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Common variations in noncoding regions of the human natriuretic peptide receptor A gene have quantitative effects

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Abstract

Genetic susceptibility to common conditions, such as essential hypertension and cardiac hypertrophy, is probably determined by various combinations of small quantitative changes in the expression of many genes. *NPR1*, coding for natriuretic peptide receptor A (NPRA), is a potential candidate, because NPRA mediates natriuretic, diuretic, and vasorelaxing actions of the nariuretic peptides, and because genetically determined quantitative changes in the expression of this gene affect blood pressure and heart weight in a dose-dependent manner in mice. To determine whether there are common quantitative variants in human *NPR1*, we have sequenced the entire human

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NPR1 gene and identified 10 polymorphic sites in its non-coding sequence by using DNA from 34 unrelated human individuals. Five of the sites are single nucleotide polymorphisms; the remaining five are length polymorphisms, including a highly variable complex dinucleotide repeat in intron 19. There are three common haplotypes 5' to this dinucleotide repeat and three 3' to it, but the 5' haplotypes and 3' haplotypes appear to be randomly associated. Transient expression analysis in cultured cells of reporter plasmids with the proximal promoter sequences of *NPR1* and its 3' untranslated regions showed that these polymorphisms have functional effects. We conclude that common *NPR1* alleles can alter expression of the gene as much as two-fold and could therefore significantly affect genetic risks for essential hypertension and cardiac hypertrophy in humans.

Introduction

Common variations in genes are increasingly recognized as important contributors to the genetic susceptibility to complex diseases such as essential hypertension and cardiac hypertrophy. Although the genetic contribution to hypertension is estimated to be 30%–60% (Ward 1990; Turner and Boerwinkle 2000; Verhaaren et al. 1991), very few genetic mutations have been linked to it in humans. Mutations in several genes are known to cause Mendelian forms of hypertension (Lifton et al. 2001), but their occurrence is too infrequent to contribute appreciably to hypertension in the population as a whole. In contrast, small differences that occur with high allelic frequencies and have additive effects are more likely to have impacts in a large population. These include quantitative alterations in gene expression governed by polymorphisms in non-coding sequences.

Even with the completion of the human genome sequence and the rapid accumulation of genome-wide single nucleotide polymorphisms (SNPs), the study of multi-genic diseases remains a great challenge (Peltonen and McKusick 2001; Cargill et al. 1999; Halushka et al. 1999). Small effects of individual polymorphisms are not readily recognized in association studies and, even when found, must be tested in animal models to determine that they are causative. Conversely, there are several gene systems in which a causative link between quantitative changes in gene expression and a cardiovascular phenotype has been demonstrated in animals but not in humans. Common polymorphisms in these genes in humans, if they have quantitative effects on expression could consequently have important implications in the population. Quantitative differences in expression of the *NPR1* gene, which codes for the natriuretic peptide receptor A (NPRA), has cardiovascular consequences in mice and is therefore such a candidate gene for hypertension and cardiac hypertrophy in humans.

NPRA is a membrane-bound guanylate cyclase that serves as the receptor for both atrial and brain natriuretic peptides (ANP and BNP, respectively) and is important in the maintenance of cardiovascular homeostasis (Chinkers et al. 1989). In response to increases in blood volume or blood pressure, ANP and BNP are released from the heart and act through NPRA in the kidneys, adrenals, and vasculature to increase natriuresis, diuresis, and vasorelaxation (Espiner 1994). Levels of ANP and BNP are elevated in both human patients and animal models of heart failure and cardiac hypertrophy (Clerico et al. 1999; Knowlton et al. 1995). In addition to its key role in blood pressure regulation, the NPRA system also influences

cardiac growth in a blood-pressure-independent manner in mice (Lopez et al. 1995; Oliver et al. 1997; Knowles et al. 2001; Kishimoto et al. 2001). Importantly, we have demonstrated that quantitative changes in *Npr1* gene expression have significant effects on both blood pressure and cardiac mass in mouse models (Knowles et al. 2001; Oliver et al. 1998). Finally, a functional deletion mutation that lies in the 5' flanking region of the human *NPR1* gene and that causes a 70% decrease in transcription of the corresponding allele has been shown to be associated with hypertension and cardiac hypertrophy in Japanese individuals (Nakayama et al. 2000).

Although cDNA coding for the human *NPR1* gene was first isolated in 1989 (Lowe et al. 1989) and the structural organization of the gene is known (Takahashi et al. 1998), the full genomic sequence has not been determined. Here, we describe the genomic sequence of the human *NPR1* gene and identify 10 common polymorphic sites in its noncoding regions. Functional analysis indicates that common haplotypes carrying these polymorphisms could determine up to two-fold differences in the levels of expression of the *NPR1* gene in the human population.

