Promoter and Terminator Analysis of 40-kDa Outer Membrane Gene (*hbp35*) in *Porphyromonas gingivalis* 381

Hideki Tanaka,¹ Koichi Hiratsuka,^{1,2} and Yoshimitsu Abiko^{1,2}

Department of ¹Biochemistry and Molecular Biology, and ²Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

Correspondence to :

Yoshimitsu Abiko

E-mail : abiko.yoshimitsu@nihon-u.ac.jp

Abstract

The 40-kDa outer membrane protein has been cloned from the periodontal pathogen *Porphyromonas gingivalis* 381 as a 2.0-kbp *EcoRI* DNA fragment. This protein was found to have several functions, including aggregation and hemagglutination. On a functional motif search, we found that this molecule carried a hemin-binding domain, and the recombinant protein expressed hemin-binding activity. We deposited the DNA sequence into the NCBI DNA database as the 35-kDa hemin-binding protein (HBP35). However, promoter and terminator of the *hbp35* gene have not been fully analyzed. In this study, we analyzed the nucleotide sequence of the *EcoRI* fragment to identify promoter and terminator regions and determined the structure of *hbp35*. A possible promoter sequence (TTGCAG in the -35 region and TATTTT in the -10 region), which were nearly identical to the predicted promoter sequences, were found upstream of the start codon (ATG). The number of bases between the regions was 17. The transcription start site (+1) was predicted to be located 53 nt upstream from the translation start codon. Palindrome analysis showed a very high matching sequence position from 1554 to 1601 on the *EcoRI* fragment. Hairpin loop and stem parts analysis predicted two typical terminators downstream of *hbp35*. Both locations partially overlapped the palindromic sequences, indicating ρ -independent transcription termination in the 3'-noncoding region. These terminator and promoter analyses therefore identified the whole gene structure of *hbp35*.

Keywords :

Porphyromonas gingivalis, gene structure, promoter, terminator, outer membrane

Introduction

Porphyromonas gingivalis is a black-pigmented, Gram-negative anaerobic bacterium that is an important etiological agent in adult periodontal disease (1). Chronic periodontitis has also been implicated in non-oral diseases, including cardiovascular diseases (2, 3), diabetes (4), osteoporosis (5) and pre-term delivery of low birth weight infants (6, 7). Therefore, prevention of periodontitis is relevant for both oral and systemic health. In the periodontal pocket, *P. gingivalis* is predominantly found as a component of complex biofilms containing multiple bacterial species, which colonize specific surfaces and result in interactive nutritional chains (8-10). *P. gingivalis* has several potentially virulent traits in-

cluding the secretion of exotoxin and proteases, and the ability to adhere to epithelial tissue, erythrocytes and other bacteria (1).

We previously cloned a gene for a 40-kDa outer membrane protein (11) that is specifically expressed in *P. gingivalis* cells (12, 13). This gene is located in a 2.0-kbp *EcoRI* fragment from the chromosome, and the protein is localized in the membrane fraction and on the extracellular vesicles released from the outer membrane (11). DNA sequence analysis revealed that the open reading frame (ORF) consists of 344 amino acid residues with a calculated molecular weight of 37,559 (14). Computer analysis suggested that the first N-terminal 21 residues function as the signal peptide for secretion, giving rise to a 323-residue mature

Fig. 1. Predicted open reading frames and DNA sequence of *EcoRI* fragment in *P. gingivalis*. A, Predicted open reading frames in *EcoRI* fragment. Arrows indicate the predicted open reading frames, and numbers (+1, +2, -2, and -3) indicate the frame. Upper and lower numbers (1 to 2001) indicate the position of bases. Vertical lines on the arrows indicate the position of initiation codes (ATG or GTG). B, DNA sequence deposited in GenBank under the accession number [AB059658](#).

polypeptide with a calculated molecular mass of 35,313 Da. In addition, hemin is able to bind to the recombinant 40-kDa outer membrane protein in a dose-dependent manner, indicating that the protein is a hemin-binding protein in *P. gingivalis* (14). Thus, the outer membrane gene and the protein were designated as *hbp35* and HBP35, respectively.

