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Generation of transgenic mice with elevated blood pressure by introduction of the rat renin and angiotensinogen genes

(hypertension/renin-angiotensin system/captopril/animal model)

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ABSTRACT **The role of the renin-angiotensin system in blood pressure control and in the development of hypertension was investigated by generating transgenic mice carrying the rat renin or angiotensinogen gene or both genes under the control of the mouse metallothionein I promoter. The systolic blood pressure was significantly elevated in transgenic mice carrying both transgenes but was maintained normally in those bearing either of the transgenes. The transgene was effectively and properly transcribed to form the mature mRNA in the transgenic mice. The production of rat renin and angiotensinogen in the transgenic mice carrying the corresponding transgene was also verified by immunoanalyses of these proteins. Furthermore, the specific angiotensin-converting enzyme inhibitor captopril was effective in reducing the elevated blood pressure of the hypertensive transgenic mice. These results indicate that the combined action of the exogenous rat renin and angiotensinogen is responsible and necessary for elevation of blood pressure in the hypertensive transgenic mice.**

The renin-angiotensin system consists of three major components: angiotensinogen, renin (EC 3.4.23.15), and angiotensin-converting enzyme (dipeptidyl carboxypeptidase I, peptidyl-dipeptide hydrolase, EC 3.4.15.1) (1, 2). Angiotensinogen is mainly synthesized in the liver and is secreted into the circulating blood. This peptide precursor is cleaved by the enzyme renin, which is expressed and secreted into the circulation from the juxtaglomerular cells of the kidney. The angiotensin I thus formed is subsequently converted to angiotensin II by angiotensin-converting enzyme. Angiotensin II acts as a potent vasoconstrictor and stimulates release of aldosterone from the adrenal cortex. The renin-angiotensin system thus plays an important role in the control of blood pressure and hydromineral balance. In our previous studies (3–7), we elucidated the structures, gene organization and regulation of angiotensinogen and renin with the aid of recombinant DNA techniques. To study how the renin-angiotensin system is involved in the regulation of blood pressure and in the development of hypertension, we have generated transgenic mice in this investigation that are capable of expressing exogenous rat renin or angiotensinogen or both under the control of the mouse metallothionein I (MT-I) promoter. We here report the generation of hypertensive transgenic mice by introducing the renin and angiotensinogen genes.

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EXPERIMENTAL PROCEDURES

Construction of Rat Angiotensinogen and Renin Transgenes. The mouse MT-I/rat angiotensinogen fusion gene (MAG-16) and the mouse MT-I/rat renin fusion gene (MRN-2) illustrated in Fig. 1 were constructed as follows. The 14.2-kilobase-pair (kbp) *Eco*RI–*Hin*dIII fragment containing the rat angiotensinogen gene (4) was subcloned in pBR322, and the 1.5-kbp *Eco*RI–*Xho* I fragment covering the 5′-flanking region of this gene was replaced with the 1.8-kbp *Eco*RI–*Xho* I fragment containing the promoter and the 5′-untranslated sequence of the mouse MT-I gene derived from pMGH (8). Subsequently, the TATA box region of the angiotensinogen gene was removed by digestion with BAL-31 nuclease after cleavage of the above *Xho* I site. For preparation of the renin fusion gene, the *Bst*NI site located 62 bp upstream from the 5′ terminus of the rat renin gene (7) was converted to the *Xho* I site, and the 1.1-kbp *Eco*RI–*Xho* I fragment containing the 5′-flanking region of the renin gene was replaced with the 1.8-kbp *Eco*RI–*Xho* I fragment of pMGH described above. The TATA box region of the renin gene was removed by digestion with BAL-31 nuclease after cleavage of the *Xho* I site. The MT-I/ renin fusion gene was then constructed by connecting the BAL-31-treated *Eco*RI–*Xba* I fragment containing the MT-I gene and renin exon 1, the *Xba* I–*Hin*dIII fragment containing renin exons 2 and 3, and the *Hin*dIII–*Sac* I fragment containing renin exons 4–9.

