Paraoxonase-1 promoter haplotypes and serum paraoxonase: a predominant role for polymorphic position −107, implicating the Sp1 transcription factor

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Accumulating data suggest that paraoxonase-1 (PON1) is a primary determinant of the antioxidant and anti-inflammatory capacities of high-density lipoproteins (HDLs). Variations in HDLs and PON1 have been shown to influence the functions of both. There is a wide spectrum of serum PON1 mass in humans, to which promoter polymorphisms make an important contribution. The present studies attempted to define: (i) the relevance *in vivo* of promoter polymorphisms by analysing haplotype structure; and (ii) molecular mechanisms implicated in promoter activity. Highly significant differences ($P < 0.0001$) in serum mass and activity were observed as a function of haplotype sequence. Of three promoter polymorphisms (−107, −824 and −907), the −107 site was shown to be of predominant importance to serum PON1. Significant increases in serum PON1 mass and activities between haplotype subgroups could be explained by unit increases in the number of high-expresser variants of the −107 site (−107C) alone. No significant contribution was observed for the −824 and −907 sites. The coding-region Leu^{55} → Met (L55M) polymorphism made an independent contribution to serum PON1 mass, which may account for variations in serum PON1 mass and activity within haplotype subgroups defined by the −107 site. A molecular basis for the effect of the −107 polymorphism on serum PON1 was indicated by the greater affinity of the high-expresser variant (−107C) for hepatocyte nuclear extracts, indicating higher affinity for transcription factors. Competition studies with oligonucleotides representing the consensus (and mutated) sequence for Sp1, and the use of Sp1 antibodies, confirmed formation of complexes between the transcription factor and the *PON1* promoter during incubation with nuclear extracts. The data underline the importance of the region containing the C(−107)T polymorphism for gene expression *in vivo*. Differences in the affinity of the −107C and −107T polymorphic fragments for nuclear extracts have been demonstrated, and coincide with their impact on gene expression. A potential role for the transcription factor Sp1 has been demonstrated, which is consistent with the disruption of an Sp1 recognition sequence by the −107 polymorphism.

Key words: atherosclerosis, haplotype, lipoprotein, oxidative stress, polymorphism, promoter.

INTRODUCTION

Paraoxonase-1 (PON1) has emerged as a primary determinant of the antioxidant and anti-inflammatory capacities of high-density lipoproteins (HDLs) [1,2]. A decrease in the PON1 content of serum HDL increases the risk and extent of lipoprotein oxidation, and is associated with a greater degree of atherosclerosis in murine models [3,4]. Conversely, transgenic mice overexpressing PON1 are protected from lesion formation [5]. Reducing the PON1 content of HDL can also transform the lipoprotein from an anti-inflammatory into a pro-inflammatory complex [6,7]. The protective functions can be recovered by replenishing HDL with PON1. These studies illustrate how variations in serum PON1 can modify these important defence processes.

There is a wide spectrum of serum concentrations and enzyme activities of PON1 in humans. An activity polymorphism, which was known for decades, has been located to a coding-region polymorphism {Gln^{192} → Arg (Q192R); [8,9]}. It affects activity towards certain non-physiological substrates, but the consequences for the physiological substrate(s) and function of PON1 are not clear. It has been suggested, however, that the glutamine and arginine (Q and R) variants differ in their hydrolytic activities towards lipid peroxides [10] and homocysteine metabolites [11]. Recently, we identified three common polymorphisms in the promoter region of the *PON1* gene, which strongly affect serum concentrations of the enzyme [12]. The polymorphisms and their impact on serum PON1 were confirmed by later, independent studies [13,14]. By means of *in vitro* expression studies, we showed that polymorphic positions −107 and −824, but not −907, could influence promoter activity [12]. The effect of the −107 site was confirmed in later studies [13,14], but Brophy et al. [14] reported that the −907, but not the −824, mutation could influence promoter activity.

The growing role attributed to PON1 in assuring protective mechanisms associated with HDL underlines the need to clarify fully the factors that control gene expression and thus modulate serum PON1 concentrations. Moreover, previous studies have emphasized the importance of ambient serum PON1 levels in defining risk of coronary disease in humans [15,16]. Whereas the polymorphisms discussed above indicate regions of the promoter of potential relevance for gene expression, the importance *in vivo* of the three polymorphisms is not clear. Such information would help to orientate studies of the *PON1* promoter. To this end, the present study has analysed *PON1* promoter haplotypes to define regions of importance to *PON1* gene expression *in vivo*, and subsequently examined the potential role of transcription factors. It demonstrates a critical role for the −107 polymorphic site, and implicates the transcription factor Sp1.

Abbreviations used: ASO, allele-specific oligonucleotides; EMSA, electrophoretic mobility-shift assay; HDL, high-density lipoprotein; L55M polymorphism, etc., Leu^{55} → Met polymorphism, etc.; PON1, paraoxonase-1; SREBP, sterol-regulatory-element-binding protein.

