Past and Current Perspectives on the Natriuretic Peptides (44041)

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It is now some 15 years since de Bold and colleagues observed the profound hypotension, diuresis, and natriuresis that occurred following injection of acid extracts of cardiac atria into anesthetized rats (1). As is now well documented, these effects were due to a peptide hormone, atrial natriuretic factor or peptide (ANF or ANP), which is stored in precursor form in atrial specific granules (for review of discovery and early research on ANP see Ref. 2). ANP is a 28–amino acid disulfide bonded peptide that forms the C terminus of a larger precursor, pro-ANP, and which is released into the circulation in response to local wall stretch induced by increased intraatrial volume (3).

Following the initial experiments of Sonnenberg and de Bold (1), several atrial natriuretic peptides were purified, sequenced, and chemically synthesized. Administration of pharmacological doses of synthetic ANP confirmed that the peptide was a potent natriuretic substance with mild hypotensive effects (4–7). Infusion of pharmacological amounts of ANP to a variety of species showed that the peptide evoked a fall in blood pressure that was sustained for a short period of time and a diuresis and natriuresis that was profound but short. Accompanying these changes was an efflux of chloride and potassium, and a fall in the hematocrit. These changes, although the result of large doses of ANP, suggested that release of ANP from the heart might be involved in the control of blood pressure and body volume.

Determination of the amino acid sequence of ANP allowed the cloning and sequencing of first the cDNA (8–10) and then the gene for ANP (10, 18). From such studies, it was clear that ANP is synthesized as a precursor, pro-ANP, of 149 to 153 amino acids depending on the species. Cleavage of the hydrophobic signal peptide and in some species of two C-terminal arginines from the precursor produces the 126–amino acid pro-ANP (1–126). The mature and circulating form of the peptide, ANP(99–126) is derived from the C-terminal 28 amino acids and is formed upon exocytosis of the atrial myocyte (19). The presence of a disulfide bond linking the two cysteine residues (Fig. 1) is essential for biological activity (20). The amino acid sequences of ANP(99–126) are identical except for position 110, which is Ile or Met depending on the species (21). The amino acid sequences of pro-ANP also exhibit a great deal of sequence identity (Fig. 2), and since conservation of primary structure is often an indication of functional importance it raises the question of the function, if any, of the first 98 amino acids of pro-ANP.

There is no doubt of the functionality of ANP (99–126). Reduction of the disulfide bond eliminates biological activity and truncation of the peptide particularly at the C-terminal end diminishes its biological potency (22). ANP(99–126) exerts its physiological actions by binding to specific receptors on cell surfaces (23), and integrity of ANP(99–126) structure is essential for function. There is evidence, however, that peptides derived from the N terminus of pro-ANP are biologically active. Vesely and his co-workers (24) have obtained evidence that in addition to ANP(1–98), three other peptides derived from the N terminus of pro-ANP circulate in the blood. These peptides—ANP(1–30), ANP(31–67), and ANP(79–88)—are designated long-acting sodium stimulator, vessel dilator, and kaliuretic stimulator, respectively, according to their reported functions (25). They have much longer plasma half-lives than ANP and circulate at higher concentrations (26–28). It is clear that these peptides do not act through the ANP receptor system (29) and there is controversy regarding whether or not they have specific binding sites (29, 30). There is much that is puzzling about their mode of action. A major criticism is that studies previously carried out with these

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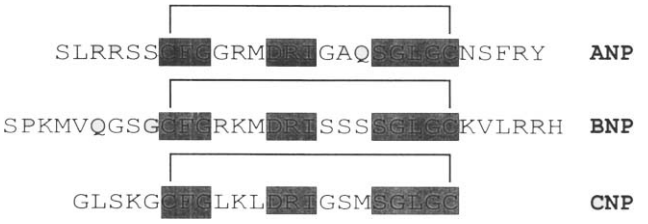

Figure 1. Amino acid sequence alignment of the mature forms of human ANP, BNP, and CNP. The shaded boxes denote conservation of identical residues. The line joining the cysteine residues signifies a disulfide bond.