Pronuclear Microinjection and *in Vitro* Mating. The 14.5-kbp *Eco*RI–*Hin*dIII fragment (MAG-16 in Fig. 1) and the 13.3-kbp *Bam*HI fragment (MRN-2 in Fig. 1) were obtained by sucrose density gradient (5–20%) centrifugation. Pronuclear microinjection was performed by using standard techniques (9). C57BL/6 female mice were superovulated and mated to C57BL/6 or C57BL/6 × BALB/c hybrid males. The fertilized eggs were recovered and male pronuclei were microinjected with the above DNA fragments. Mating between the renin and angiotensinogen transgenic mice was performed by *in vitro* fertilization techniques (10). All animals used were handled in accordance with the guidelines established by the Central Institute for Experimental Animals.

Nucleic Acid Analyses. DNA and RNA blot hybridization analyses were performed as described (5). DNA probes used were the 1097-bp *Acc* I fragment of the rat angiotensinogen cDNA pRag16 (3) and the 748-bp *Rsa* I fragment containing exon 9 of the rat renin gene (7). Primer extension analysis was performed by using 10 μg of total liver RNA and the 65-bp *Pvu* II fragment of pRag16 DNA (3) as a primer according to the method described (5).

Abbreviation: MT-I, metallothionein I.

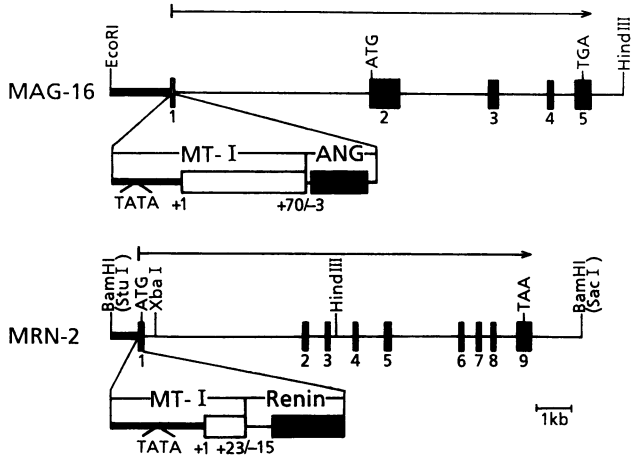

FIG. 1. Structures of the MT-I/angiotensinogen (MAG-16) and MT-I/renin (MRN-2) fusion genes. The MT-I/angiotensinogen fusion gene consists of ≈1.7 kbp of the promoter (thick line) and 70 base pairs (bp) of the 5′-untranslated region (open box) of the mouse MT-I gene and the entire rat angiotensinogen gene extending from 3 bp of the 5′-flanking region up to ≈900 bp of the 3′-flanking region. The MT-I/renin fusion gene is composed of ≈800 bp of the promoter (thick line) and 23 bp of the 5′-untranslated region (open box) of the MT-I gene and the entire rat renin gene, including 15 bp of the 5′-flanking region up to ≈1.5 kbp of the 3′-flanking region. Locations of exons of the rat genes are indicated by numbered boxes; thin lines show introns and flanking sequences of the rat genes. Translation initiation (ATG) and termination (TGA, TAA) codons and relevant restriction sites used for the transgene construction are indicated. The regions fused between the MT-I promoter and rat genes are expanded. ANG, angiotensinogen; TATA, TATA box. The direction of transcription is shown by arrows.

Immunoanalyses of Angiotensinogen and Renin. Plasma concentration of rat angiotensinogen was measured by radioimmunoassay using ^{125}I-labeled angiotensinogen and rabbit antiserum against rat angiotensinogen (11). Immunohistochemical analysis of renin was performed by using rabbit antibody against rat renin (12).

Blood Pressure Measurement. Systolic blood pressure was measured with a programmable sphygmomanometer (PS-200; Riken Kaihatsu, Machida, Japan) using tail cuff methods. Unanesthetized mice were introduced into a small holder mounted on a thermostatically controlled warming plate and maintained at 37–38°C during measurement. Ten readings were taken for each measurement. Statistical analysis for comparison of means of blood pressure was performed by using Student's *t* test.

