Isolation and characterization of an avian A₁ adenosine receptor gene and a related cDNA clone

José S. AGUILAR, Fulong TAN, Isabelle DURAND and Richard D. GREEN*

Department of Pharmacology, School of Medicine, University of Illinois at Chicago, Chicago, IL, U.S.A.

We have isolated the gene for a chick A₁ adenosine receptor along with a cDNA that codes for the same adenosine receptor. The cDNA clone was isolated from both adipose tissue and heart cDNA libraries and encodes a 324-amino acid protein with 80 % identity with mammalian A₁ adenosine receptors. Transient expression of the cDNA in human embryonic kidney (HEK) 293 cells shows that it encodes a protein that binds [³H]CCPA (2-chloro-N⁶-[cyclopentyl-2,3,4,5-³H]cyclopentyladenosine, a specific agonist radioligand, with a K_D of 5.6 ± 2.4 nM. Cyclic AMP measurements in HEK 293 cells co-transfected with the chick cDNA and a cDNA for a luteinizing hormone/choriogonadotropin receptor shows that A₁ adenosine receptor agonists

antagonize the cyclic AMP-elevating effect of bovine luteinizing hormone. Two partial genomic clones were isolated. The first contains 5'-untranslated sequence including a putative promoter region which does not contain a TATA box, an intron and the first third of the coding sequence of the A₁ adenosine receptor cDNA. The coding sequence of this partial genomic clone terminates at a second intron. The second partial genomic clone contains the rest of the coding sequence and the 3'-untranslated elements in a single exon. Thus the chick A₁ adenosine receptor gene contains one intron in the 5'-untranslated region and a minimum of one intron in the coding sequence.

INTRODUCTION

Adenosine acts at G-protein-coupled receptors to modulate a diversity of cellular and physiological functions (reviewed in ref. [1]). Biochemical and pharmacological experimentation established the existence of two types of adenosine receptor. A₁ adenosine receptors are coupled to the inhibition of adenylate cyclase [1], opening of cardiac atrial K⁺ channels [1] and the modulation of cardiac ATP-sensitive K⁺ channels [2]. A₂ adenosine receptors are coupled to the stimulation of adenylate cyclase [1]. cDNA clones for both types of receptor from several mammalian species have now been isolated and expressed. These include cDNAs for dog, rat, cow, human [3] and rabbit [4] A₁ adenosine receptors and cDNAs for A_{2a} adenosine receptors in dog and rat and A_{2b} adenosine receptors in rat (reviewed in ref. [3]). Molecular cloning recently revealed the existence of an additional adenosine receptor subtype, the A₃ adenosine receptor [5–7], which can mediate the inhibition of adenylate cyclase activity.

Although many subtypes of most G-protein-coupled receptors are known to exist, to date all the A₁ adenosine receptors that have been cloned appear to be species variations of the same receptor. Ligand-binding experiments from our laboratory suggest that bovine heart contains more than one A₁ adenosine receptor subtype [8]. In addition, we have now found that embryonic chick cardiac myocytes appear to have more than one A₁ adenosine receptor subtype that is coupled to the inhibition of adenylate cyclase [9]. We herein report the cloning and expression of a chick A₁ adenosine receptor cDNA. In addition, we report the cloning of two partial genomic clones which together code for the same A₁ adenosine receptor protein. The upstream clone, which contains a putative promoter region and an intron in the

5'-untranslated region (UTR), terminates at a second intron midway in the coding sequence; the downstream clone contains the remaining coding sequence and the 3'-UTR in a single exon.

MATERIALS AND METHODS

Probe generation by PCR

Total RNA was isolated from dog and chick brain, and polyadenylated RNA was prepared by chromatography on oligo(dT)-cellulose [10]. First-strand cDNA was synthesized using Moloney murine leukaemia virus reverse transcriptase and PCR was performed using *Taq* DNA polymerase (Promega, Madison, WI, U.S.A.). PCR was performed using oligonucleotide primers based on conserved regions of the cDNAs of dog A₁ and A₂ adenosine receptors [10] [5'-GGA ATT CAT GGA (GA)TA (TC)AT GGT (GATC)TA (TC)TT (TC)AA 1-3' and 5'-GGA ATT CCA IAT ITT IAI (AG)AA IGT (ATCG) AC (ATCG)C(TG) (AG)A-3'] and primers previously used to make the probes subsequently used to clone dog A₁ and A₂ adenosine receptors [11]. PCR products were subcloned into a pT7Blue(U) vector (Novagen, Madison, WI, U.S.A.), and bacteria containing plasmids with the fragment of the putative chick A₁ adenosine receptor were selected by colony hybridization using a dog probe as discussed in the Results section.

