Jpn. J. Infect. Dis., 57, 25-28, 2004

Short Communication

Isolation of a Promoter Region of a Secreted Metalloprotease Gene from *Microsporum canis*

Tsuyoshi Yamada[1,2], Koichi Makimura[1,2]*, Asuka Hirai[3], Rui Kano[3], Atsuhiko Hasegawa[3], Katsuhisa Uchida[1,2] and Hideyo Yamaguchi[1,2]

[1]*Teikyo University Institute of Medical Mycology and*
[2]*Genome Research Center, Teikyo University, Tokyo 192-0395 and*
[3]*Department of Pathobiology, School of Veterinary Medicine, Nihon University, Kanagawa 252-8510, Japan*

(Received July 28, 2003. Accepted November 28, 2003)

SUMMARY: *MEP3* of *Microsporum canis* encodes a 43.5 kDa extracellular keratinolytic metalloprotease, which is thought to be one of the virulence-related factors in dermatophytosis. In order to analyze the system underlying the regulation of *MEP3* gene expression, the 5′-upstream region was isolated by inverse PCR. The nucleotide sequence of a DNA fragment of about 2.1 kb containing the coding region contains putative transcription factor binding sites and transcriptional initiation points. Further analyses of the regulatory sequence may be useful for understanding the molecular basis of the coordinated expression of the various genes involved in dermatophytosis.

Dermatophytes, fungal pathogens, invade keratinized structures, such as hair, skin, and nails, causing an infectious disease called dermatophytosis (ringworm). Among the various species of dermatophytes, *Microsporum canis* is the main causative agent of dermatophytosis in animals. Infected cats and dogs are the most common sources of human infection through zoonosis (15).

The use of extracellular enzymes to penetrate through the solid structural barriers in the host appears to be a common strategy for treatment against fungal pathogens. Dermatophytes commonly gain entry into the host via hair, skin, or nails, and therefore the keratins, the major constituents of these cornified tissues, would be the first target for such enzymes. Thus, a great deal of attention has been focused on keratinolytic proteases (the keratinases) as a possible target for treatment of dermatophytosis. Several keratinases have been purified from dermatophytes, and some of their biochemical characteristics have been reported previously (1,2,13,18,19,21). However, few reports have investigated the molecular characteristics of genes encoding the keratinases, limiting our understanding of the mechanism of host invasion of dermatophytes.

An extracellular keratinolytic metalloprotease (43.5 kDa) was purified from a feline clinical isolate of *M. canis*, and its characteristics were partially identified (3). Production of this 43.5 kDa metalloprotease was shown to be induced specifically by keratin, suggesting that this enzyme may be one of the virulence-related factors involved in dermatophytosis. The N-terminal 13 amino acid sequence of the mature protein showed a high degree of similarity (about 60%) to the extracellular metalloprotease from *Aspergillus fumigatus* (10). Recently, Brouta et al. (4) isolated three metalloprotease genes (*MEPs*) from a *M. canis* genomic library, and demonstrated that *MEP3* (DDBJ/EMBL/GenBank accession number: AJ490183) encodes the 43.5 kDa metalloprotease. They also showed that *MEP3* is expressed in vivo during dermatophytic infection of experimental animals by *M. canis*. Thus, the regulation of these genes was suggested to be important in the invasion of target tissues by *M. canis*. Nucleotide sequences in the 5′ flanking region of the ATG initiation codon are often involved in the regulation of gene expression, containing both promoter and enhancer sequences that regulate transcription.

Here, we describe the isolation of a DNA fragment located upstream from the coding region of *MEP3* by inverse PCR (20). Hyphae of *M. canis* (teleomorph: *Arthroderma otae*) TIMM4092 (11,12) were cultured in flasks containing 50 ml of potato dextrose broth at 28°C. After 14 days in culture, the hyphae were collected, and the genomic DNA was isolated according to the method of Girardin and Latge (9). The nucleotide sequence of *MEP3* was used to design a pair of specific primers as shown in Fig. 1. Aliquots of 10 ng of genomic DNA were digested with *Eco*RI and circularized by self-ligation. The reaction was carried out overnight at 16°C with 1.4 Weiss units of T4 DNA ligase (Takara Bio, Shiga), in a total volume of 100 μl. Aliquots of 2 μl of the self-ligation mixture were used for amplification with a pair of primers, IPCR-SP1 (5′-TGACGACCACTACATTGGCA-3′) and IPCR-ASP1 (5′-TGGATCTTCTGAGCGGTAGC-3′). Amplification was performed by 40 cycles of PCR followed by the hot-start method (each PCR cycle was comprised of 15 sec at 94°C, 30 sec at 65°C, 4 min at 68°C). Reactions were performed in 25 μl containing 0.3 μM of the primer pair IPCR-SP1 and IPCR-ASP1, 0.2 mM deoxynucleotides, 1.5 mM $MgSO_4$, and 0.5 units of a high fidelity KOD-Plus-DNA polymerase (Toyobo Biochemicals, Tokyo) in the corresponding buffer. PCR resulted in an intense band at around 2.1 kb and only a few faint bands. The intense band was extracted from the gel and cloned in the plasmid pBluescript SK- (pBSK$^-$) (Stratagene, La Jolla, Calif., USA). Four clones were sequenced, and all of the insert sequences matched perfectly. Both the short sequence between primer IPCR-SP1 and the *Eco*RI site of the coding region, and the sequence between the initiation codon (ATG) and primer IPCR-ASP1 were consistent with those of *MEP3*. Sequencing

