Preliminary characterisation of the promoter of the human p22phox gene: identification of a new polymorphism associated with hypertension

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Abstract The p22phox subunit is an essential protein in the activation of NAD(P)H oxidase. Here we report the preliminary characterisation of the human p22phox gene promoter. The p22phox promoter contains TATA and CCAC boxes and Sp1, γ-interferon and nuclear factor κB sites. We screened for mutations in the p22phox promoter and identified a new polymorphism, localised at position −930 from the ATG codon, which was associated with hypertension. Mutagenesis experiments showed that the G allele had higher promoter activity than the A allele. These results suggest that the $-930^{A/G}$ polymorphism in the p22phox promoter may be a novel genetic marker associated with hypertension.
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1. Introduction

NAD(P)H oxidase is the major inducible source of superoxide anion ($^{\bullet}O_2^-$) in phagocytes. In these cells, this enzyme has a bactericidal function and generates $^{\bullet}O_2^-$ upon stimulation by pathogens [1]. This oxidase is a membrane-bound enzyme that catalyses a single electron reduction of molecular oxygen to form $^{\bullet}O_2^-$. It consists of a membrane-associated cytochrome b_{558}, two cytosolic components p47phox and p67phox, and the small GTPase protein rac2 [2,3]. Cytochrome b_{558}, which is the final electron transporter from NAD(P)H to molecular oxygen, consists of a large and a small subunit, gp91phox and p22phox respectively [4].

The NAD(P)H oxidase system is also considered to be the most important source of $^{\bullet}O_2^-$ in vascular cells such as smooth muscle cells (VSMCs) and endothelial cells [5–7]. Despite the similarities between vascular and phagocytic NAD(P)H oxidases, it has been suggested that vascular oxidase represents a novel family of enzymes [8].

Oxidative stress induced by $^{\bullet}O_2^-$ has been implicated in the development of hypertension [9,10]. A major component of NAD(P)H oxidase is the p22phox protein, a critical subunit that plays an essential role in NAD(P)H oxidase-dependent $^{\bullet}O_2^-$ generation in vascular cells [11]. Enhanced vascular NAD(P)H oxidase-driven $^{\bullet}O_2^-$ production is associated with upregulation of p22phox mRNA in several models of hypertension, including the spontaneously hypertensive rat (SHR) [12,13].

The gene encoding human p22phox (CYBA) is located on chromosome 16q24 and has several allelic variants [14–17]. Recently we reported the existence of five functional polymorphisms in the promoter of the p22phox gene in SHR, associated with higher transcriptional activity [18]. Thus, the first goal of this study was to perform the structural and functional characterisation of the human p22phox gene, with the final purpose of screening for new polymorphisms in its promoter region.

2. Materials and methods

2.1. Subjects

The study population consisted of Caucasian subjects who were referred to our institution for routine medical examination. The case group of hypertensives consisted of 88 subjects with elevated systolic blood pressure (SBP > 139 mm Hg) and/or elevated diastolic blood pressure (DBP > 89 mm Hg). As a control group, 68 normotensive subjects were also studied. Subjects agreed to participate in the study, which was carried out in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethical Committee of the University Clinic of Navarra.

2.2. Isolation of the p22phox gene 5′ extreme

A human gene library constructed in vector P1 was screened by conventional methods, and a positive clone was obtained. A fragment of approximately 5 kb, obtained by digestion with *Sac*I, was cloned in plasmid pUC19, thus obtaining clone p5S. Sequencing of clone p5S was performed using primers of vector pUC19 and internal primers from the cloned fragment. Sequencing reactions were carried out in both strands using the Autoread sequencing kit (Amersham).

2.3. Primer extension

Primer extension was carried out using a dye-labelled antisense oligonucleotide (5′-ACATGGCCCACTCGATCT-3′) located at positions +8 to +25 relative to the ATG codon. The oligonucleotide was hybridised with 1 µg human kidney poly(A)⁺ RNA (Clontech) and extended using Superscript II reverse transcriptase (Gibco BRL). The products of this reaction were analysed by electrophoresis on a 6%

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polyacrylamide denaturing gel on the ALF™ DNA Analysis System (Amersham) and the sizes of the products were determined by comparison with a DNA sequence obtained with the oligonucleotide used.