Library screening

Four libraries were screened: (1) a chicken brain cDNA library in λgt10 (Clontech, Palo Alto, CA, U.S.A.); (2) and (3) chicken adipose tissue and heart cDNA libraries in λZAP (Stratagene,

Abbreviations used: HEK, human embryonic kidney; CCPA, 2-chloro-N⁶-cyclopentyladenosine; [³H]CCPA, 2-chloro-N⁶-[cyclopentyl-2,3,4,5-³H]cyclopentyladenosine; UTR, untranslated region; 1 × SSC, 0.15 M NaCl plus 0.015 M sodium citrate; LH, luteinizing hormone; CG, choriogonadotropin; R-PIA, (–)-N⁶-2-(phenylisopropyl)adenosine; cAMP, cyclic AMP; CPA, N⁶-cyclopentyladenosine; NECA, 5'-N-ethylcarboxamidoadenosine.

* To whom correspondence should be addressed.

La Jolla, CA, U.S.A.); (4) a chicken genomic library in λ EMBL3 [12]. Nitrocellulose membrane (0.45 μ m pore size; Schleicher and Schuell, Keene, NH, U.S.A.) lifts were prehybridized [50% formamide, 4 $\times$ SSC (where 1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5 $\times$ Denhardt's solution (where 1 $\times$ Denhardt's is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.002% BSA), 5 mM EDTA, 0.1% SDS, 25 μ g/ml yeast tRNA, 100 μ g/ml salmon sperm DNA] for 4 h at 37 °C. Hybridization was carried out in the same medium containing 10⁶ c.p.m./ml [α -³²P]dCTP-labelled probe at 37 °C overnight. All probes were labelled using the random-primer technique [13]. The filters were washed with 1 $\times$ SSC/0.1% SDS at 37 °C for 20 min periods until no radioactivity was detected in the wash buffer (four to six times). Filters were subsequently washed twice in 1 $\times$ SSC/0.1% SDS at 60 °C for 20 min, and twice in 0.1 $\times$ SSC/0.1% SDS at 60 °C for 20 min. Filters were exposed to Kodak X-Omat-AR film for 1–3 days at –70 °C.

No positive clones were identified in the brain cDNA library. pBluescript SK plasmids were excised from positive clones from the adipose and heart libraries according to the manufacturer's instructions. Restriction analyses of the inserts were carried out and fragments were subcloned in pBluescript SK, pGEM7Z or pGEM4Z (Promega). Restriction fragments of positive genomic clones were subcloned into plasmid vectors for sequencing. In all cases both strands of DNA were sequenced using Sequenase (US Biochemical, Cleveland, OH, U.S.A.). Primers were either vector-specific or derived from previous sequence information.

Expression of A₁ adenosine receptor

The putative A₁ adenosine receptor cDNA clone was subcloned in the eukaryotic expression vector, pcDNA1 (InVitrogen, San Diego, CA, USA). Calcium phosphate precipitation [10] (10 μ g of plasmid DNA/60 mm dish) was used to transiently express the putative A₁ adenosine receptor in human embryonic kidney (HEK) 293 cells either alone for binding studies or with a rat luteinizing hormone/choriogonadotropin (LH/CG) receptor (in pcDNA1/Neo; 5 μ g of plasmid DNA/60 mm dish) for second-messenger studies.

Binding assays

Cells were rinsed with homogenization buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 0.1 M benzamidine, 0.1 mM phenylmethanesulphonyl fluoride), scraped into the same buffer, and after 15 min on ice homogenized by passage through syringe needles of decreasing size. The homogenate was centrifuged at 30000 *g* for 20 min and the pellet was resuspended in the same buffer at about 5 mg of protein/ml. Binding was performed at 37 °C for 1 h in the presence of 10 mM Hepes buffer containing 0.5 mM EDTA, 2.5 mM MgCl₂, 1 unit/ml adenosine deaminase and various concentrations of the A₁-adenosine-receptor-specific agonist radioligand, [³H]2-chloro-*N*⁶-[cyclopentyl-2,3,4,5-³H]-cyclopentyladenosine ([³H]CCPA) [14]. Bound and free ligand were separated by vacuum filtration over Whatman GF/A glass-fibre filters. Non-specific binding was defined by the addition of 10 μ M (–)-*N*⁶-2-(phenylisopropyl)adenosine (R-PIA) or by binding to equal amounts of membrane protein prepared from untransfected cells. The two methods gave identical results. Non-specific binding was about 10% of total binding when the radioligand concentration was approximately its *K_D*.

