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cDNA cloning and characterization of A3i, an alternatively spliced rat A3 adenosine receptor variant**

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Abstract **A cDNA encoding a variant form of the A3 adenosine (Ado) receptor was isolated from rat by reverse transcription of brain mRNA followed by PCR. The full-length receptor (A3i) cDNA encodes 337 amino acids and shares complete sequence identity with the rat A3 Ado receptor, except for the presence of a seventeen amino acid insert located in the second intracellular domain. In contrast to the rat A3 receptor, stable expression of A3i in CHO cells resulted in poor coupling to Gi proteins. Analysis of receptor transcripts by RT-PCR suggests that the A3 Ado receptor mRNAs are products of alternative splicing. Sequence analysis of A3 genomic DNA identified a 1.7 kb intron that is likely alternatively spliced to produce the A3 and A3i receptors.**

Key words: A3; A3i; Receptor; Adenosine; Rat; cDNA

1. Introduction

Ado receptors mediate a variety of physiological functions in the cardiovascular and central nervous system and are important regulators of allergic and immunologic responses [1,2]. Ado receptors function in part through heterotrimeric G-proteins and display subtype heterogeneity, with four known receptor subtypes, the A1 [3], A2a [4], A2b [5] and A3 Ado receptors [6]. The recently identified A3 Ado receptor, which was originally cloned from rat testis and brain cDNA libraries [7,8], exhibited about 50–60% homology with rat A1 and A2 receptors. This new receptor showed ligand binding characteristics that distinguished it from A1 or A2 receptors, with relatively high affinity for I-APNEA, I-ABA, and IB-MECA [8,9]. However, the rat receptor displayed low binding affinity for the antagonists DPCPX and XAC and had surprisingly low homology to both the sheep (72%) [10] and human receptors (72%) [11]. In contrast, the A1 and A2 Ado receptors are highly conserved between species [1]. The tissue distribution of the rat A3 Ado receptor appeared to be distinct from that of the sheep and human receptors, although previous studies in the rat relied solely on RT-PCR to detect specific RNA transcripts [6,8,10].

We have now cloned the rat A3 Ado receptor and identified several nucleotides in the sequence that differ from previously published reports. Northern blot analysis revealed evidence of alternative splicing in the rat as well as a restricted tissue distribution compared to other species. Furthermore, an alternatively spliced variant of the A3 Ado receptor containing an insert in the second cytoplasmic domain was identified and confirmed by characterization of the corresponding genomic region. This variant was named A3i to denote the presence of the in-frame insert.

2. Material and methods

2.1. Reverse transcription PCR and sequencing

mRNA was purified from rat brain and spleen using oligo-dT (Qiagen, Chattsworth, CA). Single-stranded cDNAs for brain and spleen and rat genomic DNA were obtained from Clontech (Palo Alto, CA). Reverse transcribed cDNA was subjected to PCR amplification with the following primers: A-(5'-CACATCCTGCTGAAGAAGCAACAG); B-(5'-ACTCTCGAGCTGGCTCTTTATCTGTCATGG); C-(5'-GAAAGCCAACAATACCACGACGAG); D-(5'-CAAAGAATCCGAGGTCTGACAGAG); E-(5'-GTCCAGATGCACTTCTATGCCTGC); F-(5'-GACCATGTAATCCAAGCCGACCAC); I-(GTTGTGAACTTACCGTTTTCCTCC). PCR (26–35 cycles) consisted of denaturing at 94°C for 20 s, hybridization at 60°C for 25 s and extension at 72°C for 30 s using a Perkin Elmer 480 instrument and reagents (Perkin Elmer, Foster City, CA). PCR products were concentrated on micro spin filters (Amicon, Beverly, MA) and resolved on 0.7–1.1% agarose gels.

cDNA bands of interest were purified, subjected to restriction digestion and cloned into pBluescript (Stratagene, San Diego, CA) using established protocols [12] and subjected to sequence analysis (both strands for the coding region) by the dideoxy termination method using Sequenase (USB, Cleveland, OH). The 2.3 kb fragment spanning the A3 intron region was cloned into pCRII (Invitrogen, San Diego, CA).

2.2. Northern blot hybridization

A rat multiple tissue mRNA blot (Clontech, Palo Alto, CA) was probed with the full length A3i probe, an oligo spanning the A3i insert region and a human β-actin cDNA, as previously described [6]. Autoradiography was carried out using Reflection X-Ray film and intensifying screens (Dupont, Boston, MA).

