The molecular basis for genetic polymorphism of human deoxyribonuclease II (DNase II): a single nucleotide substitution in the promoter region of human DNase II changes the promoter activity

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Abstract Deoxyribonuclease II (DNase II) levels in human vary depending on whether the individual has the *DNASE2*H* (high) allele or the *DNASE2*L* (low) allele. We examined the promoter activity of the 5′-flanking region of each of these alleles by transient transfection luciferase assay. *DNASE2*H* had 5-fold higher promoter activity than *DNASE2*L* in human hepatoma HepG2 cell. Comparison of the nucleotide sequences of the proximal promoter regions revealed a G to A transition at position −75; G and A residues were assigned to *DNASE2*H* and **L*, respectively. Since no differences were found between the open reading frame sequences of these alleles, it is likely that the A−75G transition causes the allelic difference in the promoter activity of the gene, underlying the genetic polymorphism.

Key words: Deoxyribonuclease II; Genetic polymorphism; Luciferase assay; Promoter; Transfection; Human

1. Introduction

Deoxyribonuclease II (DNase II, EC 3.1.22.1) is composed of three non-identical subunits, which are converted from a single-chain precursor by proteolytic processing [1,2]. Recently, it has been suggested that DNase II may be responsible for the internucleosomal DNA degradation that is characteristic of apoptosis [3]. We have developed a sensitive and specific method for measuring DNase II activity in biological samples [4,5]. Using this assay, we showed that DNase II activity in human urine and leukocyte lysates was bimodal in a Japanese population [6]. We then identified two alleles that correlate with DNase II activity, the dominant *DNASE2*H* (high) and the recessive *DNASE2*L* (low) alleles, which are situated at a single locus assigned to 19p13.2-p13.1 [7]. The allele frequencies were determined to be 0.632 and 0.368, respectively [6]. Recently, we cloned both complementary [8] and genomic DNAs [9] encoding human DNase II. The entire translated nucleotide sequence of *DNASE2*H* was identical with that of *DNASE2*L*, which excluded the possibility that the genetic polymorphism is attributable to alterations in the primary structure of the enzyme. However, other groups have reported that mutations or polymorphisms in the promoter region affect the promoter activity of some genes [10–12], so we suspected that a polymorphic site(s) in the promoter region of the DNase II gene was the primary cause of the observed variation in its transcriptional activity, leading to variation in the level of DNase II activity.

In this context, we examined the promoter activity of the 5′-flanking region of the genomic DNAs derived from *DNASE2*H* and *DNASE2*L*, by transient transfection luciferase assay. We identified a polymorphic site that is responsible for allelic variation in the promoter activity.

2. Materials and methods

2.1. Biological samples and cells

Genomic DNA was isolated separately from peripheral blood leukocytes collected from individuals who were homozygous for *DNASE2*H* or *DNASE2*L* typed by family studies [6], using a QIAamp Blood kit (Qiagen). The human hepatoma, HepG2 (RCB0459), and thyroid gland cancer, TCO-1 (JCRB0239) cell lines were obtained from the RIKEN Cell Bank and the Health Science Research Resources Bank, Japan, respectively. The cells were maintained in Dulbecco's modified Eagle's medium, containing 1 mM L-glutamate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) fetal calf serum (Life Technologies).

2.2. Amplification of genomic DNA

Based on the previously obtained sequence of the 5′-upstream region [9], we obtained fragments of the 5′-flanking region of the human DNase II gene, by nested PCR amplification using the GenomeWalker system (Clontech) according to the manufacturer's instructions. The nucleotides were numbered relative to the transcription start site (+1). DNA fragments of 577 bp, spanning from −505 to +72, were amplified from each genomic DNA sample, using a set of primers that correspond to the sequences from −505 to −487 and from +72 to +51 as described previously [13]: 5′-CGTGGTGTCGGGCGCCTG-3′ (sense) and 5′-AGCTGCTATGGGGCTGAGATCC-3′ (antisense). The fragments amplified were subcloned directly into a TA cloning pCR II vector (Invitrogen), and sequenced. Nucleotide sequences were determined by the dideoxy chain-termination method using a Dye Terminator Cycle Sequencing FS kit (Applied Biosystems). The sequencing run was performed on an Applied Biosystems Genetic Analyzer 310, and all of the DNA sequences were confirmed by reading both DNA strands.

