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# Transcriptional regulation of $\beta$ -defensin-2 by lipopolysaccharide in cultured human cervical carcinoma (HeLa) cells

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Received 8 October 2004; received in revised form 12 January 2005; accepted 17 January 2005

First published online 23 February 2005

#### Abstract

Human β-defensin-2 (hBD-2) is an antimicrobial peptide with a broad spectrum of antimicrobial activity against bacteria, yeast and fungi. Here, we analyzed the transcriptional regulation of hBD-2 in cultured human cervical carcinoma (HeLa) cells with or without lipopolysaccharide (LPS). DNA from position -329 to -39 in the hBD-2 promoter region contained the consensus binding sites for transcription factors, one site for nuclear factor for IL-6 expression (NF-IL6) and two sites for nuclear factor-κB (NF-κB). Reporter gene assays for promoter activity revealed that the region had the highest level of responsiveness to LPS. Furthermore, mutations in both of the NF-κB binding sites caused a significant reduction of the responsiveness to LPS, whereas mutation in the NF-IL6 binding site resulted in an elevation of the basal promoter activity. Electrophoretic mobility shift assays demonstrated that LPS induced the binding of HeLa nuclear factors to 60-bp probe containing the two NF-κB binding sites, suggesting that the sites were essential for the binding. Our results suggest that the two NF-κB binding sites contribute to LPS-mediated hBD-2 transcription while the NF-IL6 binding site represses LPS-independent hBD-2 transcription in the HeLa cells.

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Keywords:  $\beta$ -defensin-2; Transcriptional regulation; Lipopolysaccharide; HeLa cells

#### 1. Introduction

Epithelia not only serve as a physical barrier against infections, but also secrete substances that inhibit or neutralize invading microbes or their toxins. Antimicrobial peptides have recently been discovered in tissue, and one important subgroup of the peptides is the defensins, which are classified as  $\alpha$ -,  $\theta$ -, and  $\beta$ -defensins [1]. The human  $\alpha$ -defensins are expressed in a few kinds of cells such as neutrophils, macrophages, and Paneth cells of the intestine [2], and  $\theta$ -defensins have only been identi-

fied in leukocytes of rhesus macaques to date [3]. By contrast, 28 humans β-defensin (hBD) genes have been discovered by genomics-based approaches [1]; and in view of protein or mRNA level, hBD-1, -2, and -3 have been mainly detected in many tissues including secretory glands and epithelial cells [4], oral mucosa and salivary glands [5,6], and skin [7].

These hBD-2s vary in their distribution, induction, and antimicrobial properties. Among them, human β-defensin-2 (hBD-2) is induced in keratinocytes stimulated with Gram-negative or Gram-positive bacteria, *Candida albicans*, and is upregulated in inflamed epithelial tissues whereas it is poorly expressed in normal epidermal keratinocytes [8]. HBD-2 demonstrates in vitro

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antimicrobial activities against yeast and both Gramnegative and Gram-positive bacteria [9–11]. Therefore, the expression of hBD-2 might be tightly regulated in epithelial cells in response to microbial invasion.

HBD-2 mRNA expression was upregulated strongly in airway epithelial cells by lipopolysaccharide (LPS) derived from Escherichia coli [12]. In contrast to airway epithelial cells, hBD-2 mRNA was expressed weakly in human gingival epithelial cells exposed to LPS from E. coli, Fusobacterium nucleatum, and Porphynomonas gingivalis [8]. Weak expression was detected in human cervical carcinoma (HeLa) cells stimulated with E. coli LPS (our unpublished data). The findings suggest that the regulation of hBD-2 gene activation may differ in the types of epithelial cells. Recently, the hBD-2 promoter has been cloned [13], and found to contain several consensus transcription factor binding sites [14]. The hBD-2 promoter region between -324 and -180, which contains motifs that resemble nuclear factor-κB (NF-κB) and nuclear factor for IL-6 expression (NF-IL6) binding sites, has both basal and LPS-induced reporter gene activities in bovine tracheal epithelial cells (TECs) [12]. Moreover, the NF-IL6 binding site is necessary for the response to LPS in TECs [12]. However, the regulation of the hBD-2 gene in stratified epithelial cells may be different from that in simple epithelial cells such as TECs, because stratified epithelia of skin, gingiva, and cervix are continuously exposed with commensal bacteria, fungi, or viruses whereas simple epithelia of trachea and bronchus are sometimes invaded by pathogenic microbes.