2.3. DNA transfection and cAMP measurements

The 1.1 kb A3i cDNA and a 1 kb rat A3 cDNA were cloned as *Hin*dIII-*Xho*I fragments into the expression vector pcDNA3 (Invitrogen, San Diego, CA). CHO-K1 cells were stably transfected using lipofectamine reagent (Gibco, Gaithersburg, MD). Neomycin-resistant colonies were isolated following selection in 800 mg/ml G418 (Gibco, Gaithersburg, MD). To check the expression levels of transfectants, mRNA was purified from several clones and subjected to Northern blot analysis using the A3 cDNA as probe.

For cAMP assays, cells were grown to ~90% confluence in 24-well tissue culture cluster plates and washed in phosphate-buffered saline. Cells were subsequently treated with adenosine deaminase (2 U/ml) for 10 min in RPMI medium (no FCS) followed by the addition of forskolin and I-ABA for an additional 10 min. Cells were washed with PBS and assays were terminated by addition of 70% ethanol and subsequent incubation on ice for 1 h. Cells were scraped from the wells and supernatants cleared of cell debris by brief centrifugation. Supernatants were dried under a stream of nitrogen and cAMP was

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**The nucleotide sequence described in this paper has been submitted to the EMBL Data Library under the accession number X93219.

Abbreviations: I-ABA, N^6-(4-amino-3-iodobenzyl)adenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; XAC, xanthine amine congener; Ado, adenosine; kb, kilo base pairs; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR

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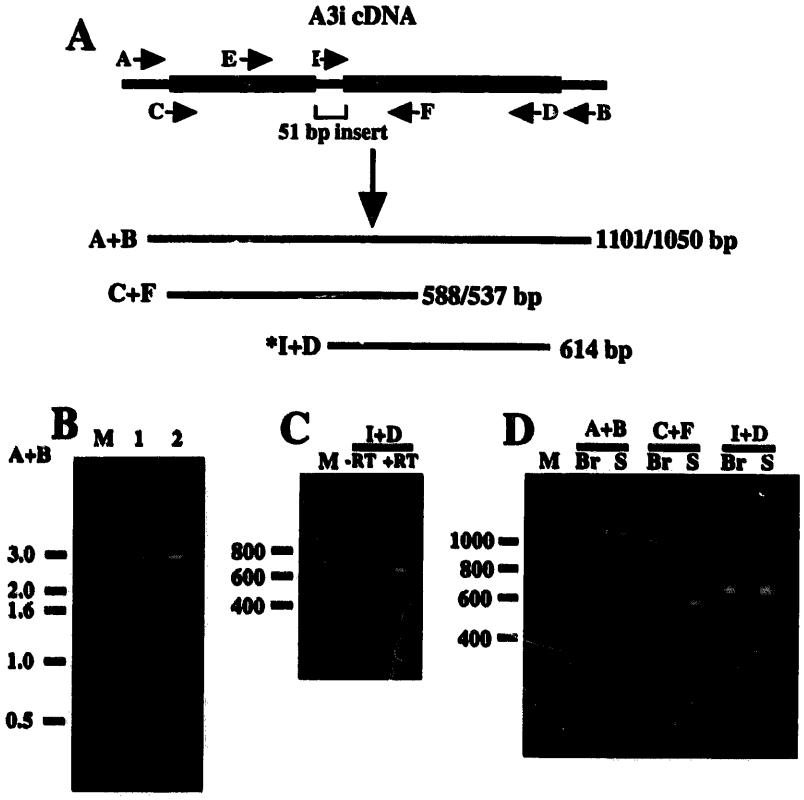

Fig. 1. (A) Schematic of the rat A3i cDNA. Primers corresponding to their positions on the cDNA are indicated by arrows and the corresponding PCR products and their sizes are shown below (M = size markers). (B) Primers A and B; Digestion products of rat A3 and A3i Ado receptors resolved on an agarose gel. (C) Primers I + D; Amplification of the 614 bp product obtained from splenic mRNA, in the presence and absence of reverse transcriptase (+RT or −RT, respectively). The lack of signal without RT rules out contamination as the source of A3i signal. (D) Primers A + B, C + F, I + D; PCR products from cDNA pools of brain (Br) and spleen (S) resolved on agarose gel.

quantified using the Titerzyme cAMP EIA kit (Perseptive Diagnostics, Inc., Cambridge, MA), in accordance with the manufacturer's instructions.

