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Human AT_1 receptor is a single copy gene: characterization in a stable cell line

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Abstract

To address conflicting reports concerning the number of angiotensin II (AII) receptor type 1 (AT_1) coding loci in vertebrates, Southern blot analysis was used to determine the genomic representation of AT_1 receptor genes in animals comprising a divergent evolutionary spectrum. The data demonstrate that the AT_1 receptor gene is present as a single genomic copy in a broad spectrum of animals including human, monkey, dog, cow, rabbit, and chicken. In contrast, members of the rodent taxonomic order contain two genes in their genomes. These two genes may have arisen in rodents as a consequence of a gene duplication event that occurred during evolution following the branching of rodents from the mammalian phylogenetic tree. In order to investigate the properties of the human AT_1 receptor in a pure cell system, the recombinant human AT_1 receptor was stably expressed in mouse L cells. An isolated cell line, designated $LhAT_1$-D6, was found to express abundant levels of recombinant receptor [430 ± 15 fmol/mg] exhibiting high affinity [K_D = 0.15 ± 0.02 nM] for [^{125}I][SAR^1, IIe^8] angiotensin II (SIA). The pharmacological profile of ligands competing for [^{125}I] SIA binding to the expressed receptor was in accordance with that of the natural receptor. Radioligand binding of the expressed receptor was decreased in the presence of the non-hydrolyzable analog of GTP, guanosine 5′-(γ-thio) triphosphate [GTPγS]. Angiotensin II evoked a rapid efflux of $^{45}Ca^{2+}$ from $LhAT_1$-D6 cells that was blocked by AT_1 receptor specific antagonists. In addition, AII inhibited forskolin-stimulated cAMP accumulation in these cells which was blocked by the AT-1 antagonist. Thus, the $LhAT_1$-D6 cell line provides a powerful tool to explore the human AT_1 receptor regulation. (Mol Cell Biochem **131:** 75–86, 1994)

Key words: angiotensin II receptor, gene, stable cell line, human

Introduction

The renin-angiotensin system plays a critical role in the control of cardiovascular homeostasis and sodium balance [1]. The active hormone of this system is AII, the actions of which are mediated by cell-surface receptors [2]. The development of pharmacological probes, including selective peptide analogs and nonpeptide recep-

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tor antagonists, has allowed the definitive identification of at least two types of AII receptors, AII receptor type 1 (AT_1) and type 2 (AT_2) [3–7]. AT_1 receptors possess high affinity for the selective nonpeptide antagonists DuP 753 [8] and SK&F 108566 [9], while the AT_2 binding site has high affinity for the selective peptide CGP42112A [10] and the nonpeptide antagonist PD123319 [11].

AT_1 and AT_2 receptor binding sites are differentially modulated by sulfhydryl agents. For example, dithiothreitol (DTT) inhibits AII binding to the AT_1 receptor whereas it has either no effect, or enhances binding to AT_2 receptors [12]. Also, AT_1 and AT_2 receptor subtypes can be distinguished based on their ability to couple to guanine nucleotide binding regulatory proteins (G-proteins). Studies indicate that AII binding to the AT_1 receptor, but not the AT_2 receptor, is decreased in the presence of guanine nucleotides, suggesting that only the AT_1 receptor is G-protein coupled [13]. To date, all the major functional roles attributed to AII appear to be mediated by the AT_1 receptor. No clear function for the AT_2 receptor has been described.

The AT_1 receptor was first isolated from rat and bovine cDNA plasmid libraries by mammalian cell expression cloning [14–21]. Subsequently, we and others isolated the human AT_1 receptor cDNA and its genomic counterpart [22–25]. The human cDNA encodes a receptor protein of 359 amino acids that has a predicted seven transmembrane topology typical of G-protein-linked receptors. Ligand binding studies of the recombinant AT_1 receptor expressed in COS cells indicated that it had the pharmacological characteristics of a naturally occurring AT_1 receptor. Recently, a flurry of reports were published that described a second form of the AT_1 receptor in both rat and mouse mRNA and genomic DNA [16–26]. The two receptor isoforms, named AT_{1A} (originally described [27]) and AT_{1B} or AT_3 [21], are highly homologous, sharing approximately 95% and 92% protein and nucleotide sequence identity, respectively. Several studies have demonstrated that these receptors are pharmacologically indistinguishable; however, they exhibit different patterns of expression in tissues and are differentially expressed in response to hormonal treatment [17, 21, 28]. The presence of two AT_1 receptors in rat and mouse raised the possibility that multiple genes also occur in humans, which could have implications in the strategy to develop receptor-specific drugs for specific cardiovascular diseases.

In this communication, we analyzed and compared the number of coding loci for the AT_1 receptor gene copy number present in a variety of animals, including one bird and seven mammalian species belonging to five taxonomic orders. Also in this study, in order to thoroughly investigate the properties of the human AT_1 receptor in a pure cell system, a mouse L-cell line was generated that stably expressed this receptor. The recombinant cell line was subsequently used for detailed characterization of the recombinant human AT_1 receptor, including analysis of ligand binding properties, coupling to G-protein, functional coupling to phosphoinositol turnover and intracellular Ca^{2+} mobilization, and adenylate cyclase inhibition.

Materials and methods

Materials

[^{125}I]Sar^1, IIe^8 Angiotensin II (SIA) [2200 Ci/mmol], [^{125}I] Angiotensin II (AII) [2200 Ci/mmol] and $^{45}CaCl_2$ [14.17 mCi/mg] were obtained from NEN Research Products (Boston, MA). [^{3}H] myoinositol (10–20 Ci/mmol) was obtained from Amersham (Chicago, IL). Peptide analogs of AII were from Sigma Chemical Co. (St. Louis, MO). GTPγS was obtained from Boehringer Mannheim (Mannheim, Germany). SK&F 108566, DUP 753, PD123319 and [SAR^1, HIS^8]AII were synthesized at SmithKline Beecham Pharmaceuticals (King of Prussia, PA). [^{125}I] cAMP radioimmuno-assay kits were from Advanced Magnetics (Boston, MA). Restriction and modifying enzymes were obtained from Boehringer Mannheim Biochemicals (IN); ZOO-BLOT, human and rat genomic DNA was purchased from CLONTECH Laboratories, Inc. (CA); nitrocellulose was acquired from Schleicher and Schuell, Inc. (NH); and ^{32}P-radionucleotides were obtained from ICN Biochemicals, Inc. (CA). TAG-IT kit was purchased from BIOS Corp. (CT).