2.4. Plasmid construction

Digestion of clone p5S with *Sac*I and *Sau*3AI released a fragment of ∼1200 bp containing the whole promoter and the codon ATG of the p22^{phox} gene. This fragment was then subcloned in 5′ position in relation to the luciferase gene, in the *Sac*I/*Bgl*II-digested pGL3basic plasmid. We thus obtained the construct c1. Deleted constructs were obtained by double digestion of c1, using *Sac*I in the plus 5′ end of the promoter and different endonucleases (*Bal*I, *Sma*I and *Apa*I) in the plus 3′ end of the promoter. The restriction ends were filled using the DNA polymerase I Klenow fragment, and the blunt edges were ligated for subsequent transformation. As a result of this approach, constructs c2, c3 and c4 were obtained. For the in vitro mutagenesis experiments we employed construct c1, which contains the G allele at the −930 polymorphic site, as a template. The mutated construct, in which the G allele at the −930 polymorphic site in the p22^{phox} promoter was replaced by the A allele, was obtained using the QuikChange Site-Directed Mutagenesis kit (Stratagene) with the primers 5′-GGGGAATAAACCAGCATTACTGCCTCCGGCCTC-CG-3′ and 5′-CGGAGGCCGGAGGCAGTAATGCTGGTTTATT-CCCC-3′. Correct orientation and sequence of the p22^{phox} promoter in all constructs were confirmed by restriction enzyme analysis, polymerase chain reaction (PCR), and DNA sequencing.

2.5. Cell culture, transfection experiments and luciferase activity

Primary VSMCs were obtained from thoracic aorta of 30-week-old SHR as previously reported [19]. The rat aortic smooth muscle cell line A7r5 was cultured in Dulbecco's modified Eagle's medium with 10% foetal calf serum supplemented with sodium pyruvate. A7r5 or VSMCs were transfected as previously reported [19] and the assays for luciferase activity were performed using Dual-Luciferase Reporter Assay System (Promega). The ratio of firefly luciferase level to *Renilla* luciferase level was used as a measurement of p22^{phox} promoter activity.

2.6. Identification of polymorphisms in the p22^{phox} promoter

To screen for mutations in the p22^{phox} promoter, genomic DNA was extracted from blood and a 1167-bp fragment containing the whole promoter was amplified by PCR using the primers 5′-CAGG-GAAGGGTCACTGTG-3′ and 5′-ACATGGCCCACTCGATCT-3′. The PCR products were sequenced directly using a Thermo Sequenase kit (Amersham) after column purification.

2.7. Genotyping

Since the A-to-G mutation of the −930$^{A/G}$ polymorphism introduces a *Bbv*I digestion site, restriction fragment length polymorphism (RFLP) was used to analyse this polymorphism in all subjects. A 650-bp fragment containing the −930 site was amplified employing the oligonucleotides F$_1$ (5′-GGAAACCACCAAGTGCCTCGGATGG-3′) and R$_1$ (5′-TCTGCACCCTGCTACCAAGGAC-3′). Digestion of the PCR product by *Bbv*I yields 504-, 85-, 57- and 15-bp fragments in the G allele, whereas *Bbv*I makes 589-, 57- and 15-bp fragments in the A allele. Genotypes were scored according to the patterns of DNA bands, and the results were confirmed by duplicate analysis. To be sure of the accuracy of the method, a subset of 650-bp fragments were sequenced.