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MATERIALS AND METHODS

Haplotyping of promoter polymorphisms

DNA was extracted from blood cells as described previously [12]. Haplotyping for positions −907, −824 and −107 was performed with the following procedure: (i) separation of alleles by PCR amplification with allele-specific primers; and (ii) separate analysis of each amplified allele by hybridization with allele-specific oligonucleotides (ASO). Each DNA sample was first subjected to two separate PCR reactions, using either sense allele-specific primer CAGCAGACAGCAGAGAAGAGAC (specific for nucleotide C at position −907) or CAGCAGACAGCAGAGAAGAGAG (specific for nucleotide G at position −907). The opposing anti-sense primer was ATCCGGATCCGGGGATAGACAAAGGGATCGATG (for conditions used, see [12]). The PCR reaction thus defined the nucleotide at position −907, and gave rise to 1027 bp PCR products, which included positions −824 and −107. The latter were analysed by hybridization with ASO [12]. Thus, for samples heterozygous at position −907, the PCR reactions resulted in two 1027 bp PCR products, each derived from an individual allele, whereas homozygous samples gave a product in only one of the PCR reactions.

Reporter gene vector, cell transfection and cell culture

DNA fragments (1027 bp) from individuals who differed in *PON1* promoter structure were obtained by PCR amplification, and were inserted upstream of the firefly luciferase reporter gene in the pGL2Basic (Promega) derivative, as described previously [12]. Cloned fragments were completely sequenced to ensure that sequence variations were limited to those under study. *Renilla* luciferase gene from plasmid pRLSV40 (Promega) was used as a control for transfection efficiency. The vectors were transfected into the hepatoma cell line HepG2 at 50 % confluence [12], and transfected cells were grown for 24 h before being harvested for analysis of firefly and *Renilla* luciferase activities [12].

Nuclear extract and electrophoretic mobility-shift assay (EMSA)

HepG2 cells were grown to confluence, harvested, and the nuclear fraction was isolated and extracted as described previously [17]. EMSA was performed as described previously [18] using a ^{32}P-labelled 22 bp fragment of the *PON1* promoter region (nt −114 to −97) with nucleotide C or T at position –107. The DNA–protein complexes were analysed on 5 % polyacrylamide gels, as described previously [18]. Double-stranded oligonucleotides representing the consensus sequence for the Sp1 recognition site (5′-ATT CGA TCG GGG CGG GGC GAG C-3′) were obtained from Santa Cruz Biochemicals (Santa Cruz, CA, U.S.A.), and a mutated form (5′-ATT CGA TCG GTT CGG GGC GAG C-3′; mutated nucleotides shown underlined) was synthesized by Qiagen (Basel, Switzerland). Anti-Sp1 antibodies (Santa Cruz Biochemicals) were employed to study binding of Sp1 from nuclear extracts to the PON1 fragment, as described previously [18].

Population analysis

Promoter haplotypes were analysed in a population recruited during the course of a study of genetic risk factors for coronary disease [19]. The population comprised 865 subjects, who gave written, informed consent to participate. The study was approved by the Ethics Committee of the Department of Internal Medicine, University Hospital, Geneva, Switzerland. Plasma and serum samples were obtained after an overnight (14 h) fast.

Paraoxonase activities and mass concentrations

PON1 enzyme activity was assayed in human serum samples as described previously [20] using, as substrates, either phenylacetate, in which case it is referred to as arylesterase activity, or paraoxon (referred to as paraoxonase activity). A control pool of human sera, independently calibrated by Dr M. Mackness (School of Medicine, University of Manchester, Manchester, U.K.), was used to standardize activity assays. Mass measurements were performed by a competitive ELISA, as described in detail previously [20].

Statistical analyses

Continuous and normally distributed variables were compared with ANOVA. The Mann–Whitney U test was used for non-normally distributed variables. The χ^2 test was employed to examine the association between categorized data.

RESULTS

We have previously demonstrated with *in vitro* expression studies that positions −107 and −824 influenced independently promoter activity [12], with each causing a 2-fold increase in activity. Given that several reports have shown wide variations (up to 6-fold) in serum concentrations [12,14] and activities [21] of PON1, independently of the Q192R activity polymorphism, an additive effect of the two polymorphisms on gene expression was considered a possibility. Expression studies were performed with promoter fragments containing different combinations of polymorphisms. As shown in Figure 1, when the high-expresser variants of −107 (C) and −824 (A) were both present, there was an approximate 4-fold increase in promoter activity, compared with a 2-fold increase in activity when only one high expresser was present. Changing the nucleotide at position −907 had no effect on activity.

To try to define the *in vivo* importance of individual promoter polymorphisms, as well as combinations of the polymorphisms, we developed a haplotyping procedure to analyse haplotypes as a function of serum concentrations and activities of PON1. The population that was studied is briefly described in Table 1.

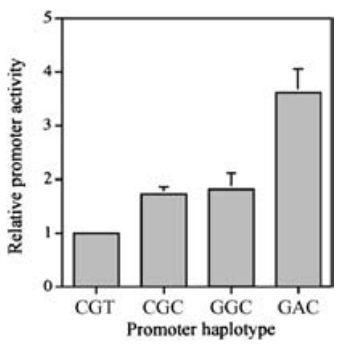

Figure 1 Activities of *PON1* promoter fragments

Promoter fragments (1027 bp) with the indicated promoter haplotypes (sequence −907/−824/−107) were cloned into expression cassettes and transfected into HepG2 cells. Promoter activities were normalized to that of co-transfected *Renilla* luciferase.

