FEBS Letters 356 (1994) 33–38

FEBS 14842

Characterization of the promoters of the guinea pig neutrophil cationic peptide-1 and -2 genes

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Received 17 October 1994

Abstract Guinea pig neutrophils contain the antimicrobial cationic peptides GNCP-1 and GNCP-2 in the granules. To understand the regulation of the gene expression, the promoters for the GNCP-1 and GNCP-2 genes were characterized. Sequencing analysis of the genomic clones showed that the nucleotide sequences of the 5'-upstream regions (1.7 kb) from exon 1 were homologous (90–93%) between the GNCP-1 and GNCP-2 genes. However, transient transfection assays using luciferase reporter gene constructs revealed that the promoter activity of GNCP-1 was 2-fold greater than that of GNCP-2. Furthermore, DNase I footprint analysis demonstrated that three regions (I, II and III) were protected on the GNCP-1 promoter, whereas only two protected regions (II and III) were identified on the GNCP-2 promoter. Together these observations indicate that GNCP-1 and GNCP-2 are encoded by homologous genes, but the expression of the GNCP-1 and GNCP-2 genes is likely to be different at the level of transcription.

Key words: Cationic peptide; Defensin; Gene expression; Promoter; Neutrophil; Guinea pig

1. Introduction

Neutrophils play a central role in protecting hosts against microbial infections. The antimicrobial systems of neutrophils can be divided into two categories [1–3]. One is the oxygen-dependent mechanism in which H_2O_2, O_2^- and HOCl are involved [1]. The other is the oxygen-independent mechanism in which granular antimicrobial proteins and peptides are involved [2,3]. The most abundant of these granular antimicrobial components is the low molecular-weight cationic peptide defensin, which shows potent microbicidal activities against bacteria, fungi and viruses [4].

In the previous study [5], we have purified two structurally homologous cationic peptides, GNCP-1 and GNCP-2, from guinea pig neutrophils, and found that both peptides are single-chain polypeptides comprising 31 amino acid residues, which differ by only one amino acid substitution at position 21 (isoleucine vs. leucine). Furthermore, we have isolated three kinds of cDNA clones (GNCP-1A, GNCP-1B and GNCP-2) encoding GNCP-1 and GNCP-2 [6,7]. The nucleotide sequences of these cDNAs are highly homologous (99%), and the deduced amino acid sequences have shown that the precursor proteins encoded by GNCP-1A and GNCP-1B cDNAs differ by only one amino acid substitution in the propeptide region, whereas the precursor proteins encoded by GNCP-1B and GNCP-2 cDNAs differ by only one amino acid substitution in the mature peptide region. Recently, we have isolated four different genomic clones, encoding GNCP-1A (clone 5), GNCP-1B (clone 10), and GNCP-2 cDNAs (clones 16 and 22) [8,9]. The nucleotide sequences of these genomic clones are highly homologous (97%) in the region sequenced, and Southern blot analysis of the guinea pig genomic DNA have revealed that clones 5 and 10, and clones 16 and 22 represent the alleles of the GNCP-1 and GNCP-2 genes, respectively [9]. Thus, GNCP-1 and GNCP-2 are encoded by homologous but different genes.

It has been shown that the GNCP genes are transcribed by the neutrophil precursor cells in the bone marrow but not by the mature neutrophils, indicating that the GNCP genes are expressed during a limited period of neutrophil maturation. However, the mechanism(s) controlling expression of the GNCP gene remains unknown. In this study, therefore, to understand the transcriptional regulation of the GNCP genes, we have characterized the promoters for the GNCP-1 and GNCP-2 genes using the genomic clones previously isolated.

2. Materials and methods

2.1. DNA sequencing

The 5'-flanking regions of the GNCP genes were sequenced using four different genomic clones encoding GNCP-1A (clone 5), GNCP-1B (clone 10), and GNCP-2 cDNAs (clones 16 and 22) which had been isolated from a guinea pig genomic library [9]. Selected restriction fragments of the genomic DNA inserts were subcloned into the plasmid vector pBluescript SK(−) (Stratagene, CA), and the nucleotide sequences of the DNA fragments were determined from both directions with sequence-specific oligonucleotide primers by the dideoxy chain-termination procedure [10], using the Taq Dye Deoxy Terminator Cycle Sequencing Kit and the 373A DNA Sequencer (Applied Biosystems, Inc., CA).

