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Posttranscriptional mRNA processing as a mechanism for regulation of human A_1 adenosine receptor expression

(alternative splicing/translational regulation/receptor regulation)

HONGZU REN AND GARY L. STILES*

Departments of Medicine and Pharmacology, Duke University Medical Center, Durham, NC 27710

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ABSTRACT **The human A_1 adenosine receptor gene contains six exons with exons 1, 2, 3, 4, and part of 5 representing 5′ untranslated regions. Reverse transcription–PCR with exon-specific primers showed two distinct transcripts containing either exons 3, 5, and 6 or exons 4, 5, and 6, with exons 3 and 4 being mutually exclusive. No mature mRNAs containing exons 1 and 2 have been detected. All human tissues that express any A_1 receptors contain mRNA with exons 4, 5, and 6. Tissues which express high levels of A_1 receptors contain mRNA with exons 3, 5, and 6. Exon 4 contains two upstream ATG codons whereas exon 3 contains none. COS cells transfected with expression vectors containing exon 4 (exons 1–6, 3–6, or Ex4–6) express much lower levels of A_1 receptors than vectors without exon 4 (exons 3, 5, and 6). Mutation of upstream ATG codons in exon 4 leads to 3- to 7-fold increased A_1 receptor expression, up to the level seen with the construct containing exons 3, 5, and 6. Thus, in human tissues "basal" levels of A_1 receptors can be expressed by use of mRNA containing exons 4, 5, and 6, but when high levels are needed, alternative transcripts with exons 3, 5, and 6 are produced.**

A_1 adenosine receptors mediate a wide range of physiological effects including inhibition of neurotransmitter release, suppression of heart rate and contractility, inhibition of lipolysis, and regulation of smooth muscle tone (1). Activation of A_1 receptors may produce a dramatic cardioprotective effect against ischemic heart damage (2). Factors which regulate A_1 receptor expression in tissues thus become critically important in understanding physiologic and therapeutic processes. We have recently cloned the human A_1 receptor gene and found it to contain six exons in which exons 1–4 and part of exon 5 represent 5′ untranslated regions, while the 3′ end of exon 5 and exon 6 represent coding and 3′ untranslated sequence (3). Although exons 1 and 2 are not expressed in mature transcripts in any human tissue studied thus far, they have been detected in a human brain cDNA library (3). Exons 3 and 4 are mutually exclusive, giving rise to two classes of mRNA containing either exons 3, 5, and 6 or exons 4, 5, and 6 (3). All human tissues known to express even low levels of A_1 adenosine receptors contain mRNA with exons 4, 5, and 6, whereas tissues such as brain, kidney, or testis, which express high levels of A_1 receptors, also contain mRNAs with exons 3, 5, and 6 (3). Thus, tissue-selective expression of exons 3, 5, and 6 may be the mechanism whereby high levels of A_1 receptors are produced. In this report, we document that exon 4 specifically inhibits the expression of the human A_1 receptor by use of two upstream ATG codons in exon 4. Mutation of these two ATG codons leads to relief of inhibition such that A_1 receptor expression equals that seen with mRNAs containing exon 3, which contains no ATG codons (3).

MATERIALS AND METHODS

Construction of Plasmids Used for Transfection. PCR cloning was used to construct a series of plasmids for transient transfection of COS-7 cells. The vector used was pCMV5 digested with *Eco*RI and *Xba* I (3). Plasmids were named according to the exons of the A_1 receptor gene that were included in the inserts; for example, the Ex356 insert includes exons 3, 5, and 6.

The template for inserts of Ex1–6, Ex3–6, Ex4–6, and Ex56 was human A_1 receptor cDNA clone 7A (3). The template for insert of Ex356 was the first-strand cDNA reverse transcribed from human testis total RNA. The downstream primer used in PCR for all five plasmid inserts was 3UTXb (5′-ACCCCTCTAGATGTGGGCTGGTGGGA-3′), which had an artificial *Xba* I site (underlined) for cloning and in which the remaining sequence was complementary to the human A_1 receptor cDNA sequence 1406–1431.