Table 1 Population characteristics

Parameter	Value
Number (*n*)	865
Men/women	634/231
Age (years)	59.8 ± 9.9
Cholesterol (mmol/l)	5.73 ± 1.21
Triacylglycerols (mmol/l)	1.58 ± 1.04
HDL/cholesterol (mmol/l)	1.16 ± 0.33
Patients	
Non-diabetic/diabetic	720/145
Cardiovascular disease-positive/-negative	678/187

Table 2 Haplotype frequencies in the population

Haplotype structure is shown in the order of polymorphisms of −907, −824 and −107.

Haplotype	*n* (%)
CGT-CGT	237 (27.3)
CGT-GAC	214 (24.7)
CGT-GGC	143 (16.5)
GAC-GAC	65 (7.5)
GAC-GGC	63 (7.3)
CGT-CGC	62 (7.2)
CGC-GAC	29 (3.3)
CGC-GGC	23 (2.6)
GGC-GGC	20 (2.3)
CGC-CGC	4 (0.5)
GGC-GGT	3 (0.3)
CGT-GGT	2 (0.2)

Other details are given in [19]. Table 2 shows the haplotype distribution in the population. Of the eight possible combinations of single allele haplotypes, five were detected, of which two haplotype combinations were rare. The predominant haplotype was CGT (−907/−824/−107; 51.8%), which, together with GAC and GGC, accounted for >90% of single allele haplotypes. For two allele haplotypes, three combinations accounted for 70% of haplotypes (Table 2). This probably reflects linkage disequilibrium between the polymorphisms, as we reported previously [12].

Serum concentrations and activities as a function of the *PON1* promoter haplotypes are shown in Table 3. Highly significant differences between haplotypes were observed for mass and both paraoxonase and arylesterase enzyme activities. Concentration and arylesterase activity increased in parallel, whereas changes in paraoxonase activity between haplotype subgroups tracked less well with concentration, due undoubtedly to the confounding effect of Q192R activity polymorphism. There was, however, a tendency for low- and high-activity haplotypes to segregate with low- and high-concentration haplotypes respectively. When paraoxonase activities for homozygous forms of the 192 polymorphism (QQ or RR) were compared between haplotypes, activities paralleled the increases in concentration (Table 3).

Several analyses of the haplotypes were undertaken to gauge the potential importance of the individual polymorphisms to gene expression, using serum PON1 concentrations as a marker. First, mean concentrations of the enzyme were expressed as a function of the number of high-expresser variants present for each polymorphism. The results are illustrated in Figure 2. Considering the −107 position, the increase in the number of high-expresser (C) variants at this site (Figure 2A) correlated with increases in mean enzyme concentrations, irrespective of the variants

Table 3 Serum PON1 concentrations and activities as a function of the promoter haplotypes

Haplotype structure is shown in the order of polymorphisms of −907, −824 and −107. Statistical differences between haplotypes were $P < 0.0001$ for concentration and enzyme activities. For paraoxonase Gln/Gln homozygotes, numbers of subjects are indicated in parentheses.

Haplotype (*n*)	Concentration (μg/ml)	Arylesterase (units/ml)	Paraoxonase (units/ml)	Paraoxonase Gln/Gln (units/ml)
GAC-GGC (63)	115.8 ± 17.7	104.7 ± 23.2	343.0 ± 218.0	168.6 ± 39.2 (31)
GAC-GAC (65)	111.9 ± 18.1	102.1 ± 28.0	268.0 ± 195.1	154.2 ± 37.2 (43)
CGC-GGC (23)	108.3 ± 20.8	99.2 ± 19.4	417.6 ± 263.2	130.2 ± 16.5 (6)
CGC-GAC (29)	108.0 ± 19.4	93.6 ± 27.7	334.4 ± 215.0	150.2 ± 28.9 (13)
GGC-GGC (20)	107.2 ± 19.0	98.5 ± 21.7	356.9 ± 251.7	148.6 ± 45.9 (10)
CGC-CGC (4)	101.3 ± 10.7	88.3 ± 14.1	344.6 ± 169.0	124.7 (1)
GGC-GGT (3)	99.5 ± 20.5	92.0 ± 21.7	401.0 ± 322.2	165.8 (1)
CGT-GGC (143)	98.9 ± 22.0	81.9 ± 24.1	286.3 ± 169.5	127.2 ± 31.5 (59)
CGT-CGC (62)	98.4 ± 21.5	81.6 ± 18.8	304.5 ± 191.1	123.6 ± 28.5 (24)
CGT-GAC (214)	95.7 ± 20.5	83.1 ± 31.7	243.6 ± 160.6	121.3 ± 33.4 (113)
CGT-GGT (2)	82.1 ± 17.0	53.2 ± 8.3	280.8 ± 69.7	–
CGT-CGT (237)	79.4 ± 18.7	64.4 ± 17.6	184.5 ± 121.3	97.0 ± 37.0 (135)

