Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 277-284

Cloning, Expression, and Characterization of a Gene Encoding the Human Angiotensin II Type 1A Receptor

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Received May 16, 1992

The human angiotensin II (AII) type 1a receptor gene and its upstream control sequence has been cloned from a human leukocyte genomic library. The promoter element CAAT and TATA sequences were found at -602 and -538, respectively, upstream from the translational initiation site. The deduced protein sequence is homologous to rat and bovine AT_{1a} receptors (94.7% and 95.3% identity). The expressed gene exhibited high-affinity AII and Dup753 binding and was functionally coupled to inositol phosphate turnover. Northern analysis of human tissues showed AT_1 receptor mRNA expression in placenta, lung, heart, liver, and kidney. Using 5' untranslated and coding sequence as probes in a Southern blot analysis, it was established that another AT_1 subtype exists in the human genome.

The peptide hormone angiotensin II (AII) stimulates a wide variety of physiological responses, including vascular smooth muscle vasoconstriction, aldosterone and catecholamine secretion, and renal response in the kidney (1 for review). Angiotensin II binds to specific membrane-bound receptors located on the cell surface. In many tissues, the signalling mechanism used to initiate these responses are mediated via activated G-proteins, although some AII-stimulated activities are not. Given the broad range of AII-stimulated effects and the differential signal transduction mechanisms, it has been proposed that many subtypes of AII receptor exist (2,3) At this time, two distinct subtypes of AII receptor have been identified by their differential affinities for peptide (CGP42112A) or nonpeptide antagonists (Dup753, PD124177) and by their sensitivities to sulfhydryl reducing agents. Angiotensin II type 1 receptor (AT_1) has nM affinity for Dup753 and very low affinities (>μM) for PD123177 and CGP42112A. Angiotensin II Type 2 receptor (AT_2) has high affinities for PD123177 and CGP42112A, but binds poorly to Dup753. In the presence of mM concentrations of sulfhydryl reducing agents such as dithiothreitol, AII binding to the AT_1 receptor is greatly reduced whereas binding to the AT_2 receptor remains

0006-291X/92 $4.00

unchanged. Recent data on the signal transduction mechanism of AT_1 and AT_2 have further distinguished the two receptor subtypes. The AT_1 receptor appears to be coupled to a pertussis toxin insensitive G-protein which stimulates PI turnover and Ca^{2+} release and, in certain tissues, is also coupled to a pertussis toxin sensitive G-protein which inhibits adenylyl cyclase (4). Experiments have failed to demonstrate G-protein coupling to the AT_2 receptor subtype (5).

Using expression cloning techniques, two groups have recently succeeded in cloning AT_1 receptor cDNAs from rat and bovine tissues (6,7). The AT_1 receptor belongs to the G protein-coupled receptor superfamily, which includes rhodopsin. The protein homology identified among members of this family to the rhodopsin sequence suggests that these proteins share a common tertiary structure of seven transmembrane domains (TMD) linked by intra- and extra-cellular loops (8). In this report, we have isolated a human genomic DNA clone which contains homology to both the rat and bovine receptor sequences. Expression studies in COS-7 cells confirm the AT_1 identity and sequence comparison suggests that the cloned receptor is the AT_{1a} subtype.

EXPERIMENTAL PROCEDURES

Materials. The human multiple tissue Northern (MTN) blot and the human genomic DNA were purchased from Clontech Laboratories, Inc. [^{32}P] dCTP was obtained from Amersham. A Geneclean DNA purification kit was obtained from Bio101, Inc. The vector pCR1000 (TA PCR Product Cloning Kit) and the expression vector pCDNA1 were obtained from Invitrogen. A random primed DNA labeling kit was purchased from US Biochemicals. All blot hybridization assays were accomplished using Hybond N membrane from Amersham.