RESULTS

The mouse MT-I/rat angiotensinogen fusion gene and the mouse MT-I/rat renin fusion gene illustrated in Fig. 1 were microinjected into fertilized mouse eggs, which were then transferred to foster mothers. Fifty-four pups were obtained from 664 eggs injected with the angiotensinogen fusion gene, whereas 120 pups were obtained from 839 eggs injected with the renin fusion gene. Blot hybridization analysis of DNAs isolated from the tails of these mice showed that 16 and 36 mice carried the angiotensinogen and the renin fusion genes, respectively. Expression of the angiotensinogen and renin transgenes of these founder animals was then examined by analysis of total liver RNA (for the procedures of RNA analysis, see Figs. 3 and 4). To induce a maximal expression of the transgenes under the control of the MT-I promoter, individual transgenic mice were supplied with water containing 25 mM $ZnSO_4$ for 2 weeks and then subjected to a partial hepatectomy for the isolation of the liver RNA. The results of the RNA analyses indicated that 10 angiotensinogen and 29 renin transgenic mice were capable of expressing appreciable but varying amounts of the respective mRNAs.

Because the liver is not a major expression site of the renin gene, production of the exogenous rat renin protein in the liver was examined by immunohistochemical analysis using the rabbit antiserum against rat renin. This antibody cross-reacts with mouse renin. Thus, immunohistochemical analysis of the nontransgenic mouse showed that the renin protein is restrictedly located in the juxtaglomerular cells of the kidney and is undetectable in the liver (Fig. 2 *a* and *b*). In contrast, the renin protein was observed in parenchymal and Kupffer cells of the liver of the renin transgenic mouse (Fig. 2*c*). In addition, RNA blot hybridization analysis showed that the rat renin mRNA was expressed in various tissues and responded to $ZnSO_4$ treatment as reported for the control of the MT-I gene expression (13). The results thus indicate that the rat renin is indeed produced in the transgenic mice under the control of the MT-I promoter.

Although the rat renin or angiotensinogen was produced in most of the respective transgenic mice (for angiotensinogen, see Table 2), none of these mice showed blood pressures higher than those of nontransgenic control mice, before or

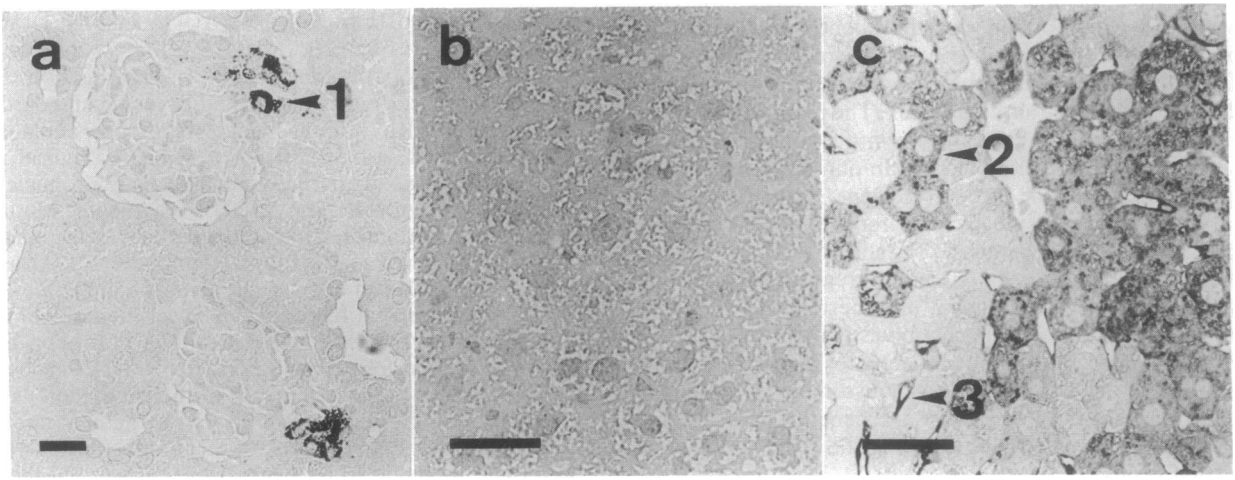

FIG. 2. Immunostaining of sections of mouse tissues with the renin antibody. Sections of the kidney (*a*) and liver (*b*) of a nontransgenic control mouse and of the liver of a renin transgenic mouse (*c*) were incubated with the antiserum against renin, followed by visualization with the peroxidase-conjugated second antibody. Positively immunostained cells (indicated by numbered arrowheads) are as follows: 1, juxtaglomerular cell; 2, parenchymal cell; 3, Kupffer cell. (Bars = 50 μm.)