Preparation of hybridization probes

Oligonucleotide primers were made corresponding to the amino and carboxy termini of the human and rat AT_1 receptors, combined with appropriate template cDNAs (previously cloned [22]), and the polymerase chain reaction [29, 30] was used to obtain DNA fragments (approximately 1100 base pairs (bp) in length) containing the complete coding region of the receptors without untranslated regions. For use as hybridization probes,

these DNA fragments were ^{32}P radiolabeled using the TAG-IT kit.

Genomic DNA blotting

Human and rat genomic DNA (15 μg) was digested to completion with restriction enzymes, fractionated by electrophoresis in 0.8% agarose gels, and transferred to nitrocellulose as described by Southern [31]. Blots were baked for two hours (h) at 80° C. Blots were prehybridized for 4 hours (h) at 68° C in hybridization buffer [5X SSPE (20X: 3 M NaCl, 0.2 M $NaHPO_4$, 0.02 M Na_2 EDTA, pH 7.4), 5X Denhardts (50X: 1 g Ficoll, 1 g polyvinylpyrrolidone, 1 g bovine serum albumin, to 100 ml H_2O), 0.25% SDS (sodium dodecyl sulfate), tRNA at 100 μg/ml]. Blots were hybridized in the same hybridization buffer (including 10% dextran sulfate), with ^{32}P-labeled DNA probes at 68° C for 18 h. Blots were washed twice at room temperature in 2X SSC (20X: 3 M NaCl, 0.3 M Na_3citrate, pH 7.0), 0.1% SDS for 15 min (m) each, once at 65° C in 2X SSC, 0.1% SDS for 20 m, and three times at 65° C in 0.5X SSC, 0.1% SDS for 20 m each. ZOOBLOTS were hybridized and washed according to the protocol recommended by CLONTECH. Blots were wrapped in Saran Wrap and exposed to Kodak SAR-5 X-ray film at – 80° C with an intensifying screen.

Construction of the human AT_1 receptor expression vector

A eucaryotic expression vector, termed pCDN, was constructed from the plasmid TND [32] for the stable integration and expression of heterologous proteins in mammalian cells. The complete 2250 base pair (bp) cDNA encoding the human AT_1 receptor, previously cloned in our laboratory, was inserted into the *Eco*RI site of the expression cassette of the plasmid pCDN creating vector pCDNhAT_1. For this vector, transcription of the cDNA is controlled by the human cytomeglovirus promoter [33] while a termination signal is provided by the bovine growth hormone 3′-flanking sequence [34]. This vector also contains a bacterial neomycin phosphotransferase gene (NEO) expression cassette for geneticin (G418) selection [35], a murine dihydrofolate reductase (DHFR) expression cassette for methotrexate (MTX) amplification [36], an amphicillin resistance gene, and origin sequences that permit replication in both *E. coli* and COS cells [37].

Transfection and stable expression of recombinant human AT_1 receptor

To produce stable transformants, 5×10^5 mouse L-cells (ATCC number CCL 1.3) were plated onto 100-mm plates in feeding media (Dulbecco's Modified Essential Medium (DMEM) supplemented with 20 mM L-glutamine, 10% fetal bovine serum (FBS) and 1% (100 units/ml) penicillin/streptomycin) and incubated overnight at 37° C in a 5% CO_2 humidified incubator. The next day, 20 μg/plate of pCDNhAT_1 DNA was introduced into mouse L-cells by the calcium phosphate procedure using a mammalian transfection kit according to the manufacturer. Following transfection, the cells were incubated at 37° C in 3% CO_2 for 24 hr, washed with warm Dulbecco's phosphate buffered saline (DPBS), fed with fresh feeding media, and maintained at 37° C in 5% CO_2. After overnight incubation, the media was removed and replaced with fresh selection media that contained 400 μg/ml G418 to select for cells that were stably transformed with the pCDNhAT_1 expression vector. Selection media was replaced twice weekly for 2–4 weeks until independent cell colonies appeared on the dishes. Cell colonies were individually picked and purified by limited dilution. The clones were finally grown in 6 well plates and a clonal cell line expressing a high level of human AT_1 receptors was identified by radioligand binding. This cell clone was named LhAT_1-D6.

Radioligand binding experiments

Monolayer cultures (175 cm^2 flasks) of LhAT_1-D6 cells were washed twice with 10 ml of DPBS, detached from the flasks with trypsin-EDTA, counted and pelleted by centrifugation at 1000 xg for 10 min. The cell pellet was resuspended at 3×10^6 cells/ml in assay buffer (50 mM phosphate, pH 7.4; 5 mM $MgCl_2$; 150 mM NaCl; 0.2 mg/ml bovine serum albumin (BSA); 5 μg/ml leupeptin; 50 μg/ml bacitracin, 100 μM phenylmethylsulfonyl fluoride). Radioligand binding assays were performed for 30 min at 30° C in 12×75 mm polystyrene tubes with 3×10^5 cells/tube (5–8 μg/protein) in a final volume of 0.2 ml assay buffer with [^{125}I]SIA or in some cases, [^{125}I] AII. Cells were incubated with 10–500 pM [^{125}I]SIA for saturation binding, or 150 pM radioligand for displacement studies