The upstream primers used in PCR were as follows: For Ex1–6, primer KS (5′-CGAGGTCGACGGTATCG-3′) (Stratagene); for Ex3–6 and Ex356, Ex3Eco (5′-TGGAAGGAATTCCTGGAGCTAGCGGCTGCTGAA-3′); for Ex4–6, Ex4Eco (5′-TTGGTGAGAATTCGCCGGGCTGGGAGCGCTGCG-3′); for Ex56, Ex5Eco (5′-GCCTGTGGAATTCATGCCGCCCTCCATCTCAGCTT-3′). All of those above primers except KS have an artificial *Eco*RI site (underlined) for cloning after PCR. The PCR product of Ex1–6 has an original *Eco*RI cloning site. The PCR was performed in 100 μl with 2 units of Vent DNA polymerase (New England Biolabs) in a Perkin–Elmer 480 thermal cycler. The cycle program used was 95°C for 1 min and 70°C for 2 min for 45 cycles.

The PCR fragments were purified with a Qiaex DNA purification kit (Qiagen, Chatsworth, CA) after separation in 1% agarose gel. Then they were digested with *Eco*RI and *Xba* I and again purified with Qiaex. The digested inserts and vector were ligated and used to transform *Escherichia coli* XL1-Blue cells (Stratagene). The isolated colonies contained the expected recombinant plasmids, which were confirmed by DNA sequencing.

Construction of Mutant Plasmids. To study the effect of upstream AUG codons in exon 4 on expression of human A_1 receptors, three mutant plasmids were constructed with PCR cloning. The mutant ATG1 has the first AUG codon in exon 4 (bases 237–239) mutated to GGG. The mutant ATG2 has the second AUG codon in exon 4 (bases 351–353) mutated to GGG. The mutant ATG12 has both of those AUG codons mutated to GGG. The primers used in PCR were as follows: For ATG1, the upstream primer was Ex4MUT (5′-CTGGGAGCGCTGCGGCGGGAGCCGGAGGACTGGGAGCTGC-3′) and the downstream primer was 3UTXb; for ATG2, the upstream primer was Ex4Eco and the downstream

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Abbreviation: DPCPX, 8-cyclopentyl-1,3-dipropylxanthine.

*To whom reprint requests should be addressed at: Duke University Medical Center, Box 3444, Durham, NC 27710.

primer was Ex54 (5'-GGGCACCAAGGGGCACGAG-GCAAGCACCCCCTGCCTGCT-3'), which is complementary to cDNA sequence 341–380; for ATG12, the upstream primer was Ex4MUT and the downstream primer was Ex54. The template used in PCR was the plasmid Ex4–6 described above, and PCR conditions were as above except that 35 cycles were programed.

The PCR fragment ATG1 was digested with *Eco*47III and *Xba* I, purified with Qiaex, and ligated to plasmid Ex4–6 that had been digested with the same enzymes. The PCR fragment ATG2 was digested with *Eco*RI and *Sty* I, purified with Qiaex, and ligated to pCMV5 digested with *Eco*RI and *Xba* I and a fragment (≈1 kb) isolated from the *Sty* I/*Xba* I digestion of Ex4–6. The PCR fragment ATG12 was digested with *Eco*47III and *Sty* I, purified with Qiaex, and ligated with a fragment isolated from the *Sty* I/*Xba* I digestion of plasmid Ex4–6 (same as for ATG2), and the same plasmid used for ATG1 ligation. The sequences of PCR-generated fragments in all three mutant plasmids were confirmed with DNA sequencing.

Transfection of COS-7 Cells. In all experiments, 15 μg of plasmid DNA was transfected into COS-7 cells as described (4).

Radioligand Binding of Transfected COS-7 Cell Membranes. About 72 hr after transfection, the cell membranes were prepared for radioligand binding (4). The procedure used was essentially the same as in ref. 4 except that the radioligand used was the ^{3}H-labeled A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX) instead of the ^{125}I-labeled agonist N^6-[2-(4-aminophenyl)ethyl]adenosine (^{125}I-APNEA).