To date, we have identified numerous associations

between this protein and the pathogenicity of *P. gingivalis* in our laboratory; HBP35 plays a role in adherence to host cells, and coaggregation with other bacteria and erythrocytes (13, 15-19). Seven ORFs are predicted in the 2.0-kb *EcoRI* fragment through ORF analysis (Fig. 1A). The predicted protein from the largest ORF, which includes nucleotides 499 to 1530 of the DNA sequence, is similar in size to the

isolated recombinant protein. However, the promoter and terminator of the *hbp35* gene have not yet been identified. Here, we report an analysis of the promoter and terminator regions, and the possible identification of the *hbp35* gene ORF.

Materials and Methods

DNA sequence of *hbp35*

The nucleic acid sequence of *hbp35* has been deposited in GenBank under the accession number [AB059658](#), and the whole DNA sequence is shown in Fig. 1B.

Computational analyses

The structure of *hbp35* was analyzed from the DNA sequence data for a 2.0-kbp *EcoRI* fragment using GENETYX-MAC software (version 16.0; GENETYX Co., Shibuya, Tokyo, Japan). Palindromic sequences were identified using the following conditions: minimum length, 10; maximum length, 50; and matching percentage, ≥ 75.00 . Hairpin loop and stem part analysis were performed using the

following conditions: matching percentage of stem parts (%), 60.0; maximum size of stem parts, 11; minimum size of stem parts, 10; maximum size of loop parts, 11; and minimum size of loop parts, 10. For promoter analysis, The -35 and -10 regions were predicted based on the constituting bases and the relative loci at the -35 and -10 regions, as well as the number of bases between both regions (20). Analysis was performed using the following conditions: sequence position, 1-498; and minimum score, 50.00. In addition, the NNPP method, which identifies eukaryotic and prokaryotic promoters in DNA sequences, was performed (http://www.fruitfly.org/seq_tools/promoter.html). The time-delay network mainly consists of two feature layers; one for recognizing the TATA-box and one for recognizing the "Initiator", which is the region spanning the transcription start site. Both feature layers are combined into one output unit, which gives output scores between 0 and 1 (21). Analysis was performed with a score cutoff of 0.80.

Start	End	Score	Promoter Sequence
322	367	0.89	gaggtgacgcccgaagcatgcgcgcgtataaattatccTcgacgagac
405	450	1.00	aagtattttgcagattttcatctatctaactgatttttggtgcCcgaaatacg
436	481	0.91	ttttggtgcccgaatacggaaaatcaaacaaaatgttttActaacaata

Fig. 2. Predicted promoters in *hbp35* gene. Promoter analysis was performed using two feature layers; one recognizing TATA-boxes and one recognizing "Initiators", which are regions spanning the transcription start site. Both feature layers were combined into one output unit, which gave output scores between 0 and 1. Predicted transcription start site is indicated by capital letters.

1	gaattccaac	cgtggaaagg	ggagatagag	cggcgcaaca	acggatcgat	cattgcgctc
61	gaaagcggaa	cggcctatgc	ctatgccctg	aataatctcc	aaagccgtgg	acgcttcttc
121	atctctccgc	aggaggaggt	ctatgccggt	caggtcgtgg	gcgagcacac	gaaggagggc
181	gatctctgcg	tcaacgtctg	caagagcaaa	aagcttacca	atatgcgtgc	ttccggtagc
241	gatgataagg	tgctactggc	tccaccggtg	gtattcagcc	tcgaagatgc	tttgaggtat
301	atcaagtatg	acgagtatgt	ggaggtgacg	ccgaaaagca	tgcgcgatgcg	taaaattatc
361	ctcgacgaga	cagaacgtaa	acgacaagga	cgataacaga	ggcaaagtat	ttgcagattt
						-35
421	catctatcta	acgtattttg	gtgcccgaaa	tacggaaaat	caaacaaaat	gtttcactaa
		-10	+1			
481	caaaaaagat	aaacgaacAT	GAAAAGATTA	TTACTCTCTG	CTGCTATCCT	AAGTAGTATG
541	GCTTTGTTTA	ATGTCAATGC	ACAAGAGTTG	AAACCTCTG	CTGACATGAA	AGGTTCTTTT