3. Results

RT-PCR was performed on rat brain mRNA using primers specific to the previously published A3 Ado receptor sequence [8] to amplify the entire coding region of the receptor (primers A and B, Fig. 1A). The 1 kb product was cloned into pBluescript vector and subjected to sequence analysis. The sequence of this PCR product was found to differ by several nucleotides from that previously reported (Fig. 2). Subsequent restriction digests of additional clones showed that one clone exhibited slightly less mobility than the expected A3 Ado receptor cDNA (Fig. 1B). Sequence analysis of the cDNA showed that the slower gel migration was due to a 51 bp insert in the coding region of the rat A3 Ado receptor (Fig. 2). In addition, the insert was found to be in frame with the A3 Ado receptor sequence, encoding 17 amino acids in the second intracellular loop of the receptor (Fig. 3). This clone, designated A3i, was the only one containing an insert among 12 clones analyzed in the A3 Ado receptor cDNA pool, suggesting a low abundance of its mRNA in brain tissue.

The possibility that A3i might be a PCR-derived non-specific product was first addressed by subjecting mRNA from rat spleen to RT-PCR with primers I and D (Fig. 1A) in the presence and absence of reverse transcriptase. Only the reverse-transcribed cDNA yielded the expected 614 bp fragment, showing the insert in A3i to be contiguous with the A3 Ado receptor mRNA and ruling out the possibility of PCR artifact in the original A3i isolation (Fig. 1C). In addition, cDNA pools from rat brain and spleen tissues were used in PCR reactions using different primer pairs designed to detect both the A3 Ado receptor and A3i. As shown in Fig. 1D, the primers C and F that span the A3 Ado receptor insert region, yielded PCR products of 537 bp and 588 bp corresponding to the A3 and A3i receptors respectively. While the yields of the PCR reactions were not quantitative, the relative intensities of the two products suggested that A3i likely represents the minor species in the cDNA pools. PCR analysis of rat adipose cDNA also demonstrated the presence of both A3 and A3i Ado receptor mRNA (data not shown).

In a separate series of PCR reactions, the full-length cDNA products of primers A and B were subjected to a second round of amplification with internal primers I and D (Fig. 1D). The presence of A3i in the A/B cDNA pool was confirmed by the 614 bp product in both brain and spleen. Together, these results strongly suggested that A3i is an authentic mRNA product that is likely present as a minor component of the A3 Ado receptor pool in these tissues.

To characterize the genomic organization of the A3 Ado receptor locus, rat genomic DNA was subjected to PCR analysis using primers E and F, spanning the insert region (Fig. 1).

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A3i   ATGAAAGCCAACAATACCACGACGAGTGCCTTGTGGTTGCAAATCACCTACATCACCATGGAGGCTGCCATTGGTCTCTGTGCTGTAGTGGGCAACATGCTGGTCATCTGGGTGGTCAAG  120
A3    ATGAAAGCCAACAATACCACGACGAGTGCCTTGTGGTTGCAAATCACCTACATCACCATGGAGGCTGCCATTGGTCTCTGTGCTGTAGTGGGCAACATGCTGGTCATCTGGGTGGTCAAG
R226  ATGAAAGCCAACAATACCACGACGAGTGCCTTGTGGTTGCAAATCACCTACGTCACCATGGAGGCTGCCATTGGTCTCTGTGCTGTAGTGGGCAACATGCTGGTCATCTGGGTGGTCAAG

A3i   CTGAACCGCACTCTGAGGACCACCACCTTCTATTTCATCGTCTCCCTAGCACTGGCTGACATTGCTGTTGGGGTGCTGGTCGTACCCTTGGCCATTGCCGTCAGCCTGGAGGTCCAGATG  240
A3    CTGAACCGCACTCTGAGGACCACCACCTTCTATTTCATCGTCTCCCTAGCACTGGCTGACATTGCTGTTGGGGTGCTGGTCGTACCCTTGGCCATTGCCGTCAGCCTGGAGGTCCAGATG
R226  CTGAACCGCACTCTGAGGACCACCACCTTCTATTTCATCGTCTCCCTAGCACTGGCAGACATTGCTGTTGGGGTGCTGGTCATACCCTTGGCCATTGCCGTCAGCCTGGAGGTCCAGATG

