Confirmation of Mutant $\alpha_1$ Na,K-ATPase Gene and Transcript in Dahl Salt-Sensitive/JR Rats

Nelson Ruiz-Opazo, Francis Barany, Kenji Hirayama, Victoria L.M. Herrera

Abstract As the sole renal Na,K-ATPase isozyme, the $\alpha_1$ Na,K-ATPase accounts for all active transport of Na+ throughout the nephron. This role in renal Na+ reabsorption and the primacy of the kidney in hypertension pathogenesis make it a logical candidate gene for salt-sensitive genetic hypertension. An adenine (A)$^{1079}$$\rightarrow$thymine (T) transversion, resulting in the substitution of glutamine with leucine and associated with decreased net $\Delta$Rb+ (K+) influx, was identified in Dahl salt-sensitive/JR rat kidney $\alpha_1$ Na,K-ATPase cDNA. However, because a Taq polymerase chain reaction analysis of genomic DNA using a $\Delta$Rb+ (K+) allele and Dahl salt-sensitive rat genomic DNA, we reexamined $\alpha_1$ Na,K-ATPase sequences using Taq polymerase error-independent amplification-based analyses of genomic DNA (by polymerase allele-specific amplification and ligase chain reaction analysis) and kidney RNA (by mRNA-specific thermostable reverse transcriptase–polymerase chain reaction analysis). We also performed modified 3' mismatched correction analysis of genomic DNA using an exoribonuclease-positive thermostable DNA polymerase. All the confirmatory test results were concordant, confirming the A$^{1079}$$→$T transversion in the Dahl salt-sensitive $\alpha_1$ Na,K-ATPase allele and its transcript, as well as the wild-type A$^{1079}$ sequence in the Dahl salt-resistant $\alpha_1$ Na,K-ATPase allele and its transcript. Documentation of a consistent Taq polymerase error that selectively substituted A at T$^{276}$ (sense strand) was obtained from Taq polymerase chain reaction amplification and subsequent cycle sequencing of reconfirmed known Dahl salt-sensitive/JR rat mutant T$^{276}$ $\alpha_1$ cDNA M13 subclones. This Taq polymerase error results in the reversion of mutant sequence back to the wild-type $\alpha_1$ Na,K-ATPase sequence. This identifies a site- and nucleotide-specific Taq polymerase misincorporation, suggesting that a structural basis might underlie a predisposition to nonrandom Taq polymerase errors. (Hypertension. 1994;4:260-270.)

Key Words • Na+-K+-transporting ATPase • mutation • polymerase chain reaction • genetics • hypertension, sodium-dependent
reverse transcriptase error in the synthesis of the Dahl S rat cDNA library. This is an important issue to resolve because clarification of the mutation is critical in determining the role of α₁ Na,K-ATPase in hypertension as well as insightful in the analysis of PCR-based and cDNA-cloning-based experimental approaches.

**Methods**

**Materials**

Inbred Dahl S/JR and Dahl R/JR rats were obtained from both the Boston University Medical School colony and from Harlan Sprague Dawley, Inc. The following are the sources for reagents used: Sequenase sequencing kit from United States Biochemical Corp; complete PCR-kits, thermostable reverse transcriptase (RT)-PCR kits, PCR cycle sequencing kits, and UITma DNA polymerase from Perkin-Elmer Cetus; synthetic oligonucleotides from Keystone Laboratories, Inc; terminal transferase from Stratagene; p6 Biospin columns from Bio-Rad; acrylamide gel solutions from National Diagnostics; and all radioisotopes from New England Nuclear Research Products, Du Pont Co. Thermostable ligase was isolated and purified by F. Barany. An MJ Research automated thermal cycler was used for all experiments.

**Animals Studied**

Inbred strains of Dahl S and Dahl R rats developed by J.P. Rapp were used. These were obtained from the colony maintained at Boston University School of Medicine (BUSM) derived from an inbred hypertensive line from J.P. Rapp. When these inbred Dahl S and R rats developed by J.P. Rapp became available from Harlan Sprague Dawley, the BUSM colony was then discontinued and Dahl S/JR and Dahl R/JR rats were obtained from Harlan Sprague Dawley. Genomic DNA from Dahl S and Dahl R rats from Harlan Sprague Dawley used in this study was obtained before 1990 and was identical to that in rats studied previously. Respective Dahl S and Dahl R kidney tissues were kept frozen at -70°C and DNAs stored at 4°C. Obtaining the need for purchase of more rats for the reconfirmation study presented here. This was a serendipitous advantage because the pedigree expansion and production lines of Harlan Sprague Dawley for Dahl S and R rats have recently been reported to be genetically contaminated (personal communication, J.R. McGlinchey and R. Russell, Harlan Sprague Dawley, 1993, and Reference 13). All DNA samples used were previously genotyped with the Pst I restriction fragment length polymorphism in the α₁ Na,K-ATPase gene showing consistent S allele homozygosity in Dahl S rats and R allele homozygosity in Dahl R rats. All animals were killed with an intraperitoneal overdose of pentobarbital sodium. Procedures were in accordance with institutional guidelines.