Fig. 3. Part of the nucleotide sequence of the 5'-coding and -noncoding regions of *hbp35* gene in 2.0-kDa *EcoRI* fragment. Sequence of the open reading frames is indicated by capital letters. Predicted promoter regions (-35 and -10) and transcription start site (+1) are also indicated.

Results

Promoter analysis

Promoter prediction analysis was performed using NNPP by considering TATA-boxes and transcription start sites. There were three candidate promoter sequences (Fig. 2). Further promoter analysis was then performed by searching for -10 and -35 regions by Genetyx-Mac. Analysis indicated that the -35 and -10 regions were TTGCAG (411 to 416) and TATTTT (434 to 439), respectively (Fig. 3). The number of bases between -10 and -35 regions was 17. Thus, the transcription start site (+1) was predicted to be cytosine and was located 53 nt upstream from the translation start site.

Palindrome analysis

In order to identify the terminator for *hbp35*, palindrome analysis was performed, as palindromic sequences typically form stem-loop hairpin structures that lead to the dissociation of RNA polymerase from the DNA template. Nine palindromic sequences were identified (data not shown). For eight of these, the matching number of bases was 10 to 13, while one candidate showed the very high matching number of 48. The best matching sequence position was from 1554 to 1601, and the matching percentage was 75.0% (Fig. 4).

Hairpin loop and stem part analysis

Terminator sequences have two intrinsic features ; a hairpin in the secondary structure, and a region that is rich in U residues at the end of the unit. Both structures are needed for termination of transcription. In this study, the 3'-noncoding region in an *EcoRI* fragment containing *hbp35* was analyzed for terminator sequences (Fig. 5). Two candidate ρ -independent transcription terminators were found in the noncoding region; the terminator started at position 1553 (Fig. 5A). The stem part and the loop part were 20 and 10 nt, respectively. The matching for the stem parts was 90.0%, and another terminator started at position 1558 (Fig. 5B). The stem part and the loop part were 16 and 8 nt, respectively. The matching of the stem parts was 100.0%. Both ter-

1601

5'- acgaaaactgcgcttctttaatgtttcataagaaggcgagtttcctt -3'

3'- TGCTTTTGACGCGGAAGAATTACAAAGTATTCTTCGCGTCAAAGGAA -5'

1554

Fig. 4. Palindromic structure in 3'-noncoding region of *hbp35* gene in 2.0-kDa *EcoRI* fragment (from 1534 to 2008) in *P. gingivalis*.

Fig. 5. Hypothetical intrinsic ρ -independent transcription terminator structures for *hbp35* in *P. gingivalis*. Hairpin loop and stem part analysis was performed in 3'-noncoding region of *hbp35* gene in 2.0-kDa *EcoRI* fragment (from 1534 to 2008).

minators formed the G-C-rich hairpin loop followed by a run of U residues. Fig. 6 shows the location of the predicted palindromic sequence and their stem-

Fig. 6. Part of the nucleotide sequence of the 3'-coding and -noncoding regions of *hbp35* gene in 2.0-kDa *Eco*RI fragment. Sequence of the open reading frames is indicated by capital letters. Palindromic sequence shown in Fig. 4 is underlined. The predicted thymine-rich sequence following the termination signal is indicated by italic letters. Tandem inverted repeat sequences, which may form a stem and loop structure, are indicated by dashed (Fig. 5A) and solid (Fig. 5B) arrows.