A3i   CACTTCTATGCCTGCCTTTTCATGTCCTGTGTGCTTCTGGTCTTCACCCACGCTTCCATCATGTCCTTGCTGGCCATTGCTGTAGACCGATACCTGCGAGTCAAGCTGACAGTCAGGTTG  360
A3    CACTTCTATGCCTGCCTTTTCATGTCCTGTGTGCTTCTGGTCTTCACCCACGCTTCCATCATGTCCTTGCTGGCCATTGCTGTAGACCGATACCTGCGAGTCAAGCTGACAGTCAG----
R226  CACTTCTATGCCTGCCTTTTCATGTCCTGTGTGCTTCTGGTCTTCACCCATGCTTCCATCATGTCCTTGCTGGCCATTGCTGTAGACCGATACCTGCGAGTCAAGCTGACAGTCAG----

A3i   TCATTTCTTGTTGTGAACTTACCGTTTTCCTCCCCCCATTCAAACAGATATAGAACGGTTACCACTCAAAGAAGAATATGGCTACTCCTGGGCCTCTGCTGGCTAGTGTCCTTTCTGGTG  480
A3    ----------------------------------------------------ATATAGAACGGTTACCACTCAAAGAAGAATATGGCTACTCCTGGGCCTCTGCTGGCTAGTGTCCTTTCTGGTG
R226  ----------------------------------------------------ATATAGAACGGTTACCACTCAAAGAAGAATATGGCTATTCCTGGGCCTCTGCTGGCTAGTGTCCTTTCTGGTG

A3i   GGACTGACCCCCATGTTTGGCTGGAATAGAAAAGTGACCTTAGAGCTCTCTCAAAACAGCTCCACCCTCTCATGCCACTTCCGTTCCGTGGTCGGCTTGGATTACATGGTCTTCTTCAGC  600
A3    GGACTGACCCCCATGTTTGGCTGGAATAGAAAAGTGACCTTAGAGCTCTCTCAAAACAGCTCCACCCTCTCATGCCACTTCCGTTCCGTGGTCGGCTTGGATTACATGGTCTTCTTCAGC
R226  GGACTGACCCCCATGTTTGGCTGGAATAGAAAAGTGACCTTAGAGCTCTCTCAAAACAGCTCCACCCTCTCATGCCACTTCCGTTCGTGGTCGGCTTGGATTACATGGTCTTCTTCAGC

A3i   TTCATCACCTGGATCCTCATCCCCCTGGTTGTCATGTGCATCATCTATCTGGACATCTTCTACATCATCCGAAACAAACTCAGTCAAAATCTGACTGGCTTCAGAGAGACGCGTGCATTT  720
A3    TTCATCACCTGGATCCTCATCCCCCTGGTTGTCATGTGCATCATCTATCTGGACATCTTCTACATCATCCGAAACAAACTCAGTCAAAATCTGACTGGCTTCAGAGAGACGCGTGCATTT
R226  TTCATCACCTGGATCCTCATCCCCCTGGTTGTCATGTGCATCATCTATCTGGACATCTTCTACATCATCCGAAACAAACTCAGTCAGAATCTGACTGGCTTCAGAGAGACGCGTGCATTT

A3i   TACGGTCGGGAGTTCAAGACCGCTAAGTCCCTGTTTCTGGTTCTCTTCTTGTTTGCCTTGTGCTGGCTGCCTTTGTCCATCATCAATTTTGTTTCCTACTTTAATGTGAAGATACCAGAG  840
A3    TACGGTCGGGAGTTCAAGACCGCTAAGTCCCTGTTTCTGGTTCTCTTCTTGTTTGCCTTGTGCTGGCTGCCTTTGTCCATCATCAATTTTGTTTCCTACTTTAATGTGAAGATACCAGAG
R226  TACGGTCGGGAGTTCAAGACCGCTAAGTCCCTGTTTCTGGTTCTCTTCTTGTTTGCCTTGTGCTGCCTGCCTTTGTCCATCATCAATTTTGTTTCCTACTTTAATGTGAAGATACCAGAG