present at the other polymorphic sites. In contrast, the increases in the frequencies of the high-expresser variants at the −824 (Figure 2B) and −907 (Figure 2C) polymorphic sites correlated less well with increases in serum PON1 concentrations. Moreover, when there were marked increases in concentrations between haplotype subgroups (Figure 2A; shown by the arrows), this corresponded to a unit increase in the frequency of the high expresser −107C variant. A second approach to the question was to compare serum PON1 as a function of haplotype variations for each polymorphic site (Table 4). When other polymorphic sites were maintained constant, only variations at the −107 site were associated with significant changes in serum concentrations and enzyme activities. This was evident when passing from 0 to 1 high expresser or from 1 to 2 high-expresser variants at position −107. In contrast, no significant changes in serum PON1 mass or activity accompanied changes in the −824 or −907 positions (Table 4). Given that diabetes and vascular disease can influence serum PON1, we also analysed subgroups of the population without these diseases ($n = 188$). The general conclusions concerning the correlation between the −107 polymorphism and serum PON1, and the lack of association between polymorphisms −824 and −907, remained the same (results not shown). This was underlined by a final analysis, in which stepwise multiple-regression analysis was employed to determine the variation in serum concentrations of PON1 attributable to the polymorphisms. In a regression model including lipid variables, age, gender, smoking status and the presence or absence of diabetes or vascular disease, haplotype structure was responsible for 25.0% of the variation in serum PON1 mass (41.8% for the model). If the haplotype variable in the model was replaced by the −107 genotype alone, the new model explained a similar degree of variation (24.4% of total variation for the genotype compared with 41.4% for model). Including the other promoter genotypes (−824 and −907) added little to this model (24.7% of total variation for the three genotypes compared with 41.5% for the model).

Within the subgroup of haplotypes that were homozygous (CC) for the −107 site (see Figure 2), there was still variation in serum mass (and activity) of PON1, which could not be explained by the −824 (mass, $P = 0.65$) or the

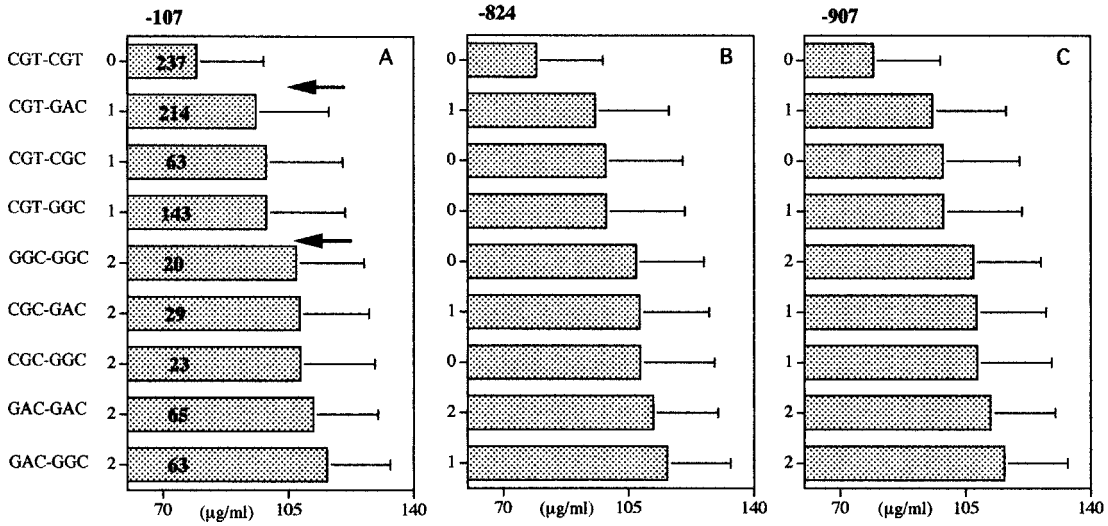

Figure 2 Mean PON1 serum concentrations as a function of haplotype structure (indicated above each panel as −107, −824 and −907 for each haplotype)

(**A**–**C**) The *y*-axis shows the number of high-expresser variants present in the haplotype for each polymorphism. The values set in the bars are the number of haplotypes per subgroup (in total, $n = 856$; subgroups with < 20 were not included). High-expresser variants are −107C, −824A and −907G. The arrows mark the unit increase in the number of high-expresser variants of the −107 site.

Table 4 Comparison of serum PON1 concentrations and activities as a function of haplotype variations

Haplotype subgroups were chosen such that only one of the polymorphic positions varied. The variable position is shown underlined. Haplotype structure is in the sequence order of −107, −824 and −907.

Polymorphism	Haplotypes	Concentrations (μg/ml)	Probability	Arylesterase (units/ml)	Probability
−107					
0 vs. 1 high expresser	CGT-CG<u>T</u> vs. CGT-CG<u>C</u>	79.4 ± 18.7 vs. 98.4 ± 21.5	$P < 0.0001$	64.4 ± 17.6 vs. 81.6 ± 18.8	$P < 0.0001$
1 vs. 2 high expressers	CG<u>T</u>-GAC vs. CG<u>C</u>-GAC	95.7 ± 20.5 vs. 108.0 ± 19.4	$P = 0.0024$	83.1 ± 31.7 vs. 93.6 ± 27.7	$P = 0.019$
−824					
0 vs. 1 high expresser	CGT-G<u>G</u>C vs. CGT-G<u>A</u>C	98.9 ± 22.0 vs. 95.7 ± 20.5	$P = 0.17$	81.9 ± 24.1 vs. 83.1 ± 31.7	$P = 0.71$
0 vs. 2 high expressers	G<u>G</u>C-G<u>G</u>C vs. G<u>A</u>C-G<u>A</u>C	107.2 ± 19.0 vs. 111.9 ± 18.1	$P = 0.30$	98.5 ± 21.7 vs. 102.1 ± 28.0	$P = 0.60$
−907					
0 vs. 1 high expresser	CGT-<u>G</u>GC vs. CGT-<u>C</u>GC	98.9 ± 22.0 vs. 98.4 ± 21.5	$P = 0.89$	81.9 ± 24.1 vs. 81.6 ± 18.8	$P = 0.92$
1 vs. 2 high expressers	<u>C</u>GC-GGC vs. <u>G</u>GC-GGC	108.3 ± 20.8 vs. 107.2 ± 19.0	$P = 0.85$	99.2 ± 19.4 vs. 98.5 ± 21.7	$P = 0.91$