Materials and methods

NPR1 sequence determination

A human genomic DNA library was made by using DNA that was partially digested with *Mbo*1, size selected, and ligated into *Bam*H1 sites of the phage vector LambdaGEM-11 (Promega). Two phage clones containing the *NPR1* gene were isolated with polymerase chain reaction (PCR)-generated fragments of exon 1 (primers 4a and 4b, Table 1) and of exon 22 (primers 20a and 20e, Table 1) as probes. The entire coding and non-coding region of the gene was sequenced at the UNC Sequencing Core Facility at least once in both directions by using overlapping subclones and primers. The complete sequence has been submitted to Genbank (accession no. AF190631). The nucleotide positions in the text and figures refer to this sequence, counting the first nucleotide of ATG codon +1 and the first nucleotide upstream of the ATG codon as –1. Nucleotide sequence comparisons were made with GCG software (Genetics Computer Group).

Identification of common polymorphisms

Human DNA samples were isolated from blood samples or from fibroblasts of unrelated individuals (Maeda et al. 1983, 1986). In some cases, the racial origin of the sample was known. The study was approved by the institutional review boad of the University of the North Carolina. A PCR-based approach was used to amplify DNA fragments covering most of the *NPR1* gene in six unrelated individuals (3 Caucasians and 3 African-Americans). Briefly, sets of overlapping primers (Table 1) were designed from appropriate exonic or intronic sequences and amplified by PCR with high-fidelity Pfu DNA polymerase (Stratagene). PCR products of approximately 1 kb were electrophoresed on agarose gels to verify their correct size, purified by using a QIAquick PCR purification kit (Qiagen), and sequenced by using the PCR primers.

Polymorphic sites so identified were further characterized in samples from an additional six Caucasians, seven blacks, and 15 people of unknown racial origin by single-stranded confirmational polymorphism analysis (SSCP). PCR products were made covering the polymorphic regions of the *NPR1* gene in a buffer containing 20 pmole each primer, 10% dimethylsulfoxide, 10 ng template DNA, *Taq* DNA polymerase (Boehringer Mannheim), 10 mM dNTPs, and 0.1 mCi ^{32}P-dCTP. After an initial denaturation step at 94°C for 2 min, reactions were cycled at 94°C for 1 min, 60–65°C for 1 min, and 72°C for 1 min, for 33 cycles. The labeled PCR product (1 μl) was diluted into 9 μl 0.1% SDS, 20 mM EDTA, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol. Samples were denatured for 10 min at 95°C, placed on ice, and separated in a 1× MDE gel matrix (FMC Bioproducts). After electrophoresis at 12 W for 3–4 h at room temperature, gels were dried and exposed overnight to Kodak films.

Reporter plasmid construction

To test the promoter function of the 5' haplotypes, oligonucleotides were designed to PCR-amplify a *Bam*H1/*Nco*1 fragment containing the promoter region that extends upstream 770 bp from the AUG start codon of the *NPR1* gene. The PCR fragments amplified from genomic DNA from individuals carrying the three 5' haplotypes were cloned into the *Bgl*2 and *Nco*1 sites of the pGL3-basic luciferase reporter vector (Promega). To test the effects of 3' untranslated region (UTR) sequences on gene expression, we made a plasmid (PGK-PL2) by inserting an *Xba*1/*Hin*d3 fragment containing the promoter for the mouse phosphoglycerorate kinase gene (PGK; Adra et al. 1987) into the pGL2-basic vector (Promega). The SV40 late poly(A) signal sequence of the PGK-GL2 plasmid was replaced by a 700-bp fragment containing the 3'UTR of the *NPR1* gene that had been amplified by PCR from each of the three 3' haplotypes. The correctness of all constructs was verified by sequencing, and sets of test plasmids were prepared simultaneously for each transfection experiment by using QIAGEN plasmid purification kits (QIAGEN). A CMVβGal plasmid containing the *Escherichia coli* galactosidase gene driven by a cytomegalovirus promoter (MacGregor and Caskey 1989) was a gift from Dr. MacGregor, at Emory University.

Reporter gene expression assay

Mouse aortic smooth muscle cells isolated from aorta of adult C57BL6 mice (Yasuda et al. 2000) and 293 cells were cultured in DMEM containing 10% fetal calf serum. Cells cultured in 3-cm wells until 70% confluent were transfected with 2 μg luciferase reporter DNA mixed with 1 μg CMVβGal DNA by using lipofectamine (Life Technologies) according to the protocol of the supplier. Cells were harvested 48 h after transfection, lysed in 300 μl of lysis reagent (Promega), and frozen at –70°C until analyzed. The lysates were centrifuged at 16,500 *g* for 10 min at 4°C, and 20 μl of the supernatant was mixed with 100 μl of Luciferase Assay Reagent (Promega). Samples were analyzed in a Monolight 2010 luminometer (BD PharMingen). To normalize the transfection efficiency, β-Gal activity was determined for each extract by using the assay system from Promega according to their protocol. In each experiment, transfection was carried out in duplicate or triplicate for each reporter plasmid, and transcriptional ability was calculated relative to the expression of the most common haplotype as 100%. Experiments were repeated three to four times, and statistical analysis by ANOVA was performed by using the JMP software package (SAS

Institute). Data was expressed as mean ± SEM, and means for all pairs were compared using Tukey-Kramer HSD.