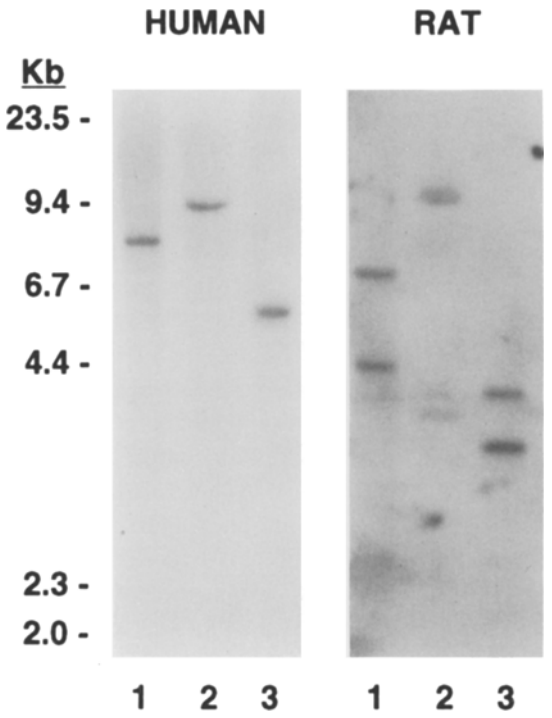


Fig. 1. Southern blot analysis of human and rat DNA. Human and rat high molecular weight DNA was digested to completion with *Bam* HI (lane 1). *Eco* RI (lane 2), and *Hind* III (lane 3) and analyzed in parallel under identical blotting, hybridization and washing conditions. The coding region of the human or rat AT_1 receptor cDNA was used as hybridization probes. Molecular weight markers are indicated in kilebases (Kb).

with increasing concentrations of unlabeled competitor. To ascertain equilibrium conditions, [^{125}I]SIA (150 pM) binding was done over a period of 90 min at 30° C. Since equilibrium was reached within 20 min of incubation (data not shown), 30 min incubation was chosen for all subsequent experiments. Binding was terminated with 3 ml wash buffer (Tris-HCl 5 mM pH 7.4 and NaCl 150 mM) and rapid filtration through Skatron filtermates [Cat. # 11734] using a Skatron cell harvester (Skatron Instruments, Norway). The bound radioligand was counted in a gamma counter. Nonspecific binding was determined in the presence of 1 μM unlabelled AII. Each experiment was done in duplicate determinations and repeated 2–3 times. Intra-experimental variation was less than 5% and inter-experimental variation was less than 10%. Results are expressed as the mean ± SE. The data for the saturation and displacement experiments were analyzed by computer-assisted nonlinear least square fitting, using the LUNDON software programs (LUNDON Software, Inc. Cleveland, OH).

Phosphoinositide hydrolysis

$LhAT_1$-D6 cells were grown in DMEM containing 10% FBS. The day before the experiment, the medium was changed to serum- and inositol-free medium containing 1.0 μCi/ml [^{3}H] myoinositol and incubated overnight. On the day of the experiment, the medium was removed, the cells were washed twice with 2 ml $DPBS^{++}$ and incubated in $DPBS^{++}$ containing 10 mM LiCl for 10 min at 37° C. The experiment was initiated by the addition of indicated concentrations of agonist, and the incubation continued for 5 min at 37° C after which the reaction was terminated by addition of 10% trichloroacetic acid. The supernatants were separated by centrifugation at 1200 xg for 10 min and then extracted with water-saturated ether (4 times, 4 ml each time). The individual inositol phosphates were separated by anion exchange chromatography as explained previously [38].

$^{45}Ca^{2+}$ efflux experiments

Efflux experiments utilizing $^{45}Ca^{2+}$ were performed as described previously [39]. In brief, $LhAT_1$-D6 cells were grown in 35 mm plates until confluent, rinsed with PSS buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, and 0.03 mM EGTA, pH 7.4), loaded with $^{45}CaCl_2$ by the addition of one ml of PSS containing 2 mCi of $^{45}CaCl_2$, and incubated for 120 min at 37° C in 5% CO_2. Following $^{45}Ca^{2+}$ loading, the cells were rapidly washed twice with Ca^{2+} free PSS to remove superficially bound radioisotope and, to establish a $^{45}Ca^{2+}$ efflux baseline, 1 ml of PSS buffer (37° C) was added to the cells, collected and replaced every two minutes for a total of 16 min. Subsequently, 1 ml of PSS buffer containing varying concentrations of either agonist in the absence or presence of antagonists (except in controls) was added and collected from the cells every two min for a period of 10 min. At the completion of the experiment, the cells were lysed with 2% SDS and radioactivity of the cell lysate and efflux solutions were measured by liquid scintillation counting. Efflux curves were generated as described previously [40]. The rate constant of $^{45}Ca^{2+}$ efflux was defined as the radioactivity released from the cells per min at time t divided by the radioactivity remaining in the cell at time t.

cAMP measurements

$LhAT_1$-D6 cells were plated at a density of 75,000 cells/well in 24 well plates. On day 3, the medium was aspirated and the cells were washed twice with 1 ml Dulbecco's phosphate buffered saline containing 0.1% glucose, 0.2% BSA, and 10 mM $MgCl_2$ ($DPBS^{++}$). Cells were then incubated in 0.4 ml $DPBS^{++}$ containing 0.5 mM isobutylmethyl xanthine (IBMX) for 15 min at room temperature in the absence or presence of 1 μM AII. At the end of 15 min, 1 μM forskolin was added to the indicated wells and the incubation was continued for another 10 min at 37° C. The reaction was stopped by the addition of 50 μl of 100% ice-cold trichloracetic acid (TCA) to each well, and cAMP in each well was measured following the RIA protocol as described (Advanced Magnetics). To test the effect of the AII antagonist, the antagonist, at indicated concentrations, was added to the cells along with AII. Each experiment was done in triplicate and repeated 2–3 times with different passages of cells.

Results and discussion

Prompted by the recent reports of multiple, closely related AT_1 receptor genes present in rat and mouse genomes [16–21], Southern blot analysis of human and rat genomic DNA was performed in order to establish and compare the number of coding loci for the AT_1 receptor. Restriction enzymes used for the analysis included *Bam*HI, *Eco*RI and *Hind*III, for which the known genes lacked restriction recognition sites within coding sequences, with the exception of the rat AT_{1A} gene, which contains a single *Eco* RI site within the center of the translated region [18]. As hybridization probes, cDNA fragments corresponding to the coding portion of the human AT_1 and rat AT_{1A} receptor genes were used. The results of the Southern analysis are shown in Fig. 1.

A single band was detected in each lane of the human genomic blot, which is suggestive of a single-copy gene in the genome having no closely related genes sharing a high degree of nucleotide sequence similarity. Alternatively, two genes may be present in the human genome but are so closely linked in tandem that the three restriction enzymes used in this analysis would each produce DNA fragments containing two gene copies. This possibility was addressed in a separate Southern blot analysis (see below).

