Isolation of Preferentially Expressed Genes in the Kidneys of Hypertensive Rats

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By differential hybridization, three complementary DNAs designated as S3, S2, and SA were isolated, and the corresponding messenger RNAs (mRNAs) were differentially expressed between the kidneys of spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats. S3 is identical to cytochrome P450 IV A2. SA encoded a protein of 546 amino acid residues, and its carboxyl terminal region had a slight homology to luciferase. No homologous sequence has been reported in S2 sequences. S3 mRNA was about four times more abundantly expressed in the kidneys of 28-day-old SHR than in those of age-matched WKY rats, but there was no difference at age 16 weeks. A low NaCl diet positively modulated the expression of the S3 gene. S2 mRNA was almost undetectable in the kidneys of 28-day-old WKY rats but was clearly detected in those of age-matched SHR. The expression level of S2 mRNA in the livers of 16-week-old SHR was about five times higher than that of age-matched WKY rats. The expression of S3 mRNA in the livers was modulated by dietary NaCl and captopril. S2 mRNA was more than 10 times more abundantly expressed in the kidneys of SHR than in those of WKY rats from age 4 weeks. With the administration of captopril, the expressions of S2 mRNA in the livers of SHR were positively modulated. Because these three genes are not only differentially expressed between SHR and WKY rats but also related to sodium metabolism or blood pressure control, the identification of these genes may provide important probes to examine the mechanisms of hypertension. (Hypertension 1991;17:161-169)

Methods

Animals

Male SHR and male WKY rats were obtained from Taconic Farms (Germantown, N.Y.). Ten-day-old and 28-day-old male rats were derived from pregnant rats obtained from Taconic Farms. Male Sprague-Dawley (SD) rats (225-250 g or 275-300 g), male Dahl salt-sensitive (DS) and salt-resistant (DR) rats of the Brookhaven strain were obtained from Harlan Sprague Dawley, Inc., Indianapolis, Ind. A high NaCl (3.15%) and a low NaCl (0.02%) rat chow were obtained from Ralston Purina Co. (Richmond, Ind.). Captopril (100 mg/kg/day) was dissolved in drinking water. Water consumption was monitored and the concentration of each drug was adjusted to provide the dose level mentioned above.

Isolation of Complementary DNA Clones

Two rat kidney complementary DNA (cDNA) libraries were prepared from poly(A)+ RNA of the kidneys of 9-week-old SD rats and 16-week-old SHR according to the method of Gubler and Hoffman3 using a cDNA synthesis kit and a cloning kit (Amer sham Corp., Arlington Heights, Ill.) with minor modifications. cDNA fragments of more than 500 base pairs were selected on a 1.1% low-melting agarose gel and ligated to Agt10 arms.
Five hundred to 1,000 plaques containing more than 90% recombinant clones were plated on one plate 15 cm in diameter, and four replica filters (Hybond, Amersham) were prepared from one plate. Up to 7,500 clones were screened in each library. Two filters were hybridized to the cDNA probes prepared from SHR kidney messenger RNA (mRNA), and the other two were hybridized to those prepared from WKY rat kidney mRNA. The cDNA probes were the single strand antisense cDNA fragments labeled uniformly with [α-32P]dCTP and were synthesized by avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) from the template mRNA using oligo(dT)12-18 as a primer, as described by Davis et al.6 Hybridization and washing conditions were the same as those in Northern blotting analysis. On the assumption that most of the genes were equally expressed between the kidneys of SHR and WKY rats, the clones that evidently gave a different signal intensity were selected and subjected to further analyses. The clones that gave a different signal intensity in the one set but not in the other set were not studied.

RNA Preparation and Analysis

Total cellular RNA was isolated according to the method of Chirgwin et al. Poly(A)+ RNA was purified over an oligo(dT) cellulose column (New England Biolabs, Beverly, Mass.). The insert cDNA fragments were labeled with [α-32P]dCTP by nick translation. The specific activity of nick-translated probes was 1.0-3.0×10^8 cpm/μg. For Northern blotting analysis, RNA was denatured by glyoxal, electrophoresed in 1.1% agarose gel, and transferred to Gene Screen Plus (New England Nuclear, Boston, Mass.) in 10x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). The blot analysis was performed using Bio Dot SF apparatus (Bio Rad, Bethesda, Md.) according to the manufacturer’s recommended method.

