Expression of the mouse and rat *mas* proto-oncogene in the brain and peripheral tissues

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Abstract **We isolated the *mas* proto-oncogene from a mouse genomic library. Sequence analysis showed that it contains an open reading frame without intervening sequences. The amino acid sequence deduced confirms the seven-transmembrane-domain structure and exhibits 97% and 91% amino acid homology with the rat and the human Mas, respectively. In mice and rats, *mas* mRNA was detected in the testis, kidney, heart, and in the brain regions: hippocampus, forebrain, piriform cortex, and olfactory bulb. Testicular *mas* mRNA from rats increases markedly during development, while cerebellar mRNA is high postnatally but completely disappears at later stages. We conclude that the product of the mouse *mas* gene may be involved in the development of the brain and testis.**

Key words: Oncogene; G-protein coupled receptor; Brain localization; Testis

1. Introduction

The *mas* oncogene was detected by an in vivo tumorigenicity assay applying NIH 3T3 cells and nude mice [1–3]. The initiation of the *mas* gene's oncogenic potential occurs during transfection and is due to a rearrangement in the 5′-flanking region. Moreover, the human *mas* gene was localized within a region on the long arm of chromosome 6, which is often rearranged in malignant cells [4]. The normal, not rearranged human *mas* gene, however, exhibits only weak transforming activity. The *mas* gene codes for a protein with seven potential transmembrane domains and may therefore induce malignant growth through signal transduction pathways like other membrane associated oncoproteins [5]. It exhibits sequence similarity to a family of sensory receptors, including the opsins [6] and the adrenergic, muscarinergic, and substance K receptors [7], with highest homology to the *mas*-related gene (*mrg*) [8] and the rat thoracic aorta (RTA) gene [9].

Jackson et al. [10] suggested that the Mas protein represents a G-protein-coupled receptor for angiotensins. They were able to show that angiotensin (ANG) II and III can initiate [^{3}H]thymidine incorporation in *mas*-expressing cells [11]. Additionally, in neuronal cells stably transfected with the human *mas* oncogene, an accumulation of inositol phosphates and the mobilization of intracellular Ca^{2+} after stimulation with ANG II and ANG III were observed [11,12]. A comparable response was demonstrated for *mas*-transfected COS-7 cells [13]. The activation of an inward current by ANG in *mas*-injected oocytes [11], however, was not inhibited by the ANG antagonists [Sar1,Val5,Ala8]-ANG II and [Sar1,Val5]-ANG II [14]. Therefore, doubts have arisen as to whether the Mas protein really is an ANG II receptor, particularly since binding of ANG to *mas*-transfected cells has never been shown. In addition, the major ANG II receptors, AT_1 and AT_2 have recently been cloned [15–18], exhibiting only 8% and 19% amino acid identity, respectively, to the Mas protein.

mas transcripts were found in the human and rat brain [10,19] with highest amounts in the hippocampus and lower amounts in the cortex and cerebellum. In situ hybridization in rat brain using radiolabeled *mas* cRNA probes showed selective expression in the forebrain and specifically in parts of the limbic system (dentate gyrus, the CA3 and CA4 areas of the hippocampus), the piriform cortex, and the olfactory bulb [20,21]. As it could be shown that *mas* expression is regulated during development [21] and by neuronal activity [22] in the rat hippocampus, it may contribute to the plasticity of this part of the brain.

The apparent tissue-specific gene expression, its possible relationship to the renin-angiotensin system (RAS), and its oncogenic properties make the Mas protein a particularly interesting molecule. In this study, we report the cloning and molecular characterization of the mouse *mas* gene, which has recently been mapped to chromosome 17 [23,24]. We provide a detailed analysis of its DNA structure and RNA distribution in rats and mice with respect to tissue-specificity, developmental control, and relationship to hypertension and sexual maturation demonstrating for the first time that there are additional sites of *mas* expression in rodents besides the brain.

2. Materials and methods

2.1. Animals

Nine-week-old BALB/c and NMRI mice as well as Wistar–Kyoto rats of different ages were used for the expression analysis. Animals had free access to food and water and were kept in alternating 12 h light and dark cycles.