after the $ZnSO_4$ treatment. We also generated homozygous transgenic mice with respect to the integrated renin or angiotensinogen transgene. However, no significant change in blood pressure was observed for these homozygous transgenic mice.

Since cleavage of angiotensinogen by renin is a crucial step for the production of the bioactive angiotensin, we attempted to generate transgenic mice expressing rat renin and angiotensinogen by mating the respective transgenic mice. Two transgenic lines, lines 108-4 and 84-4, were chosen as representatives of the angiotensinogen and renin transgenic mice, respectively, because the transgene in both lines is highly expressed in the liver and is transmissible in a Mendelian fashion. Because these two lines are heterozygous in terms of the integrated transgene, the mating of these two lines should eventually produce four different groups of siblings—namely, transgenic mice carrying the angiotensinogen and renin transgenes, those carrying one of the transgenes, and those lacking both transgenes. We characterized a total of 68 siblings and classified these mice into four groups on the basis of DNA blot hybridization analysis. Table 1 summarizes data of blood pressure measured for individual siblings at 4–5 months after birth without administration of $ZnSO_4$. This summary indicated that all but 4 animals carrying both transgenes exhibited systolic blood pressures higher than those of the other three groups by an average of 20–30 mmHg in both sexes ($P < 0.01$). Furthermore, the dual transgene carriers, including the above 4 mice, responded to $ZnSO_4$ treatment and showed an average blood pressure of 141.5 ± 8.4 mmHg ($n = 18$) after 1 month of $ZnSO_4$ treatment. In contrast, no such response to the $ZnSO_4$ treatment was observed for the other groups of siblings. These results indicated that the presence of the renin and angiotensinogen transgenes is responsible for the increment in blood pressure of the transgenic mice.

This conclusion was supported by a series of experiments conducted with some representative animals from each of the groups presented in Table 1. The animals at age 8–9 months were supplied with water containing 25 mM $ZnSO_4$ for 1 month, and their blood pressures were measured (see Table 2). Circulating blood and livers of these animals were obtained in order to conduct the following three experiments.

(*i*) The expression of the rat renin mRNA was analyzed by blot hybridization of total liver RNA (Fig. 3*a*). All of the transgenic mice containing the renin transgene (R/− and R/A lines) gave rise to a single hybridization band. The size of this mRNA corresponded exactly to that of the predicted mRNA initiated from the MT-I promoter and then properly processed to form the mature renin mRNA.

(*ii*) The expression of the rat angiotensinogen mRNA was similarly analyzed by RNA blot hybridization (Fig. 3*b*). In this case, all groups of the animals showed a hybridization band, due to cross-hybridization of the rat cDNA probe with the endogenous mouse angiotensinogen mRNA. However, the band observed for the transgenic mice containing the rat angiotensinogen gene (A/− and R/A lines) was more intense than that of the animals lacking this transgene. Furthermore, primer extension analysis was conducted to distinguish between the rat and mouse angiotensinogen mRNAs through the presence of the extended MT-I sequence in the rat mRNA (Fig. 4), and this analysis clearly indicated that the rat angiotensinogen mRNA is expressed to the amount comparable to that of the endogenous mouse mRNA in the transgenic mice bearing the angiotensinogen transgene.