2.3. Preparation of reporter constructs

The pGL3-Basic vector (Promega) was used to make various reporter constructs containing fragments of the 5′-flanking region of the human DNase II gene driving a firefly luciferase gene. A DNA fragment of 2301 bp (−2229 to +72) was isolated from each genomic DNA sample and then amplified using the Expanded™ High Fidelity PCR system (Boehringer-Mannheim) and a set of primers corresponding to the sequences from −2229 to −2205 and from +72 to +51: 5′-CCCACGCGTAGTGCAGTTGTTCCATCTTGGCTC-3′ (sense) and 5′-CCCCTCGAGAGCTGCTATGGGGCTGAGATCC-3′ (antisense), respectively. After double digestion with *Mlu*I and *Xho*I, the

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Abbreviations: DNase II, deoxyribonuclease II

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amplified fragment was ligated into the *Mlu*I-*Xho*I sites of the pGL3-Basic vector. The reporter constructs derived from *DNASE2*H* and *DNASE2*L* were named P1-H and P1-L, respectively. Each of the truncated fragments from −1243, −849, −505, −324, −271, −222, −177, −151, −136, −122 and −70 to +72 were obtained by PCR using P1-H and P1-L, or genomic DNA of each as a template, and then subcloned into the pGL3-Basic vector. These 11 products were designated P2 through P12, respectively. Four internal deletion mutants derived from P5, P5-Δ(−103/+72), P5-Δ(−103/−12), P5-Δ(−103/−69) and P5-Δ(−144/−137), were constructed using P5-H and P5-L constructs as templates, by the splicing by an overlap extension method [14]. Each of the reporter constructs was purified for subsequent transfection by using the Plasmid Midi Kit (Qiagen).

2.4. Transient transfection and luciferase assay

Transient transfections were performed by the lipofection method using Lipofectamine Plus™ reagent (Life Technologies) according to a previously described method [15]. Each of the cell lines was cotransfected with 1.0 μg of the reporter construct and 20 ng of the pRL-TK vector (Promega). Two days after transfection, the cells were harvested and lysed, and the luciferase assay in the lysate was measured. Both the firefly and *Renilla* luciferase activities in the lysate were measured by using a luminometer (Lumat LB9506, EG and G Berthold) with a Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions. The vectors pGL3-Basic and pGL3-Control (Promega) were used as promoter-less and positive controls, respectively. All transfections were performed in triplicate, with at least two different plasmid preparations.

3. Results

3.1. Transient expression of chimeric DNase II reporter gene constructs

We determined the nucleotide sequence of the 5′-flanking region of the human DNase II gene up to nucleotide position −2379 (DDBJ/EMBL/GenBank accession number AB031422). Next we prepared 12 reporter constructs containing progressive deletions of the 2301 bp fragment amplified from the 5′-flanking region of *DNASE2*H* (−2229 to +72) with the firefly luciferase gene, and studied them in transient transfection assays. Since the liver and thyroid gland show the highest levels of DNase II activity [8], we introduced the reporter constructs into hepatoma (HepG2) and thyroid gland cancer (TCO-1) cell lines, which constitutively express the DNase II gene. In both cells the P1-H construct, containing a 2301-bp promoter fragment, directed effective expression of the luciferase gene (Figs. 1 and 2). Stepwise deletions of this sequence from −2229 to −151 did not significantly affect the expression of the reporter gene. However, a drastic decrease of luciferase activity occurred when the −151 fragment was reduced to −136. Constructs P10-H to P12-H, in which further downstream sequences were deleted, from −151 to −137, −123 and −71, respectively exhibited promoter activity that was several fold higher than that of the promoter-less expression vector pGL3-Basic. These results suggest that the region from −2229 to −152 is not required for basal promoter activity, and that the regions from −151 to −137 and from −70

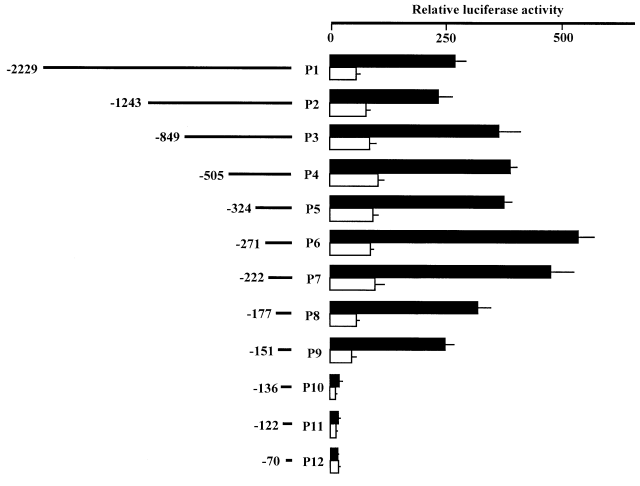

Fig. 1. Expression of luciferase activities in HepG2 cells after transient transfection. Various lengths of the 5′-flanking region of the DNase II gene derived from *DNASE2*H* (solid bars) and *DNASE2*L* (open bars) alleles were inserted upstream from the luciferase gene in a pGL3-Basic vector. The nucleotide position numbers are counted from the transcription start site (+1). The constructs (P1 to P12), pGL3-Basic and pGL3-Control were cotransfected with the pRL-TK vector into HepG2 cells by the lipofection method. The promoter activity of each construct is expressed as the luciferase activity of the construct relative to the *Renilla* luciferase activity derived from pRL-TK. Values are the mean ± S.D. The positive and promoter-less control vectors displayed relative luciferase activities of 1890 ± 140 and 6.0 ± 0.20 in HepG2 cells, respectively.