−907 (mass, $P = 0.65$) polymorphisms. However, when the coding region Leu[55] → Met (L55M) polymorphism was analysed within this CC homozygous subgroup, serum concentrations (and activity; results not shown) differed significantly among genotypes (in μg/ml: LL, 113.8 ± 27.8; LM, 107.0 ± 20.7; and MM, 99.0 ± 9.9; $P = 0.019$). This suggests an impact of the L55M polymorphism, which is independent of the −107 promoter polymorphism.

The results described above clearly designated the C(−107)T polymorphism as the focal point for the influence of the promoter on serum PON1 concentrations *in vivo*. The next set of experiments centred on this region, and in particular on the binding of extracts of nuclear proteins and the potential consequences of the C → T polymorphism. EMSA demonstrated binding of transcription factors from hepatocyte nuclear extracts to promoter fragments containing the C or T polymorphisms (Figure 3a). The C(−107)T polymorphism disrupts a recognition sequence for the transcription factor Sp1. To examine a potential role for Sp1, we employed synthetic oligonucleotides containing the Sp1 consensus binding sequence, and a mutated sequence, in EMSA studies. As shown in Figure 3(a), lane 7, when nuclear extract was incubated with the radiolabelled Sp1 consensus sequence, the pattern of bands observed during EMSA was quite comparable with that observed for the *PON1* promoter fragments containing C or T variants (lanes 2 and 5 respectively). Co-incubation of the unlabelled consensus binding sequence efficiently prevented formation of complexes between the radiolabelled *PON1* promoter fragment and nuclear proteins (Figure 3b). In contrast, the mutated form of the consensus sequence was unable to prevent formation of the complexes (Figure 3b). To confirm the presence of Sp1 in complexes formed between the promoter fragments and nuclear extract, supershift assays were performed. Antibodies against Sp1 were able to supershift complexes formed between both C and T variants of the promoter fragment and the nuclear extracts (Figure 3a, lanes 3 and 6). A comparable supershift pattern was observed when the labelled Sp1 consensus sequence was employed (Figure 3a, lane 8). As indicated in Figure 3(a), the −107C fragment appeared to produce a more intensely stained band than the T-containing fragment during EMSA analyses. This was confirmed by analysing the intensities of the bands in five independently performed EMSA experiments, which used

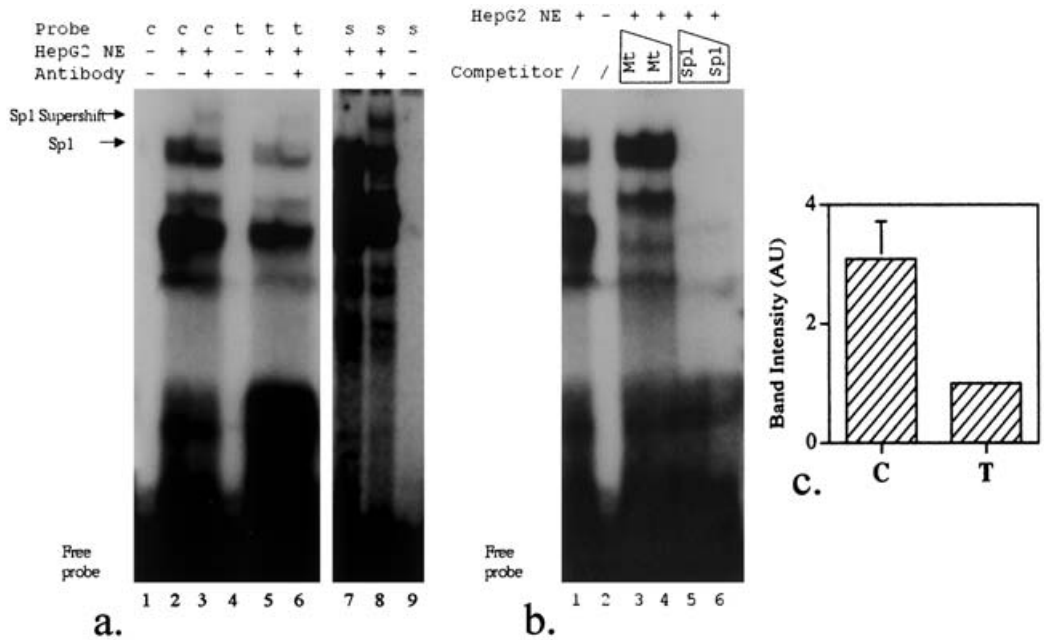

Figure 3 EMSA using labelled *PON1* promoter fragments, Sp1 consensus sequence and nuclear extracts from HepG2 cells

