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25. The contribution made by the purine O_6 and pyrimidine N_3 positions remains ambiguous. Neither the DAP·C nor the DAP·^{Me}iC pair restores binding completely to the level of the G·U pair. DAP·C and DAP·^{Me}iC are ~1.0 kcal·mol^{-1} less stable than G·U and G·^{Me}U, respectively. This might be interpreted as a major groove contact to the O_6 carbonyl of G that is disrupted upon substitution with an amine. Although we cannot completely rule out this possibility, we note that functional groups neighboring the O_6 carbonyl do not influence tertiary interaction energy, so it seems more likely that the energetic difference is derived from a less specific effect. It might result from a negative steric interaction due to the shape of the DAP wobble pairs not being completely isomorphic with G·U, or an indirect electrostatic effect in the minor groove because the hydrogen bond formed adjacent to the exocyclic amine differs between the G·U and the DAP wobble pairs (Fig. 2A). Thus, while the G·U and DAP·^{Me}iC pairs are energetically equivalent at the terminus of an isolated helix, it appears that the active site of the ribozyme is more sensitive to the subtleties of bond polarity and base shape.
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Genetic Decreases in Atrial Natriuretic Peptide and Salt-Sensitive Hypertension

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To determine if defects in the atrial natriuretic peptide (ANP) system can cause hypertension, mice were generated with a disruption of the *proANP* gene. Homozygous mutants had no circulating or atrial ANP, and their blood pressures were elevated by 8 to 23 millimeters of mercury when they were fed standard (0.5 percent sodium chloride) and intermediate (2 percent sodium chloride) salt diets. On standard salt diets, heterozygotes had normal amounts of circulating ANP and normal blood pressures. However, on high (8 percent sodium chloride) salt diets they were hypertensive, with blood pressures elevated by 27 millimeters of mercury. These results demonstrate that genetically reduced production of ANP can lead to salt-sensitive hypertension.

Essential hypertension is a heterogeneous disease in which blood pressures are harmfully high without overt cause; it affects a large number of individuals in many populations (*1*). Both genetic and environmental factors have been implicated in its etiology. Several complex physiological systems affect blood pressure, including one that is mediated by the 28–amino acid atrial natriuretic peptide (ANP) (*1*, *2*). ANP is produced mainly in the cardiac atria, where it is stored in dense granules as a large precursor (proANP), which is the major component of the granules (*2*). ANP is the COOH-terminal portion of the precursor. In response to an increase in atrial distension, ANP is released into the circulation and interacts with receptors in the vasculature, kidney, and adrenal glands. Although in pharmacological doses ANP is known to lower blood pressure and promote salt excretion, its physiologic functions have not been clearly defined and have not been proven (*3*).

Because of the actions of ANP, efforts have been made to determine whether genetic variants of the ANP system are involved in the etiology of essential hypertension. For example, plasma ANP concentrations in the children of two normotensive parents are higher than in children with a hypertensive parent, especially when the children are on high salt intakes (*4*). Several polymorphisms have been identified in the human *proANP* gene (*5*). Nevertheless, no association between two *proANP* markers and hypertension has been found in patients although they were not evaluated for their responses to dietary salt (*6*). These results indicate that the

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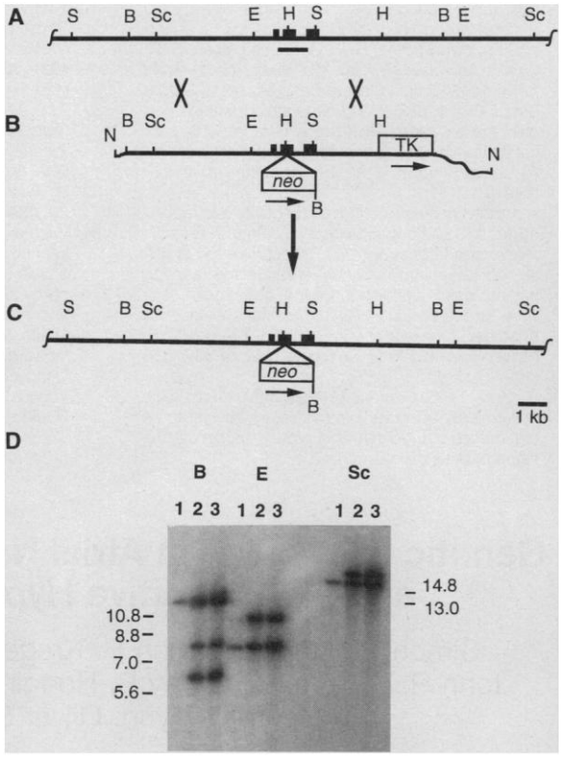