Table 1. Blood pressures of siblings obtained by mating between the renin and angiotensinogen transgenic mice

Renin gene	Angiotensinogen gene: Absence ♂ Mouse	BP, mmHg	Absence ♀ Mouse	BP, mmHg	Presence ♂ Mouse	BP, mmHg	Presence ♀ Mouse	BP, mmHg
Absence	116	101.3 ± 2.2	84	109.8 ± 4.6	65	105.1 ± 2.7	46	102.8 ± 3.4
	120	90.5 ± 1.4	85	112.3 ± 3.0	66	98.4 ± 7.2	47	112.9 ± 3.3
	123	97.7 ± 4.2	88	106.8 ± 5.3	67	109.0 ± 5.6	49	110.3 ± 1.8
	124	91.6 ± 3.3	89	110.2 ± 3.4	69	100.1 ± 2.3	58	108.9 ± 2.5
			91	112.8 ± 2.6	73	101.7 ± 4.6	86	99.7 ± 4.6
			101	108.9 ± 5.0	79	103.9 ± 2.6	94	106.7 ± 5.3
			104	101.9 ± 2.9			97	118.3 ± 4.5
			107	100.9 ± 3.3			105	101.9 ± 5.0
			108	95.9 ± 2.4				
	$n = 4$	95.3 ± 4.4	$n = 9$	106.6 ± 5.5	$n = 6$	103.0 ± 3.5	$n = 8$	107.7 ± 5.8
Presence	113	92.0 ± 1.7	82	105.8 ± 5.2	64	136.1 ± 5.0	39	134.4 ± 5.7
	114	91.6 ± 4.4	87	102.8 ± 3.2	68	119.3 ± 3.0	44	126.9 ± 5.0
	117	96.1 ± 6.9	90	108.0 ± 3.4	70	128.9 ± 6.3	45	125.9 ± 2.5
	119	95.0 ± 3.5	92	116.6 ± 1.7	74	135.1 ± 4.4	48	153.6 ± 6.8
	121	102.4 ± 3.6	95	97.2 ± 3.0	75	129.0 ± 5.9	50	142.8 ± 5.8
	126	94.3 ± 4.5	99	106.9 ± 9.6	76	126.1 ± 7.8	51	128.9 ± 4.5
			102	103.2 ± 4.6	80	128.5 ± 7.2	52	128.2 ± 7.5
			106	106.8 ± 5.1	111	117.9 ± 3.1	53	140.4 ± 5.4
			109	99.6 ± 3.7	112	121.0 ± 7.8	57	135.6 ± 5.2
					122	113.8 ± 3.8	83	132.1 ± 7.3
					127	127.8 ± 9.8	93	132.9 ± 4.0
							96	135.0 ± 4.2
							98	117.7 ± 5.2
							100	112.7 ± 3.5
							103	130.3 ± 4.1
	$n = 6$	95.2 ± 3.6	$n = 9$	105.2 ± 5.3	$n = 11$	125.8 ± 6.7*	$n = 15$	131.8 ± 9.5*

Values of blood pressure (BP) are mean ± SD; *n*, total number of mice.
*$P < 0.01$ vs. other groups of siblings.

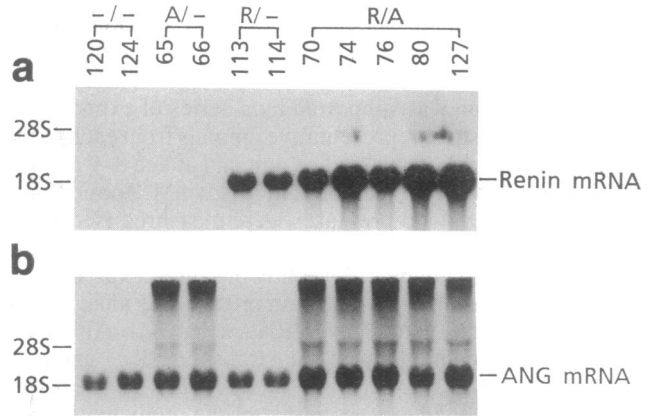

FIG. 3. RNA blot hybridization analysis of rat renin mRNA (*a*) and angiotensinogen mRNA (ANG mRNA) (*b*). A and R indicate the presence of the rat angiotensinogen and renin transgenes in each of the sibling mice, respectively; − shows the absence of the transgene. The numbers indicated correspond to the numbers of the mice listed in Table 1. Twenty micrograms of total liver RNA was analyzed on each lane.