2.2. Plasmid construction

The 1.7-kb fragments and a series of deletion mutants of the 5'-flanking regions of the GNCP genes were synthesized by polymerase chain reaction (PCR) using the sequence-specific sense and antisense primers containing an *Mlu*I site and a *Xho*I site, respectively, at the 5' end. The PCR products were digested with *Mlu*I and *Xho*I, and subcloned into the *Mlu*I/*Xho*I sites upstream from the luciferase gene in the promoterless and enhancerless PGV-B vector (PicaGene Basic Vector; Toyo Ink MFG Ltd., Tokyo). The sequences of the PCR fragments were confirmed by dideoxy sequencing. The pSV-β-galactosidase vector containing an SV40 promoter and enhancer (Promega

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The nucleotide sequences reported in this paper have been submitted to the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under Accession Numbers D37971, D37972, D37973 and D37974.

Abbreviations: GNCP, guinea pig neutrophil cationic peptide; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; kb, kilobase; bp, base pair.

SSDI 0014-5793(94)01229-6

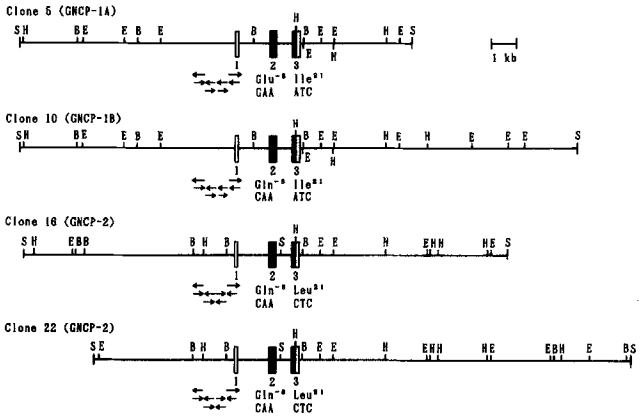


Fig. 1. Schematic structures of the GNCP genomic clones. The 15–20 kb *Sal*I DNA inserts of the GNCP genomic clones 5, 10, 16 and 22 were isolated from a size-fractionated genomic library in the vector EMBL3 [9]. Exons are shown by the numbered boxes. Filled areas indicate the coding regions, and the open areas depict the 5′- or 3′-untranslated regions of the GNCP cDNAs. Clone 5 encodes GNCP-1A cDNA (GAA, glutamic acid^{-8} and ATC, isoleucine^{21}), clone 10 encodes GNCP-1B cDNA (CAA, glutamine^{-8} and ATC, isoleucine^{21}), and clones 16 and 22 encode GNCP-2 cDNA (CAA, glutamine^{-8} and CTC, leucine^{21}). These clones encode the precursor proteins comprising 93 amino acid residues, and their amino acid sequences are the same except for glutamic acid^{-8}/glutamine^{-8} in the propeptide and isoleucine^{21}/leucine^{21} in the mature peptide [7,9]. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I. *Sal*I sites at both ends are derived from the vector. Horizontal arrows indicate the direction and length of the nucleotide sequence determined from each primer.

