed as the protein passes through the plasma membrane. The ability of the recombinant protein to be secreted by the transfected cells into the extracellular media was attractive for its ease of isolation, particularly when no serum is present in the media. Western analysis of the initial transfection indicated hAKS in the media samples, although the protein band was larger than expected (~47 kDa instead of the expected 42 kDa). It is possible that the secretion signal may not have been removed upon passage through the plasma membrane, which would result in a recombinant protein with a theoretical weight of 45.3 kDa. Because the N-terminus of native hAKS acts as a flap over the active site (Matthews et al., 1998), an extra 21 amino acids could alter how such a flap rests over the active site. Secondary structure prediction using the online secondary structure prediction program at <http://promoter.ics.uci.edu/BRNN-PRED/> indicates the Ig $\kappa$  sequence, if not detached, adds an extra helix and non-specific secondary structure to the N-terminus of the protein, and slightly changes the position of the first  $\alpha$ -helix of the native protein (Figure 11). It is unclear as to whether such secondary structure would alter the folding of the N-terminal flap, but at the very least the non-detached secretion signal would be expected to add extra bulk to the flap, which could very likely alter how it lies over the active site of hAKS. Residues near the N-terminus of hAKS, including some within the flap, interact with the adenosine molecule for binding purposes (Matthews et al., 1998), and so a

**FIGURE 11. Secondary structure prediction for recombinant and native hAKS.**Recombinant hAKS

<sup>1</sup>METDTLLLWVLLLWVPGSTGDAAPARRARRTKLTSVRENILFGMGNPLLDISAVVDKDF  
CCHHHHHHHHHHHCCCCCCCCCCCCCCCCCECCCCCEEEEECCCEEEEEECCHHH

<sup>61</sup>LDKYSLKPNQILAEDKHKELFDELVKKFKVEYHAGGSTQNSIKVAQWMIQQPHKAATFF  
HHHCCCCCCCCCECCCCCHCCHHHHHHHCCCCCEEECCCCHHHHHHHHHHHHHHHHHCCCCCEEE

<sup>121</sup>GCIGIDKFGELKRKA AEAHVDAH<sup>C</sup>YEQNEQPTGTCAACITGDNRS LIANLAAANCYKKE  
EECCCCHHHHHHHHHHHHCCCCCEEEEECCCCCEEEEEEECCCCCEEEEECCCCCCCCCCC

<sup>181</sup>KHLDLEKNWMLVEKARVCYIAGFFLTVSPESVLKVAHHA SENNRIFTLNLSAPFISQFYK  
HHCCHHHHHHHHHHHCCEEEECCCECCCCHHHHHHHHHHHHHHHHCCEEEECCCCCCCCCHHHHH

<sup>241</sup>ESLMKVMPYVDI<sup>P</sup>FGNETEAATFAREQGFETKDIKEIAKKTQALPKMNSKRQRIVIFTQG  
HHHHHHHHCCCEEECCCCHHHHHHHHHCCCCCCCCCHHHHHHHHHHHHHHHHHCCCCCEEEEEEC

<sup>301</sup>RDDTIMATESEVTAFVLDQDQKEIIDTNGAGDAFVGGFLSQLVSDKPLTECIRAGHYAA  
CCCEEEEECCCEEEEECCCCCCCCCEEECCCCCHHHHHHHHHHHHHHHHHCCCCCHHHHHHHHHHHHH

<sup>361</sup>SIIRRTGCTFPEKPDFHTRGGPEQKLISEEDLNSAVDHHHHHH  
HHHHHCCCCCCCCCCCCCCCCCCCCCHHHHHHHHHHHHHHHHHCCCCC

Native hAKS

<sup>1</sup>MTSVRENILFGMGNPLLDISAVVDKDFLDKYSLKPNQILAEDKHKELFDELVKKFKVEY  
CCCCCEEEEECCCEEEEEECCHHHHHHHCCCCCCCCCECCCCCHCCHHHHHHHCCCCCEE

<sup>61</sup>HAGGSTQNSIKVAQWMIQQPHKAATFFGCIGIDKFGELKRKA AEAHVDAH<sup>Y</sup>YEQNEQPT  
CCCCHHHHHHHHHHHHHHHCCCCCEEEEECCCCCHHHHHHHHHHHHHHHCCCCCEEEEECCCCC