Isolation of Human AT_{1a} receptor. Two degenerate primers corresponding to the 1st and 7th transmembrane regions of the rat AT_1 receptor cDNA were used to amplify related sequences from human placenta genomic DNA by PCR amplification. The amplified PCR products were subcloned into the TA vectors and sequences analyzed. The sequence of a 0.8 kb clone, human clone-1, exhibited homology to the published rat AT_{1a} sequence (1). To obtain complete coding sequence, a human leukocyte genomic library was screened using the 0.8 kb clone as a probe. One clone, designated as hAT1-1, contains a 1.8 kb *Pst*1 fragment which includes the complete 1.1 kb coding sequence and 0.7 kb of 5' untranslated region. The *Pst*I fragment was cloned into pUC19 to create the plasmid $pHAT_1$.

Expression of Human AT_{1a} receptor in COS-7 cells. The 1.8 kb *Pst*1 fragment was subcloned into the mammalian expression vector pCDNA 1. COS-7 cells were propagated in Dulbecco's modified Eagles medium with 10% fetal bovine serum in 5% CO_2. The recombinant DNA (20 μg each) were transfected into COS-7 cells ($6x10^7$ cells/ml) by electroporation techniques. After electroporation, cells were diluted 1:40 and seeded on multiple 12-well plates at $1.5x10^6$ cells per well.

Northern analysis. A blot containing Poly A^+ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas was probed using a ^{32}P

random labelled full length rat AII type 1a receptor sequence. The probe was prepared from a *Not*I *Apa*I digestion of $pHAT_1$. Approximately 30 ng of Geneclean purified fragment was labelled by [^{32}P] dCTP incorporation using Klenow enzyme and random primers. The membrane was incubated 12 hrs at 42° C in hybridization buffer (6X SSC, 5X Denhardt's reagent, 0.5% SDS, 50% deionized formamide, 20 mM Tris, pH 7.6). Seven µl of the labelled probe and 100 µl of sonicated salmon sperm DNA (20 mg/ml) was boiled 10 min, added to 12 mls of fresh hybridization buffer, and used for hybridization with the blot at 42° C overnight. The blot was washed three times in low stringency wash buffer (2X SSC, 0.1% SDS) at room temperature. A high stringency wash (0.2X SSC, 0.1% SDS) was then added and the membrane subjected to three 15 min washes at 55° C. The wrapped blot was autoradiographed for 2 days.

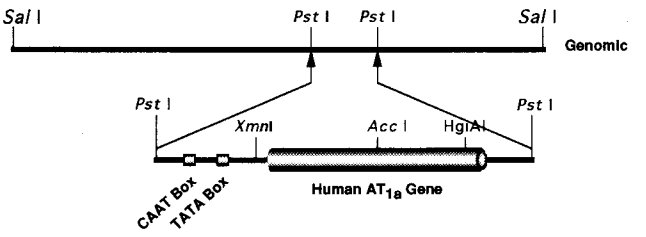
Southern analysis. Human genomic DNA was digested in duplicate with either *Pst*I or*Hin*dIII using 20 µg DNA per digest. Both sets of digests were separated via 0.7% TBE agarose gel electrophoresis, and transferred to Hybond N membrane overnight. After transfer, the blot was washed in 20X SSC and allowed to air dry. The DNA was linked to the membrane using the auto crosslink mode (120,000 microjoules/cm^2) on a Stratagene Stratalinker UV 1800 Crosslinker. The DNA probe was prepared by *Pst*I *Xmn*I digestion of pHAT2. The AT probe was prepared using the *Xmn*I-*Pst*I fragment which contains the full coding sequence of the human AT_{1a} receptor. The fragment was isolated via Geneclean and labelled as previously described. The prehybridization, hybridization, and wash conditions were the same as used in the preparation of the Northern blot.