Measurement of second-messenger response

The ability of the expressed receptors to couple to the inhibition of adenylate cyclase was tested by transiently co-expressing the

putative A₁ adenosine receptor clone and a rat LH/CG receptor [15]. Cells were transfected overnight in 60 mm dishes as described and the following day transferred to poly-L-lysine-coated 24-well plates (two to three 60 mm dishes/24-well plate). (The HEK 293 cells that we were using at this time were easily dislodged from the culture plates and treatment with polylysine or collagen was necessary to prevent the cells from 'coming up' during the subsequent procedures.) The following day the cells were incubated for 6–8 h with 1–2 μ Ci of [³H]adenine in 500 μ l of complete medium and rinsed with Hepes-buffered Dulbecco's modified Eagle's medium containing 1% BSA. The effects of LH with or without CCPA on the conversion of [³H]ATP into [³H]cyclic AMP (cAMP) was determined by a 30 min incubation at 37 °C in the presence of the phosphodiesterase inhibitors rolipram (0.1 mM) and milrinone (10 μ M). [³H]cAMP formed was isolated and quantified using standard techniques previously employed in our laboratory [16]. Data are expressed as percentage of [³H]ATP converted into [³H]cAMP and normalized against percentage conversion in the presence of LH alone.

Primer-extension analysis

The oligonucleotide, 5'-CAGGAGCAGAGAAGGAGAGCA-CGGAGCG-3', complementary to bases –414 to –444 of the cDNA clone, was labelled at the 5' end using T4 polynucleotide kinase and [γ -³²P]ATP and used for primer-extension analysis [10]. A sequencing ladder generated with the same oligonucleotide and run simultaneously on the sequencing gel was used to estimate the transcription start site.

RNA analysis

mRNAs were prepared from brain, heart, liver and adipose tissue, run on agarose-formaldehyde gels [10], transferred to nylon membranes and hybridized using the same conditions as described for library screening.

RESULTS

Initial attempts to amplify chick brain cDNA using the pair of amplimers directed to the conserved regions in the dog A₁ and A₂ adenosine receptors were unsuccessful. However, PCR with these amplimers and dog brain cDNA resulted in a 400 bp fragment which we sequenced and found to be identical with the sequence of the dog A₁ adenosine receptor previously reported (results not shown). PCR of chick brain cDNA with the primer pair previously used to clone canine adenosine receptors [11] resulted in several cDNA fragments between 400 and 700 bp. One of these amplified products, of about 500 bp, hybridized strongly with the dog A₁ adenosine receptor 400 bp fragment (results not shown). The mixed amplified products were subcloned and putative chick A₁ adenosine receptor products identified by colony hybridization using the dog probe. Several positives containing the same 474 bp insert with 77% identity with the dog A₁ adenosine receptor sequence were found. This amplified fragment was used as a probe for Northern blots and library screening.

A positive clone (CA1A) containing about 1.7 kb was isolated from the chick adipose cDNA library. The nucleotide and deduced amino acid sequence of this clone are shown in Figure 1. The longest open reading frame in this clone encodes a protein of 324 amino acids with a theoretical molecular mass of 36324 Da. Hydropathic analysis of the predicted amino acid sequence reveals the putative structure characteristic of G-protein-coupled receptors, i.e. seven putative transmembrane-spanning regions

GGGTCGCTCCGTCGCCGGTATCGGCTGCTCCGCGCTCCGTCGCTCTCTCTCTCTC -424
 TCTGCTCTCTCCCTACCAACGGAGCTCTATAACGTCGGTTTGGGGGCTGAAACGTGA -367
 GGCTTCTGGAGCCCGGGTGTGGCTGCGGAGCGAGCAACCCCAACCGGCACCGCGCC -310
 TCTCCCGCTCGGCGCGCAGCCCAATCGGTCGGTCTCTTTAAAGGGCTGCG -253
 GGAGCGCGCACCATGTGTGCTCTCGTCTCAACTGCGAGCTGCTGCGGGAGCAGC -196
 GCGTGCGGGACCCGGGGCGAGCGGAGCTCTCCGCGAGCGCTCCGCGAGCGCTCCC -139
 GCAGCCGCTCCCGCAGCGCTCCCGCAGCATGCCCGCGCTCTGCGCGCGCTGAACG -82
 CTTACAGACTCGGACACCGCAGCAGCCTCTCGGAGCGGACCAAGGTAACCGAGG -25
 GAGCGCAGAGGGGAGCGCAGCACCATTGGCAGCTCCGTGACGGCTTCCAGGCTGCC 33
M A Q S V T A F Q A A