2.2. Bacteriophage library screening

Recombinant phages of a genomic DNA library of BALB/c mice were amplified on *E. coli* strain LE 392 by the plate lysate method and blotted onto nylon membranes by the method of Benton and Davis [25,26]. 1.2×10^6 recombinant phages were analysed with a human *mas* 1.3-kb *Bam*HI/*Nsi*I genomic fragment [27]. The DNA probe was labeled by the random priming method using [α-^{32}P]dCTP and Klenow enzyme [28,29]. Hybridization with 1×10^6 cpm of the original probe was carried out overnight at 65°C in a solution containing 5 × SSC,

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5 × Denhardts, 1% SDS, and 250 μg salmon sperm DNA per ml. After hybridization, the filters were washed once for 5 min in 2 × SSC/0.1% SDS at 20°C, twice at 65°C for 15 min in the same solution, and finally in 1 × SSC/0.1% SDS for 10 min. Filters were dried and exposed to X-ray film. Positive plaques were isolated and rescreened as described above until pure preparations were obtained.

2.3. Restriction and DNA sequence analysis

DNA samples were digested with various restriction enzymes under single and double digestion conditions and were run through a 0.9% agarose gel using pulsed-field electrophoresis. Ethidium bromide-stained gels were photographed under UV-light and the DNA was blotted onto nylon filters as described by Southern [30]. Hybridization was carried out with the labeled human *mas* cDNA fragment as described above. Appropriate restriction fragments were isolated from 1% agarose gels and subcloned into the Bluescript II SK$^+$ vector (Stratagene, Heidelberg, Germany). After progressive digests with exonuclease III, DNA sequences were determined in both orientations by the dideoxynucleotide chain termination method [31] using primers complementary to the T3 and T7 promotor sequences and a modified form of T7 DNA polymerase [32].

2.4. RNase protection assay

Total RNA from tissues was isolated by homogenization in lithium chloride and urea by the method of Auffray and Rougeon [33]. A 315-bp mouse *mas* genomic DNA fragment, representing positions (−165)–(+150) relative to the translation start site, and a 615-bp rat *mas* cDNA fragment (position 258–873) were subcloned into the polylinker sites of vector Bluescript II SK$^+$ and pGEM4 (Promega, Atlanta), respectively, which are flanked by promoters for RNA polymerases. Before transcription the rat *mas* cDNA vector was cut with *Nco*I (position 600) yielding a 273-bp transcription product. The resulting plasmids were used for in vitro transcription in the presence of [α-^{32}P]UTP [34], as was a 150-bp *Sal*I/*Xba*I rat β-actin cDNA subcloned into Bluescript SK$^+$ vector. RNase protection assays were performed according to Mullins et al. [35].

2.5. In-situ hybridization

The RNA probes were synthesized using the 315-bp mouse *mas* genomic DNA fragment described above. Antisense and sense RNAs were labelled with [α-^{35}S]UTP using T3 and T7 polymerases, respectively. Hybridization conditions were the same as for the rat *mas* gene [20].

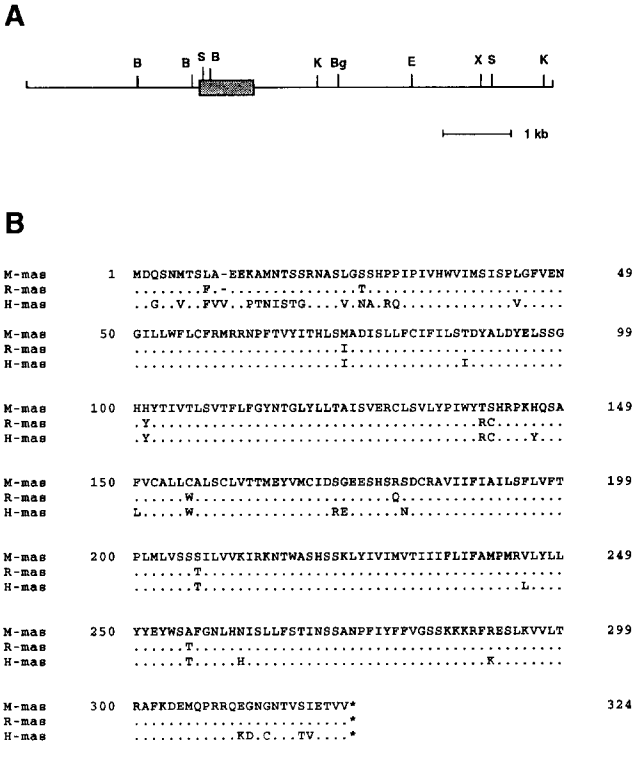

Fig. 1. Structure and sequence of the mouse *mas* gene. (A) Restriction map of mouse *mas* genomic DNA. The sequence representing the coding region of the gene is indicated by the box. The positions of relevant restriction sites are depicted as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; S, *Sac*I; X, *Xba*I. (B) Alignment of the amino acid sequences of the mouse (M-mas), rat (R-mas), and human Mas (H-mas) protein. Dots indicate amino acids identical with the mouse Mas sequence. The dashes at position 11 indicate the insertion of the amino acid valine in the human Mas. The DNA sequence of the mouse *mas* gene has been assigned the accession number X67735 in the EMBL data library.