Inositol phosphate assay in transfected COS-7 cells. After transfection, COS-7 cells were seeded on 12-well plates and incubated in medium for 24 hrs. After incubation, new medium containing [^{3}H] inositol (1 Ci/ml) without serum was added to the cells. After another 24 hrs, the cells were washed twice with 1 ml of assay buffer (EMEM with 10 mM LiCl, 20 mM HEPES, 1 mg/ml BSA) then incubated at 37° C for 1 hr with 1 ml assay buffer containing the indicated agents. To terminate the incubation, the medium was aspirated and replaced with 0.5 ml of ice cold 5% trichloroacetic acid. Total [^{3}H]-inositol phosphates were analyzed by applying the trichloroacetic acid extract directly to Dowex anion exchange columns. [^{3}H]-inositol was removed by washing with 10 ml of 5 mM inositol, and total [^{3}H]-inositol phosphates were eluted with 4 ml of 1 M ammonium formate in 0.1 M formic acid.

AII receptor binding assay. Two days after transfection, cells were washed twice with 2 ml of assay buffer (EMEM with 20mM HEPES buffer, pH 7.4, 0.1% BSA, 0.2% bacitracin). Cells were then incubated either with various concentrations of ^{125}I-labelled AII (saturation binding assay) or with 20 pM ^{125}I-labelled AII plus the indicated concentrations of compounds (displacement binding assay) for 60 min at 37° C in a 0.5 ml of assay buffer. After incubation, cells were washed twice with 1 ml ice-cold DPBS and the reaction terminated by the addition of 0.5 ml 2% SDS. Cell-bound radioactivity was determined in a gamma radioactive counter. The Kd and IC_{50} values were determined by using the Ligand computer program.

RESULTS AND DISCUSSION

The human AT_{1a} receptor gene is encoded by 1080 nucleotides (Fig. 1) and contains no introns, as shown by PCR of the region spanning from the first to

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                -681                -661                -641                -621                -601
             CTGCAGA GGCCTGGAAAACCATGGAAA AAATCCTATTCCTCAAAGTC GAGCCCTACCTCCTACGCCT CTTAAATCAATTGCCACAGT

                -581                -561                -541                -521                -501
GACCAATTTGTCAGTCACCT AAAGGCAGCTATCCTGACCA CTTAAGGGAGAATACTTATA ATCTTTATACCTAAGTAAAG ATTGTGAGAGGAAAGAAATG
 CAAT Box                                                    TATA Box
                -481                -461                -441                -421                -401
GTTTAAAGAATATGAATGTG TCACACTTCTGAGGTTAATG ATAAATGAATTGGTCCTGCT TACCTCAGGAAAAACTTTCA AGTCTTTCTGAAAAAACTAA

                -381                -361                -341                -321                -301
TTTAATTCAGTAGTATTTTC TAAGATTTAGGTTATGTTTT TAATCAATTTGGAAACCAAG ATTTACTTATAGAAAAAAAG GAAAAGGACCTAGATAGGTT

                -281                -261                -241                -221                -201
TATTCACATAGAATCCCATT TTGATCCTTCTCTGGAGTAA TACCATTTTATCAAAACGAA TTATGTTCTAAAATTTAAGT GTGCTTTCTTAGGCTTTATC

                -181                -161                -141                -121                -101
AGTTCACAGTGTTTCCTTAA GAAATATGATCCAGTATTTT TTCCTAAGACTAAAGTTGAG TTACTACGTTTATGACTGAG AAATGAATGTTTGTTAGTTT

                 -81                 -61                 -41                 -21                  -1
GTTTGTTTACAATAAGAATT TTTTCTTTACCATTTTATTT TTATTTTCCCCAGGTGTATT TGATATAGTGTTTGCAACAA ATTCGACCCAGGTGATCAAA

1                                                   51                                                  105
ATGATTCTCAACTCTTCTACTGAAGATGGTATTAAAAGAATCCAAGATGATTGTCCCAAAGCTGGAAGGCATAATTACATATTTGTCATGATTCCTACTTTATAC
MetIleLeuAsnSerSerThrGluAspGlyIleLysArgIleGlnAspAspCysProLysAlaGlyArgHisAsnTyrIlePheValMetIleProThrLeuTyr
 1                  10                  20                  30                  35