(**a**) Labelled probe was incubated with: (i) nuclear extract (NE); and (ii) nuclear extract and an antibody against Sp1. c, C-containing promoter fragment; t, T-containing promoter fragment; s, Sp1 consensus sequence; antibody, anti-Sp1 antibody; (**b**) EMSA using labelled C-promoter fragment and nuclear extract co-incubated with non-labelled Sp1 consensus sequence or mutated Sp1 consensus sequence is shown. Mt, mutated Sp1 consensus oligonucleotide; Sp1, Sp1 consensus oligonucleotide. (**c**) Shown are the staining intensities of EMSA bands (as illustrated by the lower of the arrows in **a**) obtained using equivalent concentrations of labelled C or T fragments incubated with nuclear extract alone ($n = 5$ independent analyses). AU, arbitrary units.

equivalent amounts of labelled C or T promoter fragments with nuclear extract. The C fragment consistently gave rise to a more intense band (Figure 3c), suggestive of a greater affinity of nuclear extract proteins for the C-containing promoter.

DISCUSSION

The present study is consistent with a critical role for the −107 polymorphic site of the *PON1* promoter in influencing serum PON1 concentrations and activities *in vivo*. Neither of the other two common polymorphisms (−824 and −907) appear to make a significant contribution *in vivo* to variations in serum PON1. Concurrently, the study provides evidence on molecular mechanisms involved in *PON1* promoter activity, by implicating the transcription factor Sp1. Moreover, a molecular basis for the effect of the C→T polymorphism is strongly suggested by the data, consistent with a greater affinity of nuclear proteins for the C-containing promoter fragment.

Serum PON1 mass correlated strongly with the −107 polymorphism, where a unit increase in the number of high-expresser −107 variants (the C allele) in haplotypes correlated with significant increases in serum concentrations of PON1. In contrast, polymorphisms at the −824 and −907 positions within haplotypes correlated less well with variations in serum PON1 mass and activity. When comparisons were made between haplotype subgroups that differed at one of the polymorphic sites only, significant differences in serum PON1 mass and activity were observed for changes at the −107 site alone. Finally, variations in serum PON1 mass arising from the promoter polymorphisms could be explained almost exclusively by the −107 genotypes: neither addition of genotypes arising from the −824 or −907 sites nor the use of haplotypes substantially changed the percentage variance explained by the promoter. These data suggest that the region of the *PON1* promoter in the vicinity of the −107 polymorphism should be the primary focus of attention with respect to factors influencing gene expression *in vivo*.

Studies with nuclear extracts suggest a greater affinity of the −107C variant for nuclear proteins. This is consistent with the association of −107C with higher serum PON1 mass and activity, thus providing a molecular basis for the marked influence of the polymorphism. It implies that transcription factor(s) bind to this region of the promoter, but with a preference for the −107C variant. This is consistent with our preceding expression studies [12]. The −107 site occurs within a recognition sequence for the transcription factor Sp1, as was noted previously [12,13]. The present study provides two lines of evidence (competition studies with consensus sequence oligonucleotides and the use of anti-Sp1 antibodies) for the role of Sp1 in formation of complexes with the *PON1* promoter fragment during incubation with nuclear extracts. The −107T variant disrupts the sequence, which is consistent with the results of the EMSA (lower affinity for nuclear extracts) and association of 107T with lower PON1 concentrations. Sp1 is a ubiquitous transcription factor, implicated in the expression of a wide variety of genes, and thus does not in itself give any indications as to which pathways may be involved in regulating *PON1* expression. However, one set of transcription factors with which it is also known to act in tandem are the sterol-regulatory-element-binding proteins (SREBPs) [22]. Interestingly, a sequence with similarity to binding sites for SREBP2 lies juxtaposed to the C(−107)T polymorphic region (positions −109–−120). We are currently investigating its possible role in *PON1* promoter expression.

Several preceding studies have adopted a statistical approach to analyse the relative contributions of promoter polymorphisms to serum PON1 concentration and activity [12,23,24]. These were on the basis of genotypes. However, *in vitro* expression studies have suggested that at least three polymorphic sites can influence promoter activity [12,24], whereas combinations

of high-expresser sites have an additive effect on expression (the present study). Thus, to gauge the *in vivo* relevance of the different polymorphisms, haplotype data are necessary. This was, in part, the rationale for the present study. It confirms our suggestions from earlier studies that the −107 polymorphism was of particular relevance to *PON1* gene expression [12,23], and extends it further by suggesting that this site accounts for virtually all variations of serum PON1 in the population we studied. The lack of effect of the −824 and −907 sites, despite their *in vitro* activities, may reflect sequence-context effects, which can alter their relevance to promoter activity *in vivo*.

We [25–27] and others [15,28–31] have provided evidence for an independent association between PON1 polymorphisms and the risk of coronary disease, although the latter has not been a consistent association [32–34]. However, as discussed previously [15], polymorphisms should be considered jointly with variations in serum activity in the framework of the cardiovascular role of PON1. In this context, we have previously provided evidence for an interaction between PON1 polymorphisms in their impact on coronary risk [27]. The data from the present study substantiate these conclusions by providing further evidence for an interaction between promoter and coding-region polymorphisms in defining serum concentrations of PON1. The coding-region L55M mutation has a significant influence on serum PON1, over and above the predominant impact of the −107 polymorphism. In a recent study, we provided one possible explanation for the effect of the L55M polymorphism, as we demonstrated greater susceptibility of the M allele product to proteolysis [23].