3. Results and discussion

Cloning and sequence analysis of the mouse *mas* proto-oncogene were performed to study the evolution of the gene family, to provide tools to clarify the control mechanisms of *mas* expression in the mouse, and to reveal its function by gene targeting experiments. 1.2×10^6 recombinants were screened by hybridization of a BALB/c genomic library with a full-length human *mas* DNA. Four recombinant phages were isolated. Restriction mapping was carried out with a set of restriction enzymes and revealed that each of the lambda clones contains the entire coding region of the mouse *mas* proto-oncogene (Fig. 1A). Using exonuclease-digested fragments and synthetic oligonucleotides, DNA sequencing was performed in both orientations on genomic subclones. The mouse *mas* gene contains a single continuous reading frame of 972 nucleotides which is not interrupted by an intron as it is also the case for other G-protein-coupled receptors, e.g. adrenergic, muscarinergic, serotonin, ANG II, and bradykinin receptors [36]. Fig. 1B shows the amino acid sequence of the mouse Mas protein predicted by the nucleotide analysis in comparison with the human and rat proteins. The mouse Mas protein shares 97% and 91% amino acid homology with the rat and the human Mas respectively. The mouse and rat sequences code for a protein with 324 amino acid residues, whereas the human Mas protein contains 325 amino acid residues; an additional valine is inserted at position 11 of the human protein. The greatest divergence between mouse and human Mas protein exists in the aminoterminal hydrophilic domain, with an identity of only 48%. The highest homology resides within the seven transmembrane regions and the first and second cytoplasmic loops, which is a common feature of related receptors having seven transmembrane domains, e.g. the adrenergic and substance K receptors [37,38]. The aminoterminal parts of the Mas proteins, which in other receptors of this family bind the ligand, are well conserved in their hydrophobic amino acid residues, suggesting the interaction with a similar molecule. Several potential glycosylation sites near the amino terminus and at position 271 in the predicted Mas protein sequence are identical or similar for mouse, rat, and man [1,39]. Like the other Mas proteins, also the mouse Mas protein lacks a signal sequence at the amino terminus and may therefore be integrated spontaneously into the membrane on the basis of its hydrophobicity [40].

So far, *mas* mRNA had only been detected in the brain. RNase protection assays were performed to detect *mas* mRNA in various mouse tissues. As probe, we used a ^{32}P-labeled mouse *mas* cRNA with a total length of 315 nucleotides, which is complementary to the 5′-end of the *mas* coding sequence. Fig. 2A shows the tissue distribution of *mas* transcripts in BALB/c

