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

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

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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

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.

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).

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.

Start	End	Score	Promoter Sequence
322	367	0.89	gaggtgacgccgaaaagcatgcgcatgcgtaaaattatcctTcgacgagac
405	450	1.00	aagtattttgcagatttcatctatctaacgtatttttggtgcCcgaaatacg
436	481	0.91	ttttggtgcccgaataacggaaaatcaaacaaaatgtttcActaacaaaa

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.

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1  gaattccaac cgtggaaagg ggagatagag cggcgcaaca acggatcgat cattgcgctc
61  gaaagcggaa cggcctatgc ctatgccctg aataatctcc aaagccgtgg acgcttcttc
121 atctctccgc aggaggaggt ctatgccggt caggctcgtg gcgagcacac gaaggagggc
181 gatctctgcg tcaacgtctg caagagcaaa aagcttacca atatgcgtgc ttccggtagc
241 gatgataagg tgtcactggc tccaccggtg gtattcagcc tcgaagatgc tttggagtat
301 atcaagtatg acgagtatgt ggaggtgacg ccgaaaagca tgcgcattgc taaaattatc
361 ctcgacgaga cagaacgtaa acgacaagga cgataacaga ggcaaagtat ttgcagattt
                                     -35
421 catctatcta acgtattttg gtgcccgaat tacggaaaat caaacaaaat gtttcactaa
                                     -10      +1
481 caaaaaagat aaacgaacAT GAAAAGATTA TTA CTCTCTG CTGCTATCCT AAGTAGTATG

541 GCTTTGTTTA ATGTCAATGC ACAAGAGTTG AAAACCTCTG CTGACATGAA AGGTTCTTTT

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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.

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1601
5'- acgaaaactgcgccttcttaatgtttcataagaaggcgcagtttcctt -3'
3'- TGCTTTTGACGCGGAAGAATTACAAAGTATTCTTCGCGTCAAAGGAA -5'
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1554

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

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 terminators 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-loop hairpin structures. The location of the stem-loop hairpin structures largely overlapped the palindromic sequence.

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).

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.

A promoter is a region of DNA where RNA polymerase and TF bind to initiate transcription. The “+1” indicates the base pair where transcription initiates, and is commonly referred to as the transcription start point. Base pairs upstream of the transcription start point are assigned positive numbers, while those downstream are shown with negative numbers. The core promoter consists of -10 , -35 and extended -10 and UP elements. The -10 , -35 and extended -10 elements are recognized by domains 2, 4 and 3 of the RNA polymerase σ subunit, respectively (22, 23). In our study, promoter sequence analysis by NNPP revealed three candidates, including a transcription site. Each sequence region of the promoter was separated on the 5'-noncoding region. However, the -35 and -10 regions predicted by Genetyx-Mac were unique and present on the one of the candidates, thus suggesting a promoter region including the transcription start site, and the -35 and -10 regions.

and sentences. As DNA comprises two paired strands of nucleotides, and the nucleotides always pair in the same way (Adenine with Thymine, and Cytosine with Guanine), a single-stranded sequence of DNA is said to be palindromic if it is equal to its complementary sequence read backwards. A palindromic DNA sequence often forms a stem-loop hairpin structure, which leads to the dissociation of RNA polymerase from the DNA template. In our study, a typical palindromic sequence was found from 1554 to 1601 in the 3'-noncoding region of *hbp35*, and the latter was followed by a thymine-rich sequence, suggesting that this palindromic region may function as a transcriptional termination signal.

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

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