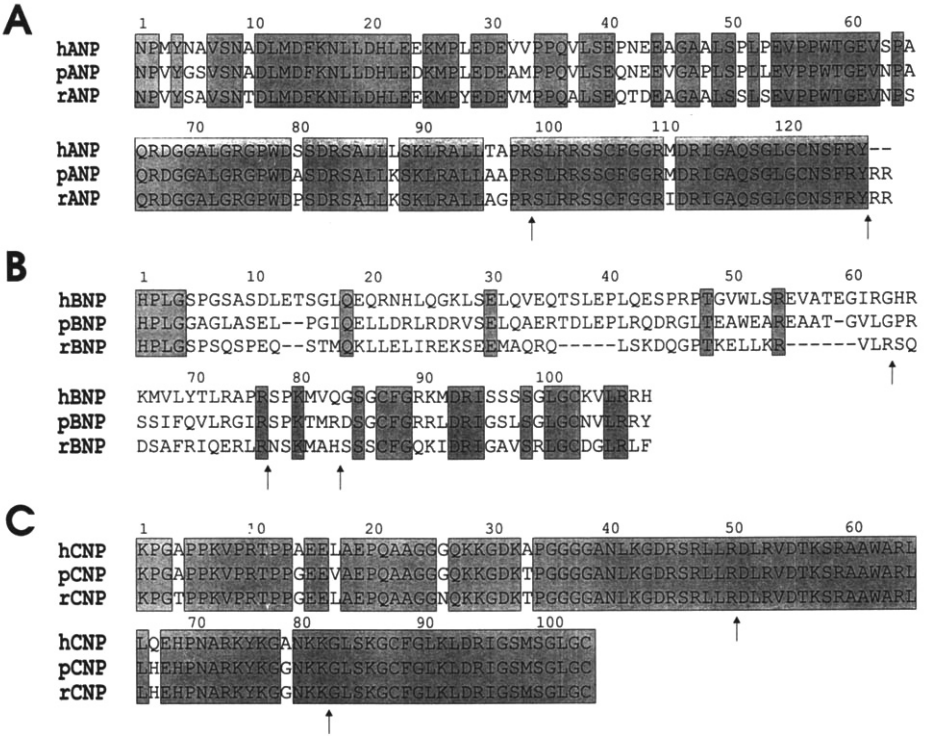

Figure 2. Amino acid sequence alignment of human pig and rat natriuretic peptide precursors. Shaded boxes indicate conservation of identical amino acid sequences. (A) Human (10), pig (11), and rat (9) pro-ANPs. (B) Human (12), pig (13), and rat (14) pro-BNPs. (C) Human (15), pig (16), and rat (17) pro-CNPs. In Panel A, the arrows denote cleavage sites for the processing of the mature hormone ANP(99–126). In Panel B, arrows denote the cleavage site for processing of BNP-45 (pig and rat), BNP-32 (pig, rat, and human), and BNP-26 (pig and rat), the mature forms of the hormone. IN Panel C, arrows denote the cleavage sites for CNP-53 and CNP-22 which are believed to be the active forms of the hormone.

peptides utilized nonhomologous species and peptides (i.e., human peptide in rat). In our own laboratory, using rat ANP(1–30 and 31–67) and rat tissues we found no evidence that either ANP(1–30) or ANP(31–67) had any biological activity. The peptides did not displace radioactive ANP from binding sites on plasma membranes and did not appear to have distinct binding sites in either lung or kidney (29). Another puzzling feature about these peptides is that they only appear to work in the intact animal. If the abdomen is opened, say, for placement of a renal catheter, then the effect of these peptides is markedly attenuated. In fact, in the rat their natriuretic effects may disappear completely without an intact abdomen (24).

The implications of the presence of these N-terminally derived peptides seems to lie in their proposed long-term effects, in contrast to the acute effects of ANP in responding to volume changes (25). Much more work needs to be done on these potentially important peptides by other workers in the field so that their exact role in volume homeostasis can be defined. An interesting and potentially important finding regarding these N-terminal peptides is that the plasma concentration of ANP(31–67) increases in early heart failure (27), even in otherwise asymptomatic people. This can therefore be used as a distinguishing feature. Indeed, Burnett and his group have used an RIA to ANP(1–25) to detect N-terminal ANPs and to use this as a marker for symptomless left ventricular dysfunction—that is, to distinguish early signs of heart failure in healthy patients with no signs of congestive heart failure (31). None of these studies, of course, speak to the mechanism of action or physiological importance of these N-terminal pro-ANP peptides. Why their concentrations are raised under some circumstances is an intriguing question and merits further investigation.

