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Research Article

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The $\alpha 1$ Na,K-ATPase Gene Is a Susceptibility Hypertension Gene in the Dahl Salt-sensitive^{HSD} Rat

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Abstract

Despite the prevalence of essential hypertension, its underlying genetic basis has not been elucidated due to the complexities of its determinants. To identify a hypertension susceptibility gene, we used an approach that integrates molecular, transgenic, and genetic analysis using Dahl salt-sensitive (S) and Dahl salt-resistant (R) rats ascertained for genotype and phenotype. To determine the role of the Dahl S Q276L $\alpha 1$ Na,K-ATPase gene variant, we developed transgenic Dahl S rats bearing the Dahl R wild-type (wt) $\alpha 1$ Na,K-ATPase cDNA directed by the cognate wt promoter region, Tg[wt $\alpha 1$]. Transgenic Dahl S rats exhibited less salt-sensitive hypertension, less hypertensive renal disease, and longer life span when compared with non-transgenic Dahl S controls. Total chromosome 2 linkage analysis of F2(S \times R) male rats detects cosegregation of the $\alpha 1$ Na,K-ATPase locus with salt-sensitive hypertension. These data support the $\alpha 1$ Na,K-ATPase gene as a susceptibility gene for salt-sensitive hypertension in the Dahl S rat model, and provide the basis for the study of the $\alpha 1$ Na,K-ATPase locus in human hypertension. (*J. Clin. Invest.* 1998; 102:1102–1111.) Key words: hypertension • genetics • Na,K-ATPase • transgenic model • cosegregation analysis

Introduction

Essential hypertension (EHT)¹ is a paradigmatic, complex, and multifactorial condition. Genes that mediate EHT will therefore be difficult to isolate and characterize, requiring multiple lines of evidence to prove their roles in EHT pathogenesis. Cognizant of these issues, delineation of a putative

EHT susceptibility gene should meet the following criteria: criteria 1, identification of a functionally significant structural mutation in the relevant gene; criteria 2, concordance of the observed genetic dysfunction with a pathophysiologic mechanism logical to the hypertension pathogenesis; criteria 3, association of the putative hypertension susceptibility gene with hypertension in validated genetic animal models or human hypertensive patients; and criteria 4, delineation of the mechanistic role in an in vivo model (1, 2). To date, no EHT susceptibility gene has been identified that meets all these criteria.

To simplify the molecular genetic characterization of an EHT susceptibility gene, we focused on one subtype of EHT, salt-sensitive hypertension (SS-EHT). We tested the hypothesis that variants of the $\alpha 1$ Na,K-ATPase gene mediate SS-EHT in a genetic rat model of hypertension, using the Dahl S hypertensive rat strain (3, 4). Because $\alpha 1$ Na,K-ATPase is the sole active Na⁺ transporter in the renal basolateral epithelia throughout the nephron (5, 6), it is a logical candidate gene to be considered in the assessment of the abnormal renal sodium handling in the Dahl S rat (7). Two of the stated four criteria (1, 2) required to define the $\alpha 1$ Na,K-ATPase gene as an EHT susceptibility gene have been met. For criteria 1, a Q276L substitution in the $\alpha 1$ Na,K-ATPase gene in inbred Dahl S rats from Harlan Sprague Dawley, Inc. (Indianapolis, IN) (Dahl S^{HSD}) has been characterized (8, 9). In contrast to the non-detection by PCR sequencing reported by Simonet et al. (10), the Q276L $\alpha 1$ Na,K-ATPase variant was confirmed in Dahl S genomic DNA by using PCR error-independent assays (polymerase allele specific amplification, PASA, and 3' mismatch correction assay) and ligase chain reaction assay; in kidney RNA by RT^h-PCR; and in cDNA clones by resequencing (9). Likewise, detection of the wild-type (wt) Q276 sequence (11) was confirmed in Dahl R genomic DNA and in resequenced cDNA clones (9).

This mutation results in decreased K⁺ (⁸⁶Rb⁺) influx detected in *Xenopus* oocyte expression experiments using both Dahl S kidney polyA⁺ RNA, as well as in vitro transcribed variant Q276L-specific cRNA transcript in contrast to control Dahl R rat kidney polyA⁺ RNA and in vitro transcribed wt Q276 cRNA transcript, respectively (8). Kinetic studies of $\alpha 1$ Na,K-ATPases in red blood cell flux experiments comparing Dahl S and Dahl R $\alpha 1$ Na,K-ATPases corroborated decreased K⁺ (⁸⁶Rb⁺) influx, and revealed normal Na⁺ transport resulting in an increased Na:K coupling ratio in the Dahl S Q276L $\alpha 1$ Na,K-ATPase variant (12). For criteria 2, simulated modeling studies have revealed that consequences of an increased Na:K coupling ratio (from 3:2 to 3:1) observed in the Q276L $\alpha 1$ Na,K-ATPase variant results in an altered set point for cellular Na⁺ metabolism, with higher sodium reabsorption at unchanged Na,K-ATPase levels in the proximal convoluted tubule, as well as in the thick ascending limb of the loop of Henle (13), thus providing a mechanistic hypothesis for increased Na⁺ reabsorption in Dahl S rats. To fulfill criteria 3 and 4, we

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1. Abbreviations used in this paper: DBP, diastolic blood pressure; EHT, essential hypertension; MAP, mean arterial pressure; nt, nucleotide; PAS, Periodic Acid Schiff; PASA, polymerase allele-specific amplification; R, salt resistant; RPA, ribonuclease protection assay; S, salt sensitive; SBP, systolic blood pressure; SS-EHT, salt-sensitive hypertension; wt, wild-type.

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addressed the following questions: does the Q276L $\alpha 1$ Na,K-ATPase variant contribute to the salt-sensitive hypertension phenotype? and does the functionally aberrant Q276L $\alpha 1$ Na,K-ATPase allele cosegregate with salt-sensitive hypertension? We addressed these questions using transgenic experiments and a standard intercross linkage analysis strategy.

Methods

Genotype and PASA analysis of Dahl S and Dahl R rats. Foundation colony Dahl S and Dahl R rats were obtained from Harlan Sprague Dawley Inc. derived from breeding pairs procured from J. Rapp (Medical College of Ohio, Toledo, OH) in 1985. Newly obtained Dahl S^{Rapp} rats from J. Rapp were obtained by and analyzed by Harlan Sprague Dawley, Inc. (Indianapolis, IN) for comparative analysis. All marker rat map pairs were obtained from Research Genetics (Huntsville, AL). Genotyping conditions were optimized and done as described (9, 14). For PASA analysis, rat spleen genomic DNA was isolated and PASA was done using primer pairs and conditions essentially as described (9) with the following modifications: the optimal stringent PCR cycling conditions were as follows: 95°C×10 min; 30 cycles of (95°C×1 min, 57°C×1 min, 72°C×1 min); extension at 72°C×7 min with 0.5 U/10 μ L of AmpliTaq Gold™ (Perkin Elmer Corp., Norwalk, CT).

Development of Dahl S transgenic rats. The transgene was constructed linking the wt $\alpha 1$ Na,K-ATPase (–1288)5' flanking region tested for functionality in tissue culture cells (15), full length 5' UT, full length 1028-amino acid-coding region, 131 bp of 3' UT of the wt $\alpha 1$ Na,K-ATPase cDNA, and 199 bp of SV40 polyadenylation signal sequences. Linearization with PvuI and HindIII restriction enzymes released the intact Tg[wt $\alpha 1$] minigene with 233 bp of vector sequence 5' to the minigene, and 237 bp of vector sequence 3' to the minigene, resulting in a total of 5,376 bp. Transgenic rats were developed as described (16) and three founders (Tg24, Tg37, and Tg48) were identified by Southern blot analysis. Only two lines were bred to homozygosity: Tg[wt $\alpha 1$]₂₄ and Tg[wt $\alpha 1$]₄₈.