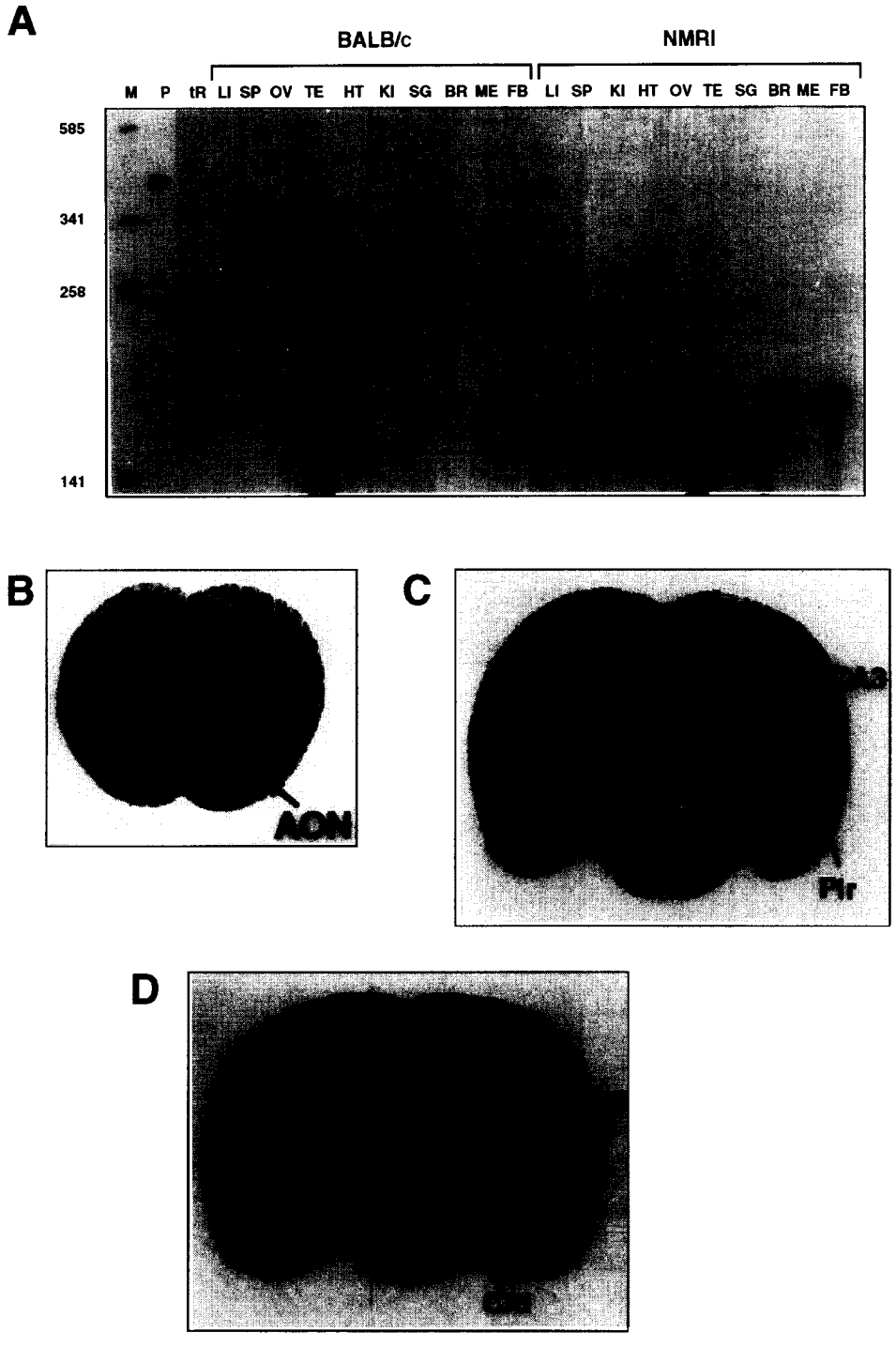

Fig. 2. *mas* mRNA in tissues of BALB/c and NMRI mice. (A) RNase protection assay. Samples of 100 μg of total RNA from brain (BR), kidney (KI), liver (LI), spleen (SP), submandibular gland (SG), heart (HT), ovary (OV), forebrain (FB), medulla oblongata (ME), and total brain (BR), 50 μg from testis (TE) and 100 μg tRNA (tR) were analysed by RNase-protection assay and loaded on the gel together with 2000 cpm of the undigested probe (P) and the molecular weight marker, ^{32}P-labeled pUC19 cut with *Sau*3A (M). The probe (P), a ^{32}P-labeled 370-nucleotide cRNA containing 315 nucleotides of the mouse *mas* gene is shortened to a protected fragment of about 200 nucleotides complementary to *mas* mRNA. (B–D) In situ hybridization of brain sections. Hybridization was performed using the same 370-nucleotide fragment labeled with [^{35}S]UTP in antisense orientation on sections of frontal cortex (B), piriform cortex (C), and hippocampus (D). Hybridization signals appear as dark grains over neurons in the anterior olfactory nucleus (AON), the piriform cortex (PIR), and the CA cells (CA1, 2 and 3) of the dentate gyrus (DG) within the hippocampus (HI).

and NMRI mouse strains. Analysis of total RNA from peripheral tissues shows abundant expression in the testis and detectable amounts of *mas* mRNA in the kidney and heart. In the liver, the spleen, the submandibular gland and the ovary, *mas* mRNA could not be detected. The *mas* gene is, however, predominantly expressed in the brain and particularly in the forebrain. In addition, the medulla oblongata contains low amounts of *mas* mRNA.