Hybridization was performed for 24–48 hours at 42°C in 50% formamide, 6x SSC, 50 mM sodium phosphate (pH 7.0), 1% sodium dodecyl sulfate (SDS); 5x Denhardt’s solution (0.1% Ficoll, 0.1% polyvinyl-pyroridone, and 0.1% bovine serum albumin [BSA]) and 100 μg/ml denatured salmon sperm DNA. Filters were washed at 63°C in 2x SSC, containing 1% SDS and then in 0.2x SSC containing 1% SDS for 30–45 minutes each. For rehybridization, filters were washed in boiling water containing 0.1% SDS for 5 minutes. The β-actin probe was a synthetic 55-oligomer corresponding to the rat β-actin gene second exon, kindly provided by Drs. Y. Morimoto and Y. Teranishi (Mitsubishi Chemical Industries, Inc., Kanagawa, Japan). The β-actin probe was phosphorus-32-labeled by T4 polynucleotide kinase according to Maniatis et al.8 When filters were hybridized to a β-actin probe, the filters were washed at 42°C in 2x SSC containing 1% SDS and then in 0.4x SSC containing 1% SDS for 30–45 minutes each. Filters were exposed to Kodak X-OMAT AR with intensifying screens (Quanta III) at −80°C. The relative signal intensities were quantitated by laser scanning densitometer (LKB, Broma, Sweden).

DNA Preparation and Analysis

High molecular weight DNA prepared from SHR and WKY rat spleens according to the method of Maniatis et al.8 were digested with a large excessive amount of various restriction enzymes, separated on 0.7% agarose gel, and transferred to Gene Screen Plus. Hybridization conditions were identical to the Northern analysis as previously described. Filters were washed at 63°C in 2× SSC containing 1% SDS for 45 minutes and then at 63°C in 0.1× SSC containing 0.1% SDS for 45 minutes. All filters were rehybridized to rat atrial natriuretic factor (ANF) cDNA9 to neglect the possible partial digestion of DNAs.

Sequencing

After subcloning various fragments into pUC19, sequence analysis was performed on both strands directly from double strand plasmid using Sequenase sequencing kit (United States Biochemical Corp., Cleveland, Ohio). Other standard techniques in molecular biology were performed according to Maniatis et al.8

Statistical Analysis

Statistical analyses were done according to the Mann-Whitney U test.10

Results

Isolation of Differentially Expressed Genes

The methodology of differential plaque filter hybridization was used to isolate the genes, the corresponding mRNAs of which were differentially expressed between the kidneys of SHR and WKY rats. Three cDNA clones were identified in a cDNA library prepared from 9-week-old SD rat kidney by hybridization with the cDNA probes prepared from the kidneys of 28-day-old SHR and WKY rats. Two of these three clones corresponded to the identical gene, which was designated as S2, and the gene corresponding to the other clone was designated as S3. The Northern blotting analyses of S2 and S3 mRNA are shown in Figures 1 and 2. The expression level of S3 mRNA in 28-day-old SHR was about four times higher than that of age-matched WKY rats (p<0.05). The expression of S3 mRNA was almost undetectable in 28-day-old WKY rats, but it was clearly detectable in SHR (p<0.05).

Another clone was obtained from a cDNA library prepared from 16-week-old SHR kidneys by hybridization with the cDNA probes prepared from the kidneys of 16-week-old SHR and WKY rats (Figure 3). This clone, designated as S4, corresponded to the mRNA species whose expression level in 16-week-old WKY rats was very low (below one tenth) as compared with that in 16-week-old SHR (Figure 4A). Some WKY rats had longer S4 mRNAs (Figure 4B).
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Developmental Analyses of Expressions of $S_3$, $S_2$, and $S_3$ Genes

The expression of the $S_3$ gene was not detected in the kidneys of 10-day-old SHR and WKY rats, and the expression levels in the 16-week-old rats were much higher than those in the 28-day-old rats in both strains (Figure 2). At age 16 weeks, no statistically significant difference was observed between the expression levels of $S_2$ mRNA in the kidneys of SHR and WKY rats (data not shown).

The expression of $S_2$ gene is also developmentally regulated. Even at age 10 days, the expression of $S_2$ mRNA was higher in the kidneys of SHR than in those of WKY rats (Figure 5). However, at age 16 weeks, no difference was observed between the expression levels of $S_2$ mRNA in the kidneys of SHR and WKY rats (data not shown).