Ribonuclease protection assays (RPAs). RPA was performed with the RPA IITM ribonuclease protection assay kit (Ambion, Austin, TX) as per manufacturer's instructions. The riboprobe was designed to span 131 bp of 3' untranslated region of the rat $\alpha 1$ Na,K-ATPase and 109 bp of SV40 sequence distinguishing the transgene transcript as a 240-nucleotide (nt) protected fragment in contrast to the 131-nt protected fragment of endogenous $\alpha 1$ Na,K-ATPase transcript. 20 μ g of total cellular RNA purified by the guanidinium-CsCl method was used for each assay.

Isolation of rat kidney rough microsomes. Membrane-bound polyosomes were isolated as described (17) using a cation (CsCl)-containing sucrose gradient. The pelleted rough microsomes were dissolved in 10 mM Tris-HCL, pH 7.4, 1 mM EDTA, and the total rough microsomal RNA was isolated by sequential phenol:chloroform (50:50) extraction followed by ethanol precipitation. 20 μ g of rat kidney membrane-bound polysomal RNA was used for RPA using the same riboprobe and experimental conditions described above.

Assessment of life span. Life span was assessed in both hemizygous and homozygous transgenic rats. Hemizygous male and female transgenic rats from three lines, along with littermate non-transgenic controls were started on a high salt (8% NaCl) diet at 6 wk of age and observed until natural death. Homozygous male and female rats from two transgenic lines, lines 24 and 48, were compared to non-transgenic Dahl S controls while fed a normal rat chow diet (0.4% NaCl), and observed until natural death. Statistical analysis was done by one-way ANOVA.

Measurement of blood pressure by radiotelemetry. Blood pressure was measured using intra-aortic abdominal radiotelemetric implants (DATASCIENCE, St. Paul, MN) obtaining non-stressed blood pressure measurements taking the average over 10 s every 5 min for 24 h (16). The 24-h average of all data points (288) over one no-entry day

at said time point after high salt challenge was used for all blood pressure measurements analyzed. The 24-h average was determined to be the best because it would account for diurnal variation, thus ascertaining accuracy. Because telemetric blood pressure signals were collected via computer, measurements were obtained without disturbance from room change, or room entry. Systolic (SBP), diastolic (DBP), and mean arterial pressures (MAP) were measured along with heart rate and activity. The protocol for transgenic and age-matched non-transgenic Dahl S rats was as follows: implant surgery at 10 wk of age; only rats with no complications after operation were used; after 12 d, baseline blood pressure levels were obtained; high salt (8% NaCl) challenge was begun at 12 wk of age and maintained for 4 wk; and transgenic and control rats were killed after 4 wk on high (8% NaCl) salt challenge (16 wk of age). The protocol for characterization of parental Dahl S and Dahl R rats, F1(S σ ×R ϕ) and F2(S σ ×R ϕ) hybrid rats was as follows: implant surgery at 8 wk of age; only rats with no complications after operation were used; after 12 d, baseline blood pressures were obtained; and high (8% NaCl) salt challenge was begun at 10 wk of age with water ad libitum. After 8 wk of high (8% NaCl) salt challenge, 24-h average SBP, DBP, MAP, and increment rise in 24-h average of SBP, DBP, and MAP were obtained per rat over one no-entry day (288 data points, 10-s recordings every 5 min).

Assessment of renal pathology. Renal tissues were fixed in 4% buffered paraformaldehyde and processed at HistoTechniques (Ohio). Serial renal sections were stained using hematoxylin-eosin, periodic acid Schiff (PAS), and Masson Trichrome stain. All glomeruli in one renal section (5 μ m) were analyzed for degree of glomerulosclerosis and mesangial matrix expansion. Age-matched control non-transgenic and transgenic male and female rats were studied after 4 wk of high salt diet challenge. Glomerulosclerosis was defined as disappearance of cellular elements from the tuft, collapse of capillary lumen, and folding of the glomerular basement membrane with entrapment of amorphous material (18). Mesangial matrix expansion was defined by the presence of increased amounts of PAS-positive material in the mesangial region (18). Renal pathology grade I, 25% of glomerulus with pathology; II, 50% involvement; III, 75% involvement; IV, 100% involvement. The extent of injury for each renal section was calculated, as the total pathology score = (1 × grade I) + (2 × grade II) + (3 × grade III) + (4 × grade IV), increasing with worse injury represented by glomerulosclerosis and mesangial matrix expansion (18). Renal sections were scored in a blind manner. Data were analyzed using non-parametric ANOVA.

Cosegregation analysis. The F2 cohort was derived from one Dahl S male and six Dahl R female rats from HSD colonies previously verified for genotype and phenotype (19). Non-stressed 24-h average blood pressure measurements were obtained by radiotelemetry, as described above. After 8 wk of high salt challenge, the F2 hybrid rats were killed and tail genomic DNAs isolated as described (16). Genotyping was performed using the following microsatellite markers: *D2mit14*; *D2mgh11* ($\alpha 1$ Na,K-ATPase); *D2mit12*; *D2mit10*; *CAMK*, and *D2mit6* (14) informative for our Dahl S σ ×Dahl R ϕ cross. Nine other markers (*D2mgh14*, *D2mit5*, *CPB*, *D2mit17*, *D2mgh15*, *D2mit13*, *D2mit20*, *D2mgh12*, and *D2mit5*) (14) were also investigated but were found to be non-polymorphic in our cross. Correlation of blood pressure parameters and genotypes for the different chromosome 2 markers was analyzed by one-way ANOVA (SigmaStat; Jandel Scientific, San Rafael, CA). Correction for multiple comparisons was not done, as parameters studied are closely related phenotypes.

Results

Ascertainment of genotype and phenotype of Dahl S and Dahl R strains. Due to the inadvertent genetic contamination of Dahl S rats at the sole commercial source resulting in subsequent contamination in our colony, our first transgenic lines

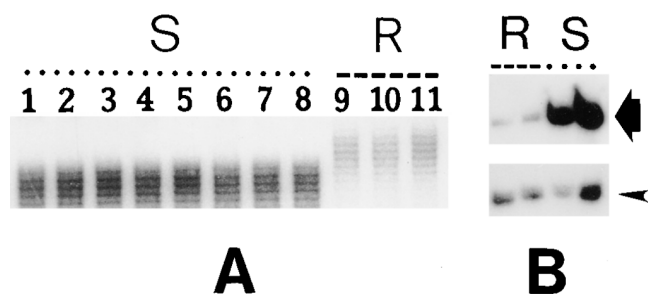


Figure 1. Genetic analysis of Dahl S^{HSD} rats. (A) Genotyping with contamination-indicative markers (20) corroborates non-genetic contamination of Dahl S^{HSD} foundation colony rats. A representative panel is shown for the R80 marker (20) demonstrating non-heterozygosity among Dahl S^{HSD} and Dahl R^{HSD} foundation colony rats; non-heterozygosity was detected in all contamination-indicative (20) markers (see Table I). The respective sizes of amplified product were: Dahl $S \neq$ Dahl R with R1041, R138, and R80 markers; Dahl $S =$ Dahl R with R721 GCA, R354. (B) PASA detection of T^{1079}/A transversion in Dahl S^{HSD} rat genomic DNA corroborates Q276L $\alpha 1$ Na,K-ATPase mutation. Comparing two Dahl R (R) and two Dahl S (S) rat genomic DNA samples, PASA analysis using primer-specific for T^{1079} detects significantly more amplified product in Dahl S rat samples (arrow) compared with Dahl R (R) rat genomic DNA samples at 57°C. Background amplified products could be expected as PASA detects a single base difference. As control, a non-specific marker, Cype (14), was used to indicate relative amounts of genomic DNA in the different samples (arrowhead). Taking the ratio of PASA-product to Cype-amplified product, Dahl S samples exhibit ratios > 1 ; whereas Dahl R samples exhibit ratios < 1 . These results indicate the presence of T^{1079} in Dahl S rat genomic DNA corroborating the Q276L $\alpha 1$ Na,K-ATPase variant as previously described (8, 9).