Fig. 1. Gene targeting. (**A**) The *proANP* locus. The black boxes represent exons. Only relevant restriction sites are shown: Sma I (S), Bam HI (B), Sac I (Sc), Eco RI (E), and Hinc II (H). (**B**) Targeting construct. The construct deletes 11 bp of exon 2 and replaces it with the neomycin resistance gene (*neo*). TK represents the thymidine kinase gene. The construct was linearized with Not I (N), and targeted cells were produced as described (*16*). Plasmid sequences are represented by wavy lines (not drawn to scale). (**C**) Targeted locus: the disrupted *proANP* locus after homologous recombination between the targeting construct and the endogenous gene. (**D**) Confirmation of targeting. Southern (DNA) blots of DNA samples from the starting ES cells (sample 1) or two independently targeted cell lines (samples 2 and 3). Digests were with Bam HI (B), Eco RI (E), and Sac I (Sc). The probe is shown as a bar as in (A). Fragments sizes are indicated in kilobases and agree with expectation. Expected fragments were also seen with Sma I (*17*).

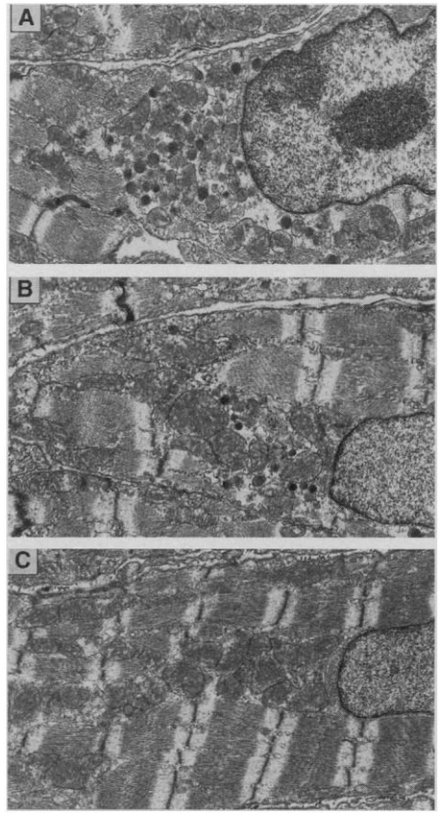

Fig. 2. Electron microscopy on atrial sections (*18*) from *proANP* (**A**) +/+, (**B**) +/−, and (**C**) −/− mice. The analysis was performed as described (*15*). Original magnification, ×14,000.

markers under investigation do not cosegregate with high blood pressure in the studied samples; however, they do not indicate whether the ANP locus can contribute to the genetic etiology of hypertension in other patients or can modulate biological responses to dietary salt. We therefore examined the effects of genetically reduced expression of the *proANP* gene on the blood pressures of mice and on the responses of mice to dietary salt.

We used gene targeting by positive-negative selection (*7*) in mouse embryonic stem (ES) cells to mutate exon 2 of the mouse *proANP* gene, which codes for the precursor of ANP (Fig. 1). Four chimeras generated from one of the targeted ES cell lines transmitted the disrupted *proANP* gene to their offspring. The chimeras were mated to mice of strain C57BL/6J (B6). Because the ES cells used for targeting (*8*) were from mice of strain 129, the offspring were 129 × B6 F_1 animals that were genetically identical except for their *proANP* genotypes and sexes. Matings between heterozygotes yielded homozygous mutant (−/−), heterozygous (+/−), and wild-type (+/+) F_2 offspring in Mendelian proportions.

Plasma ANP in animals of the three F_2 genotypes was measured by a radioimmunoassay as described (*9*). No circulating ANP was detected in the homozygous mutants (n = 5). When the mice were fed a standard salt (0.5% NaCl) diet, the amount of circulating ANP of heterozygotes (135 ± 32 pg/ml, n = 5) did not differ significantly from that in wild-type animals (148 ± 23 pg/ml, n = 7).

We then measured atrial ANP by radioimmunoassay (*9*). This analysis is a stringent test for the absence of ANP in the homozygous mutants because the atria contain more ANP than any other organ. We obtained the following values: for homozygous mutants (n = 9), undetectable amounts (<1% of the wild type); for heterozygotes (n = 5), the right atrium contained 54.3 ± 7.1 ng/mg (wet weight), the left atrium, 53.7 ± 6.2 ng/mg; and for the wild type (n = 5), the right atrium contained 114.7 ± 5.9 ng/mg, the left atrium, 112.2 ± 7.5 ng/mg.