TACATCTCCATCGAAGTGTGATCGCTTTGGTTCGGTGCCGGGAATCTCTGGTC 90
Y I S I E V L I A L V S V P G N I L V

ATCTGGGCTGTGAAGATGAACAGGCGCTCGGGATGCCACTTCTGCTTCATCGTC 147
I W A V K M N Q A L R D A T F C F I V

TCGCTGGCGGTGGCGAGCTGGCTGTGGGGCTCTGTGTCATCCCTTTGGCCATCATC 204
S L A V A D V A V G A L V I P L A I I

ATCAATATCGGACCCGAGCGAGTCTCTACAGCTGCCTCATGATGGCGTGCCCGCTC 261
I N I G P Q T E F Y S C L M M A C P V

CTCATCTCACCGAGAGCTCCATCTCGCTCTCTGCGCATCGCTGTGGATCGGTAC 318
L I L T E S S I L A L L A I A V D R Y

CTGCGAGTGAAGATCCCGCTCAGGTATAAGAGCGTGGTGACACCCCGCGAGCAGCA 375
L R V K I P V R Y K S V V T P R R A A

GTGGCCATCGCTGTGTGGATCGTCTCTTTCTGGTGGGACTACCCCAATGTTC 432
V A I A C C W I V S F L V G L T P M F

GGTTGGAATAACCTGAACAAGGTGCTGGGAGCTCGGATCTGAATGTGAGCACTCG 489
G W N N L N K V L G T R D L N V S H S

GAGTTTGTATCAAGTGCAATTCGAGAGCGTCTCATGATGGAGTACATGGTGATC 546
E F V I K C Q F E T V I S M E Y M V Y

TTCAACTTCTCTGCTGGGTCTGCGCGCCCTGCTGCTGATGCTGCTCATCTACCTG 603
F N F F V W V L P P L L L L L I Y L

GAAGTCTTCAACCTCATCGCAGCAGCTCAACAAGAGGTCTCTCCAGCTCCAAC 660
E V F N L I R T Q L N K K V S S S S N

GACCCCAAGATATTACGGGAAGGAGCTGAAGATCGCAAGTCCCTCGCGCTGGTT 717
D P Q K Y Y G K E L K I A K S L A L V

CTCTCTCTTTTGGCTGAGCTGGCTCCCTCTGCACATCTCAACTGCATCACTTTG 774
L F L F A L S W L P L H I L N C I T L

TTCTGCCATCTCTGCAAGACACCGCACATCTCACATACGCGCATCTCTCTCACC 831
F C P S C K T P H I L L T Y I A I F L T

CATGGCAACTCGGCTATGAACCCATTGTCTACGCGCTTCTAGGATCAAGAAGTTCGG 888
H G N S A M N P I V Y A F R I K K F R

ACAGCCTTCTCGAGATCTGGAACCAAGTATTTCTGCTGCAAAACCAAAAGCAGC 955
T A F L Q I W N Q Y F C C K T N K S S

AGCAGCAGCAGCTGAGACGGTCAACTAAGAGCTGAGGGTTCGCGCTGCCAGGGA 1002
S S S T A E T V N -

AGGGGGGATGAGGACCCAGGAGGGCTGAGTTTGGCATTCCCATGCAATCTGAACAG 1059
 CCCAGCCCTGAGCTGCCCGAGGGGTATTATTCTACTGTTTGGATATTGTAGCCT 1116
 GCAGTCTCTCTTTCTTTTATTACTATTATTATTATTATTATTATTATTATTATTATT 1173
 TTTTATTATTATTAGTCTGCAAGTATTATTAGGAGATGTTGTACAGATAAAGATGTG 1230
 GTTATCTTTAATGCAATTAAGTAAATGGTCATAACAAAAAAA 1276

Figure 1 Nucleotide and deduced amino acid sequence of the adipose tissue cDNA clone

Putative transmembrane-spanning regions are underlined and numbered (I–VII). ATTTA pentamers and the putative polyadenylation signal are in bold.