Since the discovery of ANP, other natriuretic peptides have been found to be present in heart and other tissues. The first of these called brain natriuretic peptide (BNP) was initially purified from pig brain (32) but subsequently found to be a cardiac peptide as well (33). It is present in the atrial granules (34) but also in the ventricles (35). A third peptide, CNP, has been isolated from brain also (36). This is believed to be a peptide of the central nervous system, although, as will be shown later, it is present and may be functional in other tissues.

Structurally, all the natriuretic peptides bear a strong resemblance to one another (Fig. 1). They are all disulfide-bonded peptides and are formed by cleavage from the C terminus of a pro-peptide precursor. Alignment of the precursors of ANP, BNP, and CNP reveals interesting features (Fig. 2). Pro-ANP is highly conserved, particularly in the C-terminal region, but there are also areas of conservation in the N-terminal side of the protein. BNP is the least conserved of all the natriuretic peptides and its precursor shows considerable variability among species. CNP is the most highly conserved of all the natriuretic peptides. The active hormone, usually the C-terminal 22 amino acids, is identical in all species, and the remainder of its precursor is 90% identical among the species. It is difficult to know what conservation of precursor sequence has to do with function. ANP, which is highly conserved, is present in the largest amounts and would seem to be the major cardiovascular and renal peptide. BNP is mainly found in cardiac ventricles, and it is increased substantially both in the ventricles and in the blood in cardiopathological states (e.g., congestive heart failure) (37). CNP is not normally found in blood although it has been detected in the circulation in some disease states (38). CNP is distributed throughout the brain in concentrations that are at least one order of magnitude higher than those of ANP and BNP, suggesting that CNP is a major peptide of the central nervous system (39). There is evidence, however, that CNP is present in the endothelial cells of blood vessels and may act as an endothelium-derived autocrine or paracrine regulator mediating vasorelaxation (40). More recently, the mRNA for CNP has been detected in seminal vesicles (41) and in ovaries (42). The significance of these findings is not clear, but it is becoming evident that CNP may play a much different role than ANP or BNP.

All of the three natriuretic peptides that are derived from the C-terminal ends of their precursors exert their physiological effects by interacting with a well-defined set of specific receptors (23). Two of these (GC-A and GC-B) are membrane-bound guanylyl cyclases, and their biological activity is mediated through the generation of cyclic GMP (43). The third receptor is not particulate guanylyl cyclase linked and is believed to serve as a clearance receptor to remove large amounts of ANP from the circulation or to store ANP and release it slowly (44). There is good evidence to suggest that ANP, BNP, and CNP react differentially with the three different receptors (45) (Fig. 3). Both ANP and BNP bind to GC-A with relatively high affinity and both can effectively stimulate GC-A to cause an increase in intracellular cGMP production with BNP being about 10-fold less potent (45). In contrast, CNP does not bind to GC-A even at very high concentrations and it does not increase intracellular cGMP. However, only CNP binds with high affinity to GC-B, and it is the only natriuretic peptide to stimulate GC-B to produce significant amounts of cGMP. This finding supports the proposition that CNP acts as an endothelial-derived relaxing peptide. Both the CNP gene and the GC-B receptor are widely expressed in vascular walls (40). All three natriuretic peptides and a wide variety of natriuretic peptide analogs bind to the clearance receptor (46), which is consistent with its role in regulating the circulating levels of all the natriuretic peptides.

Most of the research that has taken place so far has been concerned with elucidating the extent and magnitude of ANPs physiological effects and with the details of the mode of action of all of the natriuretic peptides at the cellular and molecular level. It is easy