Northern Blot Analysis. Total RNA was isolated from the transfected COS-7 cells 48–72 hr after transfection by using TriZOL reagent (GIBCO/BRL) according to the manufacturer's instructions. Poly(A)$^+$ RNA was isolated according to Celano *et al.* (5) and was loaded onto a 1% agarose/formaldehyde gel (6). After electrophoresis, RNA was transferred to a Zeta-Probe membrane (Bio-Rad) (7). The membrane was then prehybridized and hybridized as described (4). The hybridization probe, labeled by random priming, was the entire coding sequence of the human A_1 receptor. After overnight hybridization, the membrane was washed in 15 mM NaCl/1.5 mM trisodium citrate, pH 7/0.1% SDS at 60°C twice for 10 min and once for 30 min. The blot was later reprobed with random-priming-labeled human *S*-adenosylhomocysteine hydrolase cDNA (8) and washed in 150 mM NaCl/15 mM trisodium citrate, pH 7/0.1% SDS at 55°C twice for 10 min.

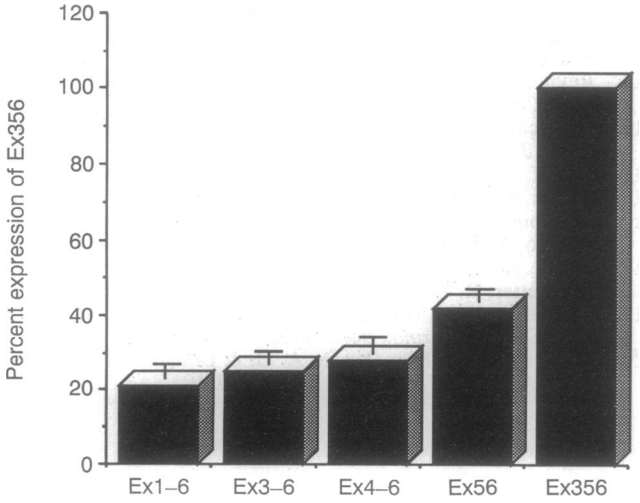

FIG. 1. A_1 receptor expression, measured as maximal [^{3}H]-DPCPX binding capacity in membrane preparations from cells transfected with pCMV5 vectors. The exons (Ex) represented in the pCMV5 expression vectors transfected into COS-7 cells are shown under each bar representing the calculated receptor expression level relative to that of Ex356 (100%). Radioligand binding was assessed as described (4). The standard error (n = 4) is indicated above each bar except for Ex356, which is expressed as 100%.

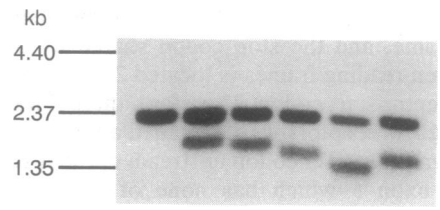

FIG. 2. Northern blot analysis of the poly(A)$^+$ RNAs isolated from COS-7 cells transfected with expression vectors with or without exon 4. RNA samples were harvested 48–72 hr after transfection and mRNA was prepared as described (4). The upper transcripts were visualized with ^{32}P-labeled human *S*-adenosylhomocysteine hydrolase cDNA and the lower transcripts were visualized with ^{32}P-labeled human A_1 receptor coding sequence cDNA. The order of samples loaded is, from left to right, untransfected COS-7 cells as negative control and cells transfected with Ex1–6, Ex3–6, Ex4–6, Ex56, or Ex356. This experiment was replicated several times.

RESULTS AND DISCUSSION

pCMV5 constructs containing various combinations of 5' untranslated regions along with coding sequence were tested for translatability in COS-7 cells. The "natural" constructs containing exons 3, 5, and 6 or exons 4–6 were compared with constructs containing only coding sequence—exons 5 and 6—or exons 1–6 or exons 3–6. These latter motifs are not found in human tissues but allow analysis of the effect exons 3 and 4 in tandem. As seen in Fig. 1, constructs without exon 4 (i.e., Ex56 and Ex356) expressed higher levels of A_1 adenosine receptors than any construct with exon 4. Ex356 expressed A_1 receptors at 3–7 times the level seen with other constructs. There are no ATG codons in exon 3. Exons 3 and 4 contain 100 and 154 bases, respectively, so that overall length is not likely to be a critical factor.