**Polymerase Allele-Specific Amplification Analysis**

Polymerase allele-specific amplification (PASA) was carried out essentially as described with the following specifications. Equal amounts of genomic DNA were used as determined by UV spectrophotometry. PCR amplifiability of all test DNA samples was ascertained by the PCR amplification of α₁ Na,K-ATPase sequences identical for both the S and R alleles using the following oligonucleotide primers: sense (+) 20-mer=5'cattgtgctacagcaatgggggtca-3' and antisense (−) 18-mer=5'gaagaaatc-3'. The antisense pair of oligonucleotide primers were used as (a) 26-mer=5'ctctggcgtgcgctcT1079-3' and (b) 22-mer=5'ggcctcgaaggagaatc-3'. The underlined sequences in the "a" oligonucleotides of the sense and antisense pairs are noncomplementary tails (non-α₁ Na,K-ATPase sequence) added on the outside of oligonucleotides so as to prevent formation of target-independent (blunt end) background ligation. Addition, single-base 3' overhang on discriminating oligonucleotides ("a") for both sense and antisense pairs was incorporated to also prevent target-independent (blunt end) background ligation. Controls consisted of 10 and 5 ng pUC19-Dahl S α₁ and cDNA; 500 ng of test genomic DNA from Dahl S and Dahl R rats, respectively, was used. The volume of reaction was 10 μL with 10 mmol/L Tris-HCl, pH 8.3, 150 mmol/L KCl, 10 mmol/L MgCl₂, 1 mmol/L dithiothreitol, and 1 mmol/L α-NAD, with primers at 1 ng/10 μL reaction and 15 U of thermostable ligase per 10 μL reaction (generously purified and provided by F. Barany). Reaction conditions were 95°Cx2 minutes and 30 cycles of 94°Cx30 seconds, 65°Cx4 minutes. The sense and antisense "b" primers were end-labeled as described. LCR products were size-fractionated on an 8% denaturing polyacrylamide gel and detected by autoradiography with intensifying screens at -80°C.

**Modified 3' Mismatched Correction Assay**

A 3' mismatched correction assay using the exonuclease-positive DNA polymerase UITma was modified to increase the sensitivity of the assay by using the presence or absence of a radiolabeled PCR-amplified product as the distinguishing parameter rather than predicted restriction enzyme digestion of the PCR-amplified product. The assay presented here was designed to detect wild-type and mutant sequences by the presence of radiolabeled PCR amplification product in contrast to minimal background radiolabeled product from mutant T1079 α₁ Na,K-ATPase sequences. The upstream sense primer was a 13-mer identical in both Dahl S and R rat α₁ Na,K-ATPase sequences: 5'-aaggggaagggccc-c'T1079-3'. This common 13-mer is then radiolabeled at the 3' end with α-[³²P]dATP by terminal transferase (Stratagene), making the primer wild-type specific after the addition of a [³²P]dATP. Tailing with terminal transferase would add several radiolabeled α-[³²P]dATPs, the length of which is critical for efficient exonuclease excision of noncomplementary 3'-terminal products by UITma DNA polymerase (exonuclease positive), depending on the allele present in the sample to be analyzed. Wild-type α₁ Na,K-ATPase sequences are predicted to anneal with radiolabeled upstream primer (13-
mer+32P- dATP) produced by the exonuclease activity of UITma DNA polymerase. Subsequent PCR amplification with this annealed 32P-3' end-labeled wild-type-specific primer then leads to the expected-sized radiolabeled PCR-amplified product (177 bp). In contrast, at optimal conditions mutant αα, Na,K-ATPase sequences will all allow the extension of all noncomplementary 32P-As added from nt 1079... thereby leaving an annealed nonradiolabeled primer leading to minimal background PCR-amplified product of the same expected size (177 bp). A common downstream antisense primer was used: 5'-acc-ttggca-gat-ggc-ta-3'.

The following specifications were critical in our experimental design: the radiolabeling of the primer, the concentration of oligonucleotide primers, the concentration of dNTPs and MgCl2 in the PCR amplification reaction, the PCR cycling conditions, the temperature of annealing to ensure efficient exonuclease activity and specific annealing, and the "bottom" wax addition. Conditions for radiolabeling were as follows: 100 μCi α32P-dATP (400 Ci/mmol), 3 nmol dATP, 90 pmol upstream 13-mer primer10661078, and Stratagene terminal transferase buffer and 18.6 U enzyme in a 50-μL volume of reaction at 37°C for 15 minutes. The enzyme was inactivated at 80°C for 10 minutes. The wild-type-specific 3' end-labeled upstream primer was then purified on a Diacell p6 column. Differential PCR amplification was performed as follows: 1 x UITma buffer, 40 μmol/L dNTPs, 1 mmol/L MgCl2, 0.3 μmol/L [α32P]dATP-added upstream 13-mer primer10661078, 0.3 μmol/L downstream primer, 200 ng genomic DNA, and 0.03 U/μL volume of reaction UITma DNA polymerase in 20 μL volume of reaction. Optimal cycling conditions were 95°Cx5 minutes; 25 cycles of 95°Cx30 seconds, 62°Cx30 seconds, and 72°Cx30 seconds; followed by extension at 72°Cx7 minutes.

For control, PCR amplification was performed at nondistinquishing PCR cycling conditions showing equivalent amplification of all samples. This was actually achieved at the manufacturer's recommended UITma cycling conditions: 25 cycles of 95°Cx30 seconds and 72°Cx90 seconds, with all chemical reagents and concentrations being identical.

RT^-PCR Analysis of Allele-Specific αα, Na,K-ATPase Transcripts

RT^-PCR was performed essentially according to the manufacturer's specifications (Perkin-Elmer Company) modified to ensure the primer extension/reverse transcription of allele-specific αα, Na,K-ATPase mRNAs by experimentally determining the optimal stringent primer extension condition for the specific antisense primer used. For the RT^-PCR of allele-specific mRNAs (mRNA-specific RT^-PCR) differing by a single nucleotide, primer extension at a range of temperatures (60°C to 70°C) was empirically tested and compared. The temperature of 70°C was documented to be optimally stringent for specific primer extension of wild-type A1079 allele-specific mRNAs alone using a 16-mer wild-type A1079-specific antisense primer with nt1080... at the 3' end: 16 mer=5'-tacagtggacaggcggc-3'. Primer-extended products, if any, were detected by PCR amplification using the following primers: (+) 20 mer=5'-tcacagtccacagcggg-3' and (−)=16-mer used for primer extension, with an expected 194 bp PCR-amplified product. The primer extension temperature for the control mRNAs (cDNA-x and common αα, Na,K-ATPase sequence) was 65°C. Oligonucleotide primers used for the control αα, Na,K-ATPase sequences were common upstream and downstream primer sequences used for each test sample (from Dahl S and Dahl R rats, respectively), primer extension at desired temperatures (60°C, 65°C, and 70°C)x15 minutes; and reaction stopped by incubation on ice. PCR amplification for detection of primer extension product was done by the addition of chelating buffer, MgCl2, and γ32P end-labeled 0.25 μmol/L sense primer; denaturation at 95°Cx2 minutes; amplification via 40 cycles of 94°Cx1 minute, 55°Cx1 minute, and 72°Cx1 minute; and extension at 72°Cx7 minutes. All RT^-PCR products were denatured in formamide and size-fractionated on a 6% denaturing polyacrylamide gel.