*Corresponding author: Mailing address: Teikyo University Institute of Medical Mycology, 359 Otsuka, Hachioji, Tokyo 192-0395, Japan. Tel: +81-426-78-3256, Fax: +81-426-74-9190, E-mail: makimura @main.teikyo-u.ac.jp

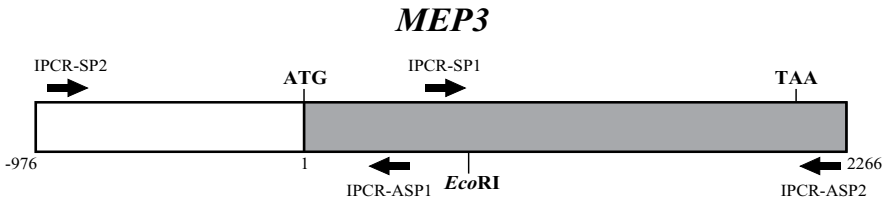

Fig. 1. Schematic representation of specific primers used in inverse PCR amplification of the 5′-untranslated region of the *MEP3* gene. An *Eco*RI cleavage site is shown on *MEP3*.

```
-976    tttttggtgg tggttttaag atttataata agctttgttt agttaaaaac gctaacctta gtttgctcca

-906    ctttgcgtgt gatttttgtt tctggtcgag gaagaaaact tcaccgtcag gatgtttctg gcgctggact

                                                        ※
-836    ttggcaaacg aacatgatgc ttgaccgagt ccagccgtgg tggaattacg atctatgtaa ggggcatccc

-766    tgtctgaggt cttatctggg cgctacgctt gtttcttgat gctcttcccc ggaccgctat caaagctgga
                                                                  (A)
-696    cttagacacg gcaatcggat atggcacatg acttttcctt ttccttttcc gctgatgtga cgcggagagc

-626    ataaagataa acagatgaag ctttgtcttc aagcttcacc agcttgggtt gtgatcgagt cttgggtgca
          (A)
-556    ggaaaccgcg ggtcgaggtt gtctgtcgaa ggcctaggtg tgggagtacg gaggactgtg tgccagtcac

-486    ggcatgaccc ggatctgctg aagctctacc agagggagaa tgaaaagaaa taccccgtat agaaacatca

-416    tgtttcttgc ttggcggaca ggccagttta atgaggcttg acacggcccg aaaattcaca aggggtggtg

-346    tgtgggtgat gagaaccgtc cagccagctg ctcgtagcta gcaggaataa tccaagcatg ggcaaaaatg

-276    cttacacacc cccgtcagcc ctcacgagac ttctctcaag taggcatgaa caaggggggg agggagagga

                                                        ※
-206    agttattcaa aggtctgaat gatacaagac ttcttagcgc cggcatgggg gcggtgattc ttctatataa
                                                                        (A)
-136    aggcattgag atatccacga aaagatatcc agaagtctca agcagagctc ctcagacgaa cagtgttctc
                 (B)          (B)
-66     tgaccccatt caccctcttt ctacctttt cttcccaccg gcgaatcagg tattccctcg gacatc

  1     ATGCACGGACTTTTGCTCGCCGGGCTTTTGGCCCTGCCCATGAATGTGCTCGCTCACCCTGCTGAGCAG-----
         M  H  G  L  L  L  A  G  L  L  A  L  P  M  N  V  L  A  H  P  A  E  Q
```

Fig. 2. Nucleotide sequence of the 5′-untranslated region of *M. canis MEP3*. Putative regions related to the TATA motif (A) and NIT2 protein binding motif (B) are shown in boldface and are underlined. Two predicted transcriptional start points are indicated by asterisks.