Results

Human NPR1 gene structure

The human *NPR1* gene spans approximately 16 kb and contains 22 exons and 21 introns. The 5' region of the gene, including both the 5'UTR and exon 1, is unique with a high G/C content of 70% (Fig. 1), which is much greater than the 41% G/C content average of the human genome (Lander et al. 2001). This region is also particularly rich in CpG dinucleotides, containing 158 within the roughly 1600 bp of the sequence (11%). For comparison, in the remaining 14.5 kb, there are only 246 CpG dinucleotides (1.7%). Another notable sequence motif is in intron 1 where an approximately 200-bp stretch of pyrimidines occurs in the sense strand. Such sequences are known to form Z-DNA and triple helices and may be involved in transcriptional regulation (Nordheim and Rich 1983). The intronic regions are interspersed with multiple repeat elements, including six Alu repeats (introns 4, 7, 8, 20), two mammalian-wide interspersed repeats (intron 20), and three elements from the medium reiteration repeats (intron 20).

Polymorphisms in the NPR1 gene

To search for common polymorphisms in the *NPR1* gene, we performed direct sequence analysis of PCR products amplified along the *NPR1* gene from three Caucasians and three African-Americans (Table 2) with primers listed in Table 1. The analyzed region from the 12 chromosomes covers approximately 10 kb DNA excluding Alu repeats. Using these data, we identified ten polymorphic sites in the noncoding sequence of *NPR1* (nos. 1–10; Fig. 1). SSCP analyses were then used to genotype a total of 10 African-Americans, 9 Caucasians and 15 individuals of unknown race (Table 2). Our early observations (Maeda et al. 1983) and more extensive studies (Wang et al. 1998) showed that base substitution polymorphisms were more frequent than length differences by a ratio of about 12:1 in the human genome. However, in the regions of the *NPR1* gene that we surveyed, we found that five of the 10 polymorphisms were length differences: a $(CT)_n$ dinucleotide repeats at nucleotide –293 in the promoter region (site 1, n=6, 10, or 11), a length difference of C_n at nucleotide 8927 in intron 14 (site 6, n=6 or 7), a highly variable number of dinucleotide repeats at nucleotide 10,955 in intron 19 (site 7), a length difference of C_n at nucleotide 14,319 in the 3'UTR (site 8, n=3 or 4), and a 4-bp insertion/deletion of AGAA at nucleotide 14,649 also in the 3'UTR (site 9). The dinucleotide repeats in intron 19 were complex and highly variable, with the *NPR1* gene being isolated in a phage clone having $(CT)_{19}GT(CT)_{15}(CA)_{13}$. The equivalent sequences from most of the PCR fragments were too complex, because of heterozygosity and because of PCR artifacts to determine the exact numbers of repeats, but they clearly differed in different individuals.

Of the five base substitutions, G/A at nucleotide –77 in the 5'UTR (site 2) and G/A at nucleotide 8812 in intron 14 (site 5) were present in several individuals (Table 2), whereas three (site 3 at nucleotide 1665, site 4 at nucleotide 1678 in intron 2, and site 10 at nucleotide 14,802 in the 3'UTR) were each seen in only one chromosome. Our finding of

relatively few SNP sites in the sequenced region is notable. In mammalian genome, the regions very rich in G/C and CpG dinucleotides, such as we have found in exon 1 of *NPR1*, are generally associated with an increased frequency of base substitution polymorphisms (Lander et al. 2001). Nevertheless, all six individuals whom we examined shared an identical sequence in exon 1 of 770 bp. The conservation of sequence included the synonymous positions in the exon suggesting that this has an importance additional to that of amino acid sequence conservation, perhaps in controlling gene expression.

Common haplotypes

Three polymorphisms, viz., $(CT)_6$ at nucleotide –293, A at nucleotide –77, and A at nucleotide 8927, were in the same three individuals, all of them being African-American. $(CT)_{10}$ and $(CT)_{11}$ were found in both African-Americans and in Caucasians. Despite the finding that nucleotides 8812 and 8927 in intron 14 were about 9 kb away from nucleotides –293 and –77 in the 5’ region, these four polymorphic sites were in strong linkage disequiliblium, and individuals with C_6 at nucleotide 8927 always had $(CT)_{10}$ at nucleotide –293. Together, the polymorphisms at sites 1–6 in the 5’ region of the gene fell into three common haplotypes, which we designated as “CT6”, “CT10”, or “CT11”, with approximate overall frequencies of 6%, 44%, and 50%, respectively (Fig. 2).