2.8. Statistical analysis

Data are expressed as mean ± S.E.M. χ^2 analysis was used to test for deviation of genotype distribution from Hardy–Weinberg equilibrium and to determine whether there were any significant differences in allele or genotype frequencies between cases and controls. Statistical significance in the luciferase activity experiments among constructs and in the clinical data among genotypes was assessed by ANOVA followed by Scheffé post-hoc test. Differences in the luciferase activity
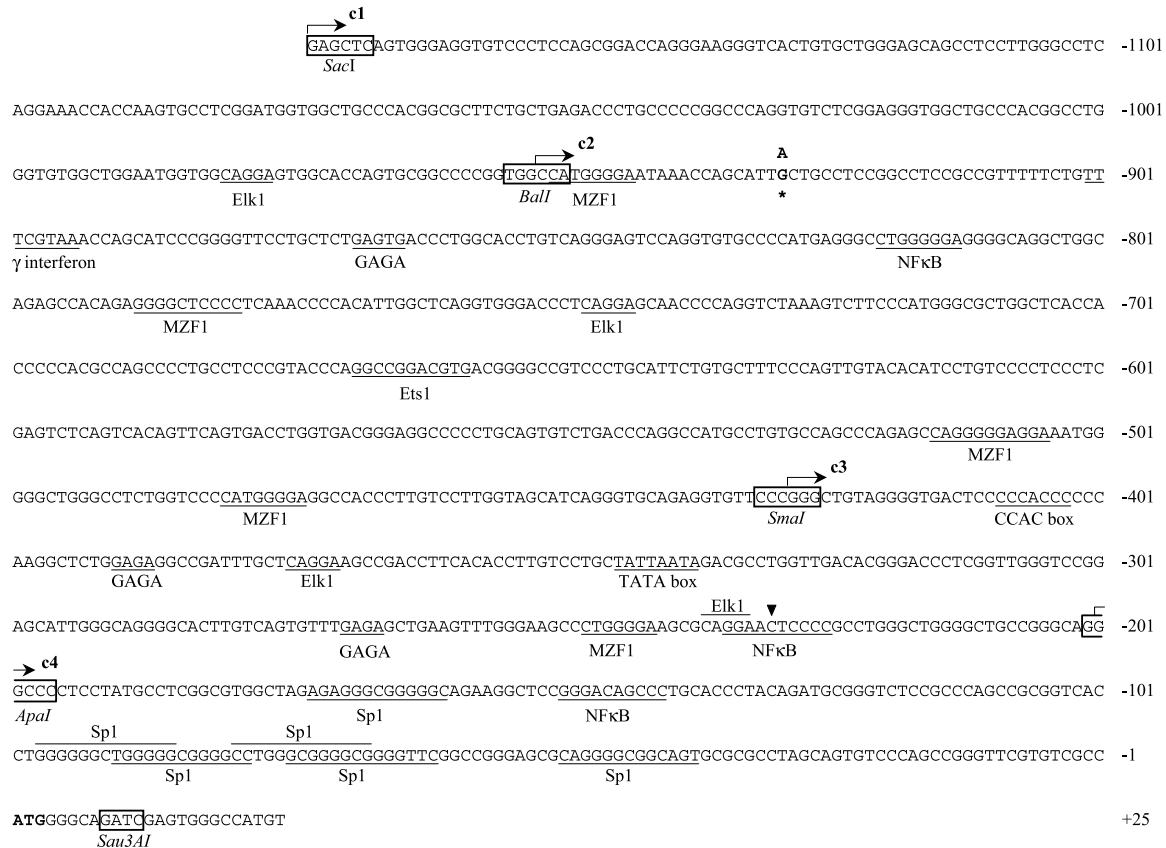

Fig. 1. Nucleotide sequence of the human p22^{phox} gene promoter. Consensus sites for transcriptional factors are underlined. The nucleotide number was counted from the ATG codon. Arrows indicate the 5′ end of the p22^{phox} promoter constructs shown in Fig. 2. The asterisk indicates the polymorphic site and the two alleles. The arrowhead (▼) indicates the transcriptional start site. The nucleotide sequence has been submitted to the GenBank data bank with accession number AY128666.
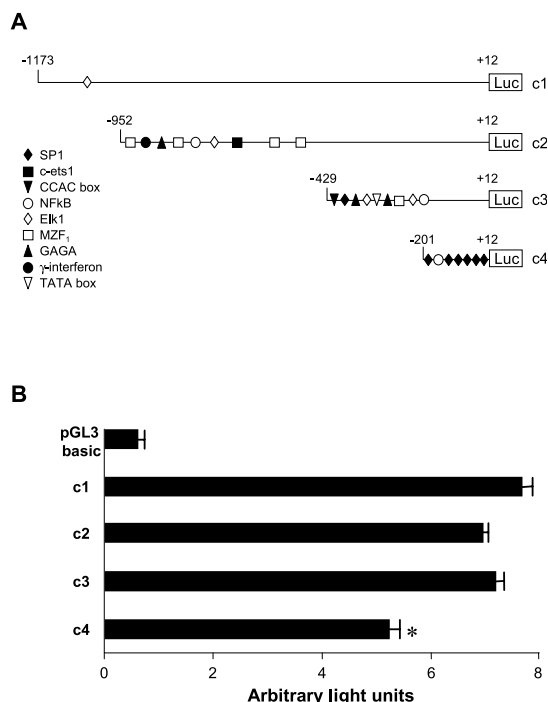
Fig. 2. A: Deletion mutants of the human p22^{phox} promoter–luciferase constructs. B: Analysis of the human p22^{phox} gene promoter. A7r5 cells were transfected with the indicated plasmids, and luciferase activity was measured. Values represent means ± S.E.M. of five separate determinations, performed in duplicate. *$P < 0.05$ compared with c1, c2 and c3.