In this study, we investigated the promoter region of hBD-2 in HeLa cells responsive to *E. coli* LPS. Furthermore, the specific binding of nuclear factors to hBD-2 promoter sequences in HeLa cells was examined by electrophoretic mobility shift assay (EMSA).

#### 2. Materials and methods

#### 2.1. Cloning of hBD-2 promoter

Human genomic DNA was amplified by nested polymerase chain reaction (PCR) using Genome Walker Kits (Clontech Laboratory Inc., Palo Alto, CA, USA). The gene-specific primers used were designed based on 5' coding region of hBD-2 cDNA (GenBank Accession No. AF040153); first primer, 5'-TCAGGAATATGAAGAGGAACGAGAAGAGA', and second primer, 5'-AAGAGGAACGAGAAGAGGAGATACAAG-3'. The PCR-based DNA fragment was cloned into the pCR-Blunt Vector (Invitrogen, Carlsbad, CA, USA), and the plasmid DNA was prepared using Plasmid Miniprep Kits (Qiagen, Hilden, Germany). A clone containing the longest insert was sequenced by the dideoxy sequencing procedure

[15] using the Automatic 377 sequencer (Perkin–Elmer, Foster City, CA, USA). The sequence was analyzed for the presence of consensus transcription factor binding sites using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCHJ.html) and SIGNAL SCAN search program (http://www-bimas.cit.nih.gov/molbio/signal/). The genomic DNA obtained was subcloned into a reporter plasmid ligated with secreted alkaline phosphatase (SEAP) (pSEAP2-Basic; Clontech), and it was used for experiments.

# 2.2. Construction of deletant of hBD-2 promoter-SEAP reporter

The deletants used were named according to the number of remaining hBD-2 promoter base pairs from 5' to the transcription start position. To obtain a series of deletants, the pSEAP2-Basic vectors containing the genomic DNA were digested using exonuclease III/Mung Bean Deletion Kit (Promega, Madison, WI, USA), and cloned again. The sequences of all constructs were confirmed by sequencing.

## 2.3. Cell culture, transfection, and reporter assay

HeLa cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 µg ml<sup>-1</sup> streptomycin, 60 µg ml<sup>-1</sup> kanamycin, and 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA). The cells at  $5 \times 10^6$  per well in 35-mm diameter plates (Corning, Corning, NY, USA) were co-transfected with 0.67 µg of hBD-2 promoter-SEAP reporter and 0.33 µg of pSV-β-galactosidase control vector (Promega) using Lipofect AMINE Plus (Invitrogen) according to the manufacturer's instructions. The plasmid DNA of each deletant was prepared using the EndFree Plasmid Maxi Kit (Qiagen). After transfection for 4 h, the cells were stimulated with 100 ng ml<sup>-1</sup> E. coli LPS (055:B5; Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The culture medium was collected and treated using Great EscAPe SEAP Chemiluminescence Detection Kit (Clontech), and then the SEAP activity was assayed quantitatively with a fluorometer (Millipore, Billerica, MA, USA). For monitoring transfection efficiency, the β-galactosidase (β-gal) activity was assayed using a luminometer (Wallac, Gaithersburg, MD, USA). The experiment was carried out three times for each construct, and the SEAP activity was normalized to the  $\beta$ -gal activity. The result of the reporter assay was indicated as a fold increase in the SEAP activity relative to that in the plasmid containing the minimum length of promoter region. A deletant exhibiting the strongest promoter activity was used for the mutation analysis and EMSA.

### 2.4. Site-directed mutagenesis and reporter assay

Point mutations were generated into the deletant for the hBD-2 promoter using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Briefly, two synthetic oligonucleotide primers, each complementary to opposite strands of the vector, were designed for the desired mutation. Incorporation of the primers into the deletant was performed by PCR using PfuTurbo DNA polymerase (Stratagene) according to the following conditions: denaturing at 95 °C for 30 s, and 18 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 1 min, and elongation at 68 °C for 8 min. After the temperature cycling, the product was treated with *Dpn*I, which digested the parental DNA template and selected for the synthesized DNA containing mutations. The plasmid DNA of each mutant was prepared using the EndFree Plasmid Maxi Kit (Qiagen). The reporter assay using the mutants was performed by the same method as mentioned above. The assay was performed three times for each construct, and results of the assays were indicated after the normalization of SEAP activity to β-gal activity.