Discussion

In contrast to eukaryotes, prokaryotic transcription occurs in the cytoplasm alongside translation. Therefore, a terminator and a promoter need to be confirmed for identification of the structural gene (24). In most genomes or sets of genetic instructions,

Bacteria use two different strategies for transcription termination based on whether RNA polymerase requires any additional factors to terminate; the ρ -dependent terminator and the ρ -independent terminator. The ρ -dependent terminator is defined by the necessity of ρ -factor, while stem-loop structures are important in prokaryotic ρ -independent transcription termination. The hairpin loop forms in an mRNA strand during transcription and causes the RNA polymerase to become dissociated from the DNA template strand. This process is known as ρ -independent or intrinsic termination, and the sequences involved are called terminator sequences.

The formation of a stem-loop structure is dependent on the stability of the resulting helix and loop regions, and helix stability is determined by its length, the number of mismatches or bulges it contains (a small number of mismatches are tolerable, particularly in long helices) and the base composition of the paired region (<http://en.wikipedia.org/wiki/Stem-loop>).

In ρ -independent transcription termination, RNA transcription stops when the newly synthesized RNA molecule forms a G-C rich hairpin loop followed by a run of U nt. When the hairpin forms, the mechanical stress breaks the weak bonds between U in mRNA and A in template DNA. This pulls the poly-U transcript out of the active site of the RNA polymerase, effectively terminating transcription. In contrast, in ρ -dependent termination, a protein factor called ρ destabilizes the interaction between the template and the mRNA, thus releasing the newly synthesized mRNA from the elongation complex (24). In this study, tandem inverted repeat sequences, that may form stem and loop structures, were found in the 3'-noncoding region (nucleotides 1274 to 1305 and 1342 to 1371). The latter was followed by a thymine-rich sequence, suggesting that these regions function as a transcriptional termination signal. These terminators may be present as either or both forms under different conditions. The locations of both terminators largely overlapped the palindromic region, indicating the presence of a ρ -independent transcription termination site in the 3'-noncoding region of this gene.

Thus, the entire gene structure of *hbp35* has been determined and this may help to elucidate more detailed functional information for this molecule.

Acknowledgments

This study was supported in part by the "Academic Frontier" Project for Private Universities, a matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology, 2007-2011, and by a Grant-in Aid for Scientific Research from the Japan Society for the Promotion of Science (B21390497).

References

1. Holt SC, Kesavalu L, Walker S, Genco CA : Virulence factors of *Porphyromonas gingivalis*. Periodontol 2000, 20 : 168-238, 1999.
2. Beck J, Garcia R, Heiss H, Vokonas PS, Offenbacher S : Periodontal disease and cardiovascular disease. J Periodontol, 67 : 1123-1137, 1996.
3. Kuramitsu HK, Kang IC, Qi M : Interactions of *Porphyromonas gingivalis* with host cells : implications for cardiovascular diseases. J Periodontol, 74 : 85-89, 2003.
4. Grossi SG, Genco RJ : Periodontal disease and diabetes mellitus : a two-way relationship. Ann Periodontol, 3 : 51-61, 1998.
5. Reddy MS : Oral osteoporosis : is there an association between periodontitis and osteoporosis? Compend Contin Educ Dent, 23 : 21-28, 2002.
6. Dasanayake AP, Boyd D, Madianos PN, Offenbacher S, Hills E : The association between *Porphyromonas gingivalis*-specific maternal serum IgG and low birth weight. J Periodontol, 72 : 1491-1497, 2001.
7. Offenbacher S, Katz V, Fertik G, Collins J, Boyd D, Maynor G, McKaig R, Beck J : Periodontal infection as a possible risk factor for preterm low birth weight. J Periodontol, 67 : 1103-1113, 1996.
8. Goulbourne PA, Ellen RP : Evidence that *Porphyromonas (Bacteroides) gingivalis* fimbriae function in adhesion to *Actinomyces viscosus*. J Bacteriol, 173 : 5266-5274, 1991.
9. Lamont RJ, Bevan CA, Gil S, Persson RE, Rosan B : Involvement of *Porphyromonas gingivalis* fimbriae in adherence to *Streptococcus gordonii*. Oral Microbiol Immunol, 8 : 272-276, 1993.
10. Maeda K, Nagata H, Yamamoto Y, Tanaka M, Tanaka J, Minamino N, Shizukuishi S : Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus oralis* functions as a coadhesin for *Porphyromonas gingivalis* major fimbriae. Infect Immun, 72 : 1341-1348, 2004.
11. Abiko Y, Hayakawa M, Aoki H, Kikuchi T, Shimatake H, Takiguchi H : Cloning of a *Bacteroides gingivalis* outer membrane protein gene in *Escherichia coli*. Arch Oral Biol, 35 : 689-695, 1990.
12. Hiratsuka K, Yoshida W, Hayakawa M, Takiguchi H, Abiko Y : Polymerase chain reaction and an outer membrane protein gene probe for the detection of *Porphyromonas gingivalis*. FEMS Microbiol Lett, 138 : 167-172, 1996.
13. Saito S, Hiratsuka K, Hayakawa M, Takiguchi H, Abiko Y : Inhibition of a *Porphyromonas gingivalis* colonizing factor between *Actinomyces viscosus* ATCC 19246 by monoclonal antibodies against recombinant 40-kDa outer membrane protein. Gen Pharmacol, 28 : 675-680, 1997.