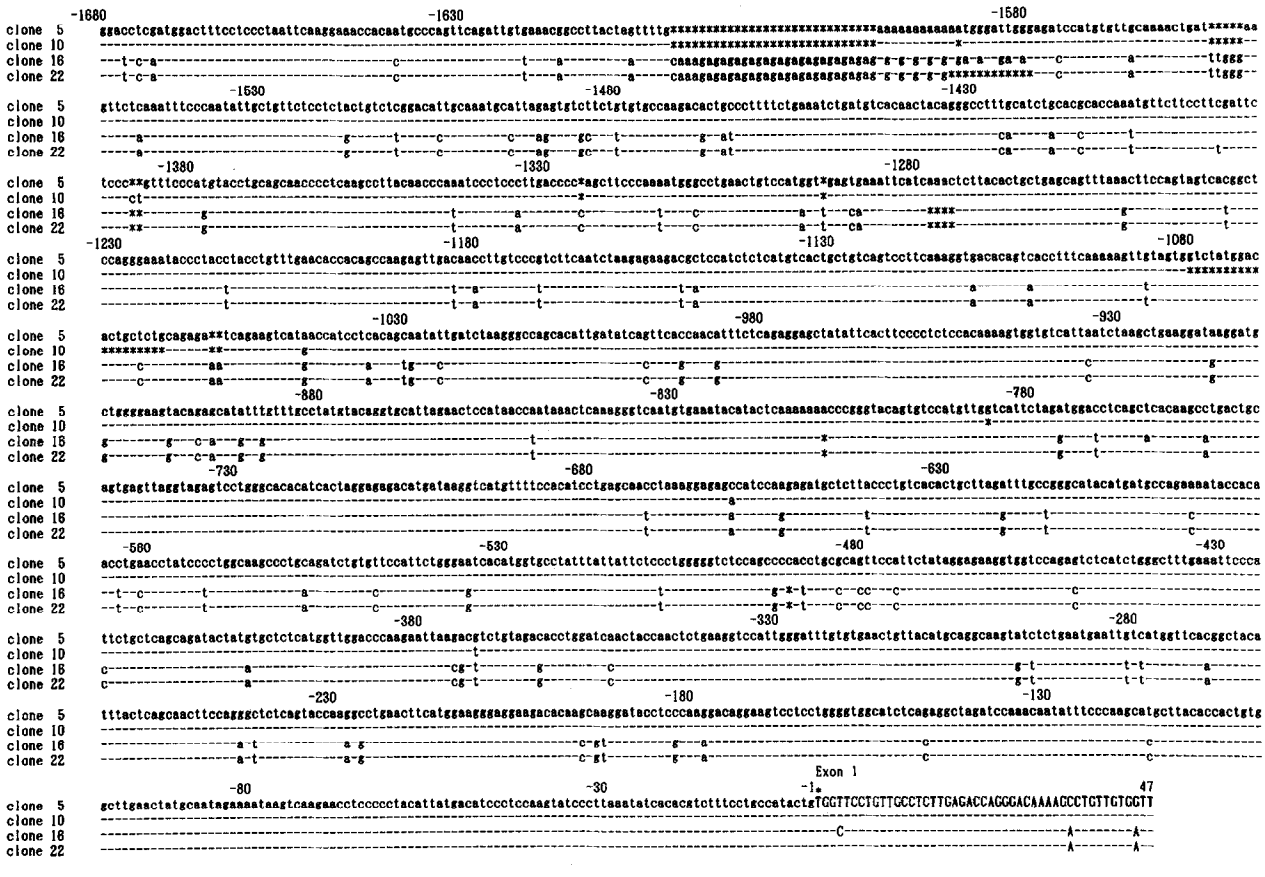
Corp., WI) was used as an internal standard for transfection efficiency, and the PGV-P luciferase vector (PicaGene Promoter Vector; Toyo Ink) containing an SV40 promoter was used as a positive control for each transfection.

2.3. Cell culture, transient transfection and luciferase assays

The cell line 104C1 (female, fetal guinea pig, transformed; ATCC CRL1405) was obtained from the Japanese Cancer Research Resources Bank (JCRB, Tokyo), and maintained in RPMI 1640 (Nissui Seiyaku Ltd., Tokyo) supplemented with 10% fetal calf serum. Nuclear run-off transcription assay [11] has revealed that 104C1 cells are constitutively transcribing the GNCP genes (data not shown). The 104C1 cells (60–80% confluent) in 60-mm tissue culture dishes were transfected with 5J μg of an appropriate luciferase construct and 4 μg of pSV-β-galactosi-J dase vector using 30 μg of the cationic lipid *N*-[1-(2,3-dioleoyloxy) propyl]-*N*,*N*,*N*-trimethyl-ammoniummethylsulfate (DOTAP; Boehringer Mannheim GmbH, Germany)[12] for 6 h in 6 ml of Dulbecco's modified Eagle's medium (DMEM; Nissui Seiyaku) without serum. After transfection, the cells were cultured in DMEM supplemented with 10% fetal calf serum for an additional 48 h, and then lysed in 25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol and 1% Triton X-100. Cell lysates were assayed for the luciferase and β-galactosidase activities using the PiCaGene Luciferase Assay System (Toyo Ink) [13] and the Galacto-Light system (Tropix Inc., MA) [13,14], respectively, in an LS 3801 liquid scintillation counter (Beckman Instruments, CA) equipped with the single photon monitor. Luciferase activities in the lysates were normalized to the β-galactosidase activities, and expressed as percentages of those transfected with PGV-P vector.

2.4. Nuclear extract preparation

Nuclear extracts were prepared from 104C1 cells and guinea pig neutrophils (>95% pure) [11], according to the method of Schreiber et al. [15], and dialyzed against 20 mM HEPES, pH 7.9, 10% glycerol, 100 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF and



1 μM pepstatin A. Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce Chemical Co., IL) and bovine serum albumin standards.