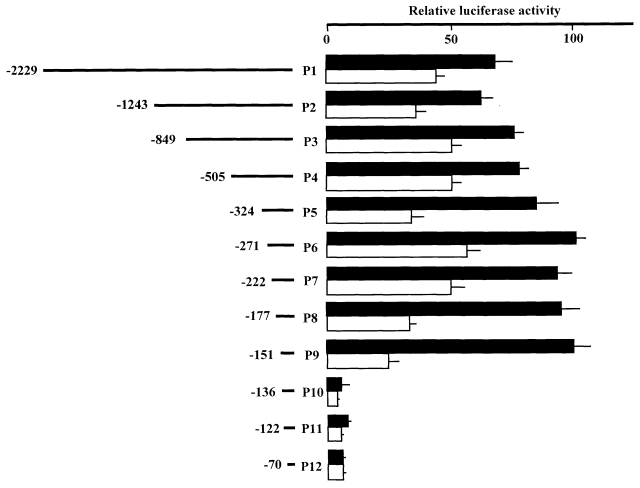

Fig. 2. Expression of luciferase activity in TCO-1 cells after transient transfection. The details are described in the legend to Fig. 1. The positive and promoter-less vectors displayed relative luciferase activities of 317 ± 24.0 and 1.0 ± 0.05 in TCO-1 cells, respectively.

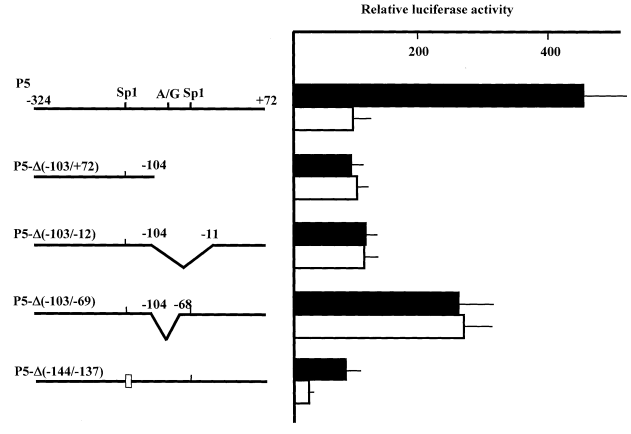

Fig. 3. Luciferase activities of the intra-promoter deletion mutants of the DNase II gene. The deletion mutants of P5-H (solid bars) and P5-L (open bars) were constructed by PCR-directed mutagenesis and were introduced into HepG2 cells. The promoter activity of each construct is expressed as the luciferase activity relative to the *Renilla* luciferase activity of the pRL-TK.

Fig. 4. Nucleotide sequence of the 5′-flanking region of the *DNASE2*L* allele. The nucleotides are numbered relative to the transcription start site (+1). The A/G transition site at position −75, which is responsible for the allelic difference in promoter activity, is shown in boldface. Another variation characterized by the length of the A-trail is indicated with a dotted underline, and was identified as $(A)_{30}$. In *DNASE2*H* this A-trail is replaced by $(A)_{12}$, as indicated over the corresponding position. Potential consensus sequences for Sp1 binding sites are underscored with wavy lines. The cap signal and the first in-frame translation initiation codon are underlined.

to +72 together comprise a minimal promoter. Since HepG2 and TCO-1 cells provided similar results in the promoter activity experiments for each of 12 constructs, the same *cis*- and *trans*-acting factors are responsible for the basal promoter activity of the gene in these cells. Next, to survey the positive regulatory elements within this region (−151 to −137 and −70 to +72), the effect of deletions within these promoter sequences was examined. A construct without the sequence from −144 to −137, which corresponds to a putative Sp1 binding site, P5-H-Δ(−144/−137) exhibited only 17% of the luciferase activity of the intact P5-H construct (Fig. 3). This indicated that the binding of a nuclear factor to this *cis*-element may be an essential event for basal promoter activity. On the other hand, deletions of −103 to +72 and −103 to −12 each reduced the promoter activity to about 20% compared to that of intact P5-H, whereas deletion of −103 to −69 elevated the luciferase activity by about 3-fold compared to the other deletion constructs (Fig. 3).