were all terminated. To perform transgenic and cosegregation studies in non-contaminated Dahl S rats, collaboration was set up with Harlan Sprague Dawley, Inc. (19) to identify non-contaminated Dahl S^{HSD} rats. In 1994, experiments were begun to ascertain non-contaminated genotype and salt-sensitive hyper-

tension phenotype of the Dahl S^{HSD} foundation colony, and, in parallel, the inbred genotype and salt-resistance phenotype of Dahl R^{HSD} rats. Using six microsatellite markers informative for the reported genetic contamination (20), foundation colony Dahl S^{HSD} and Dahl R^{HSD} rats were checked; no heterozygosity was detected (Fig. 1 A, Table I). Blood pressure phenotypes of foundation colony Dahl S^{HSD} and Dahl R^{HSD} rats were ascertained using radiotelemetric blood pressure measurements on a high salt (8% NaCl) diet begun at 10 wk of age. Severe salt-sensitive hypertension was detected in male and female Dahl S rats in contrast to salt-resistant normotension in male and female Dahl R rats (Table II). The data parallel the blood pressure phenotypes reported in the original Dahl S /JR and Dahl R /JR characterization (21). Only after this ascertainment were non-contaminated Dahl S^{HSD} and Dahl R^{HSD} rats obtained for transgenic experiments begun in 1995. Random testing of transgenic donor female and male Dahl S rats further corroborated absence of genetic contamination (data not shown).

Additionally, genotyping analysis using a panel of 97 microsatellite markers informative for Dahl S and Dahl R strains and eight markers identical in Dahl S and R strains (14, 22) was done comparing Dahl S^{HSD} and Dahl R^{HSD} rats used for our experiments, with Dahl S^{Rapp} rats obtained by Harlan Sprague Inc. from J. Rapp (21). As seen in Table I, 103 of 105 markers were identical between Dahl S^{HSD} and Dahl S^{Rapp} rats; differences were noted at two markers (D1mgh7 and D2mit5); heterozygosity was detected in the Dahl S^{Rapp} rats at D2mit13. These results document the non-genetic contamination of Dahl S^{HSD} and acceptable polymorphic differences between Dahl S^{HSD} and Dahl S^{Rapp} due to separate inbreeding over two decades.

Furthermore, we corroborated once again the presence of Q276L mutation in Dahl S^{HSD} and its absence in Dahl R rat^{HSD} genomic DNA by error-independent PCR allele-specific amplification (PASA) detecting T^{1079} in Dahl S , in contrast to non- T^{1079} in Dahl R genomic DNA (Fig. 1 B). This corrobo-

Table I. Genotype Analysis of Dahl S^{HSD} and Dahl R^{HSD} Rat Strains

Rat markers	Results
Informative for genetic contamination of Dahl S^{HSD} : GCA, R80, R138, R1041, R721, R354	No variations in S^{HSD} No variations in R^{HSD}
$S \neq R$ markers: D1: mgh6, mgh11, mgh14, mit2, mit13; D2: mit6, mit10, mit11, mit12, mit13, mit14, mit16, mit20, mit21, mgh11, mgh15, camk, UO1224; D3: mgh1, mgh17, mit3, mit13, mit14, D4: mgh15, mit2, mit9, mit10, mit19, mit20; D5: mgh12, mit4, mit7, mit9, mit10, mit11, mit13, D5Rjrl; D6: mgh9, mit2, mit3, mit8, mit9, R721; D7: mgh3, mit3, mit9, mit11, mit12, mit13, mit13; D8: mgh7, mit1, mit6; D9: mit5; D10: mgh4, mgh10, mit2, mit3, mit4; D11: mgh5, mgh6; D12: mgh1, mgh5, mit2, mit5, mit6; D13: mit1, mit3, mit5; D14: mit1, mit7; D17: mgh1, mit1, mit3, mit4; D19: mgh4, mit3; D20: mgh1; DX: mgh4 D1: cype; D2: mgh14, UO1223; D3: mit6; D4: mit17; D7: mit2; D1: mit5, mit6; D14: mit4; D15: mgh3, mgh5, mit1; D16: mit5; D17: mgh4; DX: mgh5, mit1 D1: mgh7 D2: mit5	$S^{HSD} = S^{Rapp} \neq R^{HSD}$ $S^{HSD} = S^{Rapp} = R^{HSD}$ $S^{HSD} \neq S^{Rapp} = R^{HSD}$ $S^{HSD} = R^{HSD} \neq S^{Rapp}$
$S = R$ markers: D2: mit15, mit17, mgh12, cpb; D3: mit4, D12: mit1; DX: mgh1, UO1223	$S^{HSD} = S^{Rapp} = R^{HSD}$

Markers informative for the contamination of the Dahl S^{HSD} rats (20) as well as markers reported to be informative for Dahl S and R strains were used (14, 22). Some differences from original report are detected; however, identity with Dahl S^{Rapp} rat genotype corroborates Dahl S^{HSD} as non-genetically contaminated. HSD, rats from Harlan Sprague Dawley, Inc.; S^{Rapp} , Dahl S rats obtained by HSD from Dr. John Rapp. Marker nomenclature (14, 20, 22).

Table II. Analysis of Blood Pressure Phenotype of Dahl S^{HSD} and Dahl R^{HSD}

Strain	n	SBP	ΔSBP	DBP	ΔDBP	MAP	ΔMAP
Dahl S ♂	6	220±7.1		163±7.9		183±12.1	
			(S-R) = 90.0		(S-R) = 69.3		(S-R) = 71.8
Dahl R ♂	5	130±1.3		93±1.6		111±1.5	
F1(S×R) ♂	12	152±1.4	(F1-R) = 22.0	110±1.5	(F1-R) = 16.4	130±1.5	(F1-R) = 19.3
Dahl S ♀	6	199±6.9		143±4.9		161±7.7	
			(S-R) = 78.7		(S-R) = 58.8		(S-R) = 59.0
Dahl R ♀	5	121±3.4		84±1.9		102±2.9	
F1 (S×R) ♀	5	138±5.4	(F1-R) = 16.9	98±5.0	(F1-R) = 13.4	117±5.0	(F1-R) = 15.0

Ascertainment of salt sensitivity in the Dahl S rats and salt resistance in Dahl R rats was obtained by non-stress 24-h measurements of blood pressure by radiotelemetry. SBP, systolic; DBP, diastolic blood pressure; MAP, mean arterial pressure in mmHg. Means±SEM are given; ΔBP, increment rise in BP from baseline after 8 wk of high (8% NaCl) diet; (S-R), difference between Dahl S and Dahl R mean BP; (F1-R) difference between F1 mean BP and Dahl R mean BP. For each rat, BP was measured by radiotelemetry and the average obtained of 288 data points (10-s recordings every 5 min) over 24 h in one no-entry day 8 wk after high salt (8% NaCl) challenge was begun at 10 wk.

rates previous PASA results (9). However, we note that sequencing of a Dahl S α1 Na,K-ATPase genomic DNA fragment encompassing amino acid 276 isolated from a λFix II Dahl S^{HSD} rat genomic library did not detect the A1079T transversion underlying the Q276L mutation (data not shown). In light of the consistent PASA results detecting the Q276L variant-specific T¹⁰⁷⁹ genomic DNA and previous observations demonstrating that PCR amplification reproducibly changed the Dahl S variant T¹⁰⁷⁹ to A¹⁰⁷⁹ (9), it becomes apparent that amplification of this genomic DNA region is indeed [T¹⁰⁷⁹–A¹⁰⁷⁹]-specific error prone.