Sequencing of Original M13 Clones and PCR-Amplified Templates

Single-strand templates were purified from original and new cultures of M13 subclones (5' EcoRI-Xho I fragment spanning the 5' untranslated region: amino acid [aa]105 of Dahl S αα, Na,K-ATPase cDNA and sequenced by the dyeoxy chain termination method using the Sequenase sequencing kit as per the manufacturer's specifications. The αα sequence-specific sense primer (18-mer=5'-catcggtgtagaagcagg-3') was used as sequencing primer.

To overcome difficult sequencing regions identified previously,10 deoxyinosine triphosphates were used in dyeoxy chain termination sequencing reactions (USB sequencing kit and protocol). The M13 single-strand phage stock of the reconfirmed mutant T1079 αα, Na,K-ATPase M13 subclone shown in Fig 5A, lane 1, was then diluted to 10−2, 10−3, and 10−4. One microliter of these three different dilutions was then PCR-amplified using the M13-specific (−40) universal primer as the antisense primer (17-mer=5'-gcttgccacagcggc-3') and an αα sequence-specific sense primer (18-mer=5'-catcggtgtagaagcagg-3'). PCR conditions were 50 μL volume of reaction, 0.8 μmol/L primers, 2.5 U Taq polymerase per 50 μL reaction volume, denaturation at 95°Cx2 minutes, and 40 cycles of (94°Cx1 minute, 55°Cx1 minute, and 72°Cx1 minute) extension at 72°Cx7 minutes. This 247 bp PCR-amplified product (containing 185 bp of αα, Na,K-ATPase, nt105-1190, and 62 bp of M13 sequence) was then recovered from a 2% low-melting-point agarose gel and diluted with 3 vol water, and 1 μL was used for cycle sequencing. Cycle sequencing was performed using the Perkin-Elmer cycle-sequencing kit according to the manufacturer's specifications. The 18-mer αα sequence sense primer (used in the amplification) was then labeled with γ32P-ATP (Perkin-Elmer protocol) and used as sequencing primer. Cycle sequencing conditions used were 95°Cx2 minutes, and 20 cycles of 94°Cx1 minute, 65°Cx1 minute. Sequencing products were then denatured in formamide, size-fractionated in a 6% denaturing acrylamide gel, and autoradiographed.

Conformation-Specific Differential PCR Amplification Analysis

Based on observations on the analysis of secondary structures of tRNAs, the conformation of DNA segments could also be manipulated by altering the concentration of MgCl2, thereby facilitating or hindering the annealing of primers and subsequent PCR amplification. A common 13-mer primer, identical at nt1066-1078 in both Dahl S and Dahl R rats αα, Na,K-ATPase sequences, is used as the upstream primer spanning the region of the predicted DNA secondary structure differences between the mutant T1079 (four-nt-long hairpin structure) and wild-type A1079 (three-nt-long hairpin structure) αα, Na,K-ATPase sequence. In conditions favoring the stabilization of a four-nt-long hairpin structure (low MgCl2) or three-nt-long hairpin structure (low MgCl2), one would be able to preferentially amplify the more relaxed conformation of the wild-type αα, Na,K-ATPase (three-nt-long hairpin) over the mutant αα, Na,K-ATPase (four-nt-long hairpin). In conditions that equivalently stabilize both a four- and three-nt-long structure (higher MgCl2), equivalent annealing and subsequent amplification would ensue because of the nondistinction at equilib-
rrium of different conformations between the wild-type and mutant α1 Na,K-ATPase sequence.

Experimentally, this was performed as follows: 20 μL volume of reaction; 1x UTRma buffer; 40 μmol/L dNTPs; 1 versus 2 versus 3 versus 4 mmol/L MgCl2; 15 μCi [3P]dATP; 0.3 μmol/L 13-mer upstream primer (identical to the upstream 13-mer primer used for the modified 3' mismatched correction assay above); 0.3 μmol/L common downstream primer (identical to the downstream primer used for the modified 3' mismatched correction assay); 200 ng genomic DNA; and 0.03 U UTRma polymerase per microliter volume of reaction. Cycling conditions were 95°C×5 minutes; 25 cycles of 95°C×30 seconds, 62°C×30 seconds, and 72°C×30 seconds; followed by 72°C×7 minutes extension.

Results

Confirmation of Q276L Mutant α1 and Q276 Wild-Type α1 Na,K-ATPase cDNA Sequences

We first confirmed that the mutant T1079 sequence was correct by resequencing M13 single-strand templates from old and new M13 subclones of two previously isolated overlapping Dahl S α1 cDNA clones (sequence shown and discussed in context below). We have also confirmed that R α1 Na,K-ATPase cDNA nucleotide sequences from two Dahl R/JR rats (BUSM and Harlan Sprague Dawley) are identical to wild-type α1 Na,K-ATPase cDNA sequences as originally characterized from a Sprague Dawley rat brain cDNA library3 (data not shown) as we had previously reported.10 Additionally, since the report of the contamination of Sprague Dawley colonies (Reference 13 and personal communication, R. Russell, Harlan Sprague Dawley, 1993), we also corroborated that the genomic DNA of Dahl S rats from Harlan Sprague Dawley used in the initial identification of the mutant Q276L α1 Na,K-ATPase10 and in the present reinvestigation of mutant α1 Na,K-ATPase sequences are bona fide Dahl S rats by the guanylyl cyclase A (GCA) microsatellite marker (data not shown). The GCA microsatellite marker18 is an informative marker for the genetic contamination detected in the Dahl S strain from Harlan Sprague Dawley rat brain cDNA library3, (Reference 13 and personal communication, R. Russell, Harlan Sprague Dawley, 1993).