experiments between allelic variants were assessed by Student's t-test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Structure of the p22^{phox} gene 5′ extreme

Sequence analysis of clone p5S corroborated the presence of the first four exons and three introns, which are flanked by typical splice donor and acceptor dinucleotides, with an identical sequence to the previously reported human p22^{phox} gene exon–intron organisation [14]. In addition, we identified 1173 bp located upstream of the ATG codon corresponding to the promoter region. Computer analysis of this sequence showed the presence of a TATA box and a CCAC box. Other poten-
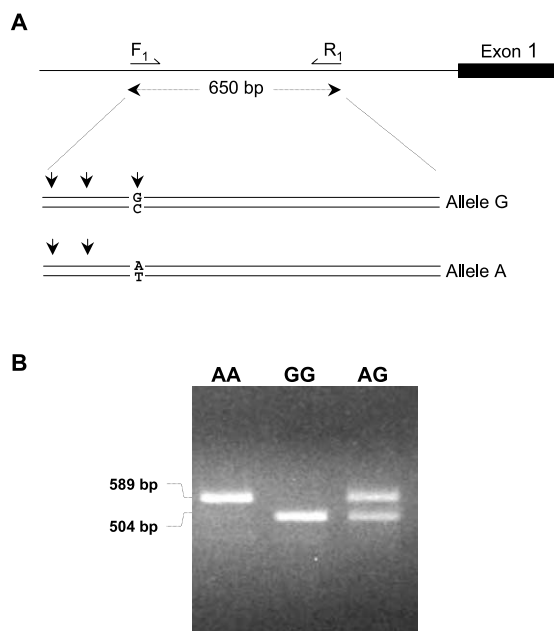

Fig. 3. A: Polymorphism $-930^{A/G}$ was genotyped by digesting with *Bbv*I a 650-bp fragment of the p22^{phox} promoter amplified by PCR with primers F$_1$ and R$_1$. Vertical arrows indicate restriction sites for the *Bbv*I enzyme. B: Representative agarose gel electrophoresis of *Bbv*I RFLP of the p22^{phox} gene.

tial binding sites for GAGA elements, γ-interferon and nuclear factor κB (NF-κB) that might transcriptionally regulate p22^{phox} gene expression were also identified (Fig. 1). It is interesting to note the presence of multiple binding sites for the SP1 transcription factor close to the ATG codon. A primer extension experiment pointed to a C located 231 nucleotides upstream of the codon ATG as the predominant site for transcription initiation (Fig. 1).

3.2. Promoter activity of the p22^{phox} gene

In order to establish if the potential promoter region was functional, a fragment of 1185 bp containing the whole promoter was subcloned in a plasmid carrying the luciferase gene (c1 clone) (Fig. 2A) and transfected in A7r5 cells. Fig. 2B shows a substantial promoter activity of construct c1 in A7r5 cells. To characterise more precisely the regions required to control promoter activity, we cloned a series of DNA fragments of the p22^{phox} promoter obtained by progressive dele-

Table 1
General clinical characteristics of normotensives and hypertensives

	Normotensives	Hypertensives	P
Gender, male/female	56/12	68/20	NS
Age, years	48 ± 1	56 ± 1	< 0.05
Body mass index, kg/m^2	25.9 ± 0.4	28.7 ± 04	< 0.05
Smokers, n	27	25	NS
Diabetes, n	4	3	NS
Systolic blood pressure, mm Hg	115 ± 1	159 ± 2	< 0.05
Diastolic blood pressure, mm Hg	76 ± 1	93 ± 1	< 0.05
Mean arterial pressure, mm Hg	89 ± 1	115 ± 1	< 0.05
Pulse pressure, mm Hg	39 ± 1	65 ± 2	< 0.05
Glucose, mg/dl	100 ± 2	101 ± 1	NS
Total cholesterol, mg/dl	236 ± 5	243 ± 4	NS
HDL cholesterol, mg/dl	54 ± 2	50 ± 2	NS
LDL cholesterol, mg/dl	159 ± 5	167 ± 5	NS
Triglycerides, mg/dl	108 ± 8	139 ± 8	< 0.05

NS: not significant.