The expression level of $S_3$ gene in the kidneys of SHR was dramatically increased from the age 28 days (Figure 6). On the other hand, the expression level of $S_3$ gene in the kidneys of WKY rats was constantly low throughout the ages examined.

Tissue Distribution of $S_3$, $S_2$, and $S_3$ Messenger RNA

The expression of these genes was investigated in several tissues including atria, ventricles, lungs, livers, adrenals, spleens, and brains. The expression of $S_3$ gene was detected in the livers of 16-week-old SHR but was not detected in the livers of 16-week-old WKY rats (Figure 7). The expression of $S_2$ gene was also detected in the livers (Figure 8). The expression level of $S_2$ mRNA in 16-week-old SHR livers was about five times higher than that in age-matched WKY rat livers. A low level expression of $S_3$ gene was observed in the liver (data not shown). The expression of these three genes was not observed in other tissues examined.

Regulation of $S_2$ and $S_3$ Gene Expressions

Because the differential expressions alone may not provide sufficient evidence for the involvement of the genes in the pathogenesis of SHR hypertension, the effect of dietary sodium on the expressions of $S_2$ and $S_3$ genes were investigated to clarify the possible involvement of these genes in sodium metabolism.
The expression of \( S_1 \) gene in the kidney was positively modulated up to 2.5-fold by low NaCl diet feeding for 2 weeks (Figure 9) \((p<0.05)\). This result was reconfirmed by \( S_1 \) nuclease mapping analysis (data not shown).

The expressions of \( S_2 \) gene in the kidney was not modulated by low NaCl diet feeding for 2 or for 4 weeks (data not shown). Conversely, the expression of \( S_2 \) gene in the liver was positively modulated by low NaCl diet feeding for 2 weeks (Figure 10A) \((p<0.05)\). This positive modulation was blocked by the administration of captopril (Figure 10B). The administration of deoxycorticosterone (DOC) decreased the expression level of \( S_2 \) gene in the liver (data not shown).

Regulation of \( S_A \) Gene Expression

The expressions of \( S_A \) gene in the kidneys of SD rats were varied among individual SD rats. In some SD rats, the expressions of \( S_A \) gene in the kidneys were hardly detected. Therefore, SD rats seemed not to be appropriate models for studying the regulation of \( S_A \) gene. This is also the case in WKY rats as shown in Figure 3. Therefore, SHR and Dahl rats, in which individual variance in the expression level of \( S_A \) gene was small in our preliminary experiments, were used.

The expression level of \( S_A \) mRNA in the kidneys of DS rats was much higher than that of DR rats (Figure 11) \((p<0.05)\). Although the expression of \( S_A \) gene in the kidneys of DS rats was not modulated by low NaCl diet feeding for 4 weeks, that of DR rats
The expression of $S_A$ gene in SHR kidneys was not modulated by low NaCl diet feeding for 2 or 4 weeks (data not shown).

The effect of the administration of captopril on the expression level of $S_A$ gene was investigated in SHR. The development of high blood pressure was completely blocked by the administration of captopril from age 4 weeks to age 9 weeks. The blood pressure of the control group was 162±8.5 mm Hg ($n=4$) and that of the captopril-treated group was 129±5 mm Hg ($n=4$).

Although the expression level of $S_A$ mRNA in the kidney was slightly increased (1.5-fold) by the administration of captopril (data not shown), that in the liver was increased up to 2.5-fold (Figure 12) ($p<0.05$).

**Sequence Analyses and Southern Blot Analyses**

The sequence analysis of the $S_3$ clone revealed that this clone is identical to the rat cytochrome P450 IV A2 mRNA.$^{11,12}$ The total of high blood pressure was completely blocked by the administration of captopril from age 4 weeks to age 9 weeks. The blood pressure of the control group was 162±8.5 mm Hg ($n=4$) and that of the captopril-treated group was 129±5 mm Hg ($n=4$).

Although the expression level of $S_A$ mRNA in the kidney was slightly increased (1.5-fold) by the administration of captopril (data not shown), that in the liver was increased up to 2.5-fold (Figure 12) ($p<0.05$).