                                                   156                                                 210
AGTATCATCTTTGTGGTGGGAATATTTGGAAACAGCTTGGTGGTGATAGTCATTTACTTTTATATGAAGCTGAAGACTGTGGCCAGTGTTTTTCTTTTGAATTTA
SerIleIlePheValValGlyIlePheGlyAsnSerLeuValValIleValIleTyrPheTyrMetLysLeuLysThrValAlaSerValPheLeuLeuAsnLeu
36                  45                  55                  65                  70

                                                   261                                                 315
GCACTGGCTGACTTATGCTTTTTACTGACTTTGCCACTATGGGCTGTCTACACAGCTATGGAATACCGCTGGCCCTTTGGCAATTACCTATGTAAGATTGCTTCA
AlaLeuAlaAspLeuCysPheLeuLeuThrLeuProLeuTrpAlaValTyrThrAlaMetGluTyrArgTrpProPheGlyAsnTyrLeuCysLysIleAlaSer
71                  80                  90                  100                 105

                                                   366                                                 420
GCCAGCGTCAGTTTCAACCTGTACGCTAGTGTGTTTCTACTCACGTGTCTCAGCATTGATCGATACCTGGCTATTGTTCACCCAATGAAGTCCCGCCTTCGACGC
AlaSerValSerPheAsnLeuTyrAlaSerValPheLeuLeuThrCysLeuSerIleAspArgTyrLeuAlaIleValHisProMetLysSerArgLeuArgArg
106                 115                 125                 135                 140

                                                   471                                                 525
ACAATGCTTGTAGCCAAAGTCACCTGCATCATCATTTGGCTGCTGGCAGGCTTGGCCAGTTTGCCAGCTATAATCCATCGAAATGTATTTTTCATTGAGAACACC
ThrMetLeuValAlaLysValThrCysIleIleIleTrpLeuLeuAlaGlyLeuAlaSerLeuProAlaIleIleHisArgAsnValPhePheIleGluAsnThr
141                 150                 160                 170                 175

                                                   576                                                 630
AATATTACAGTTTGTGCTTTCCATTATGAGTCCCAAAATTCAACCCTCCCGATAGGGCTGGGCCTGACCAAAAATATACTGGGTTTCCTGTTTCCTTTTCTGATC
AsnIleThrValCysAlaPheHisTyrGluSerGlnAsnSerThrLeuProIleGlyLeuGlyLeuThrLysAsnIleLeuGlyPheLeuPheProPheLeuIle
176                 185                 195                 205                 210

                                                   681                                                 735
ATTCTTACAAGTTATACTCTTATTTGGAAGGCCCTAAAGAAGGCTTATGAAATTCAGAAGAACAAACCAAGAAATGATGATATTTTTAAGATAATTATGGCAATT
IleLeuThrSerTyrThrLeuIleTrpLysAlaLeuLysLysAlaTyrGluIleGlnLysAsnLysProArgAsnAspAspIlePhePheLysIleIleMetAlaIle
211                 220                 230                 240                 245

                                                   786                                                 840
GTGCTTTTCTTTTTCTTTTCCTGGATTCCCCACCAAATATTCACTTTTCTGGATGTATTGATTCAACTAGGCATCATACGTGACTGTAGAATTGCAGATATTGTG
ValLeuPhePhePhePheSerTrpIleProHisGlnIlePheThrPheLeuAspValLeuIleGlnLeuGlyIleIleArgAspCysArgIleAlaAspIleVal
246                 255                 265                 275                 280