The length polymorphism of 3C or 4C at nucleotide 14,319 in the 3’UTR was found in both African-Americans and Caucasians, whereas two of the three *NPR1* genes missing AGAA at nucleotide 14,649 were found in Caucasians, and one was in an individual of unknown racial origin. The individuals with AGAA deletion had at least one 4C at nucleotide 14,319. Thus, polymorphisms at these two sites in the 3’ region of the gene constituted three common haplotypes, “3-plus”, “4-plus”, and “4-minus”, with frequencies of 39%, 57%, and 4%, respectively (Fig. 2). The three common haplotypes in the 5’ region of the gene appeared to be randomly associated with the three common haplotypes in the 3’ region of the gene. For example, “CT11” in the 5’ was seen with all three 3’ haplotypes. Similarly, “4-minus” in the 3’ region was seen with either “CT11” or “CT10” in the 5’ region. The observed frequencies in Table 2 do not differ significantly from those expected from random association between the 5’ haplotypes and 3’ haplotypes (χ^2=7.47, P=0.5), suggesting that frequent crossovers have occurred between them. A long stretch of highly variable dinucleotide repeats in intron 19 is the likely cause of frequent crossovers between the 5’ and 3’ parts of the *NPR1* gene.

Functional polymorphisms

To test whether the common genetic variants of the human NPR1 gene affected levels of gene expression, we examined the transcriptional activity of the three variants of the proximal promoter that contains the polymorphic sites 1 and 2. Mouse aortic smooth muscle (SM) cells were transfected with DNA constructs containing the three variants driving the luciferase reporter gene. SM cells expressed the *Npr1* gene, as readily demonstrated by Northern blots (not shown), even though the expression of all the reporter constructs was only 5%–10% of the expression from the PGK-GL2 control plasmid. Nevertheless, we found that the transcriptional activity of the CT6 promoter was significantly higher (145±12%) compared with the activities of CT10 (104±8%) and CT11 (100±3%) promoters

(Fig. 3A). It should be noted that the CT6 promoter also differed from others in that it had A at site 2 in the 5'UTR instead of G.

The expression levels of constructs containing the three 3'UTR variants were also assessed by transient transfection into 293 cells. The absolute luciferase activities obtained after transfection of the 293 cells were much higher than in SM cells, but the relative expression levels of different constructs were the same. The data from four experiments with SM cells and four experiments with 293 cells were therefore analyzed together with ANOVA, taking cell type and haplotype as two variants. Compared to the most common 4-plus haplotype (100±5%), the reporter constructs with 3'UTR of 3-plus and 4-minus haplotypes had significantly lower expressions of 70±6% and 78±4%, respectively (Fig. 3B). The combined effects of the promoter polymorphisms and 3'UTR polymorphisms can therefore affect the expression of the *NPR1* genes by as much as a factor of two.

Discussion

Using DNA from a relatively small number of unrelated individuals, we have identified ten polymorphic sites forming three haplotypes in the 5' and three haplotypes in the 3' noncoding regions of the human *NPR1* gene, a candidate gene for affecting the incidence and severity of essential hypertension and cardiac hypertrophy. Transient expression analysis in cultured cells has revealed that one of the 5' haplotypes, "CT6", has 45% higher promoter activity than the other two ("CT10" and "CT11"), whereas the 3'UTR of two of the 3' haplotypes, "3-plus" and "4-minus", reduces the reporter gene function by 30% compared with the "4-plus" haplotype. One of the common 5' haplotypes, with a frequency of approximately 6%, has potentially protective effects because it increases NPRA levels, while two of the common 3' haplotypes, with a combined frequency of 43%, have potentially damaging effects.

The differences in promoter sequences that we have demonstrated could clearly influence the transcriptional activity of the gene. However, it is not clear how the 12-bp versus 20-bp or 22-bp stretch of CT dinucleotides at nucleotide –293 and/or the G to A change at nucleotide –77 leads to a 45% increase in promoter function. Nucleotide sequence comparisons of human (AF190631), rat (J05677), and mouse (AJ307712) indicate that the region containing the CT dinucleotide repeats is downstream of a long homology block (–466 to –347) that contains a putative SP1 site, although the distance between this homology region and the ATG start codon is not conserved between humans and rodents. Polymorphisms in the 5' regions of genes are being increasingly recognized as important in the pathogenesis of complex diseases, including hypertension. For example, the M235T polymorphism that is associated with essential hypertension (Jeunemaitre et al. 1992) in the human angiotensiogen gene is in virtually complete linkage disequilibrium with a functional polymorphism in the promoter sequence (Inoue et al. 1997). Similarly, changes in the number of tandem repeats in the 5'UTR of the P450 prostacyclin synthase gene *CYP8A1* cause differences in its transcriptional activity, thereby moderating inflammatory processes (Chevalier et al. 2001). Moreover, rare variant chromosomes with single base changes in the promoter of the hepatocyte nuclear factor-1α that decrease transcriptional activities have been found in families with monogenic diabetes (Godart et al. 2000).