14. Shibata Y, Hiratsuka K, Hayakawa M, Shiroza T, Takiguchi H, Nagatsuka Y, Abiko Y: A 35-kDa co-aggregation factor is a hemin binding protein in *Porphyromonas gingivalis*. Biochem Biophys Res Commun, 300 : 351-356, 2003.
15. Abiko Y, Ogura N, Matsuda U, Yanagi K, Takiguchi H: A human monoclonal antibody which inhibits the coaggregation activity of *Porphyromonas gingivalis*. Infect Immun, 65 : 3966-3969, 1997.
16. Hiratsuka K, Hayakawa M, Kiyama-Kishikawa M, Sasaki Y, Hirai T, Abiko Y: Role of the hemin-binding protein 35 (HBP35) of *Porphyromonas gingivalis* in coaggregation. Microb Pathog, 44 : 320-328, 2008.
17. Hiratsuka K, Kiyama-Kishikawa M, Abiko Y: Hemin-binding protein 35 (HBP35) plays an important role in bacteria-mammalian cells interactions in *Porphyromonas gingivalis*. Microb Pathog, 48 : 116-123, 2010.
18. Hiratsuka K, Abiko Y, Hayakawa M, Ito T, Sasahara H, Takiguchi H: Role of *Porphyromonas gingivalis* 40-kDa outer membrane protein in the aggregation of *P. gingivalis* vesicles and *Actinomyces viscosus*. Archs oral Biol, 37 : 717-724, 1992.
19. Katoh M, Saito S, Takiguchi H, Abiko Y: Bactericidal activity of a monoclonal antibody against a recombinant 40-kDa outer membrane protein of *Porphyromonas gingivalis*. J Periodontol, 71 : 368-375, 2000.
20. Mulligan ME, Hawley DK, Entriken R, McClure WR: *Escherichia coli* promoter sequences predict *in vitro* RNA polymerase selectivity. Nucleic Acids Res, 12 : 789-800, 1984.
21. Harley CB, Reynolds RP: Analysis of *E. coli* promoter sequences. Nucl Acids Res, 15 : 2343-2361, 1987.
22. Murakami KS, Darst SA: Bacterial RNA polymerases: the whole story. Curr Opin Struct Biol, 13 : 31-39, 2003.
23. Borukhov S, Nudler E: RNA polymerase holoenzyme: structure, function and biological implications. Curr Opin Microbiol, 6 : 93-100, 2003.
24. Richardson JP: Rho-dependent termination and ATPases in transcript termination. Biochim Biophys Acta, 1577 : 251-260, 2002.