                                                   891                                                 945
GACACGGCCATGCCTATCACCATTTGTATAGCTTATTTTAACAATTGCCTGAATCCTCTTTTTTATGGCTTTCTGGGGAAAAAATTTAAAAGATATTTTCTCCAG
AspThrAlaMetProIleThrIleCysIleAlaTyrPheAsnAsnCysLeuAsnProLeuPheTyrGlyPheLeuGlyLysLysPheLysArgTyrPheLeuGln
281                 290                 300                 310                 315

                                                  1026                                                1050
CTTCTAAAATATATTCCCCCAAAAGCCAAATCCCACTCAAACCTTTCAACAAAAATGAGCACGCTTTCCTACCGCCCCTCAGATAATGTAAGCTCATCCACCAAG
LeuLeuLysTyrIleProProLysAlaLysSerHisSerAsnLeuSerThrLysMetSerThrLeuSerTyrArgProSerAspAsnValSerSerSerThrLys
316                 325                 335                 345                 350

                   1080                1100                1120                1140
AAGCCTGCACCATGTTTTGAGGTTGAGTGA  CATGTTCGAAACCTGTCCAT AAAGTAATTTTGTGAAAGAA GGAGCAAGAGAACATTCCTC TGCAG
LysProAlaProCysPheGluValGlu *
351                 359
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Figure 1. Nucleotide and predicted protein sequence of human angiotensin II type 1 receptor. The nucleotide numbering system indicated places the +1 position on the translational start adenine. The predicted CAAT and TATA transcriptional control sites are boxed, and the predicted transmembrane domain regions are underlined.

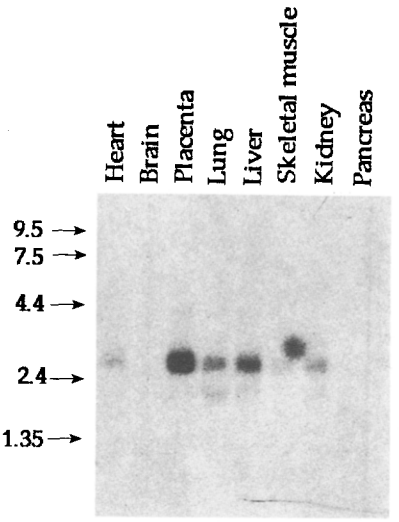
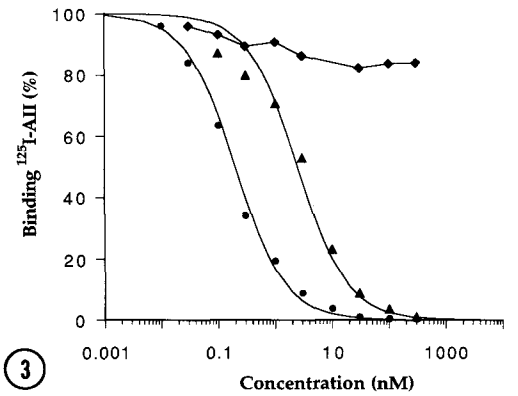


Figure 2. Northern blot analysis of Poly A+ RNA isolated from various human tissues.
Each lane contained 2 μg of Poly A+ RNA isolated from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. The blot was probed using full length rat AT_1 receptor sequence. The position of RNA markers are indicated.

seventh predicted TMD in genomic DNA (data not shown) and verified during DNA sequencing of the genomic gene. The lack of intron segments is typical of most G-protein coupled receptors. Examination of the 5' sequence for the presence



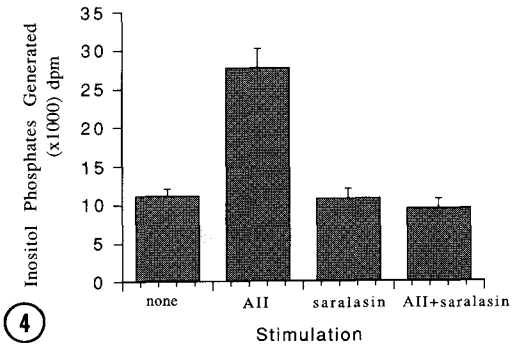


Figure 3. Competitive binding curve of [125I] angiotensin II binding to COS-7 cells transfected with cloned human AT_1 receptor.
Binding studies were done in the presence of angiotensin II (●), Dup753 (▲), or PD123177 (♦).