The $S_2$ clone encoded a long open reading frame as shown in Figure 15A and 15B. The underlined ATG was in good agreement with the Kozak consensus sequence,$^{13}$ and was also preceded by in-frame termination codons. The putative protein comprises 546 amino acid residues (Figure 15B). The underlined region has slight homology to photinus luciferin monooxygenase$^{14}$ (29.9% identity), 4-coumarate-

**Figure 7.** Expression of $S_A$ gene in livers from spontaneously hypertensive rats (SHR). Five (upper slot) and 15 (lower slot) micrograms of total liver RNAs, which were derived from three individual 16-week-old SHR and three individual 16-week-old Wistar-Kyoto (WKY) rats, were slot blotted.

**Figure 8.** Expression of $S_2$ gene in the livers. Five (upper slot) and 15 (lower slot) µg of total liver RNAs, which were derived from four 16-week-old spontaneously hypertensive rats (SHR) and four 16-week-old Wistar-Kyoto (WKY) rats, were slot blotted.

**Figure 9.** Modulation of $S_3$ gene expression by dietary NaCl. Twenty micrograms of total kidney RNAs were used, which were derived from three Sprague-Dawley (SD) rats fed with high salt diet chow for 2 weeks (H) and three SD rats fed with low salt diet chow for 2 weeks (L). Filter was rehybridized by a $\beta$-actin probe (lower panel).

**Figure 10.** Modulation of $S_2$ gene expression in the livers by dietary NaCl and by captopril. Panel A: Twenty micrograms of total liver RNAs were used, which were derived from six Sprague-Dawley (SD) rats (225–250 g) fed with high salt diet for 2 weeks (H) and six SD rats fed with low salt diet for 2 weeks (L). Aliquots of samples were separately electrophoresed in the same order and then were stained with ethidium bromide (lower panel). Panel B: Six SD rats (275–300 g) were fed with low sodium diet for 2 weeks. For the last 3 days, three rats were treated with captopril (L+Cap), and the other three rats (L) continued to drink normal water. Five (upper slots) and 15 (lower slots) µg of total liver RNAs were slot blotted.
CoA ligase\textsuperscript{15} (30.3\% identity), and enterobactin synthetase component E\textsuperscript{16} (30.3\% identity).

The Southern blot analysis of $S_4$ cDNA is shown in Figure 16. The restriction fragment length polymorphisms were observed between SHR and WKY rats by Pst I, Kpn I, HindIII, EcoRl, and Apa I.

The summary of the results is shown in Table 1. All experiments have been done at least twice to confirm the results.

**Discussion**

By applying the methodology of differential plaque filter hybridization, we have isolated three cDNA clones that hybridize to the mRNA species that are differentially expressed between the kidneys of SHR and WKY rats.

One of the problems in this approach is that the differential expression does not necessarily ensure that the gene is involved in the pathogenesis of hypertension in SHR. To gain insight into possible roles of these genes, the effects of dietary NaCl or captopril on the expression of these genes were studied. Although the modulations of a gene expression by dietary NaCl or captopril do not necessarily mean that the gene is involved in or has a direct effect on body fluid homeostasis or blood pressure control, it does provide evidence for the intimate relation of the gene with factors that are modulated by dietary NaCl or captopril. If the expression of a gene that is different between SHR and WKY rats were modulated by dietary NaCl or captopril, we could infer that the analysis of the regulation of the gene would provide useful insights into the pathogenesis of hypertension in SHR.

The sequence analysis of $S_3$ cDNA revealed that it encoded rat cytochrome P450 IV A2 mRNA.\textsuperscript{11,12} Although the specific substrate for the product of P450 IV A2 has not been determined, other members of this family (P450 IV) were noted to have $\omega$-hydroxylase activity on arachidonic acid, prostaglandin (PG) E\textsubscript{1}, PGE\textsubscript{2}, PGA\textsubscript{2}, and PGA\textsubscript{2}.\textsuperscript{17-19} The metabolites of arachidonic acid by the renal cytochrome P450-dependent monooxygenase system have been known to have important contributions to the regulation of salt and water excretion in the kidneys.\textsuperscript{20,21} This strongly suggests, although not providing direct evidence, that P450 IV A2 has some roles in water and electrolyte metabolism in the kidney. Furthermore, because the expression of P450 IV A2 mRNA in the kidney was positively modulated by low NaCl diet feeding, this enzyme may have some roles in reducing sodium excretion from the kidneys. This speculation may be supported by the fact that young SHR, in which P450 IV A2 mRNA in the kidney is more abundantly expressed than in age-matched WKY rats, retain more urinary sodium than age-matched WKY rats.\textsuperscript{22}

Although the difference in the expression of $S_2$ gene in the kidney between SHR and WKY rats was evident only at age 28 days and disappeared at age 16 weeks, the difference between the livers of SHR and WKY rats was evident even at age 16 weeks.