Development of Dahl S transgenic rat lines. Based on observations that male and female F1(Dahl S♂×Dahl R♀) rats have blood pressures closer to the Dahl R rat strain after 8 wk of high salt (8% NaCl) diet (Table II), it becomes apparent that SS-EHT in the Dahl S rat model is recessive. Accordingly, a robust transgenic design should involve the transfer of Dahl R wt Q276 α1 Na,K-ATPase gene into the Dahl S genetic background, testing its effects on salt-sensitive hypertension phenotype. To attain appropriate spatial and developmental gene regulation, the transgene design links the cognate wt α1 Na,K-ATPase promoter region, functionally validated previously (15); the Dahl R wt α1 Na,K-ATPase cDNA (8, 9); and SV40 polyadenylation signal sequences, Tg[wtα1] (Fig. 2 A). Transgenic rats were developed as described (16). Three transgenic lines were developed, Tg[wtα1]₂₄, 37, and 48. Southern blot analyses revealed intact transgene sequences in all three transgenic lines showing the predicted 2.199-kb NcoI fragment (data not shown), the predicted 4.657-kb SacI fragment (Fig. 2 B), and HindIII restriction digestion fragments greater than the microinjected 5.376-kb transgene recombinant construct hybridizing to the α1 Na,K-ATPase cDNA probe (Fig. 2 B). Different copy numbers are also noted. Other restriction fragments hybridizing to the α1 Na,K-ATPase cDNA probe are detected in both control and transgenic rat DNAs representing the endogenous α1 Na,K-ATPase gene (Fig. 2 B).

To gain insight into the relative ratio of expression of the endogenous Q276L variant versus the transgene wt α1 Na,K-ATPase, RPAs were done (Fig. 2, C–F). The endogenous Q276L α1 Na,K-ATPase variant is detected as the expected 131-nt-long partial protection fragment (Fig. 2 C) in both control (odd numbered) and transgenic (even numbered) rat

RNA samples (Fig. 2 D): heart (lanes 1 and 2), brain (3 and 4), and kidney (5 and 6). The endogenous-specific 131-nt-long fragment is likewise detected in aorta on short (Fig. 2 E) and longer exposure (Fig. 2 F). The relative levels detected are consistent with spatial expression patterns in the rat (6). In contrast, the transgene-specific α1 Na,K-ATPase expected 240-nt protected fragment (Fig. 2 C) is detected only in transgenic rat tissue RNA samples as shown in Fig. 2 D: lane 2, heart; lane 4, brain; lane 6, kidney; and Fig. 2 E, lanes 4 and 5, Fig. 2 F, lanes 4 and 5, aorta. It should be noted that the total amount of α1 Na,K-ATPase transcript is not dramatically increased by the level of transgene expression in both transgenic lines with Tg[wtα1]₂₄ exhibiting higher expression levels compared with Tg[wtα1]₄₈ transgenic line.

To determine the membrane-integrated relative protein levels of transgene-to-endogenous α1 Na,K-ATPases in the absence of an informative antibody, assessment of their respective relative levels was determined in the renal translational pool compartmentalized to kidney membrane-bound polysomes of homozygous transgenic Tg[wtα1] Dahl S rats. This was done by RPA analysis of membrane-bound polysomal RNA isolated from a homozygous transgenic Tg[wtα1] Dahl S rat kidney using the same probe depicted in Fig. 2 C. As shown in Fig. 2 G, the 240-nt protected fragment representing the transgene wt α1 Na,K-ATPase transcript is almost equivalent in amount to the 131-nt-long protected fragment representing the endogenous Q276L variant α1 Na,K-ATPase transcript (~ 40:60 ratio of transgene wt α1 Na,K-ATPase to endogenous Q276L variant α1 Na,K-ATPase). This is in marked contrast to the underrepresentation of the transgene wt RNA in the total cellular pool (Fig. 2 D). Although we do not know the precise mechanism that could account for this differential representation, it is likely that structural differences within the 3'UT between the wt (transgene) and Q276L variant (endogenous) mRNAs could account in part for their differential RNA stability when compartmentalized to the non-translational pool.

Alleviation of salt-sensitive hypertension phenotype. To test whether Tg[wtα1] transgene expression modifies the salt-sensitive hypertension phenotype of inbred Dahl S rats, we analyzed three parameters: (a) life span on a high salt (8% NaCl) and on regular (0.4% NaCl) rat chow, (b) blood pressure lev-