Additionally, the other nucleotide mutations at nt1680 and nt2922 did not result in significant amino acid changes,10 so we did not include them in our reinvestigation because they may represent DNA polymorphisms.

Confirmation of Q276L Mutant α1 Na,K-ATPase Allele in Dahl S Rats by PASA Analysis

To investigate the discrepancy of results10,11 with consideration of the likely possibility of a Taq polymerase error during PCR amplification19 or an error by reverse transcriptase during cDNA synthesis,20 we used PASA analysis, a Taq polymerase error-independent test that could test for the presence of a mutation directly in genomic DNA bypassing cloning of said DNA fragments.14,15 PASA analysis can detect single base pair mutations accurately based on homology-dependent differences in melting temperature (Tm) and hybridization or annealing stability of primer to target DNA at stringent temperatures (see "Methods"). At optimal stringent conditions, only target DNA sequences with 100% identity to the primers used will be optimally amplified. To determine whether the mutant T1079 was present in the α1 Na,K-ATPase gene of Dahl S rats, we designed our PASA analysis to detect the mutant T1079 α1 Na,K-ATPase allele by the presence of the expected 183 bp PCR-amplified product. Correspondingly, genomic DNA samples homozygous for the wild-type A1079 α1 Na,K-ATPase allele will not show the expected 183 bp PCR-amplified product. Minimal or background levels of PCR-amplified product are to be expected because of nonspecific "leaky" amplification19 due to the narrow difference in Tm between A and T.

For ascertainment of the specificity of PASA results, amplifiability of all DNA samples tested must first be shown. This is documented in Fig 1 (bottom, control panels A and C), wherein PCR amplification of α1 Na,K-ATPase gene sequences identical to both S and R alleles show the expected 244 bp control PCR-amplified product (arrow), ascertainment that all samples amplify equivalently and are of relative equivalent amounts. B and D, Mutant T1079 α1 Na,K-ATPase allele-specific PCR-amplified product, expected size 183 bp (arrow), is detected only in Dahl S rats, lanes 1 through 3 (A and B) and lanes 6 through 11 (C and D), and three Dahl salt-resistant (R) rats, lanes 4 and 5 (A and B) and lane 12 (C and D). A and C, PCR amplification of common identical sequences in the α1 Na,K-ATPase gene between Dahl S and R rats shows the expected 244 bp control PCR-amplified product (arrow), ascertaining that all samples amplify equivalently and are of relative equivalent amounts. B and D, Mutant T1079 α1 Na,K-ATPase allele-specific PCR-amplified product, expected size 183 bp (arrow), is detected only in Dahl S rats, lanes 1 through 3 (A and B) and lanes 6 through 11 (D). Because of the small difference in melting temperature between dA and T, background "leaky" amplification is observed in Dahl R rats, lanes 4 and 5 (B and D) and lane 12 (D). However, the amount of this background "leaky" PCR-amplified product is significantly lower than that observed with allele-specific PCR-amplified product, as seen in Dahl S rats, and discrepant to the documented amounts of DNA used in amplification (A and C). DNA molecular weight markers (mw) are noted to the left in base pairs.

Downloaded from http://hyper.ahajournals.org/ by guest on July 10, 2017
gent conditions for detection of mutant $\alpha_1$ Na,K-ATPase sequences, experimentally determined at 58°C annealing. PASA analysis (Fig 1, bottom, B and D) shows that genomic DNA from nine Dahl S rats homozygous for the S allele shows the expected 183 bp PCR-amplified product, whereas genomic DNA from three Dahl R rats homozygous for the R allele shows minimal, if any, PCR-amplified product. Considering the fact that equal amounts of DNA were used with similar amplifiability documented earlier, the presence of mutant $T^{1079}$ allele-specific 183 bp PCR-amplified products in Dahl S genomic DNA samples is a specific positive PASA result demonstrating the presence of the mutant $T^{1079} \alpha_1$ Na,K-ATPase allele in Dahl S rats as determined by cDNA sequencing. Likewise, the absence of the mutant $T^{1079}$ allele-specific PCR-amplified products in Dahl R genomic DNA samples is a specific negative PASA result corroborating, albeit indirectly, the presence of the wild-type $A^{1079} \alpha_1$ Na,K-ATPase allele in Dahl R rats as determined by cDNA sequencing.