connected by more hydrophilic loops [1,3]. The putative second extracellular loop contains a potential glycosylation site [1,3]. The C-terminal domain is very rich in Ser-Thr and contains two cysteines, which are potential acylation sites [1,3]. The 3'-UTR is very rich in AT residues and contains two ATTTA pentamers. A polyadenylation signal is also present. Subsequent screening of the heart cDNA library led to the isolation of three positive clones. Partial sequencing suggested that the three clones were identical. Complete sequencing of one clone showed that it is

Chick **I** MAQSVTAPQAA-----**IV** MFGWNNLNKVLGTRDLNVSHSEFVIKQFETVISME-----**V**
 Dog MPPAISAFQAA-----LFGWNLGGAQRAWAANGSGSEFVIKCEFEKVISME-----
 1 11 43 178

Chick **VII** --FRIKKFRFLQIWNQYFCCKTNKSSSSSTASTVN
 Dog --FRIQKFRVTLKIWNDFRCQPTFPFVDEDPPEAPHD
 290

Figure 2 Comparisons of deduced amino acid sequences of chick and dog A₁ adenosine receptors in three highly variable regions, the N- and C-termini and the second putative extracellular loop

Locations of putative transmembrane-spanning regions are shown in roman numerals.

Figure 3 Saturation curve and Scatchard plot (inset) of [³H]CCPA binding to putative chick A₁ adenosine receptor transiently expressed in HEK 293 cells

Points shown are means of duplicate determinations. $K_D = 3.9$ nM; $B_{max} = 720$ fmol/mg.

identical with the CA1A clone isolated from the adipose-tissue library.

CA1A has a high degree of identity with mammalian adenosine receptors. The predicted amino acid sequence is 80% identical with that of the dog A₁ adenosine receptor. Figure 2 shows the amino acid sequences of the three most divergent areas, the N- and C-terminal domains and the second extracellular loop. CA1A has a lower degree of identity (50%) with mammalian A₂ adenosine receptors. Thus CA1A appears to code for a chick A₁ adenosine receptor.

The identity of CA1A as an A₁ adenosine receptor clone was established by expression experiments. The 1.7 kb insert was subcloned into pcDNA1 and transiently expressed in HEK 293 cells. Transfected, but not untransfected, cells show specific binding of the A₁-adenosine-receptor-selective agonist [³H]CCPA. Binding is saturable and Scatchard analysis reveals a single class of site (Figure 3). In four separate experiments the K_D and B_{max} values were 5.6 ± 2.4 nM and 738 ± 234 fmol/mg of protein respectively. The binding of [³H]CCPA was displaced by the adenosine agonists R-PIA, N⁶-cyclopentyladenosine (CPA) and 5'-N-ethylcarboxamidoadenosine (NECA) (Figure 4). In three experiments the IC_{50} values (nM) for R-PIA, CPA and NECA were 5.0 ± 2.0 , 6.2 ± 0.6 and 54.0 ± 29.1 respectively. The relative potencies of these agonists in displacing the binding (R-PIA = CPA > NECA) is characteristic of A₁ adenosine receptors [1,3].

Figure 4 Inhibition of [3 H]CCPA (4 nM) binding to putative chick A₁ adenosine receptors expressed in HEK 293 cells

Points shown are means of duplicate determinations. ●, R-PIA; ○, CPA; □, NECA.

Figure 5 Effect of CCPA on LH-increased cAMP levels in HEK 293 cells transiently (a) or stably (b) expressing LH and adenosine receptors

LH (5 μ g/ml) increased cAMP 4.7- (a) and 38- (b) fold above basal levels in these experiments. Points shown are means of triplicate determinations from representative experiments.

CCPA lowered LH-elevated cAMP levels in cells co-transfected with CA1C and the LH receptor clone (Figure 5a). The experiment shown is representative of three similar experiments; the high sensitivity to CCPA is probably related to very high receptor expression in a small population of the cells. The LH receptor was in a plasmid with a neomycin-resistance gene and co-transfection of this plasmid and the CA1C-containing plasmid followed by incubation with G418 resulted in a mixed cell population stably expressing about 800 fmol of [3 H]CCPA-binding sites/mg of protein. Figure 5(b) shows one of two similar experiments on these cells in which CCPA had an IC₅₀ about

Figure 6 Northern-blot analysis of chick mRNA (1 μ g) prepared from brain (B), heart (H), liver (L) and adipose tissue (A)

10 fold higher than in the transiently expressing cells. The modest maximal response to CCPA in these cells is probably because some cells contain LH receptors but not adenosine receptors; HEK 293 cells express an endogenous β -adrenergic receptor and the maximal inhibition of isoprenaline-induced increases in cAMP is about twice that of LH (results not shown). It is thus clear that CA1C codes for a chick A₁ adenosine receptor.