In conclusion, the data reveal a critical role *in vivo* for the −107 promoter polymorphism in defining serum PON1 concentrations and activities. It provides indications of the molecular mechanisms involved in gene expression and implicates the transcription factor Sp1. By extrapolation, attention should be focused on this region as a determinant of gene expression, and thus on variations in serum PON1 mass and activity. This is of clinical relevance with respect to attempts to modulate *PON1* expression in high-risk individuals, which has potential applications in both the cardiovascular, as well as the toxicological, fields.

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REFERENCES

1 Navab, M., Hama, S. Y., Hough, G. P., Hedrick, C. C., Sorenson, R., La Du, B. N., Kobashigawa, J. A., Fonarow, G. C., Berliner, J. A., Laks, H. and Fogelman, A. M. (1998) High density associated enzymes: their role in vascular biology. Curr. Opin. Lipidol. **9**, 449–456

2 Mackness, M. I., Durrington, P. N. and Mackness, B. (2000) How high-density lipoprotein protects against the effects of lipid peroxidation. Curr. Opin. Lipidol. **11**, 383–388

3 Shih, D. M., Gu, L., Xia, Y.-R., Navab, M., Li, W.-F., Hama, S., Castellani, L. W., Furlong, C. E., Costa, L. G., Fogelman, A. M. and Lusis, A. J. (1998) Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. Nature (London) **394**, 284–287

4 Shih, D. M., Xia, Y. R., Wang, X. P., Miller, E., Castellani, L. W., Subbanagounder, G., Cheroutre, H., Faull, K. F., Berliner, J. A., Witztum, J. L. and Lusis, A. J. (2000) Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. J. Biol. Chem. **275**, 17527–17535

5 Tward, A., Xia, Y. R., Wang, X. P., Shi, Y. S., Park, C., Castellani, L. W., Lusis, A. J. and Shih, D. M. (2002) Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. Circulation **106**, 484–490

6 Castellani, L. W., Navab, M., Van Lenten, B. J., Hendrick, C. C., Hama, S. Y., Goto, A. M., Fogelman, A. M. and Lusis, A. J. (1997) Overexpression of apolipoprotein A-II in transgenic mice converts high density lipoproteins to pro-inflammatory particles. J. Clin. Invest. **100**, 464–474

7 Van Lenten, B. J., Wagner, A. C., Nayak, D. P., Hama, S., Navab, M. and Fogelman, A. M. (2001) High-density lipoprotein loses its anti-inflammatory properties during acute influenza a infection. Circulation **103**, 2283–2288

8 Humbert, R., Adler, D. A., Disteche, C. M., Hassett, C., Omiecinski, C. J. and Furlong, C. E. (1993) The molecular basis of the human serum paraoxonase activity polymorphism. Nature Genet. **3**, 73–76

9 Adkins, S., Gan, K. N., Mody, M. and La Du, B. N. (1993) Molecular basis for the polymorphic forms of human serum paraoxonase/arylesterase: glutamine or arginine at position 191, for the respective A or B allozymes. Am. J. Hum. Genet. **52**, 598–608

10 Aviram, M., Hardak, E., Vaya, J., Mahmood, S., Milo, S., Hoffman, A., Billicke, S., Draganov, D. and Rosenblat, M. (2000) Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. Circulation **101**, 2510–2517

11 Jakubowski, H., Ambrosius, W. T. and Pratt, J. H. (2001) Genetic determinants of homocysteine thiolactonase activity in humans: implications for atherosclerosis. FEBS Lett. **491**, 35–39

12 Leviev, I. and James, R. W. (2000) Promoter polymorphisms of the human paraoxonase PON1 gene and serum paraoxonase activities and concentrations. Arterioscler. Thromb. Vasc. Biol. **20**, 516–521

13 Suehiro, T., Nakamura, T., Inoue, M., Shiinoki, T., Ikeda, Y., Kumon, Y., Shindo, M., Tanaka, H. and Hashimoto, K. (2000) A polymorphism upstream from the human paraoxonase (PON1) gene and its association with PON1 expression. Atherosclerosis **150**, 295–298

14 Brophy, V. H., Hastings, M. D., Clendenning, J. B., Richter, R. J., Jarvik, G. P. and Furlong, C. E. (2001) Polymorphisms in the human paraoxonase (PON1) promoter. Pharmacogenetics **11**, 77–84

15 Jarvik, G. P., Rozek, L. S., Brophy, V. H., Hatsukami, T. S., Richter, R. J., Schellenberg, G. D. and Furlong, C. E. (2000) Paraoxonase (PON1) phenotype is a better predictor of vascular disease than is PON1 (192) or PON1 (55) genotype. Arterioscler. Thromb. Vasc. Biol. **20**, 2441–2447

16 Mackness, B., Davies, G. K., Turkie, W., Lee, E., Roberts, D. H., Hill, E., Roberts, C., Durrington, P. N. and Mackness, M. I. (2001) Paraoxonase status in coronary heart disease: are activity and concentration more important than genotype? Arterioscler. Thromb. Vasc. Biol. **21**, 1451–1457