Less attention has been devoted to assessing the effects on gene expression of polymorphisms in 3'UTR regions, but the importance of these sequences on mRNA stability and translation has long been recognized (Ross 1995). We were surprised to find that having 3C or 4C at 30 bp downstream of the stop codon in the *NPR1* transcript significantly affected expression. The nucleotide sequence of the first 100 bp in the 3'UTR is well conserved, although not identical, between humans and rodents. Possibly, this sequence influences the secondary structure of the transcript and so affects its stability or translation. In this regard, we note that a potential 27-bp stem and 6-bp loop structure with the stop codon in the loop can form in the RNA involving the sequence immediately 5' to the 3C/4C polymorphic site. The sequence with 4C, but not that with 3C, could interfere with this stem-loop formation by forming another, although less stable, stem-loop structure involving some of the same nucleotides. Stem-loop structures in the 3'UTR have been shown to be important determinants of message stability in some genes (Putland et al. 2002). C-rich sequences are in some cases important for post-transcriptional regulation as exemplified by the α2-globin and collagen α1 genes (Weiss and Liebhaber 1995; Stefanovic et al. 1997). Other examples of functional 3'UTR polymorphisms include a significant association between a common 5-bp insertion-deletion polymorphisms in an AU rich element within the 3'UTR of the protein phosphatase 1 *PPP1R3* gene and plasma glucose in Canadian Oji-Cree (Hegele et al. 1998).

Essential hypertension and cardiac hypertrophy are frequent and important causes of morbidity. Thus, the identification, in the general human population, of common haplotypes that lie within the *NPR1* gene and that potentially affect gene expression by as much as two-fold has important implications. Previously, we have shown that quantitative changes in *Npr1* expression affect blood pressure and cardiac hypertrophy (Oliver et al. 1998). In that study, the alteration of gene expression levels, achieved by varying the number of copies of the *Npr1* gene, demonstrated that blood pressure was inversely correlated with the amount of *Npr1* expression and that a two-fold difference in mRNA levels resulted in about a 10 mmHg difference in blood pressures of the animals. Our more recent observations indicate that *Npr1* expression inversely also affects cardiac mass in a dose-dependent manner (Knowles et al. 2001). The concept that *NPR1* quantitatively influences hypertension and cardiac hypertrophy in humans is further enhanced by a recent report that heterozygosity for an 8-bp deletion in the promoter region is associated with essential hypertension and cardiac hypertrophy in the Japanese population (Nakayama et al. 2000). Although this particular 8-bp deletion is not present in the small set of samples that we have studied, our analyses show the presence of far more common alleles in African-Americans and in Caucasians that affect expression levels, albeit to a lesser degree. Assuming that the 5' and 3' haplotypes that we have uncovered are randomly associated, we estimate that *NPR1* gene expression levels in the general population can differ up to two-fold (1.45 versus 0.70) as a consequence of these differences, with the upper and lower quantiles differing by about 20% (1.02 versus 0.85 with mean expression levels 0.92). If the physiological consequence of changes in *NPR1* gene expression levels in mice and humans are similar, the common *NPR1* alleles could therefore be responsible for as much as 10 mmHg in blood pressure, and for a significant part of the susceptibility to cardiac hypertrophy in the population. Further studies with a larger number of individuals are clearly needed to determine more precisely the frequencies

of the polymorphisms that we have described and the frequencies at which the 5' and 3' haptotypes recombine. An extensive population-based study will probably be required to determine whether these *NPR1* alleles do indeed account for any differences in the risk of hypertension or cardiac hypertrophy.

Genetic variations affecting complex diseases and traits are probably common in the general population and are likely to involve many genes. Small effects of individual variants, such as those that we have found in *NPR1*, may easily be masked by the heterogeneity of human populations, by gene-gene interactions, and by the effects of environmental differences. Consequently, the recognition of such small effects in genome-wide association studies will be difficult and will require a large number of subjects combined with sophisticated statistical approaches able to analyze multiple loci simultaneously (Xiong et al. 2002). The ability to go back and forth between association studies in humans and gene alteration in mice should therefore continue to play an integral part in increasing our understanding of genetic factors that influence the complex traits of mankind.

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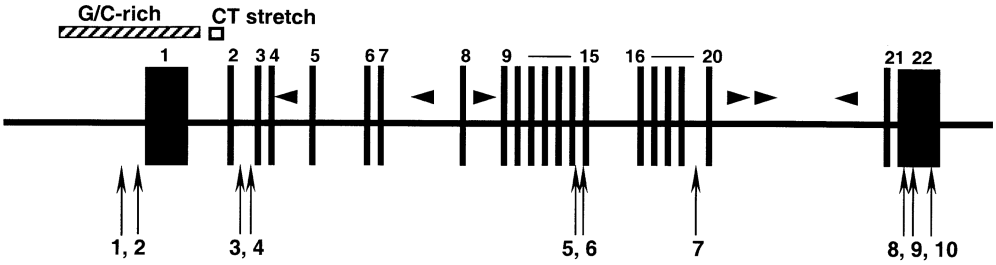

Site	Position (nt)	Polymorphism	Frequency
1	5' (-293)	$(CT)_n$ n= 6, 10, 11	3/50, 22/50, 25/50
2	5' (-77)	G , A	43/46, 3/46
3	Int 2 (1,665)	G , A	49/50, 1/50
4	Int 2 (1,678)	C , A	49/50, 1/50
5	Int 14 (8,812)	G , A	35/38, 3/38
6	Int 14 (8,927)	$(C)_n$ n= 6, 7	13/28, 15/28
7	Int 19 (10,955)	$(CT)_pGT(CT)_q(CA)_r$	highly variable
8	3'UTR (14,319)	$(C)_n$ n= 3 , 4	26/68, 42/68
9	3'UTR (14,649)	plus / minus AGAA	65/68, 3/68
10	3'UTR (14,802)	A , G	67/68, 1/68

Fig. 1.