In contrast to the human genomic blot, multiple hybridizing signals were identified in every lane of the rat

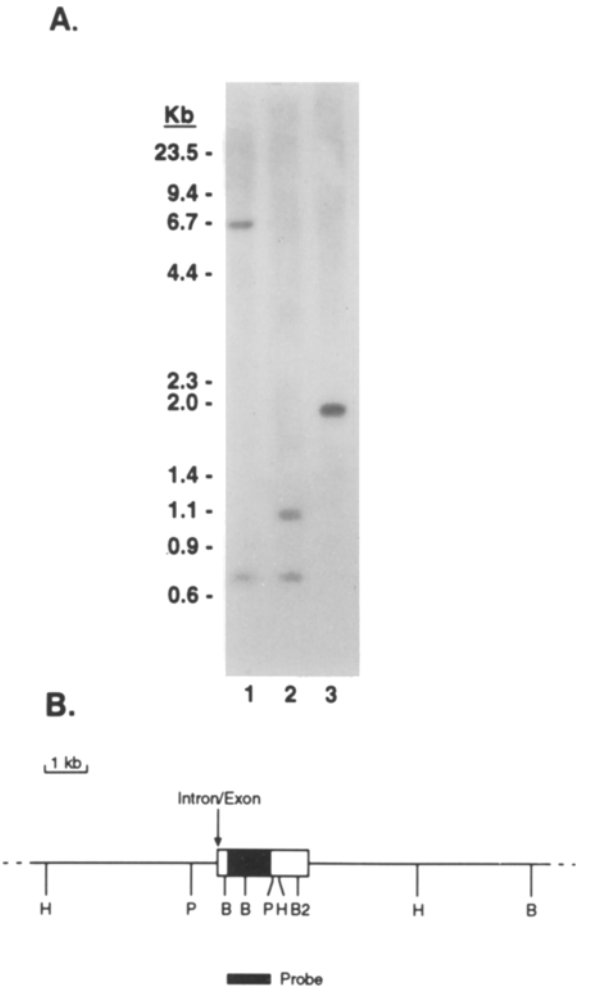


Fig. 2. Southern blot analysis and restriction map of the human AT_1 receptor gene. (A) Southern blot of human DNA digested with *Bcl* I (lane 1), *Bcl* I and *Bgl* II (lane 2), and *Pst* 1 (lane 3). Molecular weight markers are indicated in kilobases (Kb). (B) A partial restriction map of the human AT_1 receptor gene. *Black* and *open* boxes represent the coding region and noncoding regions, respectively, of the last exon of the AT_1 receptor gene. Restriction enzymes denoted include: B, *Bcl* I; B2, *Bgl* II; H, *Hind* III; P, *Pst* I. The region included within the hybridization probe is indicated by the solid bar below.

blot (Fig. 1). Two bands were present in *Bam* HI and *Hind* III digested rat DNA (lanes 1 and 3). This pattern is suggestive of the existence of two highly related copies of the AT_1 receptor gene in the rat genome, which is in agreement with the identification of two separate rat AT_1 receptor genes [17–19, 21]. Three hybridizing fragments were detected in *Eco* RI cut rat DNA (lane 2); the upper band appeared as a doublet, which was more discernible upon longer gel electrophoresis prior to blotting (see Fig. 3). This result is also consistent with the existence of two AT_1 genes in the rat genome where the

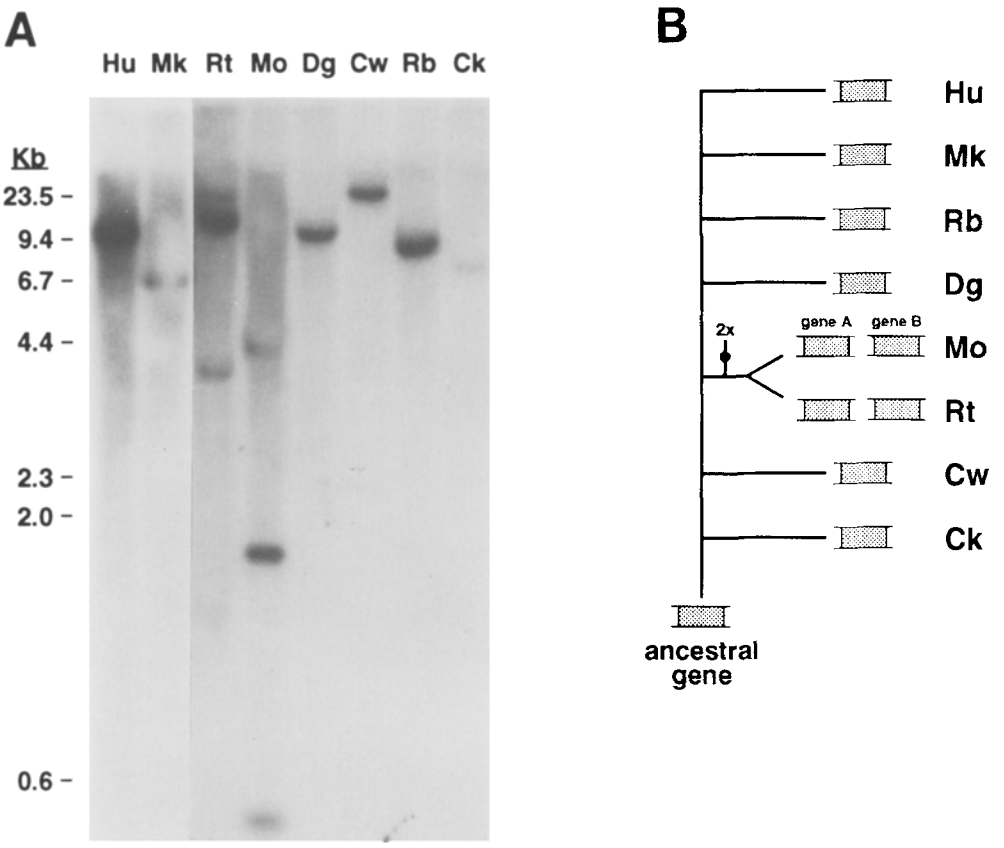


Fig. 3. (A) Multiple species Southern blot. Southern blot containing genomic DNA from nine different species digested with *Eco* RI. Mammal taxonomic order and species include: primates, human (Hu) and monkey (Mk); Rodentia, rat (Rt) and mouse (Mo); Carnivora, dog (Dg); Artiodactyla, cow (Cw); and Lagomorpha, rabbit (Rb). Class Aves representative is chicken (Ck). The coding region of the human AT_1 receptor cDNA was used as hybridization probe. The human and monkey lanes of the blot were exposed to film a shorter period of time relative to the other portion of the blot. Molecular weight markers are indicated in kilobases (Kb).
(B) The evolution of the AT_1 receptor gene. Phylogenetic relationships are constructed according to Czelusniak *et al.* [50] based on hemoglobin amino acid sequence data. The period wherein the proposed duplication event occurred is indicated by a closed circle.