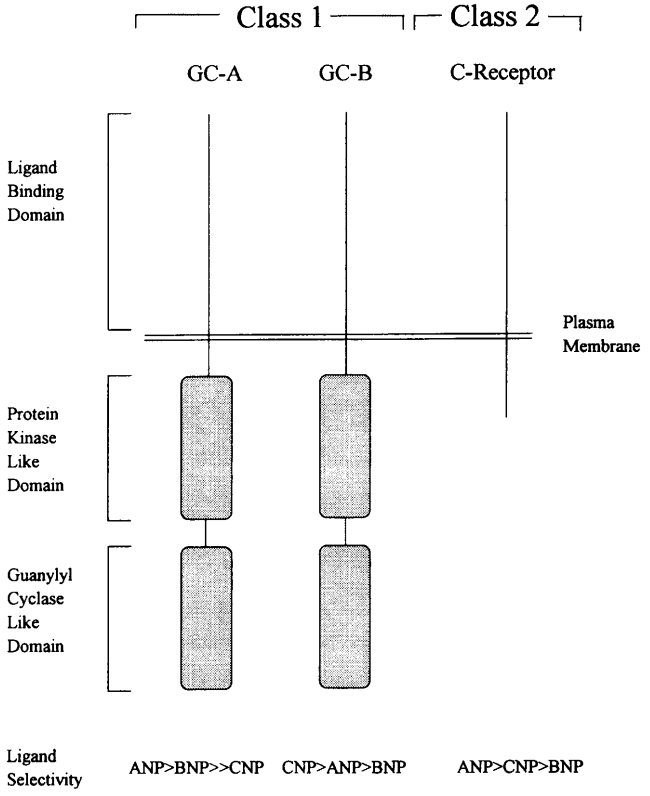

Figure 3. Diagramatic representation of the receptors for the natriuretic peptides. The ligand selectivity in terms of elicitation of cGMP and binding is indicated at the bottom of the diagram.

to see why this has been the case. The methodology for investigating the physiology of factors that affect the control of salt balance, volume control, and blood pressure are well established both in the whole animal and in isolated organ systems. Also, the tools of molecular biology advanced at such a breathtaking pace that they allowed the sequencing of the cDNA and the gene for ANP within 2 years of its purification and the determination of its amino acid sequence. The subsequent isolation, cloning, and sequencing of the cDNA for BNP and CNP and of the three natriuretic peptide receptors seem to have been almost a matter of course given the sophistication of current methods in molecular biology.

Despite all of this research, what is still left undecided is the *importance* of ANP and indeed of the other natriuretic peptides in the control of blood pressure and extracellular volume. An obvious way of deciding whether any factor is important to a system is to remove or nullify the factor, and this has been attempted in the case of ANP through the use of antagonists and specific antibodies.

A number of peptide analogs have been shown to blunt or inhibit cGMP production induced by ANP (47, 48) and a number of peptide and nonpeptide antagonists have been evaluated for their effects on diuresis and natriuresis and other parameters of ANP action (49, 50). Perhaps the most promising approach involved the use of antibodies to ANP, but the results have to some extent been conflicting. Some studies showed that the administration of anti-ANP caused increases in blood pressure and decreases in urinary sodium and water excretion (51, 52), while others did not observe these effects (53). On the basis that the effects of inhibiting ANP will be more pronounced when its levels are increased, experiments with anti-ANP have been carried out with volume expanded animals (54, 55). Infusion of ANP into rats with acute volume expansion does appear to attenuate natriuresis and diuresis in these animals. On the other hand, in chronic volume expansion induced by high dietary salt intake little change in plasma ANP occurs (56), and in rats immunized against their own ANP the natriuresis that occurred following chronic oral salt loading was comparable to that in control animals (55). It would appear, therefore, that ANP does not play much of a role in fluid homeostasis in chronic volume expansion but does seem to be a factor in acute volume expansion. None of the studies with either antagonists or ANP antibodies has answered the question as to the role of ANP in normal fluid regulation or indeed to its role in the regulation of blood pressure.

It seems clear that new approaches are required to determine the precise role played by ANP and the other natriuretic peptides in the control of blood pressure and body volume. These have been provided quite recently by the application of the technique of gene disruption or "gene knockout" in the experimental mouse. This technique, together with that of transferring extra gene copies to produce transgenic animals, has proved extremely useful in trying to determine whether or not a specific gene product is of critical importance. These new approaches to the physiology of ANP have blended the techniques of whole animal physiology so well developed in the rat with the growing body of knowledge of genetics in the mouse. So far as ANP physiology is concerned, it heralds the beginning of a new era.