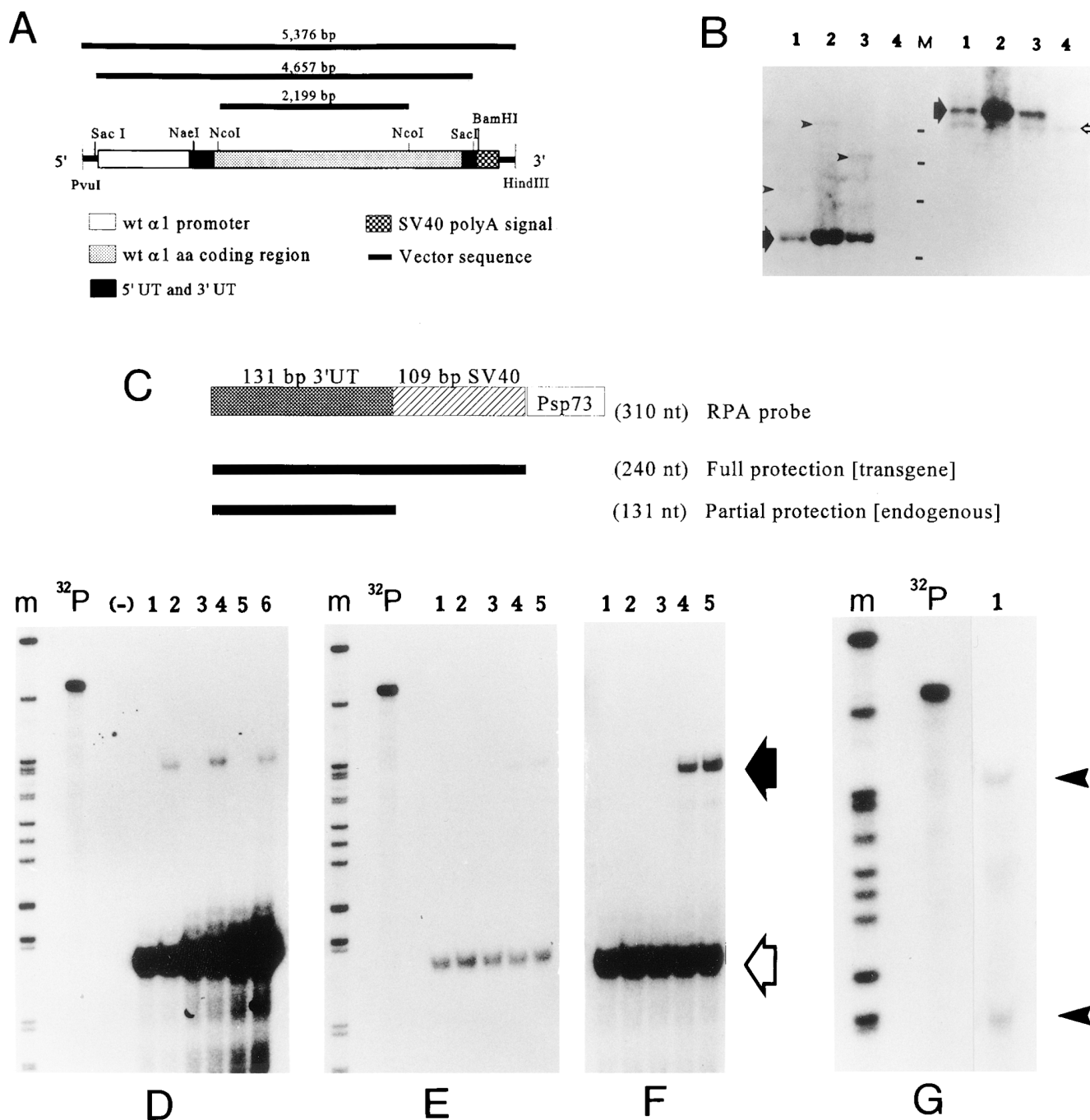


Figure 2. Molecular characteristics of transgenic Tg[wt α 1] lines. (A) The transgene construct, Tg[wt α 1], is comprised of [–1288 bp] of wt α 1 Na,K-ATPase 5' flanking regulatory region (wt α 1 promoter), linked to wt (Q276) α 1 Na,K-ATPase cDNA: spanning the entire 206-bp 5' untranslated region, full length 1,028-amino acid-coding region, and 131 bp of 3' untranslated region; linked to 199 bp of SV40 polyadenylation signal. (B) Southern blot analysis of transgenic F1 hemizygous Dahl S rats representing the three founder lines, Tg[wt α 1]₃₇ (lane 1); Tg[wt α 1]₄₈ (lane 2); Tg[wt α 1]₂₄ (lane 3), control non-transgenic Dahl S rat DNA (lane 4). M, λ Hind III molecular weight markers from top to bottom: 23,130 bp; 9,416 bp; 6,557 bp; and 4,361 bp. As shown on the left, SacI digested genomic DNA reveals intact Tg[wt α 1] transgene (4.657-kb fragment at closed arrow) detected only in the transgenic rats (lanes 1–3) and not in the non-transgenic control (lane 4). Other hybridizing SacI DNA fragments (arrowhead) in lane 1, ~7 kb; lane 2, ~24 kb; and lane 3, ~9.5 kb, indicate different random integration sites of the transgene into the genome. Additionally, different copy numbers are evident: Tg[wt α 1]₄₈ (lane 2) > Tg[wt α 1]₂₄ (lane 3) > Tg[wt α 1]₃₇ rat (lane 1). On the right, HindIII restriction digestion reveals the intact transgene (closed arrow) with a >30-kb fragment in the transgenic rat lines, absent in control (lane 4). The endogenous α 1 Na,K-ATPase HindIII fragment is smaller, ~30 kb (open arrow), and is detected in transgenic and control non-transgenic rat genomic DNA. (C) The composition of the RPA probe used to assess wt (transgene) and Q276L variant (endogenous) α 1 Na,K-ATPase RNA levels is presented: a 310-nt RPA probe comprised of 131 bp of 3' untranslated (UT) region of the α 1 Na,K-ATPase cDNA present in both transgene and endogenous α 1 Na,K-ATPase, linked to 109 bp of SV40 sequence, which is present only in the transgene; and 70-bp vector sequence. The transgene wt α 1 Na,K-ATPase RNA is expected to be 240-nt-long, distinguished from the endogenous Q276L variant α 1 Na,K-ATPase RNA, expected to be 131-nt long. (D) RPA of total cellular RNA from heart (lanes 1 and 2), brain (lanes 3 and 4), and kidney

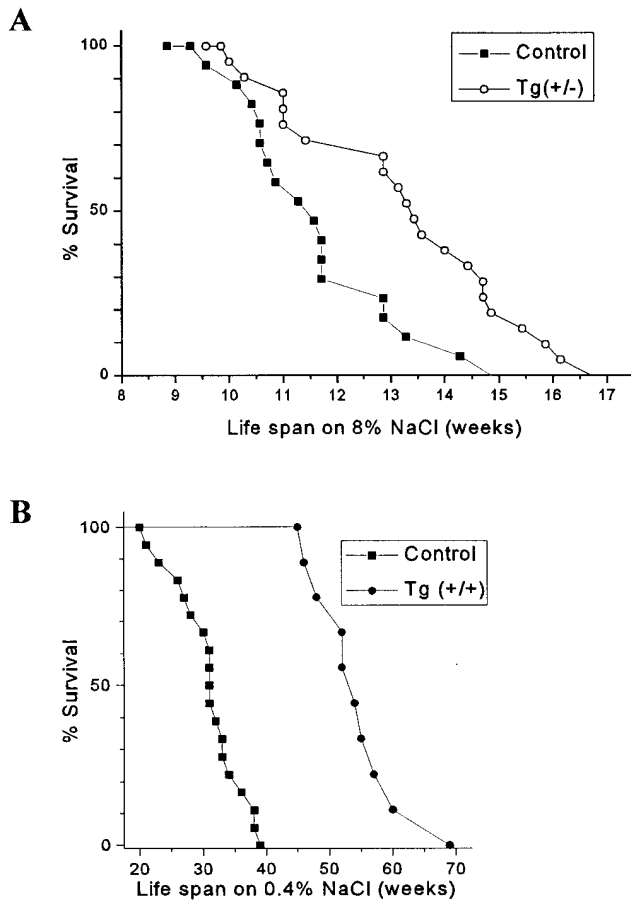


Figure 3. Life span in hemizygous and homozygous transgenic Tg[wtα1] Dahl S rats compared with control non-transgenic Dahl S rats. (A) Hemizygous male and female transgenic rats from the three Tg[wtα1] lines on high salt (8% NaCl) diet begun at 6 wk of age lived longer (13.0 ± 0.5 wk; $n = 23$) than control littermate non-transgenic (11.4 ± 0.4 wk; $n = 19$) Dahl S rats ($P < 0.01$, one-way ANOVA). (B) Homozygous male and female Tg[wtα1]₂₄ and Tg[wtα1]₄₈ rats on regular (0.4% NaCl) rat diets lived longer (54.8 ± 2.3 wk; $n = 10$), compared with non-transgenic control Dahl S rats (31.2 ± 1.2 wk; $n = 19$); $P < 10^{-9}$, one-way ANOVA.

els, and (c) hypertensive renal disease. A priori, concordance of effects in all three parameters would strongly indicate a bona fide mechanistic role for α1 Na,K-ATPase. Life span was analyzed in hemizygous transgenic rats challenged with a high salt (8% NaCl) diet at six wk of age. As seen in Fig. 3 A, hemizygous male and female rats from three transgenic lines lived

Table III. Comparison of Blood Pressure of Transgenic and Non-transgenic Dahl S Rats

BP Parameter (mmHg)	Control ♂ (n = 5)	Tg[wtα1] ₂₄ ♂ (n = 6)	Control ♀ (n = 4)	Tg[wtα1] ₂₄ ♀ (n = 6)
SBP ± SEM	231.4 ± 6.0	190.3 ± 5.4**	220.3 ± 3.6	186.8 ± 5.7‡
DBP ± SEM	179.2 ± 5.0	141.8 ± 4.7**	169.8 ± 4.2	137.2 ± 6.2‡
MAP ± SEM	203.6 ± 5.2	165.2 ± 5.0**	192.3 ± 3.4	163.0 ± 5.5‡
ΔSBP ± SEM	82.6 ± 4.2	47.8 ± 8.8*	66.8 ± 2.4	41.2 ± 4.9*
ΔDBP ± SEM	68.6 ± 3.2	38.2 ± 5.2**	52.5 ± 3.7	31.2 ± 4.2*
ΔMAP ± SEM	75.4 ± 3.5	43.5 ± 7.1*	58.0 ± 4.3	36.7 ± 4.6*

Blood pressure of homozygous transgenic Tg[wtα1]₂₄ rats were compared with age- and sex-matched non-transgenic Dahl S rats after 4 wk of high (8% NaCl) salt diet begun at 12 wk of age. BP parameters analyzed: systolic (SBP), diastolic (DBP), mean arterial pressure (MAP), as well as increment rise (Δ) in respective BP parameters after 4 wk of high salt (8% NaCl) challenge (Methods). Data were analyzed using non-parametric one-way ANOVA and one-way ANOVA on ranks. Correction for multiple comparisons was not done since the multiple phenotypes studied (SBP, DBP, MAP, ΔSBP, ΔDBP, ΔMAP) are correlated to a high degree. For each rat, BP was measured by radiotelemetry and the average obtained of 288 data points (10-s recordings every 5 min) over 24 h in one no-entry day 4 wk after high salt (8% NaCl) challenge was begun at 12 wk of age. BP parameters per rat in mmHg, millimeters mercury; n , number of rats in group; SEM, standard error of the mean; (‡), $P < 0.05$; (*), $P < 0.01$; (**), $P < 0.001$.

longer than littermate non-transgenic Dahl S rat controls. Mean life span of hemizygous Tg[wtα1] rats (13.0 ± 0.5 wk, $n = 23$) increased 14% compared with controls (11.4 ± 0.4 wk, $n = 19$), $P < 0.01$ one-way ANOVA. Upon successfully breeding Tg[wtα1]₂₄ and Tg[wtα1]₄₈ lines to homozygosity, life span was analyzed on a regular (0.4% NaCl) rat chow. As seen in Fig. 3 B, male and female homozygous rats lived longer than control non-transgenic Dahl S rats. Mean life span of homozygous Tg[wtα1]_{24,48} rats (54.8 ± 2.3 wk, $n = 10$) increased 75.6% compared with controls (31.2 ± 1.2 wk, $n = 19$), $P < 10^{-9}$ by one-way ANOVA. Improvement in mortality suggested that salt-sensitive hypertension phenotype was most likely alleviated in the different transgenic rat lines.