**Confirmation of Q276L Mutant $\alpha_1$ Na,K-ATPase Allele in Dahl S Rats by LCR Analysis**

To confirm our PASA results indicating the presence of the mutant $T^{1079} \alpha_1$ Na,K-ATPase allele in Dahl S rats, we performed LCR analysis comparing genomic DNA from Dahl S and R rats. LCR analysis also detects the presence of single base pair mutations accurately by ligating stably annealed pairs of oligonucleotide primers with 100% identity to target allele sequences, thus creating predicted-size LCR products from multiple cycles of denaturation, annealing, and ligation using thermostable ligase. We designed our LCR analysis to detect the presence of both DNA strands of the mutant $T^{1079} \alpha_1$ Na,K-ATPase allele by the presence of the expected 47-nt-long and 48-nt-long LCR products representing the sense and antisense strands of the mutant $\alpha_1$ allele (see "Methods"). Correspondingly, wild-type $\alpha_1$ Na,K-ATPase alleles will not show any LCR products. LCR analysis of genomic DNA from Dahl S and R rats, compared with dilutions of known control mutant S $\alpha_1$ cDNAs, was then done. As shown in Fig 2 (right), Dahl S genomic DNA (lane 3) has the expected double band (47-nt-long and 48-nt-long LCR products) positive result, indicating the presence of the mutant $T^{1079} \alpha_1$ Na,K-ATPase allele in Dahl S rats. To confirm that the LCR products from Dahl S genomic DNA are of the expected size, we analyzed concurrently by LCR two dilutions of Dahl S $\alpha_1$ cDNA subcloned in a plasmid vector as controls (Fig 2, right, lanes 1 and 2). As shown in Fig 2 (right), the LCR products obtained from Dahl S genomic DNA (lane 3) are of the expected size, 47 and 48 nt long, identical to the LCR products in control lanes 1 and 2. In contrast, Dahl R genomic DNA did not show any LCR product whatsoever (lane 4), even in the overexposed autoradiogram presented, consistent with the wild-type Q276 $\alpha_1$ Na,K-ATPase sequence as previously described and currently confirmed (Fig 2).

The counter experiment, detecting wild-type-specific $\alpha_1$ Na,K-ATPase sequences, did not work in the identical experimental conditions for the mutant-specific LCR analysis. Optimization of these counter experiments remains to be determined but does not subtract from the specificity of the results obtained with the mutant-specific LCR analysis. Implications as to DNA secondary structure issues are discussed below.
Intriguingly, the counterpart mutant-specific modified 3' mismatched correction assay was not optimized because the 3' labeling with α−[32P]dATP did not work efficiently at the ideal conditions found for the 3' labeling with α−[32P]dTTP by terminal transferase. This observation would be consistent with a more stable hairpin structure from nt1066-1079 with T1079 (four nt long) compared with A1079 (three nt long).

Confirmation of Wild-Type Q276 αι Na,K-ATPase Transcripts in Dahl R Rats by mRNA-Specific RT^-PCR

To investigate the presence of the wild-type A1079 αι mRNA in Dahl R and mutant uracil (U)1079 αι mRNA in Dahl S rat kidney RNAs, we designed an mRNA-specific RT^-PCR test to detect allele-specific mRNAs by using a thermostable enzyme that functions as a reverse transcriptase in the presence of Mn2^+ as well as a DNA polymerase upon the chelation of Mn2^+ and replacement with Mg2^+ (see "Methods"). Briefly, the downstream mRNA priming antisense oligonucleotide was designed with the allele-specific mRNA sequence difference at the 3' end. At the optimal discerning stringent temperature, which was experimentally determined, a primer extension product is synthesized only if there is 100% identity between the primer sequence and target mRNA. The detection of this mRNA-specific primer extension product is facilitated by PCR amplification of the expected-sized product. The absence of PCR-amplified product indicates the absence of primer extension product and hence the absence of the allele-specific mRNA sequence in question. We first documented that equal amounts of all test RNA samples had equivalent amounts of expected-sized RT^-PCR products of a control non-Na,K-ATPase mRNA (cDNA-x, Fig 4, bottom) and control αι Na,K-ATPase mRNA (αι, Fig 4, bottom). With this prerequisite validation, we then documented that wild-type allele-specific A1079 αι RT^-PCR products were detected only in Dahl R rat kidney RNAs (five rats: lanes 4 through 8, Fig 4, bottom) and not in Dahl S rat kidney RNAs (three rats: lanes 1 through 3, Fig 4, bottom) at stringent primer extension conditions (70°C). Furthermore, temperature-dependent specificity was confirmed in the RT^-PCR analysis carried out at lower primer extension temperatures (65°C and 60°C), wherein increasing amounts of "leaky" nonspecific primer extension occurred, resulting in increasing amounts of PCR-amplified product detected in the Dahl S lanes in contrast to the results obtained at 70°C.

Identification of a Site- and Nucleotide-Specific Taq Polymerase Error

To determine whether a Taq polymerase error might be the underlying cause of discrepant results between our work10 and those of Simonet et al,11 we performed Taq polymerase PCR amplification and subsequent sequencing of recently reconfirmed Dahl S αι cDNA M13 subclones. These subclones consisted of two new and one original M13 subclone,10 which, on sequencing of respective single-strand templates by the dyeoxy chain termination method with the modified T7 polymerase (see "Methods"), unequivocally showed the A1079→T transversion (Fig 5A, lanes 1 through 3, respec-
by guest on July 10, 2017 http://hyper.ahajournals.org/ Downloaded from

266 Hypertension Vol 24, No 3 September 1994

Fig 4. Thermostable reverse transcriptase-polymerase chain reaction (RT^PCR) analysis of allele-specific mRNAs confirms wild-type A^1079^a, Na,K-ATPase mRNA in Dahl salt-resistant (R) rat kidney RNA and mutant _a^1_ Na,K-ATPase mRNA in Dahl salt-sensitive (S) rat kidney RNA. Top, RT^PCR analysis was designed to detect wild-type A^1079^a, Na,K-ATPase allele-specific mRNA sequence as a 194 bp PCR-amplified product at stringent primer extension/reverse transcription conditions, with the corresponding absence of PCR-amplified product at identical stringent conditions in RNA samples with non-wild-type or mutant _a^1_ Na,K-ATPase allele-specific mRNA sequence. Bottom, three Dahl S rat kidney RNA samples (lanes 1 through 3) and five Dahl R rat kidney RNA samples (lanes 4 through 8) show equivalent amounts of RT^PCR expected-sized products (arrow) of control non-Na,K-ATPase mRNA (212 bp product cDNA-x) and of control _a^1_ mRNA sequences (181 bp product _a^1_), ascertaining that all samples are reverse-transcribed and amplified equivalently. With this preliminary ascertainment, RT^PCR was then performed at 70°C, 65°C, and 60°C. At 70°C, the expected 194 bp product is detected only in Dahl R rat kidney RNA samples, confirming the presence of the wild-type A^1079^ sequence. Correspondingly, Dahl S rat kidney RNA samples do not show the expected 194 bp wild-type RT^PCR product, indicating the absence of a mutation (non-wild-type) at nucleotide 1079 nt^1079^ of Dahl S a, Na,K-ATPase mRNAs. The temperature-dependent specificity of RT^PCR allele-specific mRNA analysis is confirmed by the detection of increasing amounts of nonspecific RT^PCR product in Dahl S RNA samples (lanes 1 through 3) at 65°C, and even more so at 60°C. DNA molecular weight (mw) markers (4X 174 Haelll digest: 310, 281, 271, 234, 194 bp) are noted at the left-most lane in the control panels of cDNA-x and _a^1_. The 194 bp marker is shown in isolation in the mRNA-specific RT^PCR results at 70°C, 65°C, and 60°C.