2.5. DNase I footprint analysis

The DNA fragments (bp −358 to −80 and bp −223 to +47 of the GNCP-1 and GNCP-2 genes) were synthesized by PCR using sequence-specific sense and antisense primers containing an *MluI* site at one end, and a *XhoI* site at the other, and used to make the probes. To label the coding strand, the DNA fragments were digested with *XhoI*, and end-labeled by filling the *XhoI* site with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP (>3,000Ci/mmol; ICN Biomedicals Inc., CA). The labeled DNA was further digested with *MluI*. For the non-coding strand, the DNA fragments were digested with *MluI*, end-labeled, and digested with *XhoI*. The probes were purified using Quick Spin columns (Boehringer Mannheim Corp., IN). Labeled DNA (approximately 5 ng, 5 × 10^7 cpm/μg) was incubated with nuclear extracts (50–80 μg) on ice for 60 min in a 50 μl binding mixture containing 20 mM HEPES, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 4 μg of double-stranded poly(dI-dC) (Pharmacia P-L Biochemicals Inc., WI), 0.5 mM EDTA, 1 mM dithiothreitol and 0.5 mM PMSF. The DNA was then digested with DNase I (8–300 ng; Takara Shuzo Ltd., Shiga) at room temperature for 90 s, and the reaction was terminated by the addition of 100 μl of stop solution (20 mM Tris-HCl, pH 7.5, 20 mM EDTA, 0.2% sodium dodecyl sulfate, 40 μg/ml sheared salmon sperm DNA and 200 μg/ml proteinase K) and subsequent incubation at 37°C for 30 min. DNA was purified by phenol/chloroform extraction and ethanol precipitation, and resolved on an 8% polyacrylamide, 8 M urea sequencing gel. Chemical sequencing reaction cleaving at purine residues (A+G) was performed on each probe to make the sequence markers [16].

3. Results and discussion

As shown in Fig. 1, four different genomic clones encoding GNCP-1 and GNCP-2 have been isolated from a guinea pig genomic library. Clone 5, encoding GNCP-1A, clone 10, encoding GNCP-1B, and clones 16 and 22, encoding GNCP-2, have been shown to represent the alleles of the GNCP-1 and GNCP-2 genes, respectively [9]. To characterize the promoters of the GNCP genes, the regions upstream from exon 1 were sequenced up to 1.7 kb (Fig. 2). The nucleotide sequences of clone 5 and 10, and clones 16 and 22 were highly homologous (>99%) in the region sequenced, and the homology between clones 5/10 and clones 16/22 was 90–93%. Next, to evaluate the promoter activity, the 1.7-kb fragments of the 5′-flanking regions of the GNCP-1 and GNCP-2 genes were fused to the luciferase gene and used in the transient transfection. All the GNCP-luciferase constructs containing the 1.7-kb upstream fragments of the GNCP-1 and GNCP-2 genes reproducibly expressed >20-fold more luciferase activities than the promoterless and enhancerless PGV-B vector (Fig. 3). Interestingly, the luciferase constructs containing the 1.7-kb upstream sequences of the GNCP-1 gene (clones 5 and 10) consistently showed 2-fold greater promoter activities than the constructs containing the 1.7-kb upstream sequences of the GNCP-2 gene (clones 16 and 22). To further localize the regulatory element(s) in the GNCP promoters, a series of 5′-deleted promoter fragments were fused to the luciferase gene and used in the transient transfection. No significant alterations in the promoter activities were observed when