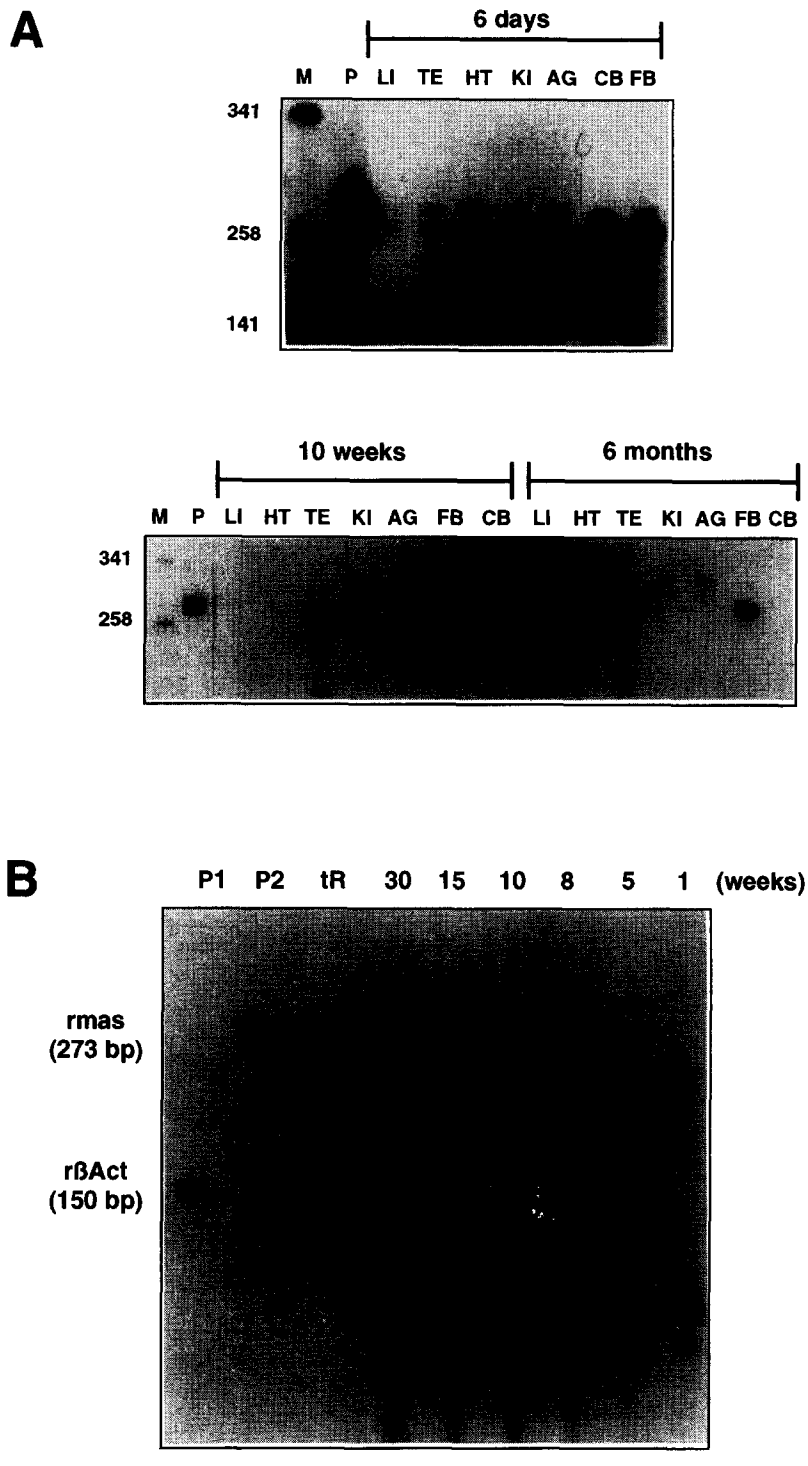

Fig. 3. Regulation of *mas* mRNA in organs of WKY rats during ontogeny. (A) Tissue survey. Animals were tested at different ages: postnatal stage (6 days), pubertal stage (10 weeks), and adult stage (6 months). By RNase-protection assay using a rat *mas*-specific probe (P), 100 μg of total RNA were analyzed of each of the following organs: liver (LI), heart (HT), kidney (KI), adrenal gland (AG), forebrain (FB), and cerebellum (CB); 50 μg total RNA were used from testis (TE) and 100 μg tRNA (tR). (B) Testis. 100 μg total testis RNA of WKY rats between 1 and 30 weeks of age were analysed by RNase-protection assay. P1: rat β-actin (rβAct) cRNA; P2: rat *mas* (rmas) probe.