Figure 6 shows a Northern-blot analysis of mRNA prepared from brain, heart, liver and fat. A message of about 1.7 kb was detected in brain and heart but not in liver or fat. Although the results with brain, heart and liver were consistently obtained, results with adipose mRNA were variable in that a message at 1.7 kb was detected in one experiment but not in several others. The reason for this is not apparent.

Screening of the genomic library with the same PCR probe resulted in one positive clone of about 20 kb. A fragment (about 2.5 kb) identified by Southern-blot analysis of restriction digests of this clone contains part of the CA1A-coding sequence including the initiation codon and extending 351 bases downstream at which point the sequence diverges at an apparent intron/exon junction (Figures 7 and 8). The region of the genomic clone 5' to the start codon contains a promoter region, as evidenced by consensus sequences for transcription factors, and an intron. The remaining sequence is identical with that in the cDNA clone. The 20 kb clone does not appear to contain the remainder of the coding sequence as the other restriction fragments do not hybridize with the probe even though the coding sequence of the positive fragment ended at a point less than half way through the probe sequence.

Primer-extension analysis located the transcriptional start site at base -815 of the genomic clone (Figure 7). Like the dopamine D1 receptor gene [17], the 5' flanking region has a high G+C content but does not contain a TATA box, features typical of housekeeping genes. Putative regulatory elements for AP2 [18], thyroid-hormone-regulatory elements [19], tumour necrosis factor [20] and Pur-1 [21] are present.

The genomic library was rescreened using a probe (an *Ava*II-*Ava*II fragment of the cDNA clone; bases 563-1014) which starts downstream from the end of the second exon in the previously isolated genomic clone. Two clones (about 10 kb) with identical restriction maps were isolated; a 3.3 kb restriction fragment that hybridized with the probe was subcloned and partially sequenced. This clone contains the remainder of the coding sequence and the entire 3'-UTR in a single exon which matches the 3' end of the second exon in the other partial genomic clone. Although there were six base differences between