### 2.5. EMSA

Double-stranded oligonucleotides were designed to cover the region of the hBD-2 promoter exhibiting the strongest SEAP activity. They were synthesized (Bex, Tokyo, Japan), and end-labeled with  $[\gamma^{-32}P]dATP$  (Amersham Bioscience, Tokyo, Japan) using MEGA-LABEL Kit (Takara, Otsu, Japan). The labeled DNA was separated from unincorporated  $[\gamma^{-32}P]dATP$  using QIAquick Nucleotide Removal Kit (Qiagen), eluted in DNase-free water, and kept at 4 °C until used for EMSA.

Nuclear extracts were prepared from HeLa cells as described previously [16] with minor modifications. Briefly,  $6 \times 10^6$  cells were stimulated with 100 ng ml<sup>-1</sup> E. coli LPS for different periods (1, 2, 4, and 6 h). The cells were washed twice with PBS at 4 °C, and recovered using a cell scraper (Becton Dickinson, San Jose, CA, USA). Nuclear protein was extracted from the cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (PIERCE, Rockford, IL, USA) according to the manufacturer's instructions. The amount of protein was measured using Protein Assay Kits (Bio-Rad Laboratories, Hercules, CA, USA) according to the method described previously [17]. Four micrograms of the extract was incubated at room temperature for 30 min in a reaction buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM  $MgCl_2$ , 4% glycerol, 0.05 mg ml<sup>-1</sup> poly(dI–dC), and 10 fmol labeled probe in a total volume of 11 µl. The DNA-protein complexes were mixed with 1 µl of 10 × loading buffer (Gel Shift Assay Systems, Promega), and then they were analyzed by electrophoresis on a 5% polyacrylamide gel using 0.5× Tris-borate-EDTA running buffer (45 mM Tris-HCl, 45 mM sodium borate, and 1 mM EDTA). The gel was dried under vacuum and visualized by autoradiography. For competition experiments, the nuclear extracts were preincubated on ice for 30 min with a 500-fold molar excess of unlabeled consensus oligonucleotides for NF-κB, NF-IL6, and AP-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

### 3. Results

# 3.1. Putative transcription factor binding sites in hBD-2 promoter

The 1.4-kbp DNA fragment isolated contained the 5'-upstream sequence of the hBD-2 cDNA. The nucleotide sequence matched completely with that of the hBD-2 genome (GenBank Accession No. AF040153). Computational analysis of the hBD-2 promoter sequence revealed the typical TATA-like box and potential DNA-binding sites specific to mammalian gene regulatory proteins (Fig. 1).

# 3.2. Deletion mutagenesis of hBD-2 promoter and SEAP assays

To determine which element of the hBD-2 promoter is important for LPS-induced transcription, a series of 5' truncated fragments of the 1.4-kbp hBD-2 promoter linked to pSEAP2-Basic plasmids were used for promoter assay. As shown in Fig. 2, the maximum basal promoter value was detected in the 1.4-kbp promoter (pro 1319), and the deletion between -1371 and -329(pro 329) decreased the value by 80%. The maximum LPS-induced value was detected in the 1.4-kbp promoter (pro 1319), and deletion from position -1019 to -329 (pro 1019, pro 704, and pro 329) resulted in a moderate decrease in the value; however, further deletion between -1371 and -39 (pro 39) decreased the value by 90%. The construct pro 329, in which similar consensus binding sites for AP-1, NF-IL6, and NF-κB were included (Fig. 1), exhibited the highest ratio of LPS-induced value to basal promoter value. Therefore, we investigated further the role of each binding site in the regulation of hBD-2 transcription by LPS.

The promoter activity of the hBD-2 mutant in the pSEAP2-Basic vector was assayed in the HeLa cells followed by incubation for 24 h with or without LPS. Mutation of the two NF-κB binding sites in tandem markedly reduced the LPS-induced value, whereas mutation at the NF-IL6 site resulted in an elevation of the basal promoter value (Fig. 3).

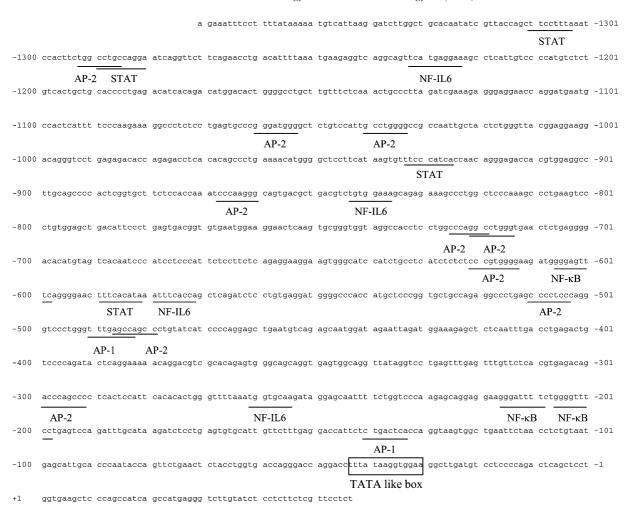


Fig. 1. Nucleotide sequence of 5'-flanking region of hBD-2 gene. Putative transcription factor binding sites (threshold score, >0.82) are underlined, and the TATA-like box is indicated.