The levels of mRNA observed with the five constructs were essentially the same (Fig. 2), indicating that the difference in receptor expression was not a consequence of differences in mRNA quantity or half-life. *S*-Adenosylhomocysteine hydrolase mRNA served as a control.

The locations of upstream ATG codons in exon 4 are shown in Fig. 3. The upstream ATG codons in exon 4 code for open reading frames of 50 and 12 amino acid residues,

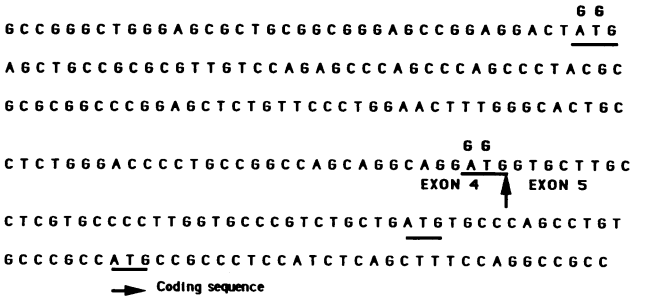

FIG. 3. Complete sequence of exon 4 and partial sequence of exon 5 of human A_1 receptor cDNA, showing the locations of point mutations. All four ATG codons including the A_1 receptor initiation codon are underlined. The first and the second upstream ATG codons in exon 4 were mutated to GGG as indicated (7). The vertical arrow indicates the border between exon 4 and exon 5. The horizontal arrow indicates the beginning of coding sequence. The sequence starting at the top represents the beginning of exon 4 (3).

respectively, which are in frame with the receptor open reading frame, and the stop codon shared by the two upstream open reading frames is located 21 bases upstream of the A_1 receptor start codon. Therefore, there is no possibility of creating A_1 receptors with extended amino-terminal tails. Also shown is an ATG codon upstream of the ATG initiation codon in exon 5 which has none of the typical Kozak consensus sequence (9) and appears to have no dramatic effect on expression as shown in Fig. 1, since its removal in construct Ex56 did not enhance A_1 receptor expression compared with that from construct Ex356. When the ATG codons in exon 4 were mutated to GGG individually and concurrently the expression of the A_1 adenosine receptor increased dramatically (Fig. 4). ATG1 is the most 5′ ATG and ATG2 is at the junction of exons 4 and 5. The effects of the mutation are additive, since the construct with both ATG codons mutated demonstrated the highest expression and closely approached the A_1 receptor level observed with the construct Ex356. This suggests that both ATG codons contribute to the repression of A_1 receptor expression. Five-prime untranslated regions which contain G+C-rich leader sequences have been shown to contribute to suppression of translation in a number systems (10). Although the G+C content of exon 4 is very high at 73%, our data show that G+C richness is not a likely source of receptor suppression since the ATG12 mutant was expressed equally as well as the Ex356 construct even though it contained the G+C-rich region. Moreover, Northern analysis of poly(A)$^+$ RNA isolated from transfected COS-7 cells showed nearly equal amounts of transcripts for all constructs in Fig. 4 (data not shown).

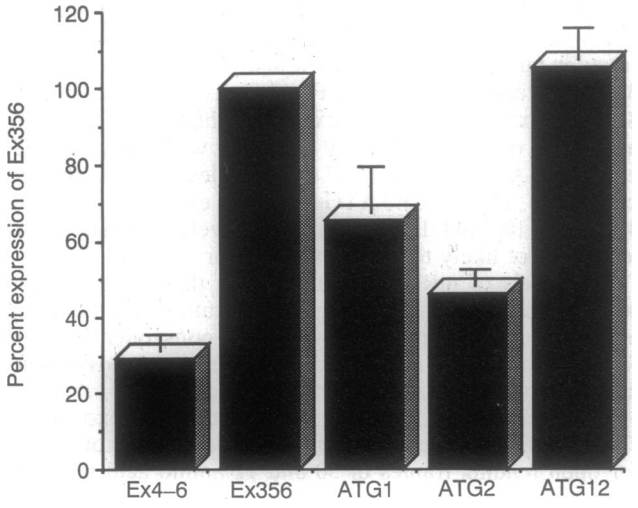

FIG. 4. Maximal [^{3}H]DPCPX binding capacity of membrane from COS-7 cells transfected with mutant vectors. The exons or the mutations in exon 4 represented in the expression vector (pCMV5) transfected into COS-7 cells are listed under each individual bar. Expression of A_1 receptor is shown as the percentage relative to the expression level of Ex356. The standard error (n = 3) is indicated above each bar except for Ex356.