Atrial sections from mice of all three genotypes were analyzed by transmission electron microscopy to evaluate the occurrence of atrial granules. The homozygous mutants had no detectable granules at ×14,000 magnification (Fig. 2) or at ×20,000 magnification, whereas the granule content in the heterozygotes was reduced. The absence of any recognizable granular structures in homozygous mutants suggests that ANP may be important for the assembly process of these atria-specific granules. Study of the homozygous mutant may therefore provide new information regarding the assembly and storage of secretory granules in endocrine cells.

Homozygous mutants were fertile and appeared normal. When maintained on intermediate salt diets (2% NaCl), they had a greater ratio of heart weight (mg) to body weight (g) (6.8 ± 0.4, n = 12) than both heterozygous (5.2 ± 0.2, n = 8, P < 0.01) and wild-type (4.8 ± 0.2, n = 9, P < 0.01) animals (*10*). Cardiac enlargement is a common feature in hypertensive animals. The increased size may be a consequence of elevated arterial pressure, although changes in growth control within the cardiovascular system are also possible (*11*). Whether the enlargement in the homozygous mutants is a direct effect of lack of ANP or is secondary to hypertension remains to be determined.

ANP lowers intravascular volume by facilitating fluid movement from blood vessels to the extracellular space and by natriuresis (*2*). Therefore, the absence of ANP may result in increased plasma volume. In support of this, the hematocrits of mutant homozygotes (45.8 ± 0.8%, n = 11) differed significantly from those of either heterozygous (48.6 ± 0.9%, n = 11, P < 0.05) or wild-type animals (49.9 ± 0.7%, n = 11, P < 0.01).

Blood pressures were measured in a "blinded" randomized fashion with either a tail-cuff or a carotid catheter (*12, 13*). Tail-cuff blood pressures were first obtained for F_2 mice of the three *proANP* genotypes that

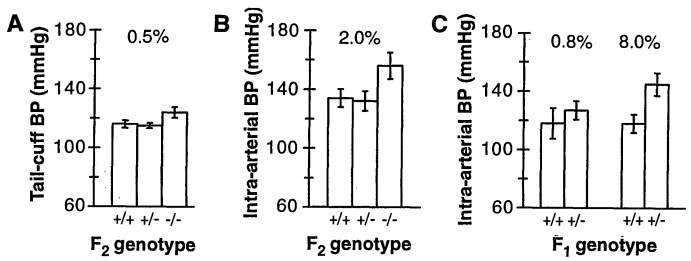

Fig. 3. Blood pressures as a function of *proANP* genotypes and dietary salt. The vertical bars show mean blood pressures (*13*); error bars indicate standard errors of the means. (**A**) Tail-cuff blood pressures of *proANP* +/+, +/−, and −/− F_2 mice fed a diet containing 0.5% NaCl. Each group consisted of eight males from 11 to 14 weeks in age. (**B**) Intra-arterial blood pressures of 11 +/+, 9 +/−, and 11 −/− F_2 males fed a diet containing 2% NaCl, from 14 to 17 weeks in age. (**C**) Intra-arterial blood pressures of +/+ and +/− F_1 mice fed diets containing 0.8% and 8% NaCl. Each group included males and females from 15 to 26 weeks in age: +/+ on 0.8% NaCl, four males plus five females; +/− on 0.8% NaCl, five males plus seven females; +/+ on 8.0% NaCl, nine males plus five females; and +/− on 8% NaCl, six males plus five females. In all experiments, there were no significant weight differences between mice of each genotype.

for 2 to 3 weeks had been fed chow containing standard salt (0.5% NaCl). Our data showed that the blood pressures of the wild-type and heterozygous animals did not differ on this diet (116 ± 2.5 mmHg and 115 ± 1.8 mmHg, respectively). However, the homozygous mutants had blood pressures that were significantly higher (124 ± 3.7 mmHg) than those of the other genotypes (difference = 8 mmHg; $P < 0.05$ for both comparisons) (Fig. 3A). There were no differences in the heart rates of the three genotypes [+/+, 745 ± 15 beats per minute (bpm); +/−, 744 ± 8 bpm; −/−, 732 ± 18 bpm].