AT_{1B} gene would account for one band, and the AT_{1A} gene would be represented as two bands in the blot since its coding region is bisected by *Eco* RI digestion.

To test the possibility that two very closely linked AT_1 receptor genes exist in human DNA, a second Southern blot analysis of human DNA was performed. This study was particularly relevant since a recent report from another laboratory claimed that a second AT_1 receptor subtype existed in the human genome [41]. The restriction enzymes *Bcl* 1, *Bgl* II and *Pst* I recognize sites within, or immediately adjacent to the coding region of the human AT_1 gene (Fig. 2), and were thus ideally suited for this analysis. The enzyme *Bcl* I recognizes a sequence beginning upstream of the receptor's translation initiation codon (ATG), and a second site 626 bp downstream of this codon within the coding region. As shown in Fig. 2 (lane 1), only two bands were present in human DNA digested with this enzyme. The smaller band corresponds to the 633 bp DNA fragment that encompasses the 5′-coding portion of the AT_1 receptor between the two restriction sites described above. The larger 6.7-kilobase (kb) base fragment contains the remaining 451 bp of the coding region as well as downstream flanking sequences. The *Bcl* I, *Bgl* II double digestion also yielded two hybridizing signals (lane 2). Since the *Bgl* II recognition site is 529 bp downstream of the coding region within the 3′-untranslated sequence of the gene [22], both bands could be accounted for; the 633 bp band corresponds to the region between the two *Bcl* I sites (same as in lane 1); the 980-bp band includes the DNA sequences between the *Bcl* I and *Bgl* II sites. Finally, the enzyme *Pst* I recognizes sites approximately 700 bp upstream and 63 bp downstream from the receptor coding region, respectively [22, 23]. As shown in Fig. 2 (lane 3), only one band was detected of an expected size of approximately 2.0 Kb.

The results of this blot analysis prove that a second AT_1 receptor gene does not immediately flank the AT_1 receptor gene and confirms our original conclusion that only one AT_1 receptor gene is present within the human genome [22]. This result contrasts the report claiming the existence of another human AT_1 subtype, a conclusion also based on Southern blot analysis [25]. It is possible that this discrepancy reflects the use of a hybridization probe that contained flanking sequences in addition to the human AT_1 receptor coding region. Accordingly, this probe might have recognized DNA fragments that share complimentarity with sequences other than those encoding the AT_1 receptor gene.

To determine the number of coding loci for the AT_1 receptor common to a wide variety of animals, a Southern blot containing DNA from multiple species was hybridized with the human AT_1 receptor coding probe. As shown in Fig. 3A, all animal genomic DNA digested with *Eco* RI yielded one hybridization signal, except for rat and mouse DNA, where three bands were detected. The pattern observed for the rat DNA was consistent with the blot in Fig. 1 and confirmed the existence of two AT_1 receptor genes in the genome. Likewise, the three bands observed within mouse DNA is in agreement with the existence of two AT_1 receptor genes; the coding region of the mouse AT_{1B} receptor gene lacks an *Eco* RI site, whereas the AT_{1A} receptor gene contains two *Eco* RI sites, which would yield at least three detectable bands in a genomic Southern blot, one of which would be 523 bp in length [20].

It is interesting to note that the intensity of the hybridization signal in chicken genomic DNA was significantly less than the signals of the other animals (Fig. 3A). Considering the more distant evolutionary relationship between birds and mammals, greater DNA sequence divergence was expected. Indeed, a turkey angiotensin II receptor was very recently described that shares about only 70% nucleotide sequence homology with mammalian AT_1 receptor coding regions [42].

Table 1. Potencies of various AII agonists and antagonists to inhibit [^{125}I]SIA binding to cloned and expressed AT_1 receptors $LhAT_1D6$ cells

	K_i (nM)	n_H
AI	23.8 ± 6	0.88 ± 0.12
AII	0.84 ± 0.2	0.98 ± 0.06
AIII	22.1 ± 3.2	0.78 ± 0.15
Sar^1, AII	0.65 ± 0.2	0.70 ± 0.18
[Sar^1, Ile^8] AII	0.25 ± 0.08	0.92 ± 0.08
[Sar^1, Ala^8] AII	0.07 ± 0.01	1.10 ± 0.15
[Sar^1, His^8] AII	8.5 ± 1.5	0.80 ± 0.08
[Sar^1, Bph^4, Phe^8] AII	240 ± 22	0.84 ± 0.12
SK&F 108566	1.38 ± 0.2	1.15 ± 0.12
DuP 753	1.83 ± 0.3	0.94 ± 0.08
PD 123319	> 10,000	

Ki values were obtained from nonlinear regression analysis using Lundon-2 and n_H values were obtained by linear regression of log-logit plots of competition curves.