<sup>121</sup>GTCAACITGDNRS LIANLAAANCYKKEKHLDLEKNWMLVEKARVCYIAGFFLHVSPESVL  
EEEEEECCCCCEEEEECCCCCCCCCHHHHHHCCHHHHHHHHHHHCCEEEECCCECCCCCHHHHH

<sup>181</sup>KVAHHA SENNRIFTLNLSAPFISQFYKESLMKVMPYVDI<sup>L</sup>FGNETEAATFAREQGFETKD  
HHHHHHHHCCCEEEEECCCCCCCCCHHHHHHHHHHHHHHHCCCECCCCCHHHHHHHHHCCCCCHC

<sup>241</sup>IKEIAKKTQALPKMNSKRQRIVIFTQGRDDTIMATESEVTAFVLDQDQKEIIDTNGAGD  
HHHHHHHHHHHHHHHHCCCCCEEEEECCCCCEEEEECCCEEEEECCCCCCCCCEEECCCCCHH

<sup>301</sup>AFVGGFLSQLVSDKPLTECIRAGHYAASIIIRRTGCTFPEKPDFH  
HHHHHHHHHHHHCCCCCHHHHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCCCCCC

Figure 11 shows the predicted secondary structure for the recombinant hAKS as compared to that predicted for the native hAKS (sequence from NCBI Protein), with the amino acid sequences in black and the predicted secondary structures in grey.

Abbreviations for the secondary structure are as follows: H = helix, E = sheet, C = non-specific structure. Predictions were performed with the online secondary structure prediction program at <http://promoter.ics.uci.edu/BRNN-PRED/>. The amino acid differences between the recombinant and native hAKS are boldfaced and underlined. None of these are predicted to significantly alter the protein's secondary structure. The Ig $\kappa$  secretion signal (N-terminus) and the *c-myc* and hexahistidine tags (C-terminus) are boldfaced, as are their corresponding predicted secondary structures. The Ig $\kappa$  leader sequence is predicted to add an extra helix (H) and non-specific structure (C) to the N-terminus of the recombinant hAKS. The *c-myc* and hexahistidine tags are predicted to add an extra helix to the C-terminus of the recombinant enzyme. It is unclear whether such additional structure may alter the function of the enzyme, but the extra helix at the N-terminus may affect the folding of the N-terminal flap that covers the active site of hAKS.



slightly bulkier N-terminus could also affect these binding interactions.

The *c-myc* epitope and hexahistidine tag attached to the C-terminus of the recombinant hAKS could also alter the structure and/or activity of the enzyme. The tags were added in order to be able to detect the recombinant protein in the extracellular media (immunoblotting with an antibody directed toward *c-myc* or the polyhistidine tag), as well as to possibly purify the recombinant protein from the media (using nickel columns, or an affinity-binding column). The *c-myc* tag was much needed for Western analysis, as there is currently no antibody available for hAKS; only a polyclonal antibody to rat AKS has been developed by the Pawelczyk laboratory at the University of Gdańsk in Poland (Sakowicz et al., 2001). Thus the *c-myc* tag was included primarily for detection of the recombinant hAKS, and the polyhisitidine tag was included primarily for purification of the recombinant hAKS using nickel column chromatography, should it be necessary. It was hoped that the additional 26 amino acids containing the *c-myc* and hexahistidine tags would not alter enzyme function significantly. However, Mg-ATP<sup>2-</sup> interacts with the C-terminal half of hAKS (Matthews et al., 1998), and extra amino acids at the C-terminus may thus alter the structure of the Mg-ATP<sup>2-</sup> binding site. The extra amino acids add an additional  $\alpha$ -helix and some non-specific secondary structure to the C-terminal tail of the recombinant hAKS, as predicted by the online program at <http://promoter.ics.uci.edu/BRNN-PRED/>, and shown in Figure 11. It is unclear whether this may alter the activity of the recombinant protein.

There is some concern regarding the terminal hexahistidine tag specifically, since the positive charges associated with histidine residues would be concentrated in this area, and could interact with an area of the enzyme that may possess an available negative

charge, altering folding of the protein or causing interactions with other AK molecules. However, output from the Statistical Analysis of Protein Sequences (SAPS) online calculator created by the laboratory of Dr. Karlin in the Department of Mathematics at Stanford University suggests no such positive charge cluster. There have been previous reports of a hexahistidine tag altering folding of a recombinant intracellular binding protein, as well as causing aggregation of a cloned receptor tyrosine kinase and subsequent prevention of ligand binding (Ramage et al., 2002). Therefore it is possible that the presence of the hexahistidine tag at the C-terminus could result in folding problems for the recombinant hAKS, or aggregation of a number of hAKS molecules, thus diminishing enzyme activity.