We then determined the mean arterial blood pressures of the same group of mice (plus a few additional F_2 animals) after feeding them a diet containing intermediate salt (2% NaCl) for at least 14 days (Fig. 3B). The mutant homozygotes again showed increased mean arterial blood pressures (156 ± 9 mmHg) relative to the other animals (+/−, 132 ± 6.7 mmHg; +/+, 134 ± 6.1 mmHg; $P < 0.05$ for both comparisons). Of the 11 homozygous mutants on the intermediate salt diet, two had mean arterial pressures of more than 200 mmHg. The blood pressures of heterozygous and wild-type animals still did not differ significantly. The heart rates of the three groups were not significantly different (+/+, 569 ± 20 bpm; +/−, 589 ± 22 bpm; −/−, 581 ± 17 bpm).

F_2 animals of the three genotypes may differ systematically at loci in addition to *proANP* if strain 129 or strain B6 differ in any closely linked genes. Thus, our F_2 data do not exclude the possibility that the elevated blood pressures of the −/− mice resulted in part from the inheritance of strain 129 alleles of a gene or genes closely linked to the *proANP* locus.

Higher dietary salt intake in humans is associated with higher blood pressure (*14*). Because a partial deficiency rather than a complete absence of ANP is more likely to occur in human patients, we determined the effects of the intake of increased amounts of salt on the blood pressures of heterozygous animals. To do this, we measured the mean arterial pressures of F_1 wild-type and heterozygous animals fed either standard chow (0.8% NaCl) or high salt chow (8% NaCl) for 2 weeks (Fig. 3C). The blood pressures of the heterozygous F_1 animals and of the wild-type F_1 animals did not differ significantly when they were fed standard chow (127 ± 6.3 mmHg for +\− as compared to 118 ± 10 mmHg for +/+; $P > 0.05$). But when the animals were fed the high salt diet, the heterozygotes became hypertensive (145 ± 7.8 mmHg, $P < 0.01$), whereas the wild-type animals did not (118 ± 6.2 mmHg). The heart rates of the four groups were not significantly different (+/+ with 0.8% NaCl, 564 ± 45 bpm; +/− with 0.8% NaCl, 553 ± 35 bpm; +/+ with 8.0% NaCl, 549 ± 25 bpm; and +/− with 8.0% NaCl, 553 ± 35 bpm).

The +/+ and +/− F_1 animals were identical except for their genotypes at the *proANP* locus. Accordingly, our F_1 data establish that genetically reduced ANP, unaccompanied by any other genetic differences linked or unlinked to the *proANP* locus, can cause salt-sensitive hypertension. Our finding that ANP modulates the blood pressure response to dietary salt will encourage the search for human genetic variants that affect the function of the ANP system. Detecting such variants may identify hypertensive patients likely to benefit from a reduced salt intake.

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13. The care of the animals and all experiments were conducted in accordance with institutional guidelines for the University of North Carolina at Chapel Hill and Queen's University, Kingston, Ontario. Tail-cuff blood pressures were measured on five consecutive days after a 7-day training period. For intra-arterial measurements, catheters were inserted into the carotid artery under general anesthesia (2 mg of ketamine and 0.2 mg of xylazine per 25 to 30 g of body weight, intramuscularly). Mean arterial pressure was calculated as the sum of two-thirds of the mean of all wave form troughs and one-third the mean of all wave form peaks. In all blood pressure experiments, mice of each genotype were analyzed on each day; the investigator did not know their genotypes (or diets in the F_1 experiment).
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18. Mice were anesthetized intraperitoneally with Avertin (0.3 ml of 2.5% drug per 25 g of body weight) and perfused with a mixed aldehyde fixative in 0.1 M sodium cacodylate buffer (pH 7.2). The whole heart was excised and immersed in fresh fixative for an additional 24 hours and then processed for electron microscopy as previously described (*15*). A total of 150 myocytes from each sample was examined.
19. The ES cell line BK4 [a subclone of E14TG2A (*18*)] was provided by B. Koller. We thank T. Coffman, L. Kester, R. Kucherlapati, R. Langenbach, C. Lawler, J. Lynch, N. Maeda, J. Pang, L. Peng, D. Rouse, I. R. Sarda, and J. Van Horne for help and advice. Supported by grants (GM20069 and HL49277) from NIH (O.S.) and from the Medical Research Council of Canada (S.C.P. and T.G.F.) and a Howard Hughes Medical Institute Physician Postdoctoral Fellowship (J.H.K.).

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