ttccaggggagatgtcatgtgtgagccctgggggggtgctcggtgccacagtggggggtgt	-1174
AP2	
ggaaaccaccctcgggtgctgcacgcctagtgcacaggagcttgggtcgggctgc	-1117
TRF	
ggtgctttgggcttggtcggttcagctccaccagagtgagcaccattcccagccca	-1060
TRF	
gggcagaaatgtgggggtttgaccattttaccctcaactctccacacactcttaaaca	-1003
Pur-1	
accaaacctgaacccccccccacagctgggagagaccattctctccgcacatttcgttg	-946
AP2	
gggacaggaatgagctccacctctctctttcccagcctccccacagctcggcatgg	-889
TFII	
ggtgcagcccggtcaacacagcgctgttcagggggtcagcgcgcccccacccggcac	-832
tgatttttgggagggcgcccgagcggtgggggctttgagcatcggggttctctccc	-775
cegggtggtccgcgcgggctgcttcggtccgggttgcgtgctccgcgctgcgtctc	-718
GTGCTCTCTCTCTCTGTCTGTCTGCCCTTACCAAGGAGCTCTATAACGCTCGGTTTG	-661
GGGGCTGAACCGTAGGGCTCTCGAGCCCGGGGTGGCTGCGGAGCGAGCAACCCCA	-604
ACCGGCACCCGCGCTCTCCCGCTGCGGCGCGAGCCCCAAATCGGGTGGTGGCT	-547
CTTTAAAGGGCTGCGGGAGCGCGCACCATGTGATGCTCGTCTCTCAACTGCGAGCT	-490
GCTTCGCGGAGCAGCGCTGTCGCGGCGACCGGGCCGAGCGAGCTCTCCGCGAGCCG	-433
TCCGCGAGCGCTGCGGAGCGCTGCGGAGCGCTGCGGAGCTGCGGAGCTGCTG	-376
CCCGCGCGCTGAACGCTCTCAGACTCGGAGCAGCGCAGCAGCTCTCTGGAGCGGG	-319
ACAACTgagcgctcttcggagctaaaggacagcagaagcgcgctcagcgccccc	-262
ctctcccccgcgctccccccaccccgcgcggtgggacacggagctccgctccc	-205
cgggtcggttgcatggagcagcgctcggggggagcggggcagcagaggtccggg	-148
ggggcgcgcgagcagctaggattatctttcccccccccgcccgagaggtccgctc	-91
ccggcaccatctcggctcgcagtgcaagttgtgacccgactccctctcggtttgcagG	-34
TAACCGAGGGAGCGCAGAGGGGAGCGCAGCACCATGGCACAGTCGTGACGGCTTTC	24
M A Q S V T A F	
CAGGCTGCCTACATCTCCATCGAAGTGCTGATCGCTTTGGTGTGCGTGCCGGGAAT	81
Q A A Y I S I E V L I A L V S V P G N	
I	
ATCTCGTCACTCTGGGCTGTGAAGATGAACAGGCGCTGCGGGATGCCACTTTCTGC	138
I L V I W A V K M N Q A L R D A T F C	
TTCACTCGTCTCGCTGCGGTCGGCAGCTGCTGCTGGGGCTCTGGTCACTCCCTTTG	195
F I V S L A V A D V A V G A L V I P L	
II	
GCCATCATCATCAATCGGACCGCAGACGGAGTCTACAGCTGCCTCATGATGGCG	252
A I I I N I G P Q T E F Y S C L M M A	
TGCCCGCTCTCATCTCTCCAGAGAGCTCCATCTCTGGCTCTCTGGCCATCGCTGTG	309
C P V L I L T E S S I L L A I A V	
III	
GATCGTACCTGCGAGTGAAGATCCCGGTGAGTgagacagtgctgctgggagtggtt	366
D R Y L R V K I P V R -	
ctgggctgggacactgggggggacagaggggacagagatgtcccgaactctttgaga	423
agcagcagcaccagcagcagcggtttggtgcagcaggaattgggggggctgagcaga	480
ggggggtcccacagcctcgtgtgcttgcgtcgtggggatcactgtgctcagctgt	537
gtatctggaaaggagcaacacgctgtgctccacacaggtttcccggtggtgaaggtg	594
aaggcactgacagccggcggaactctggggtagggatgtgacagcagtaaaagcagt	651
cctgtgtgactcctcccccaccacacagctggtttctcagggacttccccgtgtgt	708
gcaattgtattactaatattttttcaagctgaaaaatccccatccctccatccc	765
gacagcaccacagagcccttcgtgccctctcactgctttcagtgcttttctggttct	822
ttgcatcctcgtgtgctggggagcagctgcagctgtggggagcagctgtctatctaca	879
ggctctgacttcagcgcgctgactcgtttctctgtctgttccccaaaatgcaact	936
ccctctcctccctcactccctctgagcactgctggggttttggcggcagattctctg	993
cctcactcagggacctgagagcattacagctgtgcgctgttcagctgttggtgggt	1050
ttttgctcatgcaataataaacttggtccctcctggtctgtgcacagtgagattttgc	1107
ttcccacgctgtcatcctcactcccaaatccccagcgttggtgagctgtggtgtgg	1164
ttgctgctttctgatatctcttagtatgaaataacttgcaactcagtggtgccaact	1221
gtgatatttgcctcaagggggctctgtggggcagggatgtcccatcgtcgtcatgcac	1278
tttacaagagtcctccctgagatcccatatttgaactccqqaacttc	1325

Figure 7 Nucleotide sequence of 2555-base restriction fragment of partial genomic clone containing the first 351 bases of the coding sequence of the adipose A₁ adenosine receptor cDNA

The numbering begins at base 1232, the initiation codon. The deduced amino acid sequence of the coding sequence is shown with the putative transmembrane-spanning regions underlined and numbered. Nucleotide sequences found in the cDNA are in upper-case letters. Putative response elements are underlined: AP2, thyroid-hormone-regulatory elements (TRE), Pur-1, and tumour necrosis factor (TNF). The arrow indicates the putative transcriptional start site at nucleotide — 815.

the genomic sequence and cDNA sequences, minor differences in genomic DNA sequence are common and the amino acid sequence was not altered. For example, similar differences between genomic and cDNA sequences encoding the mouse high-mobility group-1 protein were recently reported and attributed to polymorphisms [22]. No attempt was made to sequence the intronic sequences at the ends of the two genomic

Figure 8 Diagram summarizing our current understanding of the structure of the chick A₁ adenosine receptor gene

Intron 1 which separates exons 1 and 2 (E1 and E2) is in the 5'-UTR. As the entire intronic sequence separating E2 and E3 has not been sequenced, it is not known for certain that the intron at the 3' end of E2 is the same intron at the 5' end of E3.

clones to determine if intervening exons are present that can code for splice variants of the receptor.