In situ hybridization allowed a more precise localization of the *mas* mRNA in the brain of NMRI mice (Fig. 2B–D). In agreement with the RNase protection assay, the highest levels of *mas* mRNA were observed in the forebrain and in areas of the hippocampus and cerebral cortex. The dentate gyrus, the CA3, CA2, and CA1 area of the gyrus hippocampi, the olfactory tubercle, the piriform cortex, and the olfactory bulb in particular are highly stained. A widespread distribution of *mas* mRNA was seen in the cortical areas, including the central cortex and the neocortex, while it was not detectable in the

medulla oblongata by in situ hybridization. Most of these areas are identically stained in mouse and rat, except for the CA regions: only CA2 and CA3 are stained in rat, while all three CA areas showed signals in mice. This distribution pattern in the brain differs substantially from that of [^{125}I]ANG II-binding sites. The highest densities for ANG II receptors have been found in the subfornical organ, several hypothalamic nuclei, including the nucleus of the solitary tract, the locus coeruleus, and the inferior olive [41,42]. On the cellular level, *mas* transcripts were confined to neuronal cells (data not shown).

The ontogeny of *mas* expression in rats and its relationship to hypertension was examined by RNase protection assays. *mas* mRNA tissue distribution in postnatal, pubertal, and adult stages of WKY rats was compared (Fig. 3A). In the postnatal brains, high signals were found in the forebrain and in the cerebellum. The signal in the forebrain remains high throughout development, while *mas* mRNA expression decreased during ontogeny in the cerebellum. These data are consistent with the findings of Martin et al. [21]. *mas* mRNA in the kidney and heart is present in young rats, but hardly detectable in adult animals. Of particular interest in this respect were the findings in the testis (Fig. 3B). *mas* transcripts were low after birth but increased at 5 weeks reaching their highest expression levels at 15 weeks of age. Similar to the expression in the forebrain, *mas* mRNA concentrations in testis then remained stable. Puberty, with its rise of testosterone, starts in rats at an age of 8 weeks [43], indicating that *mas* mRNA expression is not directly testosterone-dependent, as raised *mas* levels are already observed at an age of 5 weeks. With this expression pattern *mas* joins a growing family of proto-oncogenes expressed differentially during spermatogenesis in the testis [44]. The differential regulation of *mas* gene expression in peripheral organs during ontogeny suggests an important but up to now unknown function.

With respect to a possible role in cardiovascular regulation, we performed a detailed expression analysis of the *mas* gene in rats with and without hypertension (data not shown). Spontaneously hypertensive rats (SHRSP) and transgenic rats carrying the mouse *Ren*-2 gene, TGR(mREN2)27 [35], exhibited only slightly higher amounts of *mas* transcripts in the forebrain, kidney, and testis, but were identical in *mas* gene expression in all other tissues compared to the normotensive Sprague–Dawley rats. This makes a direct involvement of the Mas protein in blood pressure control rather unlikely without excluding long term modulating effects.

In conclusion, *mas* is clearly distinct with respect to DNA-sequence and tissue distribution of its mRNA from the typical ANG II receptors of the AT_1 subtype that mediates aldosterone secretion, vasoconstriction, and Na^+ retention, thereby increasing blood pressure, and of the AT_2 subtype with up to now unknown functions. In addition, Mas has never been shown to bind angiotensins and its tissue distribution, particularly in the brain, is quite different from the described binding sites. Taken together, these data indicate that Mas does not represent a typical ANG receptor. It has been proposed that Mas may have modulatory functions by interfering with the second messenger pathway systems used also by ANG II receptors that could lead to enhanced responsiveness of *mas*-transfected cells to ANG II via the endogenous receptor [45]. Recent results showing the conversion of a hypertrophogenic to a proliferative effect of ANG II on kidney proximal tubular cells by *mas*-transfection without change in ANG II binding characteristics corroborate this hypothesis [46]. Furthermore, the *mrg* exhibited an interaction with the ANG II signalling system in *Xenopus* oocytes [8].

The still elusive physiological function of Mas may be connected to the development and plasticity of the brain, as suggested by its influence on cell growth and its differential expression pattern during development and activity of this organ [21,22]. In addition, it may be important in the development of the testis. The availability of genomic mouse *mas* DNA makes further study of its function possible by transfecting it into tissue culture cells or ablating its expression by gene targeting in transgenic animals.

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