The first genetic studies in which the physiology of ANP was investigated involved the development of the transgenic mouse model in which plasma ANP levels are chronically elevated about 10 times above normal (57, 58). These animals are characterized by a markedly reduced arterial blood pressure, and acute volume expansion, even on low sodium intake, produces a natriuretic effect presumably due to the high circulating ANP levels. Thus, it would appear, ANP exerts a persistent hypotensive effect independent of renal functional changes but the natriuresis expected to be produced by ANP can be overcome by other mechanisms. Similar results, in an overall sense, have been observed with BNP transgenic mice in which the plasma BNP levels were elevated 10 to 100-fold (59). In these transgenic mice, the plasma concentrations of cGMP were increased and the mean arterial blood pressures were significantly lower than those of nontransgenic littermates. So far, there are no reports of CNP transgenic mice.

Given the results found for transgenic mice where the presence of an extra gene gives rise to a lowering of blood pressure, it might be expected that deletion or impairment in the ANP gene would be associated with hypertension. Recently, an experimental model was presented where, with the technique of homologous recombination, mice were generated with a disruption in the pro-ANP gene (60). This technique uses gene targeting in mouse embryonic stem cells to mutate exon 2 of the mouse pro-ANP gene. Four chimeras generated from one of the targeted ES cells transmitted the disrupted pro-ANP gene to their offspring. Mating of the animals resulted in F1 animals that were genetically identical except for their pro-ANP genotypes. Matings between heterozygotes yielded homozygous mutant (−/−) animals, heterozygous (+/−), and wild-type (+/+) F2 offspring in Mendelian proportions.

In these studies, it was shown that in mice carrying one copy of the targeted gene (heterozygous mutant [+/−]) levels of ANP in the heart were 50% of normal amounts and the number of granules was also decreased to about half that of the wild-type (+/+) animal. In the heterozygotes, however, the level of

ANP was the same as that found in the normal wild-type animals. No atrial specific granules were present in the homozygous mutants, and no ANP was found in the blood or in the atria (Table I). Homozygous mutants were fertile and appeared normal. When fed a standard salt diet (0.5% NaCl), the blood pressures of wild-type and heterozygous animals did not differ; however, the homozygous mutants (−/− animals) had blood pressures that were significantly higher than those of the other genotypes. In addition, the homozygous mutants when maintained on intermediate salt diets (2% NaCl) had a greater ratio of heart weight to body weight than both heterozygous and wild-type animals (Fig. 4). Cardiac enlargement is a common feature, of course, in hypertensive animals. However, whether the enlargement in the homozygous mutants is a direct effect of the lack of ANP or a secondary to the hypertension remains to be determined.

The absence of ANP in the homozygous animals also increased plasma volume, as shown by the hematocrits of mutant homozygotes which differed significantly from those of either heterozygous or wild-type animals (Fig. 5). It is clear, therefore, that absence of the ANP gene has a profound effect on blood pressure and intravascular volume.

Perhaps the greatest effect of disruption of the pro-ANP gene on blood pressure was shown by the administration of salt to the heterozygous and homozygous F1 animals. After feeding a diet containing intermediate salt, mutant homozygotes showed an increase in mean arterial blood pressure relative to the heterozygous and wild-type mice. It is clear, therefore, that ANP modulates the blood pressure response to dietary salt.

The +/+ and −/− F1 animals were identical except for their genotypes at the pro-ANP locus. The data show, therefore, that genetically reduced ANP *unaccompanied by* any other genetic differences linked or unlinked to the pro-ANP locus can cause salt-sensitive hypertension. One exciting aspect of this research is possible extrapolation to the situation in humans. Higher dietary intake of salt is associated in humans with higher blood pressure. Because a partial deficiency rather than a complete absence of ANP is likely to occur in humans, the effect of increased salt intake on the F1 heterozygous mice is particularly aposite. Detection of variations in the human proANP gene may identify patients likely to benefit from reduced salt intake.