Structure and polymorphisms of the human *NPR1* gene. The gene covers approximately 16 kb. *Numbered long vertical bars* Exons, *horizontal arrowheads* Alu repeats in introns, *hatched line* a G/C-rich region covering the promoter, exon 1 and 5' part of the intron 1, *open box* C/T-rich stretch in intron 1, *upward arrows with numbers* polymorphic sites. The polymorphisms and their frequencies of observation are listed *below*. The nucleotide positions in *parentheses* are based on the sequence in AF190631 (Genbank) counting the first nucleotide of the ATG codon as 1. *Frequency* indicates the number of chromosome observed/genotyped

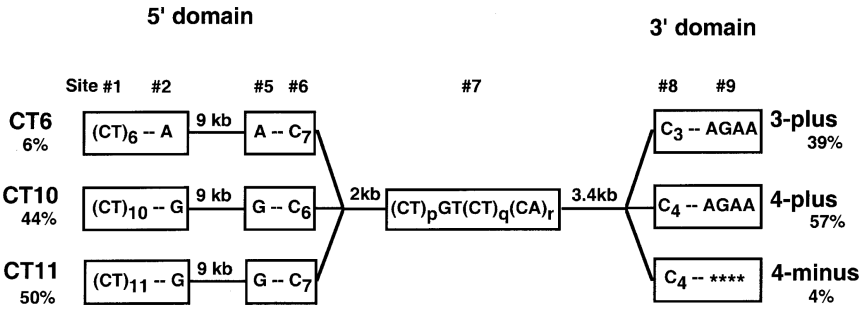

Fig. 2.

Common haplotypes of the *NPR1* gene. There are three common haplotypes, designated “CT6”, “CT10”, and “CT11”, encompassing polymorphisms 1, 2, 5, and 6 in the 5’ region of the gene (Fig. 1). There are three common haplotypes, “3-plus”, “4-plus”, and “4-minus”, in the 3’ region of the gene encompassing polymorphisms 8–10 in Fig. 1. The 5’ and 3’ haplotypes are not linked; they flank a region in intron 19 that contains a highly variable number of dinucleotide repeats. Approximate distances between the polymorphic sites are shown. The frequencies of the 5’ haplotypes are from site 1

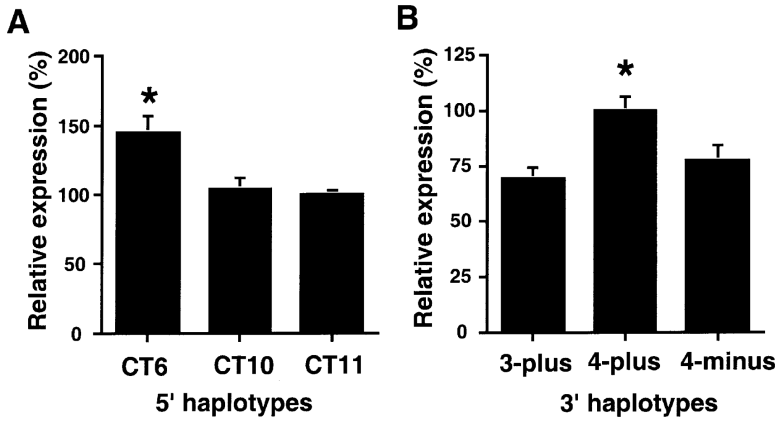

Fig. 3.

A, B Luciferase activity of reporter constructs. **A** Promoter activities were determined in transfected mouse aortic smooth muscle cells. Activity is expressed in percentages relative to the ratios of luciferase/β-galactosidase activities with the "CT11" promoter as 100%. *Bars* Means ± SE from four experiments (n=12 individual wells). P<0.001 by ANOVA. **B** 3'UTR function was examined by transfection of constructs in 293 cells (four experiments) and in mouse aortic smooth muscle cells (four experiments). Activities are expressed as percentages relative to that of "4-plus" variant as 100%. *Bars* Means ± SE from the eight experiments combined (n=18 individual wells). P<0.0001 by ANOVA. *Asterisks* in **A, B** indicate the constructs whose expression differs significantly from others