Table 2
Genotype and allele frequencies for p22^{phox} −930$^{A/G}$ polymorphism in normotensives and hypertensives

	Normotensives	Hypertensives	P
GG, n (%)	23 (33.8)	36 (40.9)	< 0.02
GA, n (%)	29 (42.6)	41 (46.6)	
AA, n (%)	16 (23.5)	11 (12.5)	
G allele frequency	0.55	0.64	
A allele frequency	0.45	0.36	

tion of the c1 clone (Fig. 2A). The resulting plasmids were transfected in A7r5 cells. As shown in Fig. 2B, constructs c2 and c3 generated signals similar to those obtained with construct c1. However, the promoter activity of construct c4 showed a significant decrease ($P < 0.05$).

3.3. Identification of a new polymorphism in the human p22^{phox} promoter

To search for polymorphisms in the 5′ flanking region of the human p22^{phox} gene, a 1167-bp fragment corresponding to the p22^{phox} promoter was amplified from genomic DNA from 20 patients with essential hypertension and sequenced as described above. Computer analysis of the p22^{phox} promoter sequences revealed the existence of A-to-G substitution at −930 from the ATG codon (Fig. 1).

3.4. Association of the −930$^{A/G}$ polymorphism of the p22^{phox} gene with essential hypertension

The clinical characteristics of subjects classified according to blood pressure values are shown in Table 1. A total of 88 hypertensives and 68 normotensives were genotyped for the −930$^{A/G}$ polymorphism by RFLP (Fig. 3). Distributions of genotypes and alleles from both groups are presented in Table 2. Genotype frequencies were consistent with the Hardy–Weinberg equilibrium law. Frequencies of genotypes for the −930$^{A/G}$ polymorphism in hypertensive patients differed significantly from those in normotensive subjects ($\chi^2 = 6.4$, $P < 0.02$). No significant differences were found in the clinical characteristics of hypertensives classified according to −930$^{A/G}$ polymorphism genotypes (Table 3).

3.5. Functional effect of the −930$^{A/G}$ polymorphism of the p22^{phox} gene

To investigate whether the G-to-A substitution had an effect on gene expression, transfection experiments on VSMCs obtained from SHR rats with each allelic promoter–reporter
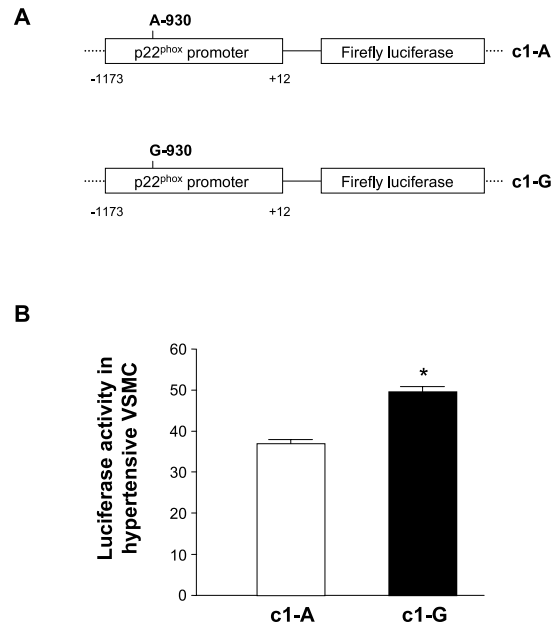

Fig. 4. A: Schematic presentation of reporter gene constructs containing the p22^{phox} gene promoter, with the only difference between the two constructs being either a G or an A base at the −930 site. B: Luciferase levels in VSMCs from SHR transfected with reporter gene constructs containing A (c1-A) or G (c1-G) allelic p22^{phox} gene promoter. Data shown are means ± S.E.M. values from six separate experiments, performed in duplicate. *$P < 0.05$ compared with c1-A.

gene construct were carried out. The G allele exhibited higher luciferase activity than the A allele in SHR cells. As shown in Fig. 4, reporter gene expression driven by the G allelic p22^{phox} promoter was 30% higher ($P < 0.05$) than reporter gene expression directed by the A allelic promoter.