(*iii*) The circulating rat angiotensinogen was measured by radioimmunoassay using the antibody specific for the rat protein (Table 2). The transgenic mice carrying the angiotensinogen transgene showed appreciable amounts of circulating rat angiotensinogen, and angiotensinogen levels in four of the five mice possessing both transgenes were higher than those of the mice carrying the angiotensinogen transgene alone. Although the statistical significance and the mechanism of the observed difference in angiotensinogen levels remain to be investigated, the results obtained indicate that rat angiotensinogen is indeed produced and secreted into the circulation. In addition to the above experiments, expression of the rat angiotensinogen and renin mRNAs in the tissues other than the liver was investigated by RNA hybridization analysis. This analysis showed that both exogenous mRNAs were expressed in various tissues (e.g., kidney, brain, intestines, and pancreas) in accordance with the report of the expression pattern of the MT-I gene (13), indicating that both transgenes undergo control of the MT-I promoter.

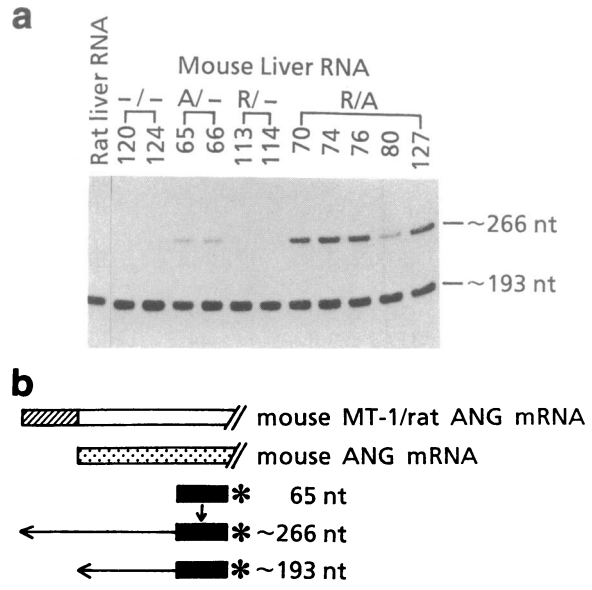

FIG. 4. Primer extension analysis of angiotensinogen mRNAs (ANG mRNAs). The explanation for RNAs analyzed on each lane of the autoradiograph (*a*) is the same as that of Fig. 3. The shaded and white boxes in *b* represent the fused MT-I mRNA and the rat angiotensinogen mRNA, respectively; the dotted box represents the mouse angiotensinogen mRNA. The black boxes indicate the primer used. The asterisks denote the ^{32}P-labeled site. nt, Nucleotides.

Table 2. Plasma concentration of rat angiotensinogen

Line*	Mouse	Angiotensinogen, μg/ml	Blood pressure, mmHg
−/−	120	ND	111.1 ± 4.9
	124	ND	108.2 ± 4.4
A/−	65	7.60	108.9 ± 5.0
	66	6.36	105.4 ± 2.7
R/−	113	ND	92.6 ± 2.6
	114	ND	97.7 ± 2.4
R/A	70	10.32	154.8 ± 4.9
	74	12.60	142.9 ± 8.0
	76	12.24	139.3 ± 7.9
	80	7.21	143.1 ± 6.6
	127	17.44	142.0 ± 4.5

Values of blood pressure are mean ± SD. ND, not detectable.
*A, R, and − designations are defined in the legend to Fig. 3.

Captopril is a potent inhibitor of angiotensin-converting enzyme and is known to reduce hypertensive blood pressure caused by the abnormality of the renin-angiotensin system (2). Thus, the effect of captopril on blood pressure was investigated to examine the involvement of the renin-angiotensin system in the hypertensive transgenic mice. Administration of captopril decreased high blood pressure of the transgenic mice carrying the two transgenes to the normal level within a day (Fig. 5). Thus, all of the results described here indicate that the combined expression of the exogenous rat renin and angiotensinogen is responsible and necessary for the increment in blood pressure of the hypertensive transgenic mice.

