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 S₄ 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. S₂ 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. S₃ 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 S₃ 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 Hoffman using a cDNA synthesis kit and a cloning kit (Amersham 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 \([\alpha -32P]dCTP\) 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.\(^7\) Poly(A)\(^+\) RNA was purified over an oligo(dT) cellulose column (New England Biolabs, Beverly, Mass.). The insert cDNA fragments were labeled with \([\alpha -32P]dCTP\) 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. 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) cDNA\(^9\) to neglect the possible partial digestion of DNAs.

**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) cDNA\(^9\) to neglect the possible partial digestion of DNAs.

**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 S\(_3\) and the gene corresponding to the other clone was designated as S\(_2\). The Northern blotting analyses of S\(_1\) and S\(_2\) mRNA are shown in Figures 1 and 2. The expression level of S\(_3\) mRNA in 28-day-old SHR was about four times higher than that of age-matched WKY rats (p<0.05). The expression of S\(_1\) 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 S\(_4\), 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 S\(_4\) mRNAs (Figure 4B).
FIGURE 1. Northern blot of S3. Twenty micrograms of total kidney RNAs were used, which were derived from three individual 28-day-old male spontaneously hypertensive rats (SHR) and three individual 28-day-old male Wistar-Kyoto (WKY) rats. Positions of ribosomal RNAs (28s, 18s) are indicated. Exposure time was 3 hours. Filter was rehybridized to a β-actin probe (lower panel).

Developmental Analyses of Expressions of S3, S2, and S3 Genes

The expression of the S3 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 S2 mRNA in the kidneys of SHR and WKY rats (data not shown).

The expression of S3 gene is also developmentally regulated. Even at age 10 days, the expression of S3 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 S3 mRNA in the kidneys of SHR and WKY rats (data not shown).

The expression level of S3 gene in the kidneys of SHR was dramatically increased from the age 28 days (Figure 6). On the other hand, the expression level of S3 gene in the kidneys of WKY rats was constantly low throughout the ages examined.

FIGURE 2. Northern blot of S3. Twenty micrograms of total kidney RNAs were used, which were derived from three individual 28-day-old spontaneously hypertensive rats (SHR), three individual 28-day-old Wistar-Kyoto (WKY) rats, one 16-week-old SHR and one 16-week-old WKY rat. Positions of ribosomal RNAs were indicated. Exposure time was 6 hours. Filter was rehybridized to β-actin probe (lower panel).

Tissue Distribution of S3, S2, and S3 Messenger RNA

The expression of these genes was investigated in several tissues including atria, ventricles, lungs, livers, adrenals, spleens, and brains. The expression of S3 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 S2 gene was also detected in the livers (Figure 8). The expression level of S2 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 S3 gene was observed in the liver (data not shown). The expression of these three genes was not observed in other tissues examined.

Regulation of S2 and S3 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 S2 and S3 genes were investigated to clarify the possible involvement of these genes in sodium metabolism.

FIGURE 3. Isolation of S3. Four replica filters were prepared from one plate. Two filters were hybridized to the complementary DNA probe prepared from the kidneys of 16-week-old Wistar-Kyoto (WKY) rats (panel A), and the other two were hybridized to those prepared from the kidneys of 16-week-old spontaneously hypertensive rats (SHR) (panel B). Clone S3 is indicated by circle. Exposure time was 24 hours.
The expression of S3 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 S1 nuclease mapping analysis (data not shown).

The expressions of S2 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 S2 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 S2 gene in the liver (data not shown).

Regulation of S4 Gene Expression

The expressions of S4 gene in the kidneys of SD rats were varied among individual SD rats. In some SD rats, the expressions of S4 gene in the kidneys were hardly detected. Therefore, SD rats seemed not to be appropriate models for studying the regulation of S4 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 S4 gene was small in our preliminary experiments, were used.

The expression level of S4 mRNA in the kidneys of DS rats was much higher than that of DR rats (Figure 11) (p<0.05). Although the expression of S4 gene in the kidneys of DS rats was not modulated by low NaCl diet feeding for 4 weeks, that of DR rats...
was positively modulated up to 2.5-fold by low NaCl diet feeding for 4 weeks ($p<0.05$).

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 Southern blot analysis of S$_3$ cDNA is shown in Figure 13A. As was reported, multiple bands were observed, which means the S$_3$ gene is one of the members of a multigene family.