A3i   ATTGCAATGTGCCTGGGCATCCTGTTGTCCCATGCGAACTCCATGATGAACCCTATTGTCTACGCCTGCAAAATAAAAAAGTTCAAAGAAACCTACTTTGTGATCCTCAGAGCTTGCAGG  960
A3    ATTGCAATGTGCCTGGGCATCCTGTTGTCCCATGCGAACTCCATGATGAACCCTATTGTCTACGCCTGCAAAATAAAAAAGTTCAAAGAAACCTACTTTGTGATCCTCAGAGCTTGCAGG
R226  ATTGCAATGTGCCTGGGCATCCTGTTGTCCCATGCGAACTCCATGATGAACCCTATTGTCTACGCCTGCAAAAATAAAAAAGTTCAAAGAAACCACTTTGTGATCCTCAGAGCTTGCAGG

A3i   CTCTGTCAGACCTCGGATTCTTTGGACTCGAACCTTGAACAGACTACTGAGTAG  1014
A3    CTCTGTCAGACCTCGGATTCTTTGGACTCGAACCTTGAACAGACTACTGAGTAG
R226  CTCTGTCAGACCTCGGATTCTTTGGACTCAAACCTTGAACAGACTACTGAGTAG
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Fig. 2. Nucleotide sequence alignment of the rat A3 Ado receptor coding regions. A3i and A3 are the sequences obtained from RT-PCR of brain mRNA. R226 is the previously published A3 sequence [8]. Dashed lines mark the position of the A3i insert region. Nucleotide differences are indicated by (●) below the specific nucleotide. Note that R226 has an insertion of A at position 863 and a deletion of T at position 884 to put the sequence back in frame.

A 2.3 kb band was subsequently cloned and subjected to sequence analysis (Fig. 4). Results showed that the A3 locus contained a 1.7 kb intron located immediately upstream of the insert region. Furthermore, the insert was found to be contiguous with the 3′ exon immediately downstream. Analysis of the sequences surrounding the 51 bp insert identified consensus splice donor and acceptor sequences that enable the A3 transcript to be alternatively spliced (Fig. 4).

To analyze the distribution of the A3i receptor in various tissues, an oligonucleotide corresponding to the 51 bp insert region of A3i cDNA was used as a hybridization probe in a rat multiple tissue mRNA blot. However, no bands were detected (data not shown), indicating a low level of expression for this mRNA. In contrast, Northern blot analysis using the full-length A3i cDNA as probe (which hybridized to both A3 and A3i) detected A3 transcripts primarily in three tissues (Fig. 5). Three transcripts of 2.0, 2.3 and 3.0 kb were present in the spleen. A single weak band at 3.0 kb was found in the lung and a highly expressed 1.8 kb transcript was present in the testis. Although the original clone was identified in the brain, Northern blot analysis was not sensitive enough to detect transcripts in this tissue. The different band sizes detected in these tissues suggests that transcripts of the A3 gene are subject to extensive mRNA splicing.

To further characterize the A3i receptor, cDNAs encoding both the rat A3 and A3i receptors were subcloned into the eukaryotic expression vector pcDNA3 and stably expressed in CHO cells. Membrane fractions were prepared from the highest expressing clones and analyzed for their ability to bind the A3-specific ligand I-ABA as previously described [11]. Unfortunately, specific binding was quite low (generally $< 30\%$), and the total specific counts were insufficient to determine reliable binding affinities of various ligands (data not shown). Because of low specific binding in membrane preparations, a cAMP functional assay was used to test G protein coupling of the receptor in intact transfectants. As shown in Fig. 6, I-ABA modestly decreased forskolin-induced cAMP accumulation ($P = 0.029$; $n = 4$), although the efficacy was substantially less than that observed with the A3 Ado receptor. No suppression of cAMP was observed in CHO cells transfected with the neomycin resistance gene alone (data not shown).

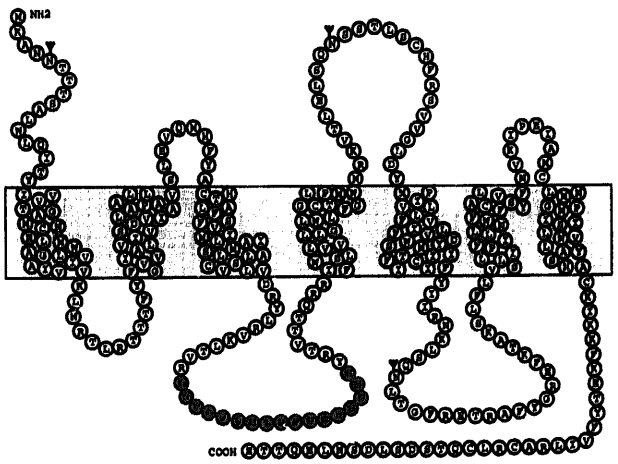

Fig. 3. The predicted amino acid sequence and transmembrane topology of the A3i Ado receptor. Putative *N*-glycosylation sites are indicated. The 17 amino acid insert in the second intracellular domain that is absent in the rat A3 receptor is shaded.