Because the expression of $S_2$ gene in the liver was positively modulated by low NaCl diet feeding, the expression of this gene in the liver may be regulated by some factors that are induced in the sodium-depleted state, and this positive modulation by low NaCl diet feeding was blocked by the administration of captopril. This result suggests that the expression of $S_2$ gene in the livers of SD rats is dependent on the renin-angiotensin-aldosterone system. Because the expression level
FIGURE 13. Southern blot analysis of $S_3$ (panel A) and $S_2$ (panel B). High molecular weight DNAs obtained from spontaneously hypertensive rats (SHR) (left lane) and Wistar-Kyoto (WKY) rats (right lane) spleens were digested with Pst I (P), Sac I (S), HindIII (H), and EcoRI (E) and were electrophoresed on 0.7% agarose gel. Molecular weight marker was HindIII-digested DNA.

FIGURE 14. Sequence analysis of $S_2$ clone.

The difference in the expression of the $S_A$ gene between SHR and WKY rats is most marked among the three genes, and as shown in Figure 4B, some WKY rats express the longer $S_A$ mRNA in the kidneys. Because more than two bands were detected with every one of the restriction enzymes used in the Southern blot analysis with $S_A$ cDNA as a probe, another sequence closely related to $S_A$ gene may exist in the rat genome. Thus, the mRNA species detected in SHR livers may not necessarily be the transcripts of $S_A$ gene. Even in the kidney, at least three mRNA species of different lengths were detected (Figure 4B). Further study will be necessary to clarify these problems.

Because the expression levels of the $S_A$ gene in the kidneys of SD rats and WKY rats were varied among individual rats, it seemed very difficult to assess the regulation of $S_A$ gene in the kidney by using these two strains. Therefore, SHR, DR, and DS rats were used in which individual variances seemed to be small in our preliminary experiments.

Interestingly, the expression levels of $S_A$ gene in the kidneys of DS rats were almost comparable with those of SHR, and those of DR rats were comparable with those of WKY rats. Although the modulation of $S_A$ gene expression in the kidney by dietary NaCl was

<table>
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<th>Gene</th>
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<th>Number in 10,000 clones</th>
<th>SHR-WKY</th>
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<td>S&gt;W</td>
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<td></td>
<td>16W</td>
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<td>S&gt;W</td>
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<td>8</td>
<td>S&gt;W</td>
<td>H=L (SHR)</td>
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SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; Insert, the length of complementary DNA (cDNA) of each clone; Number in 10,000 clones, the number of positive clones that hybridized to each cDNA fragment in the kidney cDNA library prepared from 16-week-old SHR; ND, not detected; DR, observed in Dahl salt-resistant strain.
not observed in SHR and DS rats, it was regulated negatively in DR rats when they were fed a high salt diet for 4 weeks. In the livers of SHR, SA gene expression was positively modulated by the administration of captopril for 5 weeks. These facts, although not conclusive, strongly suggest that the expression of SA gene is regulated or influenced by some factors that have an intimate relation with the sodium metabolism or the blood pressure control.

Although the carboxy terminal region of the deduced amino acid sequences of the SA clone has slight homology to photinus luciferin monooxygenase,14 the precise function of the SA gene product remains to be determined. However, the segregation analysis using the restriction fragment length polymorphisms observed in SA genes between SHR and WKY rats will determine whether the SA gene is responsible for hypertension in SHR.

Finally, we would like to comment on the methodological aspect of the present study wherein we have used a simple +/- screening method and not a subtractive screening method.23 That is, only relatively high abundantly expressed mRNAs were screened. Moreover, we have not screened a kidney cDNA library prepared from WKY rats. Therefore, it is highly likely that there are an additional number of genes that are differentially expressed between the kidneys of the two strains. Further sophisticated study may be necessary.

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