DISCUSSION

We here report the cloning and characterization of a chick A₁ adenosine receptor. This is the first non-mammalian adenosine receptor cloned and the first adenosine receptor cloned from an adipose-tissue cDNA library. In addition, it is the first A₁ adenosine receptor cloned from a heart library. The chick clone has many of the characteristics common to mammalian A₁ and A₂ adenosine receptors. These include a consensus sequence for N-linked glycosylation in the second exofacial loop, histidine residues in TM6 and TM7 and a cysteine (in this case actually two) in the carboxyl tail (reviewed in refs. [1,3]). Two of the hypervariable regions, the second exofacial loop and the C-terminus are also hypervariable among the mammalian receptors. A potentially important difference between the chick receptor and mammalian receptors is the presence in the former of five serines and four threonines in the carboxyl tail. Whereas the C-terminal regions of mammalian A₂ adenosine receptors have many serine and threonine residues, the corresponding regions of mammalian A₁ adenosine receptors are devoid of serines and possess a single threonine (with the exception of the dog receptor which contains an additional threonine). It will be interesting to determine if these multiple potential phosphorylation sites are involved in the marked receptor down-regulation of chick cardiac A₁ adenosine receptors that occurs in response to prolonged exposure to agonist [23,24].

The predicted amino acid sequence encoded by the chick clone shares about 80 % identity with mammalian A₁ adenosine receptors. Although this may seem low in comparison with more than 90 % identity between cloned mammalian A₁ adenosine receptors [3], it cannot be concluded that we have cloned an A₁ adenosine receptor subtype different from the previously cloned mammalian A₁ adenosine receptor. For comparison, chick M₂ and M₄ muscarinic receptors are 83–86 % identical with their mammalian counterparts at the amino acid level [25,26], and the identity of the turkey β -adrenergic receptor with mammalian β -adrenergic receptors is 59 % [27]. We have postulated that embryonic chick myocytes contain two A₁ adenosine receptor subtypes or A₁ and A₃ adenosine receptors that are capable of coupling to adenylate cyclase [9]. Although the K_D we reported for [³H]CCPA binding to A₁ receptors in detergent-permeabilized myocytes (about 1 nM) was a bit lower than that in the present studies, the fact that we cloned the A₁ adenosine receptor cDNA from a chicken heart cDNA library would suggest that these two receptors are one and the same.

An additional potentially important characteristic of the chick A_1 adenosine receptor clone reported here is the presence of AT-rich sequences including ATTTA pentamers in the 3'-UTR. AT-rich regions and ATTTA pentamers (AU and AUUUA in mRNA) in the 3'-UTR of mRNAs have been implicated in the regulation of translational efficiency and mRNA stability [28] and references therein). Interactions between AU and AUUUA-rich regions of the mRNA for β_2 -adrenergic receptors and RNA-binding proteins have been implicated in agonist-induced down-regulation of β_2 -receptor mRNA and β_2 -receptor protein [29]. The A_1 adenosine receptor encoded by CA1A is down-regulated by about 50 % when HEK 293 cells stably expressing the receptor are treated overnight with 10 μ M R-PIA. (J. S. Aguilar and R. D. Green, unpublished work). At present we have no data to indicate whether this down-regulation is due to receptor phosphorylation and/or destabilization of mRNA. The presence of five AUUUA pentamers in a rat dopamine D_1 cDNA clone has been noted [17] but their effect on mRNA stability has not been reported.

It is clear that the partial genomic A_1 adenosine receptor genes isolated carry the message for the A_1 adenosine receptor encoded by the cDNA clone isolated. It thus appears that the gene for the A_1 adenosine receptor that has been cloned has a minimum of two introns (Figure 8). While this work was in progress it was reported that rabbit and human A_1 adenosine receptor genes contain an intron at the same location of the coding sequence [4,30]. The 5'-UTR of the human gene differs markedly from the avian gene in that it contains a minimum of four introns. The promoter region of the human gene has not been identified. It remains to be determined whether other chick A_1 adenosine receptor exons exist so that alternatively spliced A_1 adenosine receptors can be expressed.

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