17 Schreiber, E., Matthias, P., Muller, M. M. and Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res. **17**, 6419

18 Palmer, G., Manen, D., Bonjour, J. P. and Caverzasio, J. (2001) Species-specific mechanisms control the activity of the Pit1/PIT1 phosphate transporter gene promoter in mouse and human. Gene **279**, 49–62

19 James, R. W., Leviev, I. and Righetti, A. (2000) Smoking is associated with reduced serum paraoxonase activity and concentration in coronary artery disease patients. Circulation **101**, 2252–2257

20 Blatter Garin, M.-C., Abbot, C., Messmer, S., Mackness, M. I., Durrington, P., Pometta, D. and James, R. W. (1994) Quantification of human serum paraoxonase by enzyme-linked immunoassay: population differences in protein concentrations. Biochem. J. **304**, 549–554

21 La Du, B. N. (1992) Human serum paraoxonase/arylesterase. In Pharmacogenetics of Drug Metabolism (Kalow, W., ed), pp. 51–91, Pergamon Press, New York

22 Edwards, P. A., Tabor, D., Kast, H. R. and Venkateswaran, A. (2000) Regulation of gene expression by SREBP and SCAP. Biochim. Biophys. Acta. **1529**, 103–113

23 Leviev, I., Deakin, S. and James, R. W. (2001) Decreased stability of the M54 isoform of paraoxonase as a contributory factor to variations in human serum paraoxonase concentrations. J. Lipid Res. **42**, 528–535

24 Brophy, V. H., Jampsa, R. L., Clendenning, J. B., McKinstry, L. A., Jarvik, G. P. and Furlong, C. E. (2001) Effects of 5′ regulatory-region polymorphisms on paraoxonase-gene (PON1) expression. Am. J. Hum. Genet. **68**, 1428–1436

25 Ruiz, J., Blanché, H., James, R. W., Blatter, M. C., Charpentier, G., Morabia, A., Passa, P. and Froguel, P. (1995) The polymorphism (Gln → Arg192) of the high-density lipoprotein-bound enzyme paraoxonase is an independent cardiovascular risk factor in non-insulin dependent diabetic patients. Lancet **346**, 869–872

26 Blatter Garin, M.-C., James, R. W., Dussoix, P., Blanché, H., Passa, P., Froguel, P. and Ruiz, J. (1997) Paraoxonase polymorphism Met→Leu^{54} is associated with modified serum concentrations of the enzyme. A possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes. J. Clin. Invest. **99**, 62–66

27 James, R. W., Leviev, I., Ruiz, J., Passa, P., Froguel, P. and Garin, M.-C. (2000) The promoter polymorphism T(–107)C of the paraoxonase *PON1* gene is a risk factor for coronary heart disease in type 2 patients. Diabetes **49**, 1390–1393
28 Serrato, M. and Marian, A. J. (1995) A variant of human paraoxonase/arylesterase (HUMPONA) gene is a risk factor for coronary heart disease. J. Clin. Invest. **96**, 3005–3008
29 Zama, T., Murata, M., Matsubara, Y., Kawano, K., Aoki, N., Yoshini, H., Watanabe, G., Ishikawa, K. and Ikeda, Y. (1997) A 192Arg variant of the human paraoxonase (*HUMPONA*) gene polymorphism is associated with an increased risk for coronary artery disease in the Japanese. Arterioscler. Thromb. Vasc. Biol. **17**, 3565–3569
30 Pfohl, M., Koch, M., Enderle, M. D., Kühn, R., Fällhase, J., Karsch, K. R. and Häring, H. U. (1999) Paraoxonase 192 Gln/Arg gene polymorphism, coronary artery disease, and myocardial infarction in type 2 diabetes. Diabetes **48**, 623–627
31 Salonen, J. T., Malin, R., Tuomainen, T. P., Nyyssonen, K., Lakka, T. A. and Lehtimaki, T. (1999) Polymorphism in high density lipoprotein paraoxonase gene and risk of acute myocardial infarction in men: prospective nested case-control study. Br. Med. J. **319**, 487–489
32 Antikainen, M., Murtomaki, S., Syvänne, M., Pahlman, R., Tahvanainen, E., Jauhiainen, M., Frick, M. H. and Ehnholm, C. (1996) The Gln-Arg191 polymorphism of the human paraoxonase gene (HUMPONA) is not associated with risk of coronary artery disease in Finns. J. Clin. Invest. **98**, 883–885
33 Herrmann, S.-M., Blanc, H., Poirier, O., Arveiler, D., Luc, G., Evans, A., Marques-Vidal, P., Bard, J.-M. and Cambien, F. (1996) The Gln/Arg polymorphism of human paraoxonase (PON 192) is not related to myocardial infarction in the ECTIM study. Atherosclerosis **126**, 299–304
34 Ko, Y. L., Ko, Y. S., Wang, S. M., Hsu, L. A., Chang, C. J., Chu, P. H., Cheng, N. J., Chen, W. J., Chiang, C. W. and Lee, Y. S. (1998) The Gln-Arg 191 polymorphism of the human paraoxonase gene is not associated with the risk of coronary artery disease among Chinese in Taiwan. Atherosclerosis **141**, 259–264

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