Figure 4. Effects of angiotensin II on inositol phosphate synthesis in COS-7 cells transfected with the cloned human AT_1 receptor.
Angiotensin II was added to a final concentation of 1 μM, and saralisin was added to 10 μM final concentration.

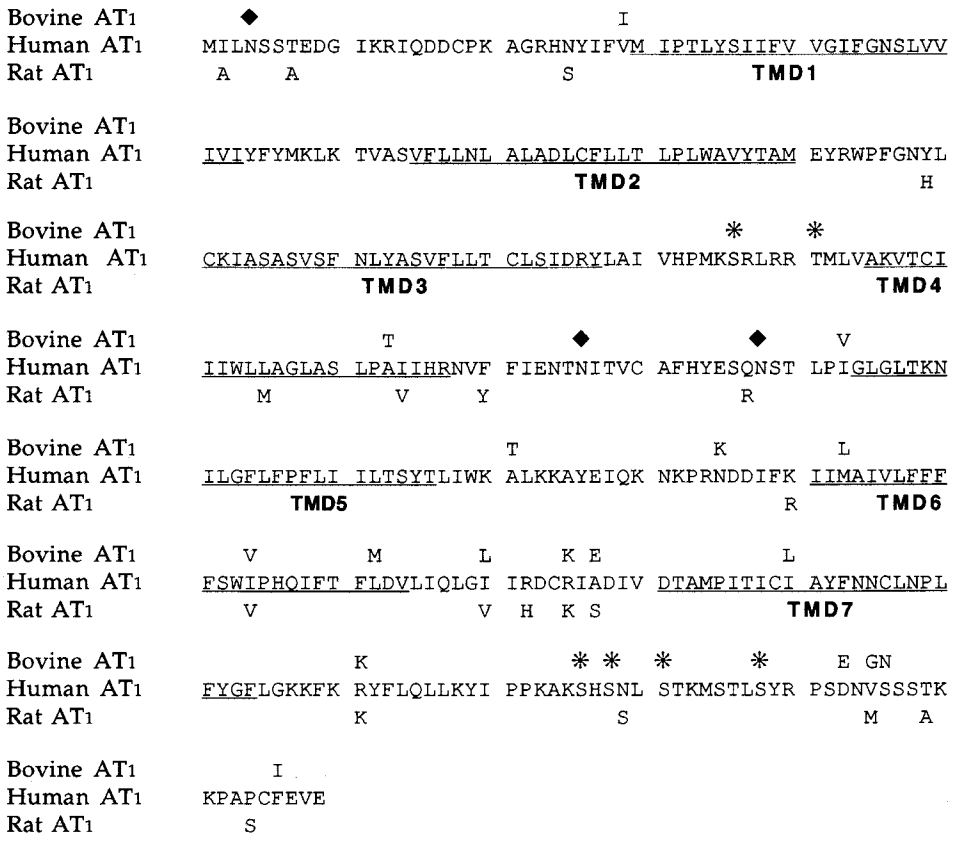


Figure 5. Alignments of the predicted protein sequences of rat (6), bovine (7), and human angiotensin type 1 receptors.
Amino acid dissimilarities between the human and rat/bovine sequence are indicated above the human sequence. The residues forming the predicted transmembrane domain segments are underlined. Possible sites of glycosylation are designated by (♦), and possible sites of phosphorylation are indicated (✳).

of control consensus sites revealed the presence of both CAAT and TATA boxes (Fig. 1) found at -602 and -538 (numbered with respect to the translational start codon adenine). A series of adenines located 25-35 bp downstream of the TATA site defines the putative transcriptional initiation start, although no sequence in this region corresponds to "CA" consensus nucleotides most frequently found.