Finally, the three amino acid changes resulting from single base changes in the hAKS gene sequence may alter the enzyme activity. Table 2 in the Results section summarizes the mutations, and where they are located within the protein. The His→Thr change at the residue corresponding to residue 173 of the native hAKS is not expected to greatly alter structure or activity. In fact, it appears that this residue is Thr 173, and not His 173, in a number of species (Matthews et al., 1998). It was discovered upon a NCBI Protein search that two variants of hAKS exist, one with a Thr at residue 173 (accession: AAB50235; GI: 1906011), and a variant with a His at that position (accession: NP\_001114; GI: 4501943). The Genbank sequence encoding the His variant was used to create the theoretical vector sequence to which sequencing results were compared. The X-ray crystal structure of hAKS indicates that Thr173 is near the active site, interacting with Trp416 residue in the adenosine-binding site, and that the adenosine binding ability

of the enzyme is in tact. It is unlikely that the possible His→Thr change at 173 is the cause of the lack of activity of recombinant hAKS.

The Tyr→Cys change at the residue corresponding to residue 112 of the native hAKS might alter protein tertiary structure. The Tyr residue is conserved across species (Matthews et al., 1998); this does not necessarily suggest relevance in terms of activity though. However, Cys residues can be involved in disulfide bonds within a protein, or two protein molecules, in adequately oxidizing conditions. The cytoplasm of cells is usually not sufficiently oxidizing to encourage the formation of disulfide bonds, but often times extracellular conditions are sufficiently oxidizing to support the formation of disulfide bridges; this mechanism appears to have developed throughout evolution in order to allow secreted proteins to contend with hostile extracellular conditions and maintain their proper tertiary structures. Therefore, although the Tyr residue does not exist near the binding sites for adenosine or Mg-ATP<sup>2-</sup>, a change to a Cys at that position produces the potential for disulfide bridge formation. If the recombinant hAKS were to remain in the cytoplasm as native hAKS does, this mutation may be of little consequence. However, because the recombinant hAKS was designed to be secreted from the transfected cell, it is possible that extracellular oxidizing conditions could encourage disulfide bonds between Cys residues in the native hAKS structure, as well as between the Cys mutation and other existing Cys residues in the recombinant hAKS. Such disulfide bonding could make the hAKS structure more rigid overall, or could alter the binding site(s) of the enzyme if a disulfide bond was formed with the Cys123 residue involved in binding the ribose group of adenosine. However, the AK activity assays included 2-mercaptoethanol, a reducing agent which should inhibit disulfide bond

formation in the protein. Therefore, it is unlikely that the Tyr112→Cys mutation is the cause of the diminished enzyme activity of the recombinant hAKS.

Finally, perhaps of most obvious relevance, is the Leu→Pro mutation at the residue corresponding to residue 220 of native hAKS protein. Although the Leu is not conserved across all species, its physical-chemical properties are conserved across species (Matthews et al., 1998); a similar amino acid could take its place without altering the protein significantly. However, Pro does not share the physical-chemical properties of Leu, and so the Leu→Pro mutation may have structural and functional implications. Pro residues are limited conformationally because of their cyclic nature, and are sometimes able to introduce kinks into the secondary structure of proteins, and thus could also alter the tertiary structures. Because Asn223, Glu224, and Thr225 are involved in hydrogen-bonding or non-specific interactions with the phosphate groups of ATP in Mg-ATP<sup>2-</sup>, and because Glu226 is involved in hydrogen-bonding with the Mg of Mg-ATP<sup>2-</sup>, a slight change in the conformation of amino acid 220 (Leu→Pro) could alter the Mg-ATP<sup>2-</sup> binding site significantly, decreasing the stability of the interaction of hAKS with Mg-ATP<sup>2-</sup>, and thus diminishing the ability of the enzyme to phosphorylate the bound adenosine (Matthews et al., 1998). Although the Leu→Pro mutation may not result in such drastic alterations of enzyme structure and activity, it is one of the many possible reasons why our recombinant hAKS lacks full kinase activity.