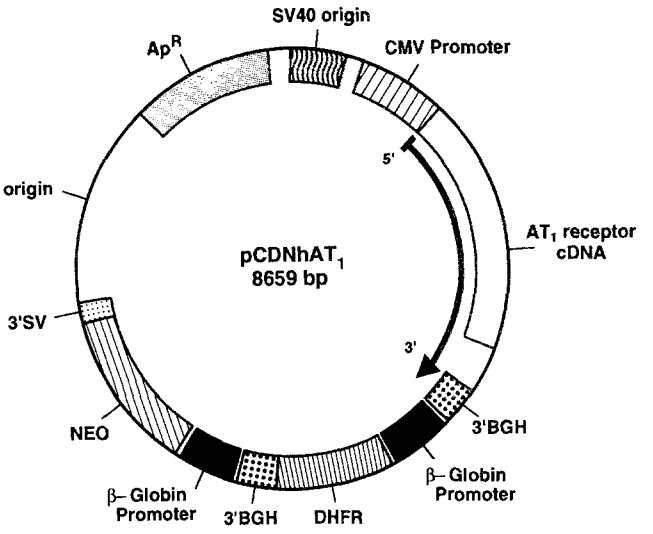


Fig. 4. Circular map of the pCDNAT$_1$ vector used for human AT_1 receptor expression in mouse L cells. Arrow denotes the AT_1 cDNA transcript. Abbreviations: SV40 origin, Simian virus 40 replication origin and enhancer; CMV Promoter, human cytomeglovirus promoter; AT_1 receptor cDNA, human AT_1 receptor cDNA; 3' BGH, bovine growth hormone polyadenylation signal; β-Globin Promoter, mouse β-globin promoter; DHFR, mouse dehydrofolate reductase gene; NEO, bacterial neomycin phosphotransferase gene; 3' SV, Simian virus 40 early polyadenylation signal; origin, plasmid origin of replication.

The multiple species blot result indicates that the AT_1 receptor gene is represented as a single genomic copy in animals across a broad evolutionary spectrum, with the exception of rat and mouse, which have two gene copies. We hypothesize that multiple AT_1 receptor genes within the rodent taxonomic order originated by gene duplication and subsequent divergence from a common rodent ancestral gene. A similar proposal has been suggested to explain the existence of two copies of the preproinsulin gene in rats where both human and chicken each contain only a single gene copy [41]. As depicted in Fig. 3B, it is possible that the AT_1 receptor gene doubling event occurred following the branching of the rodent order from the mammalian phylogenetic tree, but prior to the speciation of rat and mouse. This hypothesis is in agreement with the conclusion of a report based on Southern blot and computer analysis of AT_1 receptor genes from just three species: human, rat and mouse [43]. However, very recently, we and Ji, H., *et al.* simultaneously re-

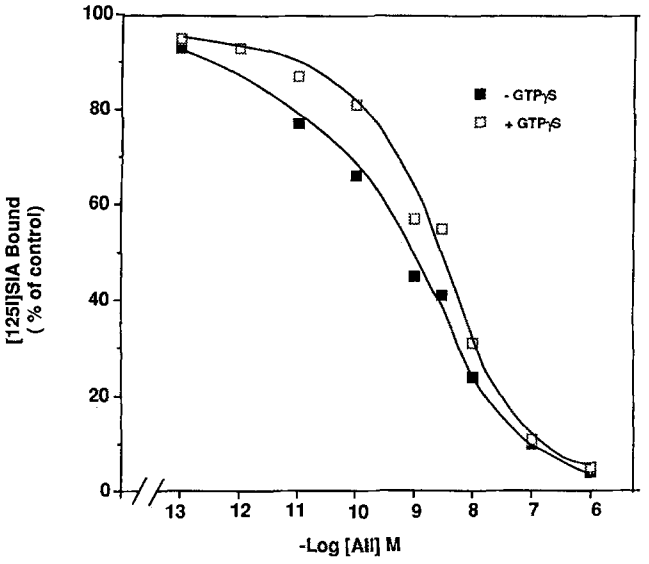


Fig. 5. Effect of GTPγS on AII binding to $LhAT_1$-D6 cell membranes. [^{125}I]SIA was displaced by increasing concentrations of unlabelled AII in the presence [□] or absence [■] of 100 μM GTPγS. Data points are the average of two separate experiments.

ported the cloning of two different angiotensin II receptor genes from *Xenopus laevis* [44, 45]. These receptors appeared to be pharmacologically similar, although they differ approximately 11% in protein sequence. As proposed for the two rodent AT_1 receptor genes, perhaps gene duplication of a primordial gene of the amphibian taxonomic order gave rise to two similar genes in *Xenopus laevis*. Conversely, it is possible that a gene duplication event occurred very early in evolution prior to the branching of amphibians from the bird and mammal phylogenetic trees and, for some yet undetermined reason, two genes were conserved in both rodents and amphibians (at least *Xenopus laevis*) and one gene was conserved and the other eliminated in other animals during evolutionary divergence.

For eucaryotic expression of the human AT_1 receptor cDNA, the vector $pCDNhAT_1$ was constructed as described in Materials and methods and is schematically shown in Fig. 4. Since this vector was found to express recombinant human AT_1 receptor when transiently introduced into COS cells (data not shown), it was transfected into mouse L-cells. The bacterial neomycin phosphotransferase (NEO) gene was used as a selectable marker which allowed cells that stably expressed the receptor to grow in medium containing the antibiotic G418. Approximately twenty clonal cell lines that expressed the AT_1 receptor at various levels were identified by [^{125}I]SIA binding. No specific [^{125}I]SIA binding was detected in untransfected cells (data not shown). One cell line, $LhAT_1$-D6, was found to express high levels of receptor and was therefore chosen for further study.

Radioligand binding assays using $LhAT_1$-D6 cells were performed to examine the pharmacological characteristics of the recombinant human AT_1 receptor. [^{125}I]SIA binding to $LhAT_1$-D6 cells was specific (approx. 90%) and saturable. Scatchard analysis indicated a single class of high affinity binding site with an apparent dissociation constant (Kd) of 0.15 ± 0.02 nM, and maximum binding (B_{max}) of 430 ± 15 fmol/mg protein (data not shown).