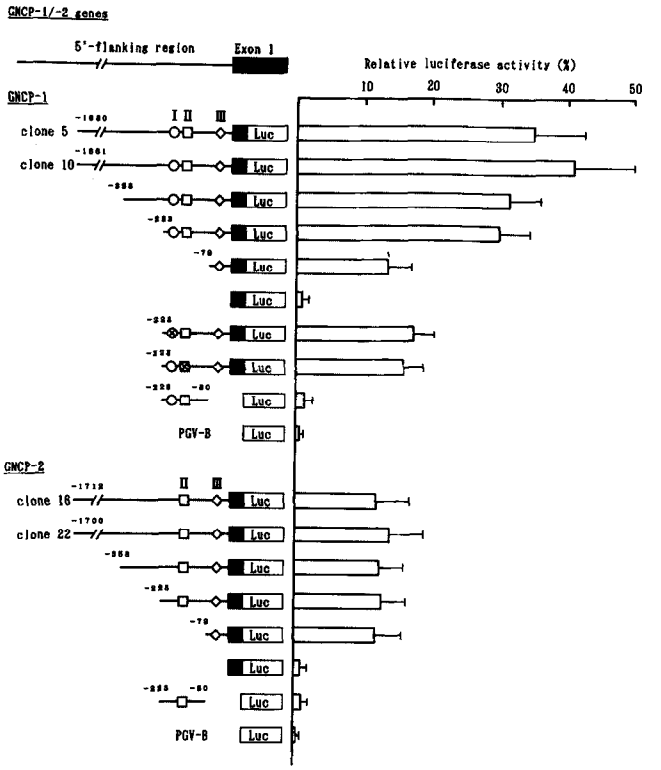


Fig. 3. Promoter activities of the 5′-flanking regions of the GNCP-1 and GNCP-2 genes. The 1.7-kb fragments (−1.7 kb to +47 bp) and a series of the deletion mutants of the GNCP-1 and GNCP-2 genes were inserted upstream of the luciferase gene of the promoterless and enhancerless PGV-B vector (Luc). The stippled boxes upstream of the luciferase gene represent sequences of exon 1 (+1 to +47 bp, where +1 denotes the previously identified transcription start site [9]). The nucleotide sequences of clones 5/10 (GNCP-1 gene) and clones 16/22 (GNCP-2 gene) were the same in the regions from −358 to −1 bp, respectively. Therefore, the deletion mutants common to each gene were synthesized and used in the transfection assay. PGV-P luciferase vector containing an SV40 promoter was used as a positive control. An appropriate luciferase construct was co-transfected with pSV-β-galactosidase vector, an internal standard for transfection efficiency, into the guinea pig 104C1 cells using DOTAP. After 48 h of incubation, the cell extracts were assayed for the luciferase and β-galactosidase activities. Following normalization for transfection efficiency, the luciferase activities were expressed as percentages of those with PGV-P vector. Data represent the mean ± S.D. of 3–7 separate experiments. Regions I, II and III correspond to the sequences identified by DNase I footprinting (Figs. 4 and 5). The mutations are indicated by an X; the sequences of region I (−213 to −191 bp) and region II (−180 to −164 bp) of the GNCP-1 promoter were replaced with the GNCP-2 sequence and the irrelevant sequence (ATGCACTGGATCGCATC), respectively, by PCR using the specific primers, and the DNA fragments containing the mutated sequences were fused to the PGV-B vector for the transfection assay. DNase I footprint analysis indicated that the mutated regions I and II of the GNCP-1 promoter were not protected from DNase I digestion by the 104C1 nuclear extracts (data not shown).

the sequences between −1.7 kb and −224 bp were deleted from the GNCP-1 promoter. However, the deletion of the region between −223 and −80 bp resulted in the reduction of the

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Fig. 2. The nucleotide sequences of the 5′-flanking regions of the GNCP-1 and GNCP-2 genes. The nucleotide sequences of clone 5 numbered from the putative transcription start site (marked with an asterisk) are shown. The transcription start site had been determined by primer extension analysis [9]. Nucleotides comprising exons are in upper-case letters, and those comprising the 5′-flanking regions are in lower-case letters. The sequences of clones 10, 16 and 22 are also shown for comparison, and the sequences identical with those of clone 5 are shown by dashes. Asterisks are inserted to maximize the homology.