A number of alterations in method could be made in future attempts to produce an easily isolated, purified, and detected recombinant hAKS. Initially, HEK 293 cells will be transfected again with pSecTag2 B/AKS plasmid DNA and both media and cytosolic protein samples will be evaluated for presence of the recombinant protein using western

analysis and AK activity assays. If the pSecTag2 B/AKS vector does encode an inactive hAKS, efforts to correct the problem will be undertaken; site-directed mutagenesis will be performed on the existing pSecTag2 B/AKS plasmid to correct the single base mismatches which cause the Tyr→Cys and Leu→Pro mutations. Transfection of HEK 293 with the corrected pSecTag2 B/AKS, and subsequent western analysis and AK activity assays, will be performed in order to determine if the mutations do indeed have a significant effect on enzyme activity. Should cloning be re-attempted from the beginning (ie. PCR), it may be wise to forego the N-terminal secretion signal, and instead rely on other methods for isolating the recombinant protein from the cell, such as column chromatography of cytosolic protein from transfected cells. As well, it may be wise to also eliminate the hexahistidine tag and rely instead on affinity binding columns for purification of the recombinant protein. In light of the number of possible problems encountered in our attempt to produce a recombinant hAKS, the best choice would seem to be to clone the hAKS gene sequence (free of mismatch errors) into a vector that attaches only an antigenic epitope, like *c-myc*, to one of the termini. A single antibody-binding epitope would allow both detection in western analysis and isolation/purification of the recombinant hAKS using an affinity binding column, or immunoprecipitation, in which the antibody would be bound to a solid support.

It would be valuable in our research, and that of other adenosine laboratories, to re-attempt production of an easily-manipulated recombinant hAKS until reliable antibodies directed toward hAKS are available. Such an enzyme, once produced in its desired form, would greatly aid the study of the role of AK in the regulation of

endogenous adenosine levels, and could be used to further ascertain the regulation of AK activity by cellular signalling molecules like PKC.

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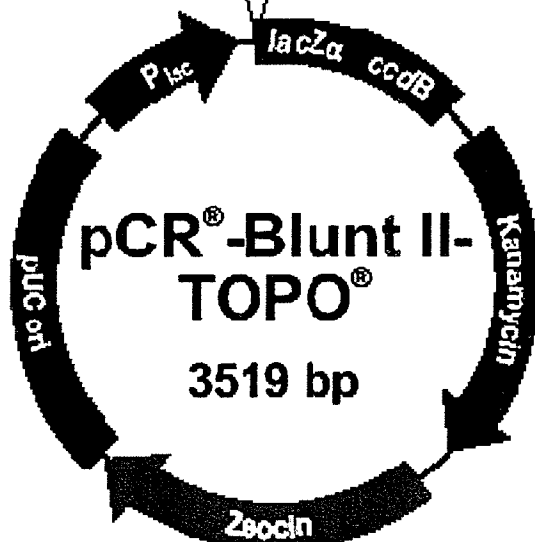
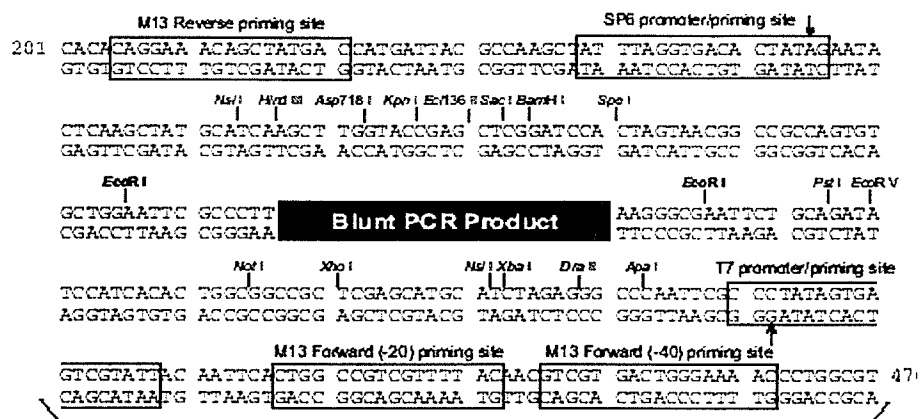
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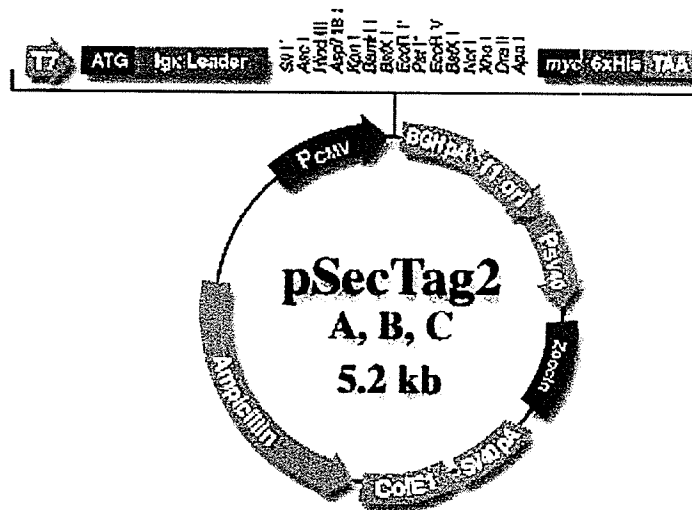
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## 7. APPENDIX