Blood pressure measurements were then analyzed comparing homozygous male and female transgenic Tg[wtα1]₂₄ rats with non-transgenic age-matched Dahl S control rats. As seen in Table III, group means of 24-h SBP, DBP, and MAP levels in both male and female transgenic Tg[wtα1]₂₄ rats were consistently lower than blood pressure levels detected in age-matched control non-transgenic Dahl S rats. Likewise, the levels of increment rise in blood pressure parameters, SBP, DBP,

Figure 2 legend (Continued)

(lanes 5 and 6) of homozygous transgenic Tg[wtα1] (lanes 2, 4, and 6) and control non-transgenic (lanes 1, 3, and 5) Dahl S rats. (–), control yeast RNA; ³²P, RPA radiolabeled probe; m , molecular size markers in base pairs from top to bottom: pBR322 DNA-MspI digest: 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110 bp. (E) RPA of total aortic RNA from homozygous transgenic (lanes 4 and 5) and age-matched control (lanes 1, 2, and 3) non-transgenic Dahl S rats. (F) Longer exposure (10×) of E. (closed arrow), 240-nt protected fragment indicative of wt transgene α1 Na,K-ATPase mRNA; (open arrow), partial protection 131-nt fragment, indicative of endogenous Q276L variant α1 Na,K-ATPase mRNA. (G) Assessment of wt (transgene) and Q276L variant (endogenous) α1 Na,K-ATPase RNA levels in membrane-bound polysomes of homozygous Tg[wtα1] rats. The identical RPA probe (shown in C) was used to perform RNase protection assays (RPA) on membrane-bound polysomal RNA isolated from homozygous Tg[wtα1]₂₄ Dahl S rat kidney (lane 1). ³²P, RPA probe; m , molecular size markers in base pairs from top to bottom: pBR322 DNA-MspI digest: 404, 307, 242, 238, 217, 201, 190, 180, 160, 147 bp. Top (arrowhead), 240-nt protected fragment indicative of wt transgene α1 Na,K-ATPase mRNA; bottom (arrowhead), partial protection 131-nt fragment, indicative of endogenous Q276L variant α1 Na,K-ATPase mRNA.

and MAP, after 4 wk of high salt challenge were also significantly lower in both male and female transgenic Tg[wt α 1]₂₄ rats (Table III).

To assess potential improvement in EHT-induced target organ damage, comparison of PAS-stained renal sections from five transgenic rat kidneys representing homozygous transgenic Tg[wt α 1]₂₄ and Tg[wt α 1]₄₈ rats and from four control non-transgenic rat kidneys was done. Low magnification revealed significant differences between transgenic rat kidney sections and control non-transgenic rat kidney sections. No differences were noted between sexes. As shown in Fig. 4, a greater number of magenta PAS-positive abnormal glomeruli are seen in a representative control rat kidney section (Fig. 4 A) compared with a representative transgenic kidney section (Fig. 4 B), indicating less hypertensive renal disease in transgenic rats. This was corroborated by quantitative analysis of renal pathology based on the scoring system described by Raji et al. (18), wherein glomeruli are graded for degree of mesangial thickening and glomerulosclerosis. As shown in Fig. 4, a glomerulus with 25% mesangial thickening and/or glomerulosclerosis is grade I (Fig. 4 D); grade II is 50% pathologic involvement (Fig. 4 E); grade III, 75% involvement (Fig. 4 F); and grade IV, 100% pathologic involvement (Fig. 4 G), in contrast to a normal glomerulus (Fig. 4 C). A total pathology score is calculated with worse severity correlated with higher pathology scores (18). Analysis of renal sections from four control non-transgenic rats (628 total glomeruli scored) compared with five transgenic rat kidney sections (1,213 total glomeruli scored) for severity of mesangial thickening and glomerulosclerosis revealed a 52% decrease in Raji renal pathology score in transgenic rat kidneys compared with control rat kidneys, $P = 0.0025$ (non-parametric ANOVA) Table IV.

This decrease in renal pathology in transgenic Tg[wt α 1] Dahl S rats is consistent with the observed improvement in life span and the alleviation of SS-EHT. More importantly, the concordance of improvement in three measures, life span, blood pressure, and hypertensive renal disease, as well as the 40:60 transgene: endogenous α 1 Na,K-ATPase ratio provide evidence to meet our third criteria for the role of the α 1 Na,K-ATPase gene in SS-EHT.

Intercross linkage analysis. To fulfill criteria 4, cosegregation analysis was done on fifty F2(S σ° \times R ϕ) hybrid male rats phenotyped for SBP, DBP, MAP, heart rate, and activity by radiotelemetry at baseline (10 wk of age) and after 8 wk of high (8% NaCl) salt challenge. These 50 F2 rats were genotyped at 15 markers that spanned chromosome 2 (14). ANOVAs comparing phenotypes across the three genotypic categories for each informative marker locus (6 out of 15) were

Table V. Correlation of Chromosome 2 Genotype and Blood Pressure

Locus	BP	Genotype			(SS-RR)	
		RR	RS	SS	Δ	P value
D2mit6	Δ DBP	22.9 \pm 2.84	16.6 \pm 1.43	22.7 \pm 2.39	-0.2	0.9542
	Δ SBP	28.7 \pm 3.84	23.6 \pm 1.75	31.3 \pm 3.28	2.6	0.3943
	Δ MAP	26.0 \pm 3.37	20.1 \pm 1.63	27.1 \pm 2.80	1.1	0.4628
	(n)	(14)	(26)	(10)		
D2Mit10	Δ DBP	16.3 \pm 2.27	19.4 \pm 1.87	22.9 \pm 2.16	6.6	0.0439
	Δ SBP	22.0 \pm 2.72	26.4 \pm 2.46	31.0 \pm 2.57	9.0	0.0188
	Δ MAP	19.3 \pm 2.47	23.0 \pm 2.23	27.1 \pm 2.34	7.8	0.0305
	(n)	(12)	(25)	(13)		
D2Mit12	Δ DBP	16.9 \pm 2.27	19.2 \pm 2.16	22.3 \pm 1.84	5.4	0.0695
	Δ SBP	23.0 \pm 2.72	25.7 \pm 2.80	30.6 \pm 2.30	7.6	0.0401
	Δ MAP	20.0 \pm 2.47	22.4 \pm 2.55	26.6 \pm 2.04	6.6	0.0466
	(n)	(13)	(21)	(16)		
D2Mgh11 (α 1)	Δ DBP	15.5 \pm 1.78	19.0 \pm 2.09	23.2 \pm 1.88	7.7	0.00920
	Δ SBP	21.1 \pm 2.07	25.2 \pm 2.59	32.3 \pm 2.39	11.2	0.00268
	Δ MAP	18.4 \pm 1.84	22.1 \pm 2.40	27.9 \pm 2.13	9.5	0.00376
	(n)	(11)	(23)	(16)		
D2Mit14	Δ DBP	15.6 \pm 1.78	19.0 \pm 2.08	23.2 \pm 1.88	7.6	0.00920
	Δ SBP	21.1 \pm 2.08	25.2 \pm 2.58	32.3 \pm 2.40	11.2	0.00268
	Δ MAP	18.4 \pm 1.84	22.1 \pm 2.40	27.9 \pm 2.13	9.5	0.00376
	(n)	(11)	(23)	(16)		
CAMK	Δ DBP	16.8 \pm 1.83	20.4 \pm 2.21	20.3 \pm 2.04	3.5	0.2488
	Δ SBP	21.5 \pm 2.07	27.1 \pm 2.74	29.0 \pm 2.64	7.5	0.0548
	Δ MAP	19.2 \pm 1.88	23.9 \pm 2.50	24.7 \pm 2.42	5.5	0.1223
	(n)	(11)	(21)	(18)		