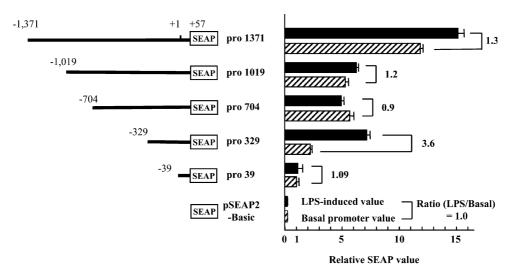


Fig. 2. Promoter activity of hBD-2. (Left) Schematic diagram of the five hBD-2 reporter constructs containing promoter fragments of different lengths cloned into the pSEAP2-Basic vector. The numbers in the names of the constructs indicate their respective lengths in nucleotides. (Right) The relative SEAP value is indicated as a fold increase in the SEAP activity for each construct relative to that without LPS for the pro 39 construct. LPS-induced value, the cells were incubated with LPS; basal promoter value, the cells were incubated without LPS. Error bars indicate SD of three independent assays.

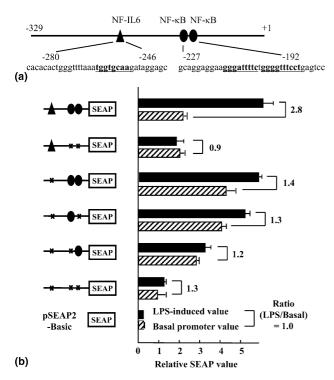


Fig. 3. Effect of mutations in NF-κB and NF-IL6 binding sites on hBD-2 promoter activity. (a) Schematic representation of the hBD-2 promoter including NF-κB and NF-IL6 binding sites. The transcription start site is numbered +1. Consensus sequences for NF-IL6 and NF-κB are indicated in bold and with underlines. (b) Promoter activity of hBD-2 mutants. (Left) Schematic diagram of the mutant. The wildtype or mutant construct was transfected into HeLa cells ( $5 \times 10^6$ ), and the cells were incubated for 24 h with or without LPS. The point mutations generated are as follows: 4xx, 5'-gcaggaggaagg-CattttctggCgtttcctgagtcc-3'; \*\*\*, 5'-cacacactgggttttaaaGggtTcaGgataggagc-3'; -x x x , 5'-cacacactgggttttaaaGggtTcaGgataggagc-3' and 5'-gcaggaggaaggCattttctggCgtttcctgagtcc-3'; \*\*•, 5'-cacacactgggttttaaaGggtTcaGgataggagc-3' and 5'-gcaggaggaaggCattttctggggtttcctgagtcc-3'; \*\*\*, 5'-cacacactgggttttaaaGggtTcaGgataggagc-3' and 5'gcaggaggaaggattttctggCgtttcctgagtcc-3'. Capital letters show nucleotides replaced for mutation. Right: The relative SEAP value is indicated as a fold increase in the SEAP activity for each construct relative to that without LPS for the pro 329 construct containing point mutations for NF-IL6 and NF-κB (-\*\*\* SEAP). The pro 329 construct is shown in Fig. 2. LPS-induced value, the transfected cells were incubated with LPS; basal promoter value, the transfected cells were incubated without LPS. Error bars indicate SD of three independent assays.

# 3.3. Oligonucleotides and their specific interaction with nuclear extract

Six kinds of 60-bp oligonucleotides covered the similar consensus binding sites for AP-1, NF-IL6, and NF- $\kappa$ B in the hBD-2 promoter region (Fig. 4(a)) which exhibited the highest ratio of LPS-induced value to basal promoter value (Fig. 2). They overlapped each other for 10 base pairs.