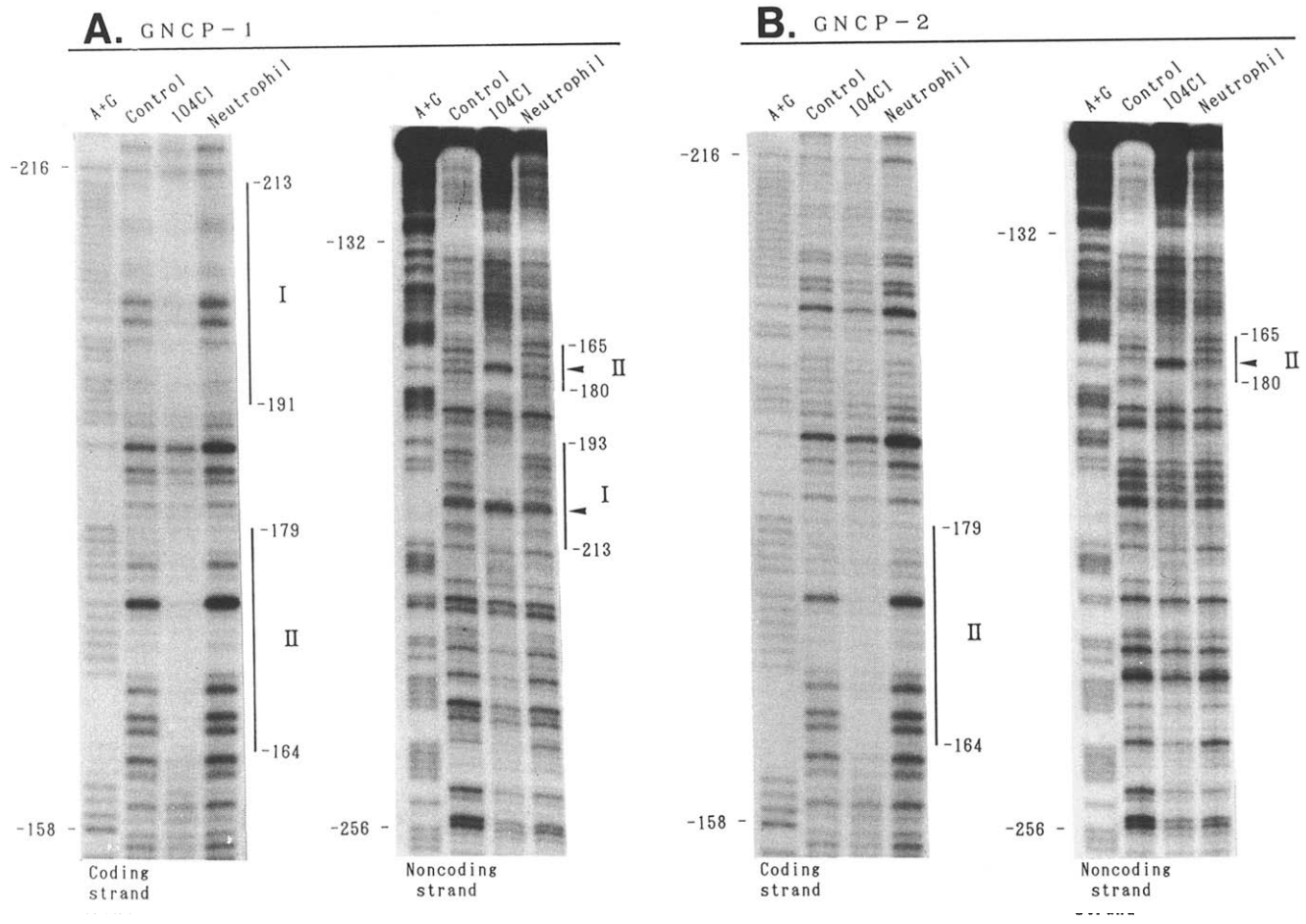

Fig. 4. DNase I footprint analysis of the GNCP-1 and GNCP-2 promoter regions (I). The coding and non-coding strands of the 279-bp fragments (−358 to −80 bp) of the GNCP-1 (A) and GNCP-2 (B) promoters were end-labeled, and incubated on ice for 60 min in the absence (control) or the presence of 104C1 (50 μg) or neutrophil (80 g) nuclear extracts. The DNA was digested with 8 ng (control), 200 ng (104C1) or 300 ng (neutrophil) of DNase I for 90 s, and the products were analyzed on an 8% sequencing gel. A+G is a marker produced by purine-specific Maxam–Gilbert sequencing reaction. The sequences protected by the 104C1 nuclear extracts are indicated with vertical lines. Arrowheads indicate DNase I hypersensitive sites.

promoter activity to the levels of the GNCP-2 promoters. Further deletion to −1 bp markedly decreased the promoter activity to the level of PGV-B. In contrast, the promoter activities of GNCP-2 were not changed by the deletion of the region between −1.7 kb and −80 bp. The deletion of the GNCP-2 promoter to −1 bp resulted in the almost complete loss of the luciferase activity, as observed with the GNCP-1 promoter. As seen in Fig. 2, the nucleotide sequences of the GNCP-1 and GNCP-2 genes are the same in the regions from −79 to −1 bp, whereas there are several variations in the nucleotide sequences in the regions from −223 to −80 bp. These observations indicate that the −79 to −1 bp regions likely contain the common sequences which are required for the promoter activities of both GNCP-1 and GNCP-2, while the −223 to −80 bp regions appear to contain the sequences which are involved in the difference in the promoter activities of GNCP-1 and GNCP-2.