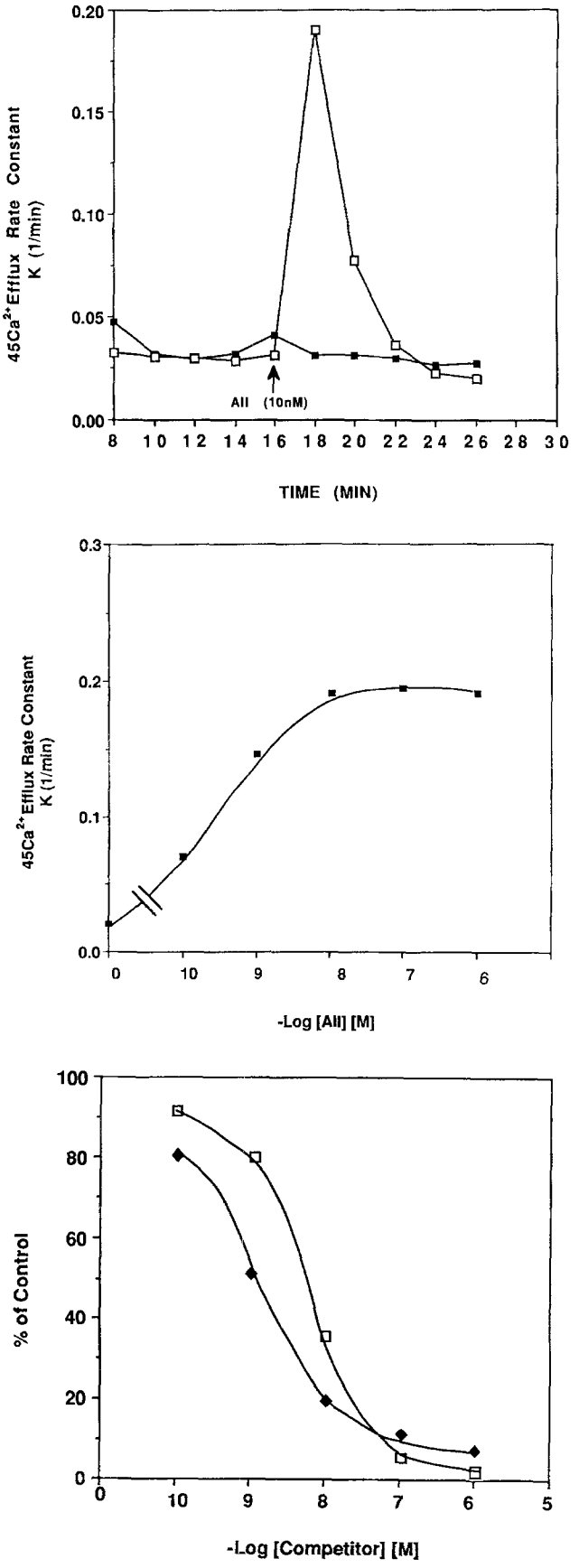
The pharmacological profile of ligand binding to $LhAT_1$-D6 cells was determined by analysis of competition binding experiments. The K_i and n_H values for the competitors are listed in Table 1. The rank order of potencies for the compounds tested is in good agreement with that obtained with transiently expressed COS-7 cells [22] as well as the endogenous AT_1 receptor in rat mesenteric artery membranes. In all cases, the compounds displayed monophasic competition curves indicative of interaction with a single class of binding sites.

Human AT_1 receptor possess several features that are common with other G-protein linked receptors. G-pro-

Table 2. Effects of AII [Sar^1, Ile^8] AII and SK&F 108566 on inositol phosphate formation in $LhAT_1D6$ cells

	DPM ± SE		
	IP_1	IP_2	IP_3
Basal	2456 ± 58	175 ± 15	68 ± 8
AII (1 μM)	3021 ± 88	435 ± 22	173 ± 12
[Sar^1, Ile]AII (3 μM)	2352 ± 62	168 ± 12	72 ± 3
AII (1 μM) + [Sar^1, Ile^8]AII (3 μM)	2508 ± 110	190 ± 18	78 ± 4
SK&F 108566 (10 μM)	2400 ± 30	178 ± 16	70 ± 6
AII (1 μM) + SK&F 108566 (10 μM)	2418 ± 45	185 ± 15	72 ± 5

Inositol phosphates were measured as explained in the Methods.



tein coupled receptors typically exist in interconvertible high and low affinity states depending on G-protein association [46]. The high agonist affinity state where the receptor is coupled to G-protein is sensitive to GTP. In this study, we examined the ability of AII to compete for specific [^{125}I]SIA binding to LhAT$_1$-D6 membranes in the absence or presence of stable GTP analog. GTPγS. As shown in Fig. 5, the competition curve for AII was shifted to the right in the presence of 100 μM GTPγS, resulting in an increase in the IC_{50} value from 0.8 ± 0.2 nM in the absence of GTPγS to 3.0 ± 0.3 nM in the presence of GTPγS. Analysis of the competition binding data presented in Fig. 5 by Lundon 2 program indicated a shallow competition curve for AII with K_ds of 0.6 and 21.5 nM in the absence of GTPγS, whereas in the presence of GTPγS, the curve was shifted to the right and the calculated Kd was 1.2 nM. This result suggests that the AII receptor expressed in LhAT$_1$-D6 cells is coupled to G protein.

It is well established that AII receptors of the AT$_1$ subtype are efficiently coupled to the stimulation of phospholipase C and mobilization of intracellular Ca^{2+} [47]. In this study, we examined the effect of AII on phosphoinositide turnover and the mobilization of intracellular calcium in LhAT$_1$-D6 cells. As shown in Table 2, exposure of [^{3}H] myoinositol-labelled cells to AII (1 μM) for 5 min in the presence of 10 mM LiCl resulted in a significant increase in the accumulation of inositol mono, di and triphosphates. Addition of [Sar^1, Ile^8]AII (3 μM) or SK&F 108566 (10 μM) prior to the exposure to AII inhibited AII-mediated inositol phosphates accumulation. In order to determine whether AII-mediated increase in inositol phosphates accumulation is coupled to the release of intracellular calcium, we measured AII-mediated $^{45}Ca^{2+}$ efflux in these cells. Figure 6A depicts the time course of 10 nM AII-mediated $^{45}Ca^{2+}$ efflux from LhAT$_1$-D6 cells preloaded with $^{45}Ca^{2+}$. Immediately following AII exposure, there was a rapid increase in the rate of $^{45}Ca^{2+}$ efflux from cells which reached a peak within 2 min; thereafter the efflux rate diminished rapidly to basal level. The dose response relations for the

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Fig. 6. (A) Effect of AII on $^{45}Ca^{2+}$ efflux from LhAT$_1$-D6 cells. (A) AII (10^{-8} M) was added at 16 min as indicated by the arrow [open squares]. Closed squares represent the basal values. Tracing is representative of four experiments. (B) The concentration-response curve for AII-induced $^{45}Ca^{2+}$ efflux in LhAT$_1$-D6 cells. Each point represents the rate constant for the peak response. (C) The effect of varying concentrations of [Sar, Ile^8]AII [◆] and SK&F 108566 [□] on AII- (10 nM) mediated $^{45}Ca^{2+}$ efflux. Data points are the average of two separate experiments.