To examine the interaction of *cis*-acting elements of the hBD-2 promoter with HeLa nuclear factors, the <sup>32</sup>P-labeled oligonucleotides were used as probes for

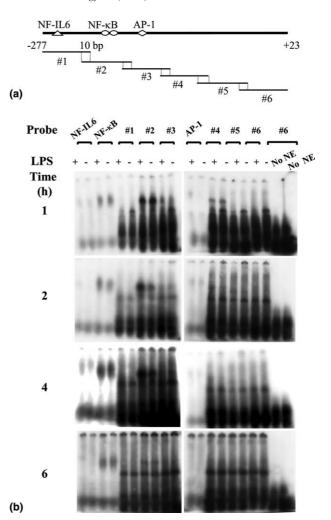


Fig. 4. Synthetic oligonucleotides and their interaction with nuclear factors. (a) Schematic representation of synthetic oligonucleotides used for EMSA. The sequence positions corresponding to the wild-type oligonucleotides span –277 to –218 (#1), –227 to –168 (#2), –177 to –118 (#3), –127 to –68 (#4), –67 to –18 (#5), and –27 to +23 (#6) in the hBD-2 gene. (b) EMSA using a series of oligonucleotides as shown in (a). The <sup>32</sup>P-labeled double-stranded oligonucleotides were incubated with nuclear extract prepared from HeLa cells incubated for 1, 2, 4, and 6 h with or without LPS. Three independent assays were performed, and a typical result is shown. No NE, sample without nuclear extract.

EMSA. The #2 oligonucleotides, of which sequence encompassed the two NF-κB consensus binding sequences, bound proteins in the nuclear extract (Fig. 4(b)). The DNA-protein complex was increased by stimulation with LPS, and was the same size as the complex of the NF-κB probe and nuclear extract (Fig. 5(a)). No apparent complex was detected for other probes (#1, 3, 4, 5, and 6) whereas oligonucleotides for NF-κB or NF-IL6 bound with the nuclear extract (Fig. 4(b)). We further analyzed whether the consensus sequence for NF-κB was critical for formation of the DNA-protein complex. In the competitive EMSA, the complex of radio-labeled #2 probe and extract was competed by a 500-fold molar excess of unlabeled

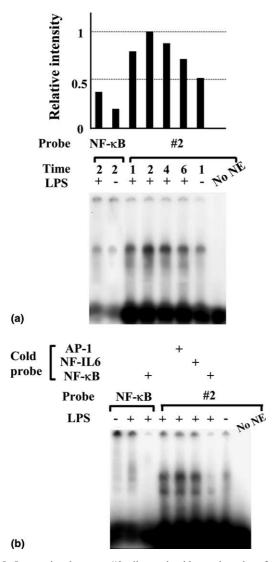


Fig. 5. Interaction between #2 oligonucleotides and nuclear factors. (a) EMSA using #2 and NF- $\kappa$ B oligonucleotides. No NE, sample without nuclear extract. (b) Competitive EMSA. A 500-fold molar excess of cold probe was added to each sample prior to formation of the DNA–protein complex. No NE, sample without nuclear extract.

NF-κB probe whereas the DNA-protein complex was not affected in the case of the other unlabeled probes (Fig. 5(b)).

#### 4. Discussion

In this study, we have performed a functional analysis of the hBD-2 promoter to gain insight into the mechanism for the regulation of hBD-2 transcription in a stratified epithelial cell line, HeLa. Using a series of sequentially deleted hBD-2 promoters (-1371 to +57) ligated into pSEAP2-Basic plasmid for expression in HeLa cells, the minimal promoter region in response to LPS was found to be located in the sequence from position -392 to -39 relative to the transcription start

position (Fig. 2). The region contained similar consensus binding sites for transcription factors, one site for NF-IL6 and two sites for NF-κB, and the responsiveness to LPS was markedly reduced by point mutations at the NF-κB binding sites (Fig. 3). These findings suggest that the NF-kB binding sites contribute to LPS-mediated hBD-2 transcription in HeLa cells. Similar regulation by the NF-κB binding sites has been shown in the murine macrophage cell line RAW264.7 [18]. Interestingly, mutation in the NF-IL6 binding site in the region between -392 and -39 resulted in an elevation of the basal promoter activity, and the mutation retained the moderate LPS-induced activity (Fig. 3(b)). The result suggests that the NF-IL6 binding site represses the hBD-2 transcription in the HeLa cells without LPS, and abolishment of the binding site released the repression of the promoter activity.