(3) 

Fig 5. Gels show detection of a site- and nucleotide-specific Taq polymerase error resulting in the artificial reversal of Dahl salt-sensitive (S) _a^1_ cDNA mutant T^1079^ to wild-type A^1079_ _a^1_. Lane 1, wild-type A^1079_ allele detected in original M13 single-strand phage stock of subclone 1 (Fig 5A, lane 1) for PCR amplification. One-microliter aliquots of increasing tenfold dilutions of the Dahl S _a^1_ cDNA M13 subclone 1 were PCR-amplified using the M13 (--40 bp) universal primer as the downstream primer and an _a^1_ Na,K-ATPase–specific oligonucleotide as the sense primer (see ‘Methods’). The expected 247 bp PCR-amplified products, spanning the questioned aa , were then size-separated, isolated, and sequenced using Taq polymerase–based cycle sequencing. As shown in Fig 5B, increasing dilutions of the mutant T^1079_ _a^1_, Na,K-ATPase M13 single-strand phage stock, representing an increasing ratio of amplified to original DNA, showed increasing amounts of wild-type A^1079_. For the M13 sample diluted to 10^-4, the ratio of erroneous wild-type (wt) A^1079_ and correct mutant (mt) T^1079_ sequencing product was 9:1:10 compared with an approximately 2:1:1 ratio for the M13 sample diluted to 10^-5. Neither cytosine (C) nor guanine (G) was substituted; substitution was limited to adenine (A) (Fig 5).

The reproducibility of this Taq polymerase error was also observed in the sequencing of PCR-amplified frag-
ments of α1 Na,K-ATPase αgt11 CDNA clones known to have the Q276L mutation (data not shown). Our report of a reproducible nucleotide- and site-specific Taq DNA polymerase error is not unique. Reproducible Taq polymerase errors have been reported involving the p53 gene.21-22

Possible Secondary Structure Mechanism Involved in the T^{1079}→A Transversion

The demonstration of a Taq polymerase error specific as to the error site and nucleotide substituted suggests the hypothesis that the chemical microenvironment, secondary structure, or both of this DNA region play a role in the predisposition to Taq polymerase error, as well as in the selection of the dNTP substituted in error. Secondary structure formation of single-strand DNA by hydrogen bonding of palindromic sequences (complementary base pairs along an axis of symmetry) resulting in stem loop/hairpin loop structures23,24 could be expected to impede if not preclude the said palindromic segment from serving as a single-strand template for DNA polymerization or cause the termination of polymerization in in vitro PCR amplification conditions. Analysis of the predicted putative secondary structures of the identical DNA segment of the α1 Na,K-ATPase gene, nt1066-1083, demonstrates a four-nt-long palindromic involving T^{1079} per se that could result in a more stable hairpin structure than the three-nt-long palindromic in the wild-type A^{1079} allele, which, of note, does not involve A^{409} (Fig 6). The predicted noninvolvement of A^{409} in a four-nt-long semistable hairpin structure could therefore favor the propagation of the erroneous substitution of dA at nt1079 over T by any DNA polymerase, the incidence of which becomes increasingly significant especially in and proportional to the degree of exponential polymerization that occurs in Taq polymerase PCR amplification as shown in Fig 5.

To determine whether putative secondary structural differences between the wild-type A^{1079} sequence and the mutant T^{1079} α1 Na,K-ATPase sequence do play a role in the observed sequence-specific PCR amplification and cycle sequencing “idiomsyncrasies” of Dahl S and Dahl R α1 Na,K-ATPase alleles, we analyzed sequence-specific, hence conformation-specific, differential PCR amplification efficiencies in conditions that would elicit conformation-specific effects (Fig 7, top). If the hypothesis is true that secondary DNA structure does play a role, one would expect that different amounts of PCR products, reflecting differential PCR amplification efficiencies, would be obtained using a common primer that spans the sequence-specific DNA secondary structures by varying the concentration of MgCl₂ (see “Methods” for conformation-specific differential PCR amplification). As seen in Fig 7 (bottom), PCR amplification performed at 1 mmol/L MgCl₂ showed less PCR amplification product in Dahl S rats compared with Dahl R rats, indicating that the equilibrium of DNA secondary structure favors the more stable (four-nt-long) hairpin structure in the mutant T^{1079} sequence (Fig 7, bottom), thus hindering primer-DNA template annealing and subsequent amplification. In contrast, the wild-type sequence in Dahl R rats, which is a relatively less stable (three-versus four-nt-long) hairpin structure (Figs 6 and 7), is predicted to be predominantly in a more open DNA secondary structure at 1 mmol/L MgCl₂, resulting in more efficient annealing and PCR amplification. With increasing amounts of MgCl₂ (2 through 4 mmol/L MgCl₂) the distinction between Dahl S and Dahl R rats became less prominent, almost being equal at 4 mmol/L MgCl₂. These results indicate that distinction between a three-nt-long and four-nt-long hairpin structure is nullified at 4 mmol/L MgCl₂, both hairpin structures being favored equivalently, thus exhibiting equivalent PCR amplification efficiencies. With primer spanning identical sequences (nt1066-1079) and stopping just short of nt1079, as well as identical PCR conditions for both Dahl S and Dahl R genomic DNA samples, the difference in PCR amplification with different MgCl₂ concentrations could be attributed only to sequence-specific differences and their resultant conformation differences.