Cosegregation of increment rise in diastolic, systolic, and mean arterial pressure by genotype for different loci on rat chromosome 2 in F2(S σ° \times R ϕ) male rats fed a high salt diet for 8 wk. Peak correlation is noted at the α 1 locus (D2mgh11) and at the locus marked by D2 mit14 marker, < 2.2 cM away (14). SS, homozygous for Dahl S allele; RR, homozygous for Dahl R allele; SR, heterozygous; P value, probability based on one-way analysis of variance of difference in Δ BP between SS and RR genotypes; (Δ BP), increment rise in BP after 8 wk of high salt diet from baseline BP; SEM, standard error of the mean; DBP, diastolic blood pressure; SBP, systolic blood pressure; MAP, mean arterial pressure in mmHg; n, number of rats. Correction for multiple comparisons was not done as parameters studied are closely related phenotypes.

carried out. As seen in Table V and Fig. 5, the most significant ANOVA results were detected at the α 1 Na,K-ATPase locus (D2mgh11) and at the D2mit14 marker, 2.2 centimorgans (cM) away, for SBP ($P = 0.00268$), DBP ($P = 0.00920$), MAP ($P =$

Table IV. Quantitative Analysis of Renal Pathology

Group (n)	% Normal \pm SEM	% Gr. I \pm SEM	% Gr. II \pm SEM	% Gr. III \pm SEM	% Gr. IV \pm SEM	Pathology score \pm SEM
Control (4)	20.1 \pm 5.2	31.7 \pm 5.6	16.3 \pm 4.4	13.5 \pm 2.2	18.3 \pm 4.3	178.2 \pm 15.0
Transgenic (5)	41.7 \pm 4.5	38.8 \pm 1.8	12.4 \pm 2.8	6.0 \pm 1.2	1.1 \pm 0.5	85.8 \pm 9.9
Probability	0.0170	NS	NS	0.0070	0.0016	0.0025

Quantitative analysis of renal pathology (18) comparing transgenic Dahl S rats ($n = 5$) and control non-transgenic rats ($n = 4$). PAS-stained renal sections were graded in a blind manner for mesangial thickening and glomerulosclerosis; total of 628 glomeruli for control non-transgenic rats; 1,213 glomeruli for transgenic rats. Scoring of pathology was done as described (Fig. 4). Data were analyzed using non-parametric ANOVA; NS, not significant; SEM, standard error of the mean; n, number of rats represented in the analysis.

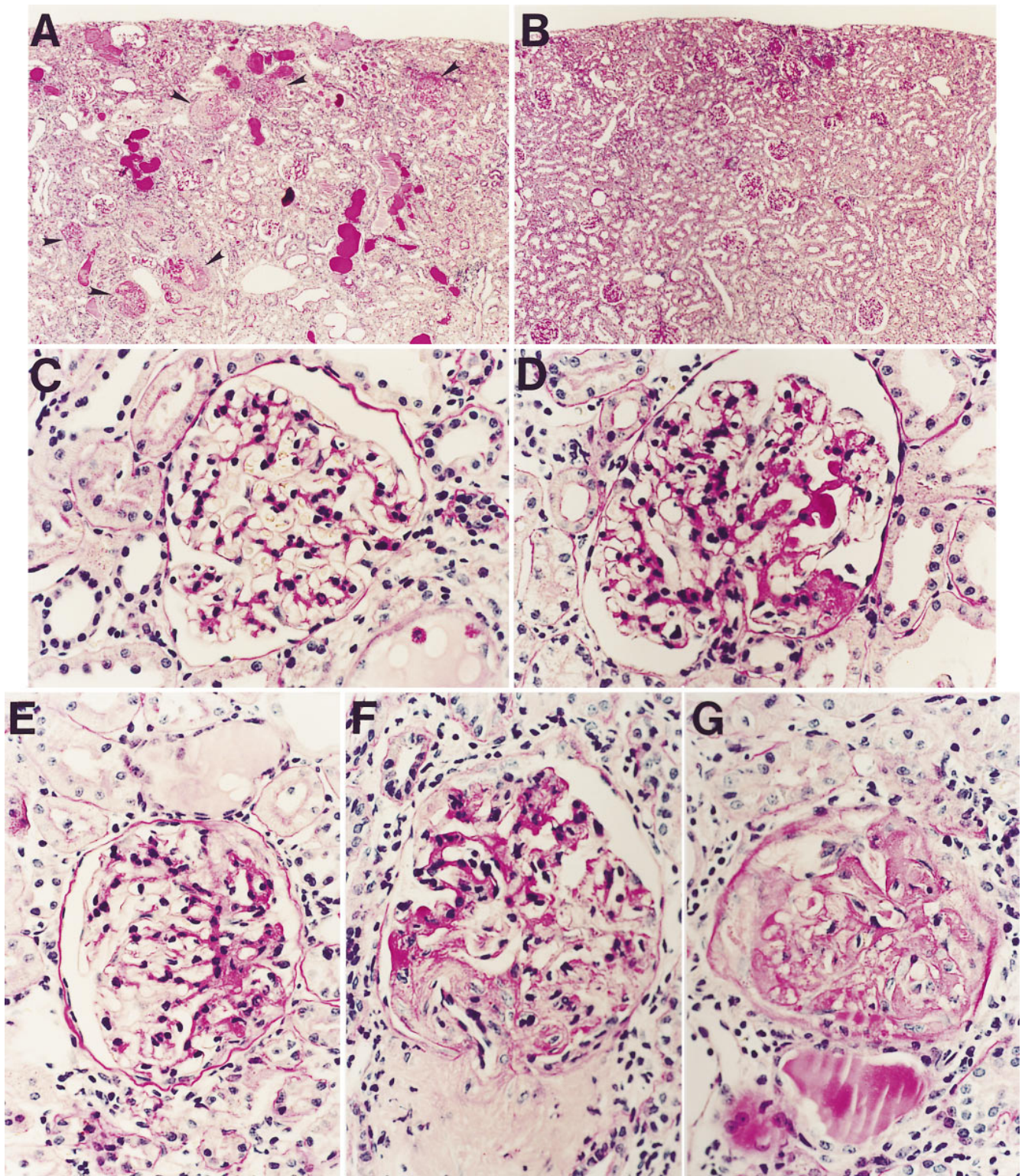


Figure 4. Comparative analysis of degree of hypertensive renal disease. Representative PAS-stained renal sections of non-transgenic Dahl S rat kidney (A) show more severe renal pathology after 4 wk of high salt diet compared with age-matched transgenic Dahl S rat kidney (B). Low power magnification reveals more hyaline casts and abnormal glomeruli with intensely PAS-positive mesangial thickening and glomerulosclerosis per unit area in non-transgenic rat kidney section (A) compared with transgenic rat kidney section (B). Abnormal glomeruli with grade IV Raij pathology score lesions are marked (arrowhead). C, D, E, F, and G show high power magnification demonstrating different grades of glomerular pathology used as parameters for quantitation of extent of renal pathology based on the Raij pathology score (18). (C) normal glomerulus; (D) grade I glomerular pathology with mesangial thickening and/or glomerulosclerosis covering 25% of glomerulus; (E) grade II glomerular pathology involving 50% of glomerulus; (F) grade III glomerular pathology involving 75% of glomerulus; (G) grade IV glomerular pathology, 100% involvement. Calculation of the total pathology score = $[1(\% \text{ grade I}) + 2(\% \text{ grade II}) + 3(\% \text{ grade III}) + 4(\% \text{ grade IV})]$ indicates worse renal pathology with increasing scores.