4. Discussion

In the present study we have characterised the promoter region of the human p22^{phox} gene for the first time. The human p22^{phox} promoter possesses a typical TATA and a CCAC box and several potential consensus sequences for transcriptional factors, such as GAGA elements, γ-interferon and NF-κB. Furthermore, we have identified several SP1 binding sites in close proximity to the ATG codon that can also play a significant role in p22^{phox} promoter activity. The construct c1, which contained 1173 bp upstream and 12 bp downstream

Table 3
p22^{phox} −930$^{A/G}$ genotypes and clinical characteristics in hypertensives

	AA	AG	GG	Significance
Gender, male/female	10/1	29/12	29/7	NS
Age, years	55 ± 3	55 ± 1	56 ± 1	NS
Body mass index, kg/m^2	27.8 ± 1.1	28.1 ± 0.5	29.8 ± 0.7	NS
Diabetes, n	1	2	0	NS
Systolic blood pressure, mm Hg	153 ± 6	162 ± 4	156 ± 3	NS
Diastolic blood pressure, mm Hg	86 ± 2	94 ± 2	94 ± 2	NS
Mean arterial pressure, mm Hg	108 ± 2	117 ± 2	115 ± 2	NS
Pulse pressure, mm Hg	66 ± 7	68 ± 4	62 ± 3	NS
Glucose, mg/dl	103 ± 4	102 ± 2	99 ± 2	NS
Total cholesterol, mg/dl	237 ± 9	241 ± 7	246 ± 7	NS
HDL cholesterol, mg/dl	48 ± 4	51 ± 3	49 ± 2	NS
LDL cholesterol, mg/dl	160 ± 14	160 ± 6	177 ± 9	NS
Triglycerides, mg/dl	135 ± 18	154 ± 13	124 ± 12	NS

NS: not significant.

of the ATG codon, was used for luciferase reporter gene assays. In this regard, a substantial promoter activity of construct c1 proved the functionality of this promoter. However, we observed a significant decrease in the promoter activity of construct c4. Deletion analysis of the promoter region revealed that TATA and CCAC boxes and several putative binding sites such as Elk1, GAGA and NF-κB, situated between positions −201 and −429, may be important for the basic promoter activity.

The main finding of the current study is the identification of a new polymorphism within the p22[phox] gene promoter, located at position −930 from the ATG codon. Genotyping studies of this polymorphism in cases and controls showed a significant increase in G allele frequency in hypertensives. Our group has recently reported the presence of functional polymorphisms in the p22[phox] promoter that resulted in a higher transcriptional activity of the p22[phox] gene in SHR [18]. In the same study we showed that VSMCs obtained from the aorta of SHR exhibit a characteristic hypertensive phenotype. Thus, these cells represent a good in vitro model for the study of the effects of hypertensive stimuli on the allelic variants of the −930[A/G] polymorphism and hence on the promoter activity. In this regard, our findings showing a higher reporter gene expression driven by the G allelic p22[phox] promoter than that of the A allelic promoter in SHR VSMCs would suggest that this polymorphism is functionally relevant. In a multifactorial chronic disease such as hypertension, a p22[phox] transcriptional activity 30% higher for the G allele than for the A allele could be expected to play a significant role in this trait. Thus, these observations suggest that the −930[A/G] polymorphism may be involved in the control of the expression of the p22[phox] gene under hypertensive conditions, although new studies are necessary to address the influence of genotypes on p22[phox] mRNA expression.

Recent findings have claimed the potential relevance of p22[phox] gene overexpression in hypertension [20]. An increased p22[phox] mRNA expression leading to a greater NAD(P)H oxidase-driven $^{\bullet}O_2^-$ production has been reported in SHR with diminished nitric oxide availability and endothelial dysfunction [12]. On the other hand, we have shown that p22[phox] polymorphisms are able to regulate p22[phox] expression and consequently NAD(P)H oxidase activity in SHR [18]. It is, thus, tempting to speculate that the polymorphism of the p22[phox] gene reported here may upregulate the expression and activity of NAD(P)H oxidase in hypertension. Although further functional studies are required to test this hypothesis, its significance is underlined by clinical data, indicating the occurrence of increased $^{\bullet}O_2^-$ production in hypertensives [21,22]. Nevertheless, we cannot discard the possibility that −930[A/G] polymorphism could be in linkage disequilibrium with another functional polymorphism.

In summary, we have characterised the genomic structure of the human p22[phox] gene promoter. In addition, we have identified the −930[A/G] polymorphism in the p22[phox] promoter

that appears to be a novel genetic marker associated with hypertension. Since this is a pilot study, it is necessary to confirm these preliminary results in further studies. Nevertheless, our findings show that expression of the p22[phox] mRNA in hypertension depends on the presence on the G or A allele for this polymorphic site.

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