of these clones led to identification of the 976 bp nucleotide sequence (DDBJ/EMBL/GenBank accession number: AB125268) (Fig. 2). Finally, in order to confirm that the identified nucleotide sequence was actually located upstream from the coding region of *MEP3*, PCR was carried out on the genomic DNA using a pair of primers, IPCR-SP2 (5′-CTGGCGCTGGACTTTGGCAAACGAAC-3′) and IPCR-ASP2 (5′-GCTATGTACAGACGCATTCCTAA-3′). Three programs, HC-TATA (Wheat & Barley Genomics Program, http://125.itba.mi.cnr.it/. webgene/wwwHc-tata.html), Neural Network promoter (Berkeley Drosophila Project, http://www.fruitfly.org/seq-tools/promoter.html), and The TFSEARCH (Parallel Application TRC Laboratory, RWCP, http://www.cbrc.jp/research//db/ TFSEARCH.html), were used to determine the transcription-related elements or motifs. We detected a sequence of three TATA-like elements (-711, -629, and -147) and two putative transcriptional start points (-792 and -153), but no CAAT box in the sequence. Interestingly, the putative NIT-2 binding motifs (consensus, TATCTA) were localized around this region (-125 and -111) (Fig. 2), suggesting that regulatory genes homologous to *nit-2* may be involved in controlling the expression of *MEP3*. *nit-2* is a major positive-acting regulatory gene of the nitrogen control circuit in the fungus *Neurospora crassa*, the product of which is necessary for the expression of a set of genes encoding enzymes that allow the organism to utilize a number of secondary nitrogen sources (nitrate, purine, amino acids, etc.) under conditions of low nitrogen availability (6-8,14,17). These enzymes include extracellular proteases, as well as nitrate and nitrite reductases, and purine catabolic enzymes. As the surfaces of the keratinized tissues that dermatophytes parasitize are poor in primary nitrogen sources, such as ammonia and glutamine, the tissues themselves could be secondary nitrogen sources. Thus, dermatophytes may produce a set of extracellular keratinolytic proteases, such as the *M. canis* 43.5 kDa metalloprotease, in part to cleave keratins into metabolically usable nitrogen compounds for their growth.

A. fumigatus has only a single extracellular metalloprotease gene in its genome (10). We isolated a cDNA clone encoding a polypeptide identical to *MEP3* from *M. canis* strain TIMM4092 and estimated its copy number in the genome by Southern blotting analysis. Aliquots of 5 μg of genomic DNA digested with *Eco*RV, *Pst*I, or *Xba*I, that have no cleavage sites within the cDNA, were subjected to electrophoresis on 1.0% agarose gels, and transferred onto Hybond N^+ membranes (Amersham Biosciences, Piscataway, N.J., USA). Hybridization was carried out using an ECL direct nucleic

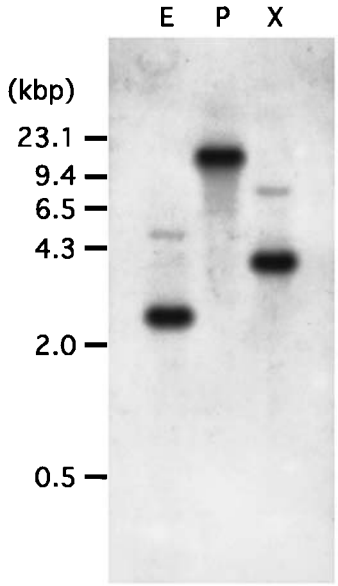

Fig. 3. Southern blotting analysis of genomic DNA from *M. canis*. Total genomic DNA samples (5 μg/lane) digested with *Eco*RV (E), *Pst*I (P), or *Xba*I (X) were separated on a 1.0% agarose gel. Hybridization was performed using a partial fragment corresponding to nucleotide positions 374 to 1453 of *MEP3* cDNA as a probe. DNA standard fragment sizes are indicated on the left.

acid labeling and detection system (Amersham Biosciences). When the partial cDNA fragment (nucleotide position 374 to 1453 in the cDNA of *MEP3*) was used as a probe, only an intense hybridization signal was observed in all DNA digests (Fig. 3). Upon digestion with both *Eco*RV and *Xba*I, hybridization signals were detected at less than 4.0 kb. A similar pattern was also obtained in another six clinical isolates from dogs and cats in Japan (data not shown). As the probe used here was specific to *MEP3*, it was suggested that the *MEP* gene exists as a single copy in the genome of *M. canis*. Various extracellular proteases produced having different proteolytic activities assist the invading fungi to penetrate through host barriers and are thought to be virulence factors. Thus, the multiplicity of the extracellular proteases, such as the *MEP* gene product, gives the fungal pathogens a competitive advantage in overcoming the complicated host defense systems against individual enzymes. It has been reported in *Candida* spp. that production of several different proteases encoded by a multiple gene family contributes to pathogenic infection (5,16,22). Therefore, development of a multiple gene family of metalloproteases in *M. canis* suggests the potential importance of these enzymes in fungal pathogenicity.