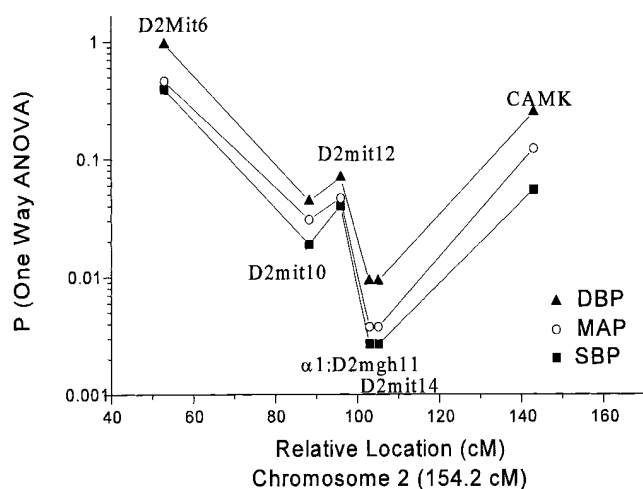


Figure 5. Cosegregation analysis of $\alpha 1$ Na,K-ATPase locus with salt-sensitive hypertension. Total chromosome 2 scan analyzing marker cosegregation with salt-sensitive hypertension in an F2(Dahl S \times R) cohort ($n = 50$ males) measured as increment rise in 24-h average systolic (SBP), diastolic (DBP) and mean arterial pressures (MAP) obtained after 8 wk of high (8% NaCl) salt diet. Markers informative for the Dahl S and R hybrid cross are marked along their respective relative location on chromosome 2 in centimorgans (cM) based on the rat map (14). Greatest significance is seen with the $\alpha 1$ Na,K-ATPase locus (*D2mgh11* marker) and the *D2mit14* marker, < 2.2 cM away. In contrast to other studies (22), *D2mit12* and *CAMK* markers do not cosegregate with salt-sensitive hypertension. Correlation trends along chromosome 2 are parallel for SBP, DBP, and MAP.

0.00376). The fact that all three blood pressure measures provide similar results is in contrast to other F2 cosegregation studies that have detected cosegregation with one blood pressure parameter but not with the others, e.g., locus cosegregation with DBP and pulse pressure, but not with SBP or MAP (23). These results suggest that the $\alpha 1$ Na,K-ATPase locus meets criteria 4.

Discussion

Because of the inadvertent genetic contamination of the Dahl S^{HSD} strain (19, 24), transgenic studies were performed using only Dahl S^{HSD} rats from the foundation colonies at Harlan Sprague, Inc. confirmed as to genotype and salt-sensitive phenotype (19). The F2 intercross was also done using Dahl S^{HSD} and Dahl R^{HSD} rats confirmed for both genotype and phenotype. Unequivocal observations are thus ascertained (19).

The results obtained from the transgenic and cosegregation studies fulfill the requirements of the criteria set out to definitively assign the $\alpha 1$ Na,K-ATPase gene as a susceptibility gene for hypertension using the Dahl S^{HSD} genetic hypertension rat model. The concordance of improvement not just in all measures of blood pressure, but also in renal disease and life span, provides holistic support strengthening the ascertainment of the mechanistic role of $\alpha 1$ Na,K-ATPase in salt-sensitive hypertension as modeled in the Dahl S^{HSD} rat. Our results suggest that phenotypic differences observed in the transgenic Tg[wt $\alpha 1$] rats are most likely due to the functional heterozygosity of wt and Q276L variant $\alpha 1$ Na,K-ATPases rather than an additive overabundance of $\alpha 1$ Na,K-ATPases. This is consistent with the observation made from blood pressure data of

F1(S \times R) rats, indicating that hypertension is a recessive trait and that normotension is a dominant trait.

Other genetic studies have documented previous linkage of the $\alpha 1$ Na,K-ATPase locus. The $\alpha 1$ Na,K-ATPase locus was found to be the closest candidate gene in a total chromosome 2 scan analyzing two F2 cohorts, one involving the Dahl S \times Milan normotensive strain, and one involving Dahl S \times Wistar Kyoto normotensive strain (22). Recent studies on chromosome 2 analyzing F2 crosses derived from the stroke-prone spontaneously hypertensive rat and the normotensive Wistar-Kyoto rat (25) and derived from the spontaneously hypertensive rat and the Wistar-Kyoto rat (26) have also detected a QTL for high blood pressure close to the $\alpha 1$ Na,K-ATPase locus. Our cosegregation study independently confirms these previous results and with $P < 0.003$, meets the required nominal $P < 0.01$ criterion for confirmed linkage (27).

The pronounced improvement in blood pressure ($\sim 40\%$) by the transgenic manipulation of a single gene suggests that hypertension, being polygenic, does not, most likely, follow a simple additive model of genetic inheritance, but rather involves a di- or multigenic interaction within a polygenic context. With normotension being dominant, transgenic experiments designed to correct hypertension in the inbred hypertensive strain would be more robust in investigating the effects of interacting hypertension susceptibility genes rather than F2 intercross studies with polymorphic markers as shown in our study.

The improvement of multiple pathogenic events in transgenic Tg[wt $\alpha 1$] Dahl S rats is consistent with observations in human hypertensive patients, wherein lowering of blood pressure has been shown to decrease mortality and target organ complications (28). The greater reduction in the degree of renal pathology (50%) and greater improvement in life span (75.6%) compared with blood pressure parameters ($\sim 40\%$) seen in the transgenic rats could be attributed to an "early" intervention, as the transgenic rats have the corrective transgene from one-cell embryo stage—a finding which promotes the value of early preventive interventions for some complex diseases. Additionally, the $\alpha 1$ Na,K-ATPase gene might play a role in hypertensive renal complication pathogenesis that is distinct from its role in hypertension pathogenesis, and/or a threshold phenomenon might be involved in the pathogenesis of hypertensive target organ complications.

The inability to detect the A¹⁰⁷⁹–T¹⁰⁷⁹ transversion in Dahl S rat genomic DNA via amplification-based methods and sequencing of genomic clones underscores the importance of a multifaceted analysis of such refractory mutations encompassing structural and functional approaches. The demonstration, therefore, of functionally significant differences between Dahl S and Dahl R $\alpha 1$ Na,K-ATPases and, more significantly, the partial correction of salt-sensitive hypertension in the Dahl S rat via transgenesis support the contention that the Q276L mutation exists in Dahl S rats as shown by error-independent assays and that it plays a role in salt-sensitive hypertension. The observation of an amplification error-prone genomic DNA region raises the question that other mutations might be similarly refractory to detection by conventional amplification-based methods. Amplification-independent assays provide an alternative and suitable approach to structurally assess these "refractory" mutations.

Altogether, our results demonstrate that the Na,K-ATPase locus is a SS-EHT susceptibility gene and showcases the

strength of a “forward genetics approach”—testing functionally significant variant alleles at biologically relevant loci (1, 29) -- as was done recently in the study of the variant serotonin transporter as a gene contributing to neuroticism (30). It also indicates the value of a multifaceted molecular genetic approach (1, 31), wherein transgenic rat experiments in an inbred model organism might allow one to deduce the role of a gene in complex disease pathogenesis. The success in the significant alleviation of salt-sensitive hypertension by the manipulation of a single gene validates the potential for gene therapy for complex cardiovascular diseases and other multifactorial disorders. Moreover, the proposed criteria and approach in animal models provide evidence that make analogous studies of homologous human genes in hypertension compelling.

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