7.1 The pCR<sup>®</sup>-Blunt II-TOPO<sup>®</sup> Vector

The above pCR<sup>®</sup>-Blunt II TOPO<sup>®</sup> vector was used for initial insertion of the hAKS PCR product. Note the Hind III (base 276; A↓AGCTT) and Xho I (base 376; C↓TCGAG) contained in the vector sequence. As well, note the two EcoR I sites surrounding the insertion site (bases 325 and 343; G↓AATTC).

## 7.2 The pSecTag2 B Expression Vector



## pSecTag2 B Multiple Cloning Site

enhancer region (3' end)

699 AATGGGAGTT TGTTTTGGCA CCAAATCAA CGGGACTTTC CAAAATGTCG TAACAACCTCC

759 GCCCCATTGA CGCAAATGGG CGGTAGGCGT GTACGGTGGG AGGTCTATAT AAGCAGAGCT  
 CAAT TATA 3' end of hCMV  
 putative transcriptional start T7 promoter primer binding site

819 CTCTGGCTAA CTAGAGAACC CACTGCTTAC TGGCTTATCG AAATTAATAC GACTCACTAT

879 AGGGAGACCC AAGCTGGCTA GCCACC ATG GAG ACA GAC ACA CTC CTG CTA TGG  
 Met Glu Thr Asp Thr Leu Leu Leu Trp  
 Ig κ-chain leader sequence

932 GTA CTG CTG CTC TGG GTT CCA GGT TCC ACT GGT GAC GCG GCC CAGCCC  
 Val Leu Leu Leu Trp Val Pro Gly Ser Thr Gly Asp  
 Signal cleavage site

980 GCCAGGCGC GCGCGCCG TACGAAG CTTGGTACCG AGCTCGGATC CACTCCAGTG TGGTGGG  
 Hind III Asp718 I Kpn I BamHI BstX I\* EcoR

1041 ATTCTGCAGA TATCCAGCAC AGTGGCGGCC GCTCGAGGAG GGCC GAA CAA AAA CTC  
 Pst I EcoRV BstX I\* Not I Xho I Dra II Apa I  
 Glu Gln Lys Leu  
 myc epitope Polyhistidine tag

1098 ATC TCA GAA GAG GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT  
 Ile Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His His His His His

1146 CAT TGA GTTAAACCC GCTGATCAGC CTCGACTGTG CTTCTAGTT GCCAGCCATC  
 His \*\*\*  
 BGH reverse priming site

1202 TGTTGTTTGC CCCTCCCCCG TGCCTTCCTT GACCCTGGAA GGTGCCACTC CCACTGTCTT  
 BGH poly (A) addition site

1262 TTCCTAATAA AATGAGGAAA TTGCATCGCA TTGTCTGAGT AGGTGT

Above is the pSecTag2 B vector used as the expression vector in cloning hAKS. Note the Hind III (base 1002; *A*↓*AGCTT*) and Xho I (base 1072; *C*↓*TCGAG*) used to insert the hAKS clone. Also note the Igκ leader sequence, *c-myc* epitope, and polyhistidine tag. The boxed bases (bases 989 – 996) are a variable region designed to make cloning with the proper reading frame for *c-myc* and polyhistidine tags more convenient; the pSecTag2 vector kit came with variants A, B, and C, each of which possessed a different number of bases in the boxed region (4, 8, and 12, respectively). Primers were designed to anneal to the T7 promoter and BGH reverse priming sites for sequencing purposes.