Table 1

Primers used in *NPR1* polymorphism analysis

Primer no.		Sequence		Location	Nucleotide position[a]	Direction	Note
1a	5'	ggatcccaaaccagcacacc	3'	5'	1	Forward	–
1b	5'	ccagactcagtgacagaaga	3'	5'	285	Reverse	–
2a	5'	gatcctggattggctcttc	3'	5'	252	Forward	–
2b	5'	agtaccacggctaccgtca	3'	Exon 1	928	Reverse	–
3a	5'	gcacgctacaaacacacact	3'	5'	412	Forward	SSCP sites 1, 2
3b	5'	ctgtagctgcgacggtct	3'	5'	657	Reverse	SSCP site 1
3c	5'	accgcagcaggcgagcgct	3'	Exon 1	793	Reverse	SSCP site 2
4a	5'	agcgctcgcctcgctgcggt	3'	Exon 1	775	Forward	–
4b	5'	catggtccgcagcagcctggt	3'	Exon 1	1510	Reverse	–
5a	5'	accaggctgctgcggaccatg	3'	Exon 1	1491	Forward	–
5b	5'	gggagctgcagatgtaga	3'	Exon 2	2219	Reverse	–
6a	5'	gggatgggcaggatgtcag	3'	Exon 2	2361	Forward	SSCP sites 3, 4
6b	5'	cattgcctaattcccagtg	3'	Intron 2	2582	Reverse	SSCP sites 3, 4
6c	5'	tcctccatggtgaagttg	3'	Exon 3	2984	Reverse	–
7a	5'	gacccagataatcccgagta	3'	Exon 3	2902	Forward	–
7b	5'	agtgcccagcagggatgatt	3'	Intron 4	3730	Reverse	–
8a	5'	atagcagtggcgatcggga	3'	Exon 5	4219	Forward	–
8b	5'	tcagaatgccgagcaagga	3'	Exon 7	5500	Reverse	–
9a	5'	cttcttgctcggcattctga	3'	Exon 7	5481	Forward	–
9b	5'	tcagccggctgcctgcactc	3'	Exon 8	6772	Reverse	–
10a	5'	tatcccaggaagatgcagct	3'	Exon 8	6655	Forward	–
10b	5'	tgccttatctttgtgtcacc	3'	Intron 8	7410	Reverse	–
11a	5'	cagcctggatgacacaatga	3'	Intron 8	7332	Forward	–
11b	5'	agtgagtaccggaacatcca	3'	Exon 12	8759	Reverse	–
12a	5'	ccagcaggacattctcgga	3'	Exon 12	8704	Forward	–
12b	5'	ggcaatctcctgaaggatga	3'	Exon 14	9435	Reverse	–
13a	5'	atcatccttcaggagattgc	3'	Exon 14	9415	Forward	SSCP sites 5, 6
13b	5'	aactcctccaggtgactct	3'	Exon 15	9825	Reverse	SSCP sites 5, 6

Primer no.		Sequence		Location	Nucleotide position[a]	Direction	Note
14a	5'	agagtcacctggaggagtt	3'	Exon 15	9807	Forward	–
14b	5'	ccagttcctccagattgttg	3'	Exon 16	10,719	Reverse	–
15a	5'	ctgctcaatgacctgtacac	3'	Exon 18	11,155	Forward	–
15b	5'	gactccattcttgaggctgt	3'	Exon 20	12,063	Reverse	–
15c	5'	ttcgcaagggaaacttgtcc	3'	Intron 19	11,717	Forward	SSCP site 7
15d	5'	tggacaatccaagacaggca	3'	Intron 19	11,904	Reverse	SSCP site 7
16a	5'	tggtgggactgaagatgc	3'	Exon 20	11,993	Forward	–
16b	5'	tcatcgcaacctctgcct	3'	Intron 20	12,581	Reverse	–
17a	5'	agaggttgcgatgagcca	3'	Intron 20	12,569	Forward	–
17b	5'	actcctggccttaagtga	3'	Intron 20	13,376	Reverse	–
18a	5'	tgctgtaatcccagcacttt	3'	Intron 20	13,319	Forward	–
18b	5'	gccactaatcacatgtgcct	3'	Intron 20	14,558	Reverse	–
19a	5'	aggcacatgtgattagtggc	3'	Intron 20	14,539	Forward	–
19b	5'	aaggagccagtaggtccgaa	3'	Exon 22	15,066	Reverse	–
20a	5'	ttcggacctactggctcctt	3'	Exon 22	15,047	Forward	SSCP site 8
20b	5'	tggcacctctgttgcttct	3'	Exon 22	15,159	Reverse	SSCP site 8
20c	5'	ccttctagacccttgtagaac	3'	3'	15,778	Reverse	–
20d	5'	gccttgctaccctgtgactt	3'	Exon 22	15,426	Forward	SSCP sites 9, 10
20e	5'	gtctctgctcatggtag	3'	Exon 22	15,652	Reverse	SSCP sites 9, 10

[a]Nucleotide position of first residue corresponds to the sequence AF190631

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Table 2

Polymorphisms seen in individuals. (*ND* not determined; *AA* African-American, *C* Caucasian, *U* unknown). *Site 1* is the number of (CT) dinucleotides. *Sites 6 and 8* are the number of C residues