With the exception of the *M. canis MEPs*, genes encoding the keratinolytic proteases have not been isolated from dermatophytes. Therefore, very little is known about how the production of these enzymes is induced in response to external keratin stimuli. The data obtained here may be the first step in obtaining a deeper understanding of the molecular basis for coordinated expression of the various genes involved in dermatophytosis.

REFERENCES

1. Apodaca, G. and McKerrow, J. H. (1989): Purification and characterization of a 27000-Mr extracellular proteinase from *Trichophyton rubrum*. Infect. Immun., 57, 3072-3080.
2. Asahi, M., Lindquist, R., Fukuyama, K., Apodaca, G., Epstein, W. L. and McKerrow, J. H. (1985): Purification and characterization of major extracellular proteinases from *Trichophyton rubrum*. Biochem. J., 232, 139-144.
3. Brouta, F., Descamps, F., Fett, T., Losson, B., Gerday, C. H. and Mignon, B. (2001): Purification and characterization of a 43.5 kDa keratinolytic metalloprotease from *Microsporum canis*. Med. Mycol., 39, 269-275.
4. Brouta, F., Descamps, F., Monod, M., Vermout, S., Losson, B. and Mignon, B. (2002): Secreted metalloprotease gene family of *Microsporum canis*. Infect. Immun., 70, 5676-5683.
5. De Bernardis, F., Arancia, S., Morelli, L., Hube, B., Sanglard, D., Schafer, W. and Cassone, A. (1999): Evidence that members of the secretory aspartyl proteinase gene family, in particular SAP2, are virulence factors for *Candida vaginitis*. J. Infect. Dis., 179, 201-208.
6. Fu, Y. H. and Marzluf, G. A. (1987): Characterization of *nit-2*, the major nitrogen regulatory gene of *Neurospora crassa*. Mol. Cell. Biol., 7, 1691-1696.
7. Fu, Y. H. and Marzluf, G. A. (1990): *nit-2*, the major positive- acting nitrogen regulatory gene of *Neurospora crassa* encodes a sequence-specific DNA-binding protein. Proc. Natl. Acad. Sci. USA, 87, 5331-5335.
8. Fu, Y. H. and Marzluf, G. A. (1990): *nit-2*, the major nitrogen regulatory gene of *Neurospora crassa* encodes a protein with a putative zinc finger DNA-binding domain. Mol. Cell. Biol., 10, 1056-1065.
9. Girardin, H. and Latge, J. P. (1994): DNA extraction and quantification. p. 5-9. *In* Maresca, B. and Kobayashi, G.S. (eds), Molecular Biology of Pathogenic Fungi. 2nd ed. Telos Press, New York.
10. Jaton-Ogay, K., Paris, S., Huerre, M., Quadroni, M., Falchetto, R., Togni, G., Latge, J. P. and Monod, M. (1994): Cloning and disruption of the gene encoding an extracellular metalloprotease of *Aspergillus fumigatus*. Mol. Microbiol., 14, 917-928.
11. Makimura, K., Tamura, Y., Mochizuki, T., Hasegawa, A., Tajiri, Y., Hanazawa, R., Uchida, K., Saito, H. and Yamaguchi, H. (1999): Phylogenetic classification and species identification of dermatophyte strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J. Clin. Microbiol., 37, 920-924.
12. Makimura, K., Tamura, Y., Murakami, A., Kano, R., Nakamura, Y., Hasegawa, A., Uchida, K. and Yamaguchi, H. (2001): Cluster analysis of human and animal pathogenic *Microsporum* species and their teleomorphic states, *Arthroderma* species, based on the DNA sequences of nuclear ribosomal internal transcribed spacer 1. Microbiol. Immunol., 45, 209-216.
13. Mignon, B., Swinnen, M., Bouchara, J. P., Hofinger, M., Nikkels, A., Pierard, G., Gerday, C. H. and Losson, B. (1998): Purification and characterization of a 31.5 kDa keratinolytic subtilisin-like serine protease from *Microsporum canis* and evidence of its secretion in naturally infected cats. Med. Mycol., 36, 395-404.
14. Nahm, B. H. and Marzluf, G. A. (1987): Induction and *de novo* synthesis of uricase, a nitrogen-regulated enzyme in *Neurospora crassa*. J. Bacteriol., 169, 1