To determine the promoter sequences which interact with any nuclear proteins, DNase I footprint analysis was performed using the upstream fragments of the GNCP genes (the 279-bp fragments from −358 to −80 bp and the 270-bp fragments from −223 to +47 bp) and nuclear protein extracts prepared from 104C1 cells and mature neutrophils. As shown in Figs. 4A and 5A, three protected regions (I–III) were identified on both the coding and non-coding strands of the GNCP-1 promoter using the 104C1 cell nuclear proteins. Region I spanned from −213 to −191 bp, region II spanned from −180 to −164 bp, and region III spanned from −61 to −33 bp. In contrast, only two protected regions, corresponding to the regions II and III, were identified on the coding and non-coding strands of the GNCP-2 promoter using the 104C1 nuclear extracts (Figs. 4B and 5B). Interestingly, no protected regions were observed on either the GNCP-1 or the GNCP-2 promoters using neutrophil nuclear extracts. The GNCP genes are transcribed in 104C1 cells but not in mature neutrophils [11]. Therefore, it is likely that nuclear proteins which interact with the GNCP promoters are not expressed in the mature neutrophils. The locations of the protected sequences are summarized in Fig. 6. There are several differences in the nucleotide sequences of the GNCP-1 and GNCP-2 promoters, and region I was not observed on the GNCP-2 promoter. On the other hand, regions II and III were identified on both the GNCP-1 and GNCP-2 promoters. Region I is purine-rich and appears to contain a sequence (GAGGAA) similar to the recognition site for the nuclear factor PU.1 which has an important role in regulating the myeloid-specific expression of the CD11b and lysozyme genes [17,18]. Region II contains the glucocorticoid regulating element-like sequence