ID	Racial origin	Site 1 5'	Site 2 5'	Site 3 Intron 2	Site 4 Intron 2	Site 5 Intron 14	Site 6 Intron 14	Site 7 Intron 19	Site 8 3'UTR	Site 9 3'UTR	Site 10 3'UTR	Note
1	AA	6/11	G/A	G/G	C/C	G/A	7/7	–	3/3	AGAA/AGAA	A/A	Initial screening
2	AA	10/11	G/G	G/G	C/C	G/G	6/7	h	3/4	AGAA/AGAA	A/A	Initial screening
3	AA	10/11	G/G	G/G	C/A	G/G	6/7	i	3/4	AGAA/AGAA	A/A	Initial screening
4	AA	10/11	G/G	G/G	C/C	G/G	6/7	g	3/4	AGAA/AGAA	A/A	SSCP analysis
5	AA	6/11	G/A	G/G	C/C	G/A	7/7	h	3/3	AGAA/AGAA	A/A	SSCP analysis
6	AA	10/11	G/G	G/G	C/C	G/G	ND	l	3/4	AGAA/AGAA	A/A	SSCP analysis
7	AA	11/11	G/G	G/G	C/C	G/G	ND	Y	3/4	AGAA/AGAA	A/A	SSCP analysis
8	AA	6/11	G/A	G/G	C/C	G/A	7/7	–	3/4	AGAA/AGAA	A/A	SSCP analysis
9	AA	11/11	G/G	G/G	C/C	G/G	7/7	v	3/4	AGAA/AGAA	A/A	SSCP analysis
10	AA	11/11	G/G	G/G	C/C	G/G	7/7	a	3/4	AGAA/AGAA	A/A	SSCP analysis
11	C	10/11	G/G	G/G	C/C	G/G	6/7	r	4/4	AGAA/AGAA	A/A	Initial screening
12	C	10/10	G/G	G/A	C/C	G/G	6/6	i	4/4	AGAA/AGAA	A/A	Cloned in lambda
13	C	11/11	G/G	G/G	C/C	G/G	ND	a	3/3	AGAA/AGAA	A/A	SSCP analysis
14	C	10/11	G/G	G/G	C/C	G/G	ND	b	3/4	AGAA/AGAA	A/A	SSCP analysis
15	C	11/11	G/G	G/G	C/C	G/G	ND	l	3/4	AGAA/– –	A/A	SSCP analysis
16	C	10/10	G/G	G/G	C/C	G/G	6/6	E	4/4	AGAA/AGAA	A/G	Initial screening
17	C	10/10	G/G	G/G	C/C	G/G	6/6	–	4/4	AGAA/AGAA	A/A	SSCP analysis
18	C	10/10	G/G	G/G	C/C	G/G	6/6	–	4/4	AGAA/– –	A/A	Initial screening
19	C	10/11	G/G	G/G	C/C	G/G	6/7	–	3/4	AGAA/AGAA	A/A	SSCP analysis
20	U	10/11	G/G	G/G	C/C	ND	ND	–	4/4	AGAA/AGAA	A/A	SSCP analysis
21	U	10/11	G/G	G/G	C/C	ND	ND	–	3/4	AGAA/AGAA	A/A	SSCP analysis
22	U	10/10	G/G	G/G	C/C	ND	ND	–	4/4	AGAA/AGAA	A/A	SSCP analysis
23	U	ND	ND	G/G	C/C	ND	ND	–	4/4	AGAA/AGAA	A/A	SSCP analysis
24	U	10/11	G/G	G/G	C/C	ND	ND	–	3/4	AGAA/AGAA	A/A	SSCP analysis
25	U	ND	ND	G/G	C/C	ND	ND	–	3/4	AGAA/AGAA	A/A	SSCP analysis
26	U	ND	ND	ND	ND	ND	ND	–	3/3	AGAA/AGAA	A/A	SSCP analysis
27	U	ND	ND	ND	ND	ND	ND	–	4/4	AGAA/AGAA	A/A	SSCP analysis

ID	Racial origin	Site 1 5'	Site 2 5'	Site 3 Intron 2	Site 4 Intron 2	Site 5 Intron 14	Site 6 Intron 14	Site 7 Intron 19	Site 8 3'UTR	Site 9 3'UTR	Site 10 3'UTR	Note
28	U	10/11	ND	ND	ND	ND	ND	–	4/4	AGAA/AGAA	A/A	SSCP analysis
29	U	10/11	ND	ND	ND	ND	ND	–	3/4	AGAA/AGAA	A/A	SSCP analysis
30	U	ND	ND	ND	ND	ND	ND	–	4/4	AGAA/AGAA	A/A	SSCP analysis
31	U	ND	ND	ND	ND	ND	ND	–	3/4	AGAA/AGAA	A/A	SSCP analysis
32	U	ND	ND	ND	ND	ND	ND	–	3/4	AGAA/– –	A/A	SSCP analysis
33	U	ND	ND	ND	ND	ND	ND	–	3/4	AGAA/AGAA	A/A	SSCP analysis
34	U	ND	ND	ND	ND	ND	ND	–	4/4	AGAA/AGAA	A/A	SSCP analysis