Retrospectively, our previous observations of difficulties with dideoxy chain termination sequencing with Klenow enzyme and modified T7 polymerase necessitating sequencing reactions with deoxyinosine triphosphates for optimal nucleotide sequencing analysis are consistent with the hypothesis of DNA secondary structure formation in this segment of the α1 Na,K-ATPase gene. Additionally, the failure to achieve optimal results at the identical experimental conditions for both sets of mutant- and wild-type-specific oligonucleotide primers for different types of analyses—PASA, LCR, modified 3’ mismatched correction assay, and RT^-PCR—would also be consistent with distinct DNA/RNA secondary structures that distinguish the mutant T^{1079} from the wild-type A^{1079} DNA/RNA sequences. This is not to say, however, that the cognate test does not work but rather that it necessitates more study to define the different sets of in vitro experimental conditions necessary for optimal results of the counter allele-specific test. Alternatively, acknowledging that the possible involvement of DNA secondary structure and/or chemical microenvironment is hypothetical, albeit logically deduced, the reproducible misincorporation by Taq polymerase may be due to factors far more complex than currently proposed and is clearly beyond the scope of this report. These putative complex factors are most likely involved.
Discussion

The molecular confirmation of the Q276L mutation in the Dahl S α1 Na,K-ATPase allele by three separate Taq polymerase error-independent tests of genomic DNA and RNA and the correlation of this mutation with a functionally significant alteration delineate the α1 Na,K-ATPase gene as a stage II candidate hypertension gene. Determination of its mechanistic role or roles in the etiology of hypertension or its ensuing target-organ complications is imperative. Because of the complexities of hypertension as a multifactorial and multiorgan disease, mechanistic analyses must be done by strategic transgenic animal experiments ensuring the necessary integrative analysis.

Carefully designed cosegregation genetic studies must also be carried out to delineate the complex genetic mechanisms that might be in play, addressing issues specific to gender, gene interaction, different blood pressure parameters, and target-organ complications. The cosegregation study by Rapp and Dene reported failure of cosegregation of the α1 Na,K-ATPase locus with hypertension when F1 male and female data were combined. However, when the data are analyzed separately, cosegregation of the mutant S α1 Na,K-ATPase allele with hypertension in F1 males is evident but not in F1 females. This could represent gender-specific differences, which nullify conclusions drawn from combined male and female data. Alternatively, since the male F2 subgroup exhibited the expected mendelian ratio of 1:2:1 and the female subgroup did not, non-cosegregation in F2 female rats might be caused by a serendipitous insufficient number reflected in a deviant mendelian ratio of 10:8:1 and/or erroneous genotyping, as suggested by the deviant mendelian ratio and different patterns of PstI restriction digestion DNA fragments when Southern blots performed by Rapp and Dene are compared with those reported by Herrera and Ruiz-Opazo. Intriguingly, a trend for cosegregation is also apparent in backcross (F1 × Dahl S) females but not in backcross (F1 × Dahl S) males. Another confounding factor in this study is that durations of salt challenge and age at analysis were different between male and female rats within a cohort, ie, F2 or backcross. Hence, given these apparent inconsistencies and the complexities of hypertension delineated in other cohort studies such as complex gene interaction and gender-specific pathogenetic differences, the conclusion of Rapp and Dene and referral by Deng and Rapp to failure of the α1 Na,K-ATPase locus to cosegregate with hypertension in Dahl rats is premature.

Nevertheless, confirmation of the molecular structural data must be taken in the context that supportive physiological evidence has recently been obtained substantiating the existence of the Q276L mutant in a comparative study of α1 Na,K-ATPases in Dahl S and Dahl R rat red blood cells. This kinetic flux study of α1 Na,K-ATPases, the sole erythrocytic isozyme, delineated the identical phenotypic alteration as was identified in Xenopus laevis oocyte expression studies: a twofold decrease in 86Rb+ (K+) influx in Dahl S rat α1 Na,K-ATPases. Further kinetic analysis of α1 Na,K-ATPase in red blood cells revealed that Na+ efflux is unaltered, resulting in an augmented Na+K+ coupling ratio in Dahl S rats (3.5:1) in contrast to Dahl R rats (2:1). This ratio differs from the "canonical" 3:2 ratio of Na+:K+ ions transported by Na,K-ATPase established in humans. A difference in K+ (86Rb+) influx was
Intuitively, the resultant effect of an augmented Na⁺-K⁺ coupling ratio in Dahl S α₁ Na,K-ATPases, in contrast to Dahl R α₁ Na,K-ATPases, is a higher number of Na⁺ ions transported per pump cycle of the α₁ Na,K-ATPase. Since α₁ Na,K-ATPase is the sole renal isozyme in rat kidney³⁴ and Na,K-ATPases are responsible for all active Na⁺ transport across the basolateral membrane of tubular epithelia throughout the nephron, as well as providing the "downhill" gradient for all apical Na⁺ transporters and channels in their respective tubular nephric segments,³⁴ an irrefutable link between a valid molecular mechanism for increased Na⁺ reabsorption mediated through a mutant α₁ Na,K-ATPase and salt-sensitive hypertension typified in Dahl S rats is hypothesized.

In summary, the molecular corroboration of mutant Q276L α₁ Na,K-ATPases, supported by concordant reports of altered α₁ Na,K-ATPase transport kinetics³⁵ and homeostatic regulation³³,³⁴ in Dahl S rats in contrast to Dahl R rats, provides the mandate to investigate the putative genetic mechanisms involved.