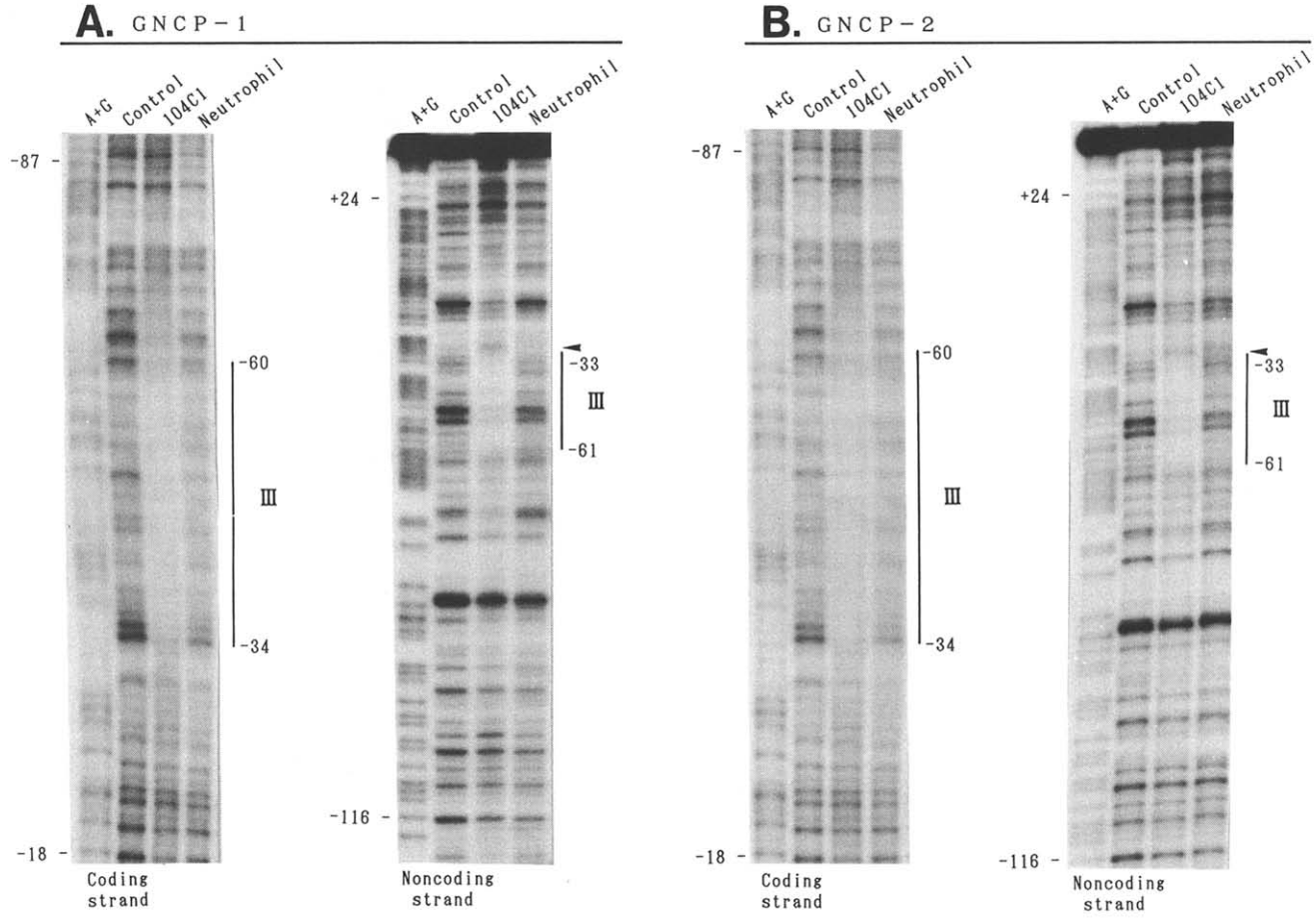

Fig. 5. DNase I footprint analysis of the GNCP-1 and GNCP-2 promoter regions (II). The coding and non-coding strands of the 270-bp fragments (−223 to +47 bp) of the GNCP-1 (A) and GNCP-2 (B) promoters were end-labeled, and further treated as described in the legend to Fig. 4.

(AGAACAN$_3$TGTTCT)[19], and region III seems to contain the pyrimidine-rich sequence which is assumed to be a regulatory element of the myeloperoxidase and neutrophil elastase genes [20].

As shown in Fig. 3, region I was present on the GNCP-1 promoter but not on the GNCP-2 promoter, and the deletion of the sequences (−223 to −80 bp) containing region I resulted in a decrease of the promoter activity. Furthermore, a mutation of region I which had been introduced by replacing the GNCP-1 sequences with the GNCP-2 sequences, reduced the promoter activity nearly to the level of the GNCP-2 promoter (Fig. 3). Thus, region I appears to be a positive regulatory element for the GNCP-1 promoter. Region II was present on both the GNCP-1 and GNCP-2 promoters. However, the deletion of region II did not affect the GNCP-2 promoter activity (Fig. 3), suggesting that region II is not important for the GNCP-2 promoter. In contrast, the mutation of region II resulted in a 2-fold decrease of the GNCP-1 promoter activity, suggesting that not only region I but also region II is involved in the difference in the promoter activities of GNCP-1 and GNCP-2. Region III was present on both the GNCP-1 and GNCP-2 promoters, and the deletion of region III brought about the almost complete loss of the promoter activities. Furthermore, the 5′-upstream sequences (−223 to −80 bp) of the GNCP-1 and GNCP-2 promoters containing regions I and II or region II did not show significant promoter activity by themselves (Fig. 3). Together these observations indicate that region III might be a common regulatory element which is involved in the basal activities of the GNCP-1 and GNCP-2 promoters.

Recently, we have found that neutrophils contain approximately 2-fold more GNCP-1 than GNCP-2 [5]. In this study, we have shown that the promoter activity of GNCP-1 is 2-fold

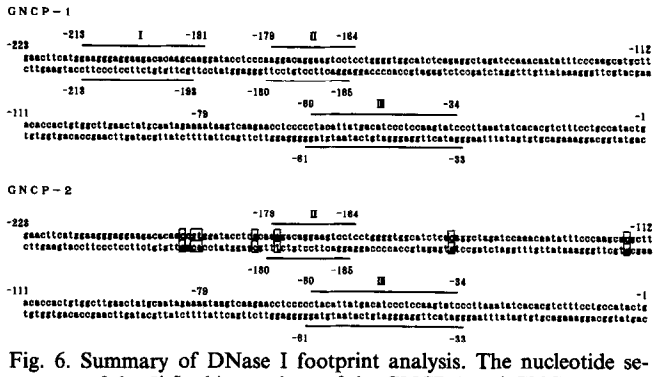

Fig. 6. Summary of DNase I footprint analysis. The nucleotide sequences of the 5′-flanking regions of the GNCP-1 and GNCP-2 genes are shown from −223 to −1 bp. Nucleotide sequences which are different from those of GNCP-1, are boxed. The regions protected from DNase I digestion by the 104C1 nuclear extracts are indicated by horizontal lines for the coding and non-coding DNA strands above and below the nucleotide sequences, respectively. The three regions of protein–DNA interactions are designated I–III.

greater than that of GNCP-2, although the nucleotide sequences of the two promoters are homologous. These observations suggest that the difference in the expression of the GNCP-1 and GNCP-2 genes is likely regulated at the level of transcription. The GNCP gene is transcribed in the neutrophil precursor cells in the bone marrow but not in the mature neutrophils [11]. Analysis of nuclear protein factors interacting with the GNCP promoters will elucidate the transcriptional regulation of the GNCP genes during differentiation and maturation of the bone marrow progenitor cells.

Acknowledgements: This work was supported in part by the Science Research Promotion Fund from the Japan Private School Promotion Foundation, and the grant from the Ministry of Education, Science and Culture, Japan.

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