3.2. Demonstration of allelic differences in DNase II gene expression

When expressed in HepG2 and TCO-1 cells, the P1-L construct derived from *DNASE2*L* exhibited 20% and 60%, respectively, of the luciferase activity of the P1-H construct. Furthermore, although stepwise deletion from −2229 affected the luciferase gene expression of all the constructs from these alleles in the same manner, the constructs (P2-L to P11-L) derived from *DNASE2*L* had consistently lower promoter activity compared to the corresponding constructs (P2-H to P11-H). These results indicate that the DNase II gene exhibits allelic differences in its promoter activity. Indeed, individuals with the high-activity allele, *DNASE2*H*, have higher levels of DNase II activity in their urine, leukocytes and semen than do individuals who are homozygous for the low-activity allele, *DNASE2*L* [4,6]. We previously reported that the observed difference in DNase II activity in biological samples is not due to variation in the primary structure of the DNase II protein, because there is no variation in the coding sequence [9]. Therefore, the difference in the promoter activities of *DNASE2*H* and **L*, which results in the production of high and low amounts of the gene transcript, respectively, is likely to be the cause of the genetically determined activity levels of DNase II.

3.3. Identification of the nucleotide substitution that generates the allelic difference in DNase II promoter activity

Comparison of the nucleotide sequences of all constructs P4-H and P4-L through P12-H and P-12L, respectively, identified variations at two positions: different length of A-trails at position −336 and an A/G transition at −75 (Fig. 4). P4-H, derived from *DNASE2*H*, carried $(A)_{12}$ at −336 and a G residue at −75, whereas the P4-L, derived from *DNASE2*L*, had $(A)_{30}$ and an A residue. However, even constructs P5 through P11, in which the A-trail around −336 was deleted, exhibited allelic differences in promoter activity (Figs. 1 and 2), indicating that variation in the length of the A-trail at this position does not regulate DNase II gene transcription. On the other hand, all of the constructs containing G residue at −75 had higher luciferase activities than their A counterparts. Next, to ascertain whether the A/G transition at −75 influences DNase II promoter activity, three internal deletion mutants of the P5-H and P5-L constructs, P5-Δ(−103/+72), P5-Δ(−103/−12) and P5-Δ(−103/−69), all of which lack the region carrying the nucleotide substitution site were studied in transition transfection assays (Fig. 3). Although the original constructs P5-H and P5-L showed different promoter activities, as expected, all the internal deletion mutants produced similar levels of activity irrespective of their origin. Therefore, deletion of the nucleotide substitution site resulted in loss of allelic specificity of promoter activity. On the basis of these data, we concluded that the A/G transition at position −75 alters the promoter activity, and that the presence of a G residue at this position brings about greater promoter activity than that does an A residue.

To confirm that there is a single nucleotide substitution at −75, DNA fragments of 577 bp, containing this site, that had been directly amplified from *DNASE2*H* and **L* were subcloned separately. Eight independent subclones of each allele were isolated and sequenced; all of the clones derived from *DNASE2*H* had a G residue, whereas an A residue was identified in the clones from *DNASE2*L*.

4. Discussion

Our data suggest that a substitution of A for G at position −75 of the DNase II gene promoter may increase DNase II gene expression, leading to higher levels of DNase II activity underlying its genetic polymorphism. It is well documented that a single nucleotide substitution, deletion or insertion in a gene promoter can drastically affect transcriptional activity that is mediated by transcription factors [10,16]. In particular, a single nucleotide substitution in the middle of a *cis*-acting element can interfere with the efficient binding of the corresponding nuclear factors, resulting in reduced promoter activity; examples are the genes encoding low density lipoprotein receptor [17], protein C [18], and aldehyde dehydrogenase [19]. However, the sequences around the substitution site in the DNase II gene exhibit no similarity to any of a large variety of transcription factor consensus sequences [20]. Changes to the spacing and sequences between adjacent transcription factor binding sites can also affect promoter activity [10,21]. Indeed, the substitution site at −75 is located between two regions responsible for the basal promoter activity of the DNase II gene.

The 5′-flanking region of the DNase II gene exhibits several features that are characteristic of housekeeping genes [9]. In particular, although there are no canonical TATA or CCAAT box consensus sequences in this area, three potential binding sites for transcription factor Sp1 [22] are present at positions −45, −144 and −499. It is relatively common for at least one Sp1 binding site to be located within the promoter of a housekeeping gene [23] and multiple Sp1 binding sites arranged in tandem and close to each other in a promoter region may interact synergistically in vivo to activate transcription [17]. The results of our intra-promoter deletions suggest that both of *cis*-acting elements present in the regions from −151 to −137 and from −68 to −12 contribute to basal promoter activity of the DNase II gene. In fact, two Sp1 binding sites are situated at positions −45 and −144, within the regions of interest. Identification of the transcriptional factors involved in the transcriptional regulation of the human DNase II gene is currently underway in our laboratory.

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