EMSA indicated that LPS induced binding of the 60bp oligonucleotides encompassing the two NF-κB consensus binding sequences with nuclear factors prepared from HeLa cells (Figs. 4(b) and 5(a)). Competitive EMSA using unlabeled oligonucleotides showed that the consensus sequence for NF-kB was essential for the binding to the nuclear factors (Fig. 5(b)). Moreover, the promoter region containing the two NF-κB binding sites had the highest responsiveness to LPS in reporter gene activity (Fig. 2). Together, these results suggest that the two NF-κB binding sites contribute to LPS-mediated hBD-2 transcription in HeLa cells. In contrast to NF-κB, no apparent DNA-protein complex was detected for the 60-bp probes encompassing the consensus sequence for NF-IL6 (Fig. 4(b)). This may be due to the different DNA binding activity between the two probes, because the #2 probe contained two sites for NF-κB, whereas the #1 probe did one site for NF-IL6 (Fig. 4(a)).

Interestingly, the NF-IL-6 binding site is likely to repress the hBD-2 gene activation, in the absence of LPS stimulation, in the HeLa cells, because mutation at the site resulted in an elevation of the basal promoter value (Fig. 3(b)). Diamond et al. [12] has reported that the NF-IL-6 binding site positively regulates both basal and LPS-induced hBD-2 gene activation in the TECs. The mechanisms responsible for the different promoter activity of the NF-IL6 binding site between the HeLa cells and TECs are currently unknown.

After our submission, Vora et al. [19] reported the regulation of hBD-2 expression by Toll-like receptor (TLR) signaling in intestinal epithelial cells (Caco-2, T84, and SW480). Activation of the hBD-2 promoter by LPS stimulation differs in these cell types, and its level depended on expression of both TLR4 and its accessory molecule MD-2. The hBD-2 promoter region located between –938 and –229, containing the consensus sequence for NF-κB, has moderate LPS responsiveness in Caco-2 and T84 followed by cotransfection with

both TLR4 and MD-2. Furthermore, the promoter with a mutation in the NF-κB site exhibits significantly reduced LPS-dependent hBD-2 expression in these transfected cells. In this study, the similar promoter region had the highest level of responsiveness to LPS in HeLa cells (Fig. 2), and the responsiveness to LPS was markedly reduced by point mutations at the two NF-κB binding sites (Fig. 3). Our results are consistent with the findings in the intestinal epithelial cells cotransfected with TLR4 and MD-2. Moreover, TLR4/MD-2 complex has shown to be required for higher responsiveness to LPS in the HeLa cells [20,21]. Taken together, TLR4/ MD-2-mediated activation of signaling cascade is likely to be required for expression of hBD-2 in not only these intestinal epithelial cells but also the HeLa cells. In addition, the hBD2 promoter activity, except for the construct pro 329, were poorly upregulated by LPS (Fig. 2). The poor upregulation may be due to the low expression level of TLR4–MD-2 complex in the HeLa cells.

Deletion of the hBD-2 promoter from position -1019to -329 demonstrated a moderate LPS inducibility but less than full-length promoter (Fig. 2). The similar reduction in LPS inducibility of the hBD-2 promoter has been reported in both Caco-2 and T84 cells [19]. Tandem STAT-like sequences are located in hBD-2 promoter from -1312 to -1281 (Fig. 1), and they may play a role in the regulation of the promoter activity. Because, it has been reported that STAT proteins are key regulatory proteins that bind to two tandem γ-interferon-activated site motifs within an IL-2 response element (positive regulatory region III [PRRIII]) in the human IL-2Rα promoter [22]. Moreover, the formation of a tetrameric Stat5 complex is essential for the IL-2-inducible activation of PRRIII [23]. The tandem STAT-like sequences would act as enhancers of the hBD-2 promoter; therefore, the missing of the sequences may cause the moderate LPS inducibility in the hBD-2 promoter.

In conclusion, the hBD-2 promoter region, containing the consensus binding sites for NF-IL6 and two NF- $\kappa$ Bs, exhibited the highest level of responsiveness to LPS in terms of reporter gene activity. The two NF- $\kappa$ B binding sites contributed to the responsiveness to LPS, and both sites were important for formation of the DNA-nuclear factor complex. The mutation in the NF-IL6 binding site resulted in an elevation of the basal promoter activity of the region. These results suggest that the two NF- $\kappa$ B binding sites contribute to LPS-mediated hBD-2 transcription and the NF-IL6 binding site represses LPS-independent hBD-2 transcription in the HeLa cells.

### Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research B (No. 14370710 to S.T.), and a Grant-

in-Aid for Exploratory Research (No. 16659579 to F.M.), from the Japan Society for the Promotion of Science, and Kobayashi Magobe Memorial Foundation, Ryobi Teien Foundation, and Inamori Foundation (F.M.).

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