Receptor regulation is known to occur in response to hormonal, environmental, or developmental changes and to provide tissue specificity (1). Regulation has been demonstrated at the level of protein modification, transcription, and message stability (1). These data represent an example where alternative forms of receptor mRNA are utilized to regulate the level of receptor in a tissue-specific manner, presumably by translational suppression. Upstream ATG codons are well known to be involved in the translational regulation of gene expression (11–13). These ATG codons tend to be inhibitory and are found in transcripts which code for critical regulatory proteins such as protooncoproteins, transcription factors, and inflammatory mediators (10, 14–20). However, this repression is constant and is not modulated with alternative transcripts or in a tissue-specific manner. Thus, although inhibition of translation by upstream ATG codons, G+C-rich secondary structure, or leader length is well documented in yeast and some higher organisms, this specific regulation of the human A_1 adenosine receptor by use of a selective exon-induced repression of expression (most likely translation) appears to provide another mechanism for receptor control.

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1. Stiles, G. L. (1992) *J. Biol. Chem.* **267**, 6451–6454.
2. Ely, S. W. & Berne, R. M. (1992) *Circulation* **85**, 893–904.
3. Ren, H. & Stiles, G. L. (1994) *J. Biol. Chem.* **269**, 3104–3110.
4. Olah, M. E., Ren, H., Ostrowski, J., Jacobson, K. A. & Stiles, G. L. (1992) *J. Biol. Chem.* **267**, 10764–10770.
5. Celano, P., Vertino, P. M. & Casero, R. A., Jr. (1993) *BioTechniques* **15**, 26–28.
6. Tsang, S. S., Yin, X., Guzzo-Arkuran, C., Jones, V. S. & Davison, A. J. (1993) *BioTechniques* **14**, 380–381.
7. Chomczynski, P. (1992) *Anal. Biochem.* **201**, 134–139.
8. Coulter-Karis, D. E. & Hershfield, M. S. (1989) *Ann. Hum. Genet.* **53**, 169–175.
9. Kozak, M. (1986) *Cell* **44**, 283–292.
10. Kozak, M. (1992) *Annu. Rev. Cell Biol.* **8**, 197–225.
11. Mueller, P. P. & Hinnebusch, A. G. (1986) *Cell* **45**, 201–207.
12. Reynolds, G. A., Basu, S. K., Osborne, T. F., Chin, D. J., Gil, G., Brown, M. S., Goldstein, J. L. & Luskey, K. L. (1984) *Cell* **38**, 275–285.
13. Reynolds, G. A., Goldstein, J. J. & Brown, M. S. (1985) *J. Biol. Chem.* **260**, 10369–10377.
14. Horiuchi, T., Macon, K. J., Kidd, V. J. & Volanakis, J. E. (1990) *J. Biol. Chem.* **265**, 6521–6524.
15. Kozak, M. (1987) *Nucleic Acids Res.* **15**, 8125–8148.
16. Kozak, M. (1991) *J. Cell Biol.* **115**, 887–903.
17. Maglione, D., Guerriero, V., Rambaldi, M., Russo, G. & Persico, M. G. (1993) *Growth Factors* **8**, 141–152.
18. Marth, J. D., Overell, R. W., Meier, K. E., Krebs, E. G. & Perlmutter, R. M. (1988) *Nature (London)* **332**, 171–173.
19. Pekhletsky, R. I., Chernov, B. K. & Rubtsov, P. M. (1992) *Mol. Cell. Endocrinol.* **90**, 103–109.
20. Williams, N. P., Hinnebusch, A. G. & Donahue, T. F. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7515–7519.