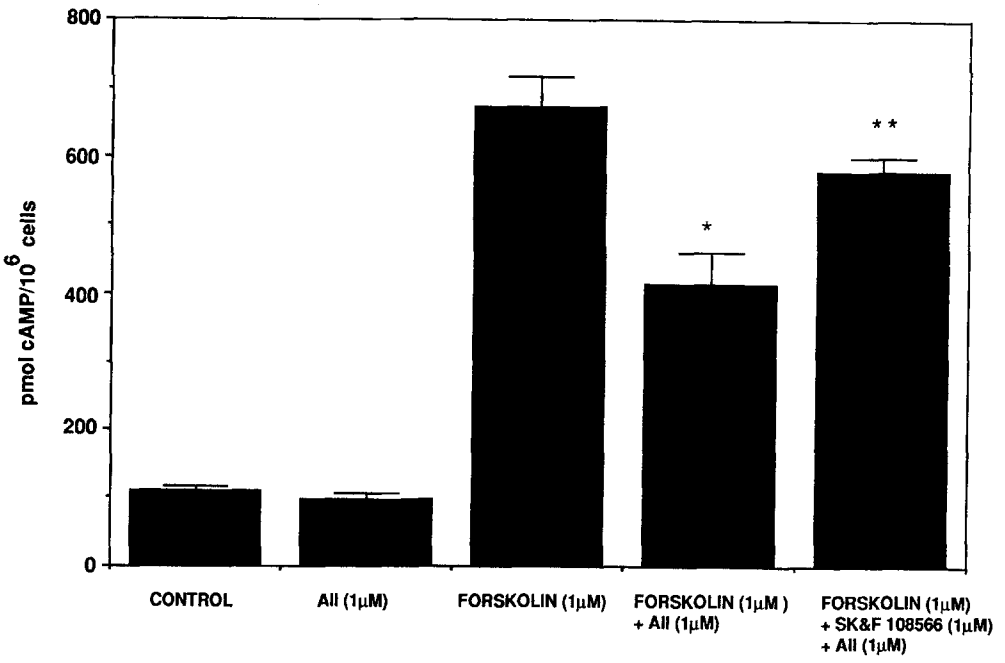

Fig. 7. Effect of AII on forskolin-mediated cAMP accumulation and the effect of AII antagonist on AII-mediated inhibition. Cells in 24 well plates were treated in the absence or presence of AII or AII and AII antagonist, and then challenged with forskolin. cAMP levels were quantitated by RIA. The data shown are mean ± SEM of three determinations. *p = < 0.005 (significantly different from forskolin alone); **p = < 0.05 (significantly different from forskolin + AII).

peak $^{45}Ca^{2+}$ efflux in response to AII treatment is shown in Fig. 6B. The efflux of $^{45}Ca^{2+}$ was significantly increased from basal levels, which reached a plateau at 10 nM AII concentration, exhibiting an effective dose (ED_{50} value) of 0.2 nM. This relative potency value of AII to induce Ca^{2+} release from $LhAT_1$-D6 cells is similar to its potency to displace radiolabeled AII binding. For further evaluation, $LhAT_1$-D6 cells preloaded with $^{45}Ca^{2+}$ were pretreated with varying concentrations of receptor antagonists [Sar^1, Ile^8]AII or SK&F 108566 and subsequently exposed to AII. Both antagonists inhibited AII-mediated $^{45}Ca^{2+}$ efflux in a concentration-dependent manner (Fig. 6C). The observed IC_{50} values were 2 nM for [Sar^1, Ile^8]AII and 8 nM for SK&F 108566. Together, these observations support the conclusion that Ca^{2+} mobilization in response to AII treatment is a receptor-mediated event.

Studies have shown that AII is involved in multiple signal transduction pathways [47–49]. For example, in hepatocytes AII is involved in the activation of phospholipase C as well as the inhibition of adenylate cyclase. Recently, there were two reports [48, 49] on signal transduction mediated by the rat AT_{1A} receptor cDNA stably expressed in Chinese hamster ovary cells. Ohinishi *et al.* [48] have demonstrated that the recombinant AT_1 receptor can independently activate both phospholipase C and dihydropyridine-sensitive voltage-dependent Ca^{2+} channels, as well as inhibit adenylate cyclase. In contrast, Webb *et al.* [49] have shown that the recombinant rat AT_{1A} receptor expressed in CHO cells is functionally coupled to activation of phospholipase C but not adenylate cyclase activity. To examine the functional coupling of the human AT_1 receptor to adenylate cyclase, we studied the effect of AII on forskolin-mediated accumulation of cAMP in LhAT-D6 cells. As shown in Fig. 7, forskolin stimulated cAMP accumulation by 6–7 fold over basal. Addition of 1 μM AII significantly inhibited the forskolin-induced cAMP accumulation by 43%. SK&F 108566 reversed AII-mediated inhibition by 65–70%. These results indicate that the human AT_1 receptor is functionally coupled to inhibition of adenylate cyclase. Similar results were obtained when the human AT_1 receptor was stably expressed in CHO cells (data not shown).

In conclusion, the data in this report indicate that for humans and most other animals, the AT_1 receptor is encoded by a single gene. No other gene exists in humans, as occurs in rodent and amphibian orders, that closely resembles the AT_1 receptor at the nucleotide level. Accordingly, the AT_2 receptor gene, in addition to yet undiscovered AII receptors, is predicted to share limited protein sequence homology with the AT_1 receptor. In

addition, we have characterized and established a stable cell line expressing human AT_1 receptor subtype. We have demonstrated that AII activation of the expressed receptor results in mobilization of Ca^{++} and inhibition of adenylate cyclase, which indicates that the receptor is capable of activating multiple effector pathways. The availability of a stable expression system specific for human AT_1 receptor will provide a useful source to study the molecular mechanism of AII-receptor function and regulation. This work will facilitate the development of additional AT_1 receptor-specific antagonists that may be of therapeutic value in the treatment of blood pressure and heart failure.

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