DISCUSSION

In this investigation, we have reported the generation of transgenic mice with elevated blood pressure by introducing the rat renin and angiotensinogen genes under the control of the mouse MT-I promoter. Involvement of the exogenous renin-angiotensin system in the increase of blood pressure was verified by effective expression of the two transgenes and the presence of appreciable amounts of circulating rat

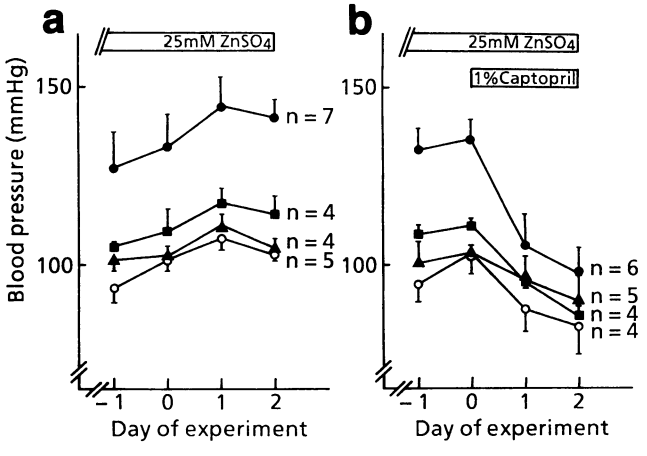

FIG. 5. Effect of captopril on systolic blood pressure of transgenic mice and their siblings. Some R/A (●), A/− (■), and R/− (▲) transgenic mice and nontransgenic mice (○) listed in Table 1 at age 8–9 months were supplied with 25 mM $ZnSO_4$ in drinking water for 1 month. (*a*) The same supply was continued for 2 days. (*b*) After blood pressure measurement on day 0, the animals were provided with water containing 25 mM $ZnSO_4$ and 1% captopril. *n*, Number of animals used. Values represent the mean ± SD.

angiotensinogen in the hypertensive mice. In addition, although the circulating rat renin was not measured because of the cross-reactivity of the antibody with the endogenous mouse renin, production of the rat renin in the liver was proved by immunohistochemical analysis. Furthermore, the effective reduction of elevated blood pressure by captopril supports the conclusion that the elevated blood pressure results from the combined action of two exogenous components of the renin-angiotensin system.

It is notable that introduction of either the renin or the angiotensinogen gene did not increase blood pressure in the resultant transgenic mice. It has been reported that mouse angiotensinogen is not cleaved by rat renin (14). Thus, one possible explanation for the above observation is that the combination of two homologous components is necessary for the effective production of the biologically active angiotensin. However, it has also been reported that rat angiotensinogen is cleaved by mouse renin (14). Thus, alternatively, feedback regulation of the natural gene may occur when the other component is elevated by introduction of its transgene, and the control of the two transgenes by the MT-I promoter may be responsible for elevating blood pressure in the hypertensive transgenic mice.

Noteworthy also is the observation that the increase in blood pressure of our transgenic mice is modest compared to that of hypertensive rats, such as spontaneously hypertensive rats (15) and Dahl salt-sensitive hypertensive rats (16), which both show >180 mmHg of blood pressure. Interestingly, genetically hypertensive mice (17) developed in a manner similar to the spontaneously hypertensive rats show 140–150 mmHg of blood pressure, which is comparable to the values of our transgenic mice. This difference between mouse and rat hypertension may reflect a species-specific difference in responsiveness to the elevation of blood pressure. Alternatively, because control of blood pressure is thought to be a polygenic trait, additional genetic factors may be required for elevating blood pressure to the maximal level.

The transgenic mice we developed will provide a unique opportunity to investigate not only the functions of the renin-angiotensin system *in vivo* but also the molecular and biochemical nature of hypertension that is caused by known genetic factors. Furthermore, the approach described here will be applicable to the generation of hypertensive animals involved in the human renin-angiotensin system and will provide a valuable model system for biochemical studies of human hypertension.

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