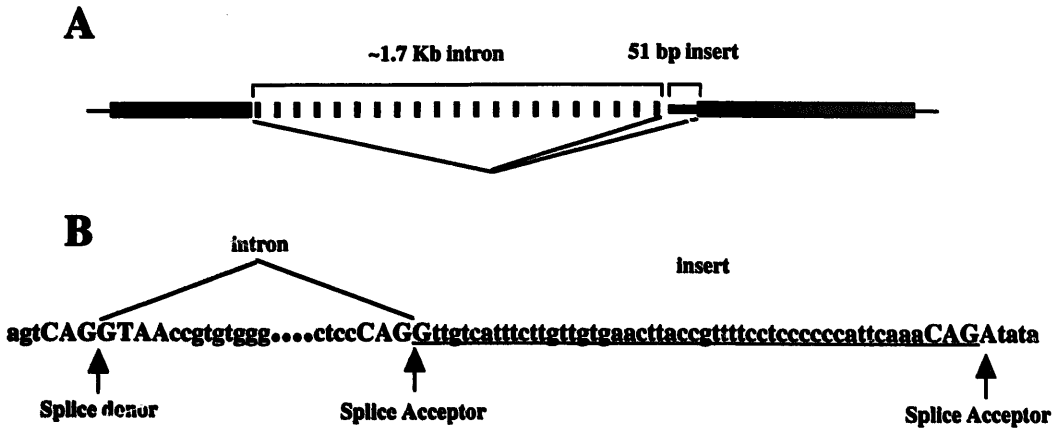

Fig. 4. Schematic of the rat A3 genomic region. (A) The splice junctions between the exons and the pattern of alternative splicing are indicated. The position of of the 51 bp insert is indicated by a solid bar. (B) The consensus sequences corresponding to the splice donor and acceptor sites are in capital letters and the insert sequence is underlined. Arrows denote the splice junctions.

4. Discussion

Diverse physiological functions are modulated by the actions of adenosine via a family of G-protein-coupled surface receptors [2]. To date four different classes of Ado receptors have been cloned from several species [1]. The A2a and A2b receptor subtypes are positively coupled to adenylate cyclase via stimulatory G proteins [4,5], whereas the A1 and A3 subtypes couple to Gi proteins and suppress cAMP production [1,11]. Other signal transduction mechanisms have also been identified for various Ado receptors, including phospholipase C and nitric oxide for the A3 receptor [13,14] and potassium channels for the A1 and A3 receptors [15,16]. The pharmacology of the A3 receptor is the least well characterized of the Ado receptor family, although recent data implicate it in cardiac preconditioning, mast cell degranulation, and suppression of TNF-alpha production by macrophages [17–19].

During the process of cloning the A3 receptor cDNA from rat brain mRNA, we obtained a second larger clone that appeared to encode an alternatively spliced variant of the A3 Ado receptor. The cDNA had complete homology to the sequence of the rat A3 Ado receptor that was cloned simultaneously by RT-PCR of rat brain mRNA except for the presence of a 17 amino acid insert in the second intracellular loop. We also noted, however, that a number of nucleotide differences existed between the sequences of these receptors and that reported for the original rat A3 Ado receptor [8]. The differences are likely sequencing errors in the original report and were also observed by Linden [17]. An extensive PCR analysis of mRNA from various rat tissues as well as commercially obtained cDNA pools showed the presence of the A3i transcript in all tissues tested, indicating that isolation of the A3i clone was not a PCR artifact. This was confirmed by the identification of genomic region encoding the insert region and its splicing sequences. Sequence analysis showed that the intron was immediately adjacent to the third trans-membrane, which is analogous to the organization of the A3 locus previously reported in the human genome [20]. Furthermore, since PCR analysis of the rat A3 gene using primers C and D (Fig. 1) yielded a 2.3 kb fragment (data not shown), our results suggest that there is likely only a single intron within the rat gene coding region, as with the human homolog [20].