More importantly, the documentation of a site- and nucleotide-specific Taq polymerase error reiterates the necessity to verify all mutations identified using PCR-amplified products by Taq polymerase error-independent tests. Confirmation of DNA mutations in the respective mRNA must also be done, when feasible, by mRNA-specific RT²⁻PCR analysis and characterization of corresponding cDNA clones. The nucleotide sequence analysis of several PCR-derived clones does not rule out Taq polymerase error-induced sequences because cloning itself introduces another variable in the ratio of erroneous to bona fide PCR-amplified mutant clones. Given the realization that molecular structural characterization and genetic linkage analysis of a mutant allele could be inherently problematic; it is thus becoming more evident that concordant functional evidence of a phenotypic alteration becomes a necessary parameter in the study of mutant candidate genes, especially in polygenic disorders. Inasmuch as errors by other DNA polymerases and reverse transcriptase also occur,²⁰ albeit at lower frequencies than observed in PCR amplification by Taq polymerase,¹⁹ multiple confirmatory tests are also necessary in the molecular determination of wild-type and/or mutant sequences from cloned material. Finally, some DNA point mutations, although seemingly random, most likely have a structural basis to their inception. The elucidation of this hypothesis as a concept would aid in the evaluation of mutations in candidate genes in complex polygenic diseases.

Acknowledgments

N.R.-O. is an American Heart Association Established Investigator. This work was supported by grants HL-47124 and GM-41337 from the National Institutes of Health, Bethesda, Md.

References

2. McDonough AA, Geering K, Farley RA. The sodium pump needs other DNA polymerases and reverse transcriptase also occur, albeit at lower frequencies than observed in PCR amplification by Taq polymerase, multiple confirmatory tests are also necessary in the molecular determination of wild-type and/or mutant sequences from cloned material. Finally, some DNA point mutations, although seemingly random, most likely have a structural basis to their inception. The elucidation of this hypothesis as a concept would aid in the evaluation of mutations in candidate genes in complex polygenic diseases.

Acknowledgments

N.R.-O. is an American Heart Association Established Investigator. This work was supported by grants HL-47124 and GM-41337 from the National Institutes of Health, Bethesda, Md.

References

2. McDonough AA, Geering K, Farley RA. The sodium pump needs other DNA polymerases and reverse transcriptase also occur, albeit at lower frequencies than observed in PCR amplification by Taq polymerase, multiple confirmatory tests are also necessary in the molecular determination of wild-type and/or mutant sequences from cloned material. Finally, some DNA point mutations, although seemingly random, most likely have a structural basis to their inception. The elucidation of this hypothesis as a concept would aid in the evaluation of mutations in candidate genes in complex polygenic diseases.

Acknowledgments

N.R.-O. is an American Heart Association Established Investigator. This work was supported by grants HL-47124 and GM-41337 from the National Institutes of Health, Bethesda,Md.

References

2. McDonough AA, Geering K, Farley RA. The sodium pump needs other DNA polymerases and reverse transcriptase also occur, albeit at lower frequencies than observed in PCR amplification by Taq polymerase, multiple confirmatory tests are also necessary in the molecular determination of wild-type and/or mutant sequences from cloned material. Finally, some DNA point mutations, although seemingly random, most likely have a structural basis to their inception. The elucidation of this hypothesis as a concept would aid in the evaluation of mutations in candidate genes in complex polygenic diseases.

Acknowledgments

N.R.-O. is an American Heart Association Established Investigator. This work was supported by grants HL-47124 and GM-41337 from the National Institutes of Health, Bethesda, Md.

References

2. McDonough AA, Geering K, Farley RA. The sodium pump needs other DNA polymerases and reverse transcriptase also occur, albeit at lower frequencies than observed in PCR amplification by Taq polymerase, multiple confirmatory tests are also necessary in the molecular determination of wild-type and/or mutant sequences from cloned material. Finally, some DNA point mutations, although seemingly random, most likely have a structural basis to their inception. The elucidation of this hypothesis as a concept would aid in the evaluation of mutations in candidate genes in complex polygenic diseases.

Acknowledgments

N.R.-O. is an American Heart Association Established Investigator. This work was supported by grants HL-47124 and GM-41337 from the National Institutes of Health, Bethesda, Md.

References

2. McDonough AA, Geering K, Farley RA. The sodium pump needs other DNA polymerases and reverse transcriptase also occur, albeit at lower frequencies than observed in PCR amplification by Taq polymerase, multiple confirmatory tests are also necessary in the molecular determination of wild-type and/or mutant sequences from cloned material. Finally, some DNA point mutations, although seemingly random, most likely have a structural basis to their inception. The elucidation of this hypothesis as a concept would aid in the evaluation of mutations in candidate genes in complex polygenic diseases.

Acknowledgments

N.R.-O. is an American Heart Association Established Investigator. This work was supported by grants HL-47124 and GM-41337 from the National Institutes of Health, Bethesda, Md.

References

2. McDonough AA, Geering K, Farley RA. The sodium pump needs other DNA polymerases and reverse transcriptase also occur, albeit at lower frequencies than observed in PCR amplification by Taq polymerase, multiple confirmatory tests are also necessary in the molecular determination of wild-type and/or mutant sequences from cloned material. Finally, some DNA point mutations, although seemingly random, most likely have a structural basis to their inception. The elucidation of this hypothesis as a concept would aid in the evaluation of mutations in candidate genes in complex polygenic diseases.


Confirmation of mutant alpha 1 Na,K-ATPase gene and transcript in Dahl salt-sensitive/JR rats.
N Ruiz-Opazo, F Barany, K Hirayama and V L Herrera

Hypertension. 1994;24:260-270
doi: 10.1161/01.HYP.24.3.260

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 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:
http://hyper.ahajournals.org/content/24/3/260