An approximately 500-nucleotide sequence was determined in the S$_2$ clone, and no homologous sequence was found in Genbank and EMBL data bases. The partial nucleotide sequence and the deduced amino acid sequence are shown in Figure 14. Only this frame can encode a long open reading frame. The Southern blot analysis of S$_2$ cDNA is shown in Figure 13B. Since the 3' portion of S$_2$ cDNA contained repetitive sequence, a 1.0 kb 5' portion of S$_2$ cDNA was used as a probe. No genetic polymorphism was observed between SHR and WKY rats in both S$_2$ and S$_3$ genes.

The S$_A$ 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-
CoA ligase$^{15}$ (30.3% identity), and enterobactin synthetase component E$^{16}$ (30.3% identity).

The Southern blot analysis of $S_A$ cDNA is shown in Figure 16. The restriction fragment length polymorphisms were observed between SHR and WKY rats by $Pst$ I, $Kpn$ I, $HindIII$, $EcoRI$, 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.$^{11,12}$ 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) E1, PGE2, PGA1, and PGA2.$^{17-19}$ 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.$^{20,21}$ 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.$^{22}$

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, 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

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**Figure 11.** Expression of $S_A$ gene in Dahl rats. Twenty micrograms of kidney total RNAs were used. DS-L, Dahl salt-sensitive rats fed with low salt diet for 4 weeks; DS-H, Dahl salt-sensitive rats fed with high salt diet for 4 weeks; DR-L, Dahl salt-resistant rats fed with low salt diet for 4 weeks; DR-H, Dahl salt-resistant rats fed a high salt diet for 4 weeks; W, 16-week-old Wistar-Kyoto rats; S, 16-week-old spontaneously hypertensive rats. Salt feeding experiments were started with 6-week-old Dahl rats. Filter was rehybridized to a $\beta$-actin probe (lower panel).

**Figure 12.** Modulation of $S_A$ gene expression in livers from spontaneously hypertensive rats (SHR) by captopril. Twenty micrograms of total liver RNAs were used, which were derived from four control SHRs (−) and four SHR treated with captopril for 5 weeks (+). Aliquots of the samples were separately electrophoresed and were stained with ethidium bromide.
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Figure 13. Southern blot analysis of S3 (panel A) and S2 (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.

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 1. Summary of Results

<table>
<thead>
<tr>
<th>Gene</th>
<th>Insert (kb)</th>
<th>Number in 10,000 clones</th>
<th>SHR-WKY</th>
<th>High-low/captopril</th>
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<td></td>
<td></td>
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<td>Kidney</td>
<td>Liver</td>
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<td>S3</td>
<td></td>
<td></td>
<td>4W</td>
<td>S&gt;W</td>
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<td></td>
<td></td>
<td>S&gt;W</td>
<td>S=W</td>
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<tr>
<td></td>
<td>0.6</td>
<td></td>
<td>16W</td>
<td></td>
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<tr>
<td></td>
<td>1.8</td>
<td>80</td>
<td>S=W</td>
<td></td>
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<tr>
<td>S2</td>
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<td>4W</td>
<td>4W</td>
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<td></td>
<td>S&gt;W</td>
<td>ND</td>
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<td>16W</td>
<td>16W</td>
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<td>1.5</td>
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<td>S&gt;W</td>
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</table>

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.
FIGURE 15. Sequence analysis of Sα clone. Panel A: Nucleotide sequence of Sα complementary DNA (cDNA). Putative initiation codon (ATG) and termination codon (TAA) are underlined. Panel B: Deduced amino acid sequence of Sα cDNA.

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, Sα gene expression was positively modulated by the administration of captopril for 5 weeks. These facts, although not conclusive, strongly suggest that the expression of Sα 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 sequence of the Sα clone has slight homology to photinus luciferin monooxygenase, the precise function of the Sα gene product remains to be determined. However, the segregation analysis using the restriction fragment length polymorphisms observed in Sα genes between SHR and WKY rats will determine whether the Sα 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. 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.

Acknowledgments

We are very grateful to Drs. Y. Morimoto and Y. Teranishi (Mitsubishi Chemical Industries, Inc.) for...
providing us with a β-actin probe, and to Doris Bruce-Bloss for typing the manuscript.

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Key Words • kidney • spontaneously hypertensive rats • cytochrome P450 • captopril • Dahl rats • luciferin
Isolation of preferentially expressed genes in the kidneys of hypertensive rats.
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Hypertension. 1991;17:161-169
doi: 10.1161/01.HYP.17.2.161

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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