Although the cDNA for A3i was stably expressed in CHO cells, transfectants displayed weak binding characteristics compared to the A3 receptor (data not shown). Coupling to G proteins was observed when analyzed for the receptor's ability to inhibit forskolin-stimulated cAMP production, but suppression of cAMP was weak compared to the A3 Ado receptor. Poor receptor coupling can contribute to low specific binding, a phenomenon that has been previously described for G-coupled receptors [1,2].

Using PCR analysis on mRNA isolated from a variety of rat tissues, Zhou et al. [8] previously showed that the A3 Ado receptor, in contrast to the A1 and A2 receptors, was only weakly detectable in the cortex and striatum, with the highest expression in the testis. Since no direct analysis of A3 transcripts had been previously reported, we analyzed mRNA from rat tissues by Northern blot analysis. The A3i Ado receptor probe (which hybridized to both A3 and A3i) detected 3 different sized transcripts in the spleen and a smaller, highly abundant transcript in the testis. These studies support the notion that the rat A3 receptor gene is subject to extensive alternative splicing.

Despite extensive PCR analysis, no variant A3 receptors were detected in human tissues (data not shown). This may not be surprising since, unlike the rat, the human and sheep

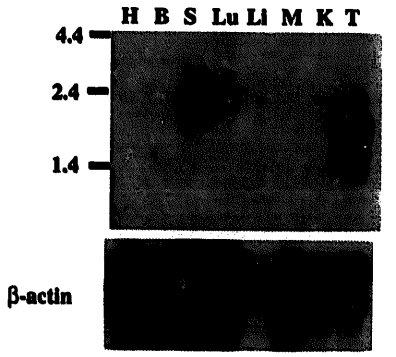

Fig. 5. Northern blot hybridization of rat tissues with the A3i cDNA probe. Heart (H), brain (B), spleen (S), lung (L), liver (Li), muscle (M), kidney (K) and testis (T). The length of size markers (in kb) is indicated to the left. The blot was stripped of the rat A3i Ado receptor probe and rehybridized with a human β-actin cDNA probe as control (below). Autoradiography was carried out with intensifying screens for 17 h (A3i) and 1 h (β-actin).

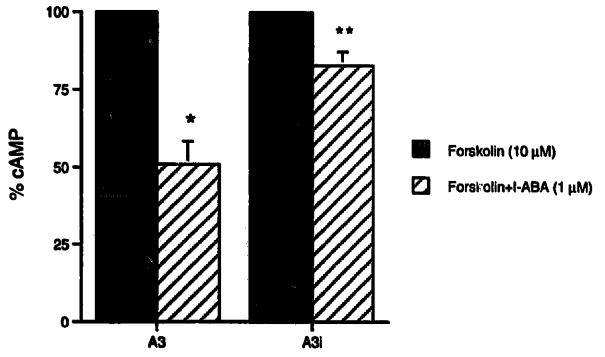

Fig. 6. Inhibition of cAMP accumulation by the A3 Ado receptor agonist I-ABA in forskolin-stimulated CHO cells expressing the A3 or A3i Ado receptors. Cells were treated with forskolin (10 μM) and I-ABA (1 μM) and cAMP measured using an immunoassay. Data are presented as the mean ± S.E.M. of 4 separate experiments and expressed as a percentage of forskolin-induced cAMP. $^{*}P = 0.008$, $^{**}P = 0.029$ compared to forskolin alone.

A3 receptor transcripts are of uniform length in different tissues [7,10]. Furthermore, the recent cloning of the human A3 gene identified only a single intron, with a structural motif indicative of a gene that does not undergo alternative splicing [20]. This contrasts with extensive splicing that occurs in the human A1 receptor [21].

The location of the insert in A3i suggests that this sequence might be involved in interactions with specific intracellular components that mediate receptor signaling. It also raises the possibility that the pharmacology of A3 observed in rat tissues might not have a corollary in humans since a homologue was not identified. Moreover, the existence of variant A3 receptors in the rat may contribute to the observations by Fozard et al. on the role of A3 receptors in hemodynamic responses [22]. Given the widely divergent cDNA sequences, tissue distribution, and patterns of alternative splicing, the relevance of these non-human models to human A3 function should be viewed with caution.

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