Northern analysis of mRNA isolated from various human tissues determined that the human AT_1 receptor gene is strongly expressed as a 3.3 kb mRNA band in placenta, with lower levels found in lung, liver, heart, and kidney (Fig. 2). A 2.3 kb band is also present in lung, with lower levels in the liver. Interestingly, our data demonstrated strong hybridization with the higher kb band, whereas Murphy et al. (6) found comparable bands in several rat tissue mRNAs, with the strongest hybridization to the 2.3 kb band.

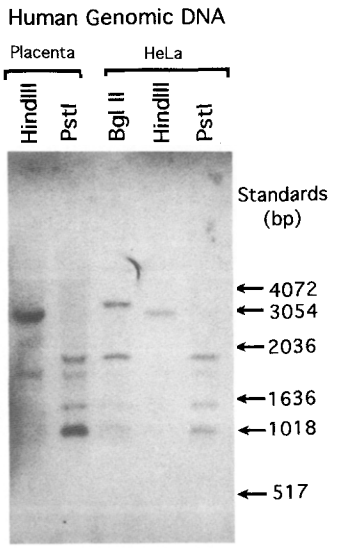


Figure 6. Southern blot of human genomic DNA using a human AT_1 probe. Aliquots of 20 μg of HeLa and placental genomic DNA were digested with *Bam*HI, *Pst*I, or *Hin*dIII. The position of the DNA markers are indicated.

To establish the identity of the cloned receptor as the AT_1 subtype, competitive binding studies using [^{125}I]angiotensin II and the compounds Dup753 and PD123177 were carried out (Fig. 3). The apparent Kd for AII (0.2 nM), and the IC_{50} values for Dup753 (2.5 nM) and PD123177 (>> 1 μM) were comparable with values expected for an AT_1 receptor. Studies of the effect of AII on COS-7 cells transiently expressing the cloned receptor gene displayed a strong stimulation of PI turnover as demonstrated by the increase in inositol phosphate in the presence of 1 μM AII (Fig. 4). The addition of 10 μM of saralasin, an AII antagonist, completely blocked the AII response. These results confirm that the cloned receptor gene codes for a fully functional AT_1 receptor.

Comparison of the deduced human AT_{1a} receptor sequence with the published rat and bovine AT_1 receptor sequence revealed 94.7% and 95.3% homology, respectively. Possible sites of glycosylation and phosphorylation are highly conserved between all three proteins (Fig. 5). Based on the protein sequence alignment, extracellular loop 3 and the C-terminal tail contain the highest sequence variability. Using a comparison of the human receptor protein with published rat AT_{1a} receptor and the newly cloned rat AT_{1b} receptor sequence (9), we have concluded that the cloned receptor is the AT_{1a} receptor subtype.

To determine if additional AT_1 subtypes exist, digested HeLa and placental genomic DNA were probed with an *Xmn*I-*Pst*I fragment containing the human AT_{1a} receptor gene (Fig. 6). Using *Pst*I digested HeLa and placental genomic DNA, we observed a 1.8 kb band as predicted from the cloning and sequencing data, with

additional bands at 1.4 and 0.9 kb. Although the hydridization pattern was similar for the *Pst*I HeLa and *Pst*I placental genomic DNA, the intensity of the 0.9 kb HeLa band was much greater than the equivalent band found in placenta. In *Bgl*2 digested HeLa DNA, a ~3.3 kb and a 1.8 kb band were seen, with a faint band located at 0.9 kb. The *Hind*III digestion of both sets of genomic DNA produced a ~3.0 kb band which strongly hybridized to the probe. A second band was detected at 1.7 kb. The existance of multiple bands in all restriction digests probed suggests that more than one subtype of AT_1 receptor is encoded in the human genome.

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