As discussed recently by Smithies and Maeda (61), gene targeting experiments when properly designed can test the effects of a precise genetic change completely free from the effects of differences in any other genes. They allow proofs of causation. However, in examining physiological control mechanisms such as those involved in the maintenance of blood pressure and volume control or diseases such as hypertension where there are multiple genetic and environmental components the generation of animal models becomes more problematic. The distinction between causation and correlation becomes difficult under these circumstances and strict adherence to al-

Table I. Plasma and Atrial ANP Levels in Mice with ANP Gene Disruption

Genotypes	ANP+/+	ANP+/−	ANP−/−
Plasma ANP (pg/ml)	148 ± 23 (n = 7)	135 ± 32 (n = 5)	Undetectable (n = 5)
Rt. atrial ANP (ng/mg)	115 ± 6 (n = 5)	54 ± 7[a] (n = 5)	Undetectable (n = 9)
Lt. atrial ANP (ng/mg)	112 ± 8 (n = 5)	54 ± 6[a] (n = 5)	Undetectable (n = 9)

Values are mean ± SEM and were analyzed with an analysis of variance followed by Tukey's protected t test.
[a] The value of +/− mice is significantly lower than that of +/+ mice at $P < 0.05$.

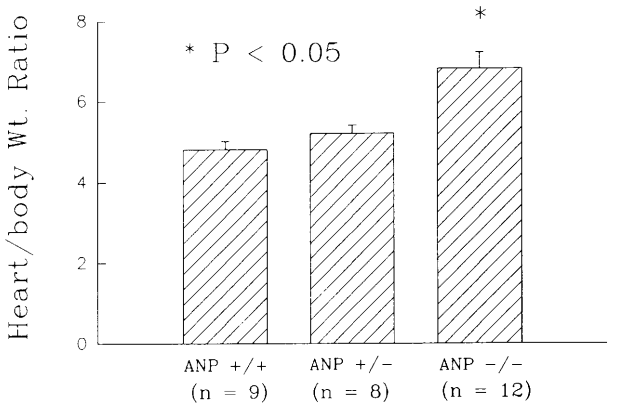

Figure 4. Heart (mg)/body weight (g) ratio of ANP +/+, +/−, and −/− mice. Values are mean ± SEM and were analyzed with an analysis of variance followed by Tukey's protected t test. *The value of −/− mice is significantly higher than that of +/+ and +/− mice at $P < 0.05$.

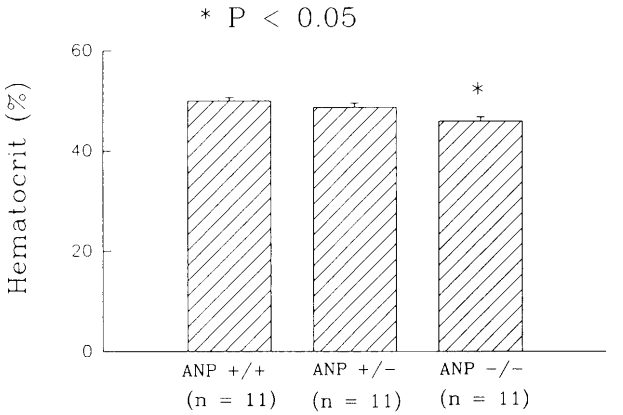

Figure 5. Haematocrit (%) of ANP +/+, +/−, and −/− mice. Values are mean ± SEM and were analyzed with an ANOVA followed by Tukey's protected t test. *The value of −/− mice is significantly lower than that of +/+ and +/− mice at $P < 0.05$.

lowing measurement of only the effects of varying one variable at a time is absolutely essential. Gene targeting experiments allow this to be done.

Much of our present understanding of the mechanisms involved in control of blood pressure and body volume have come from whole animal studies in the rat. The physiology of this animal is well understood, and despite its size even complex surgical procedures can be performed, thus obviating the need for a larger animal. Animal models of hypertension (e.g., the spontaneously hypertensive rat (SHR) and the Dahl rat), produced by genetic inbreeding, have also been very useful in gaining insight into the physiological basis of elevated blood pressure and the effects of high and low salt in the diet. While the rat has an advantage in terms of its physiological manipulability, correlating physiological characteristics with their genetic components is not easy in this animal. The SHR and Dahl rats are useful models, but their genetics are complex and ill-understood. Indeed, the genetics of the rat *per se* are not well developed nor are techniques for its genetic manipulation.

In contrast, the genetics of the mouse are well understood and far in advance of those of the rat. It is incumbent, therefore, on physiologists to rapidly adapt the techniques so well established for the rat to this smaller animal. For the elucidation of multifactorial genetic diseases and for understanding regulatory control mechanisms, a combination of mouse genetics and physiology hold great promise. The fact that we know so much about the genetics of the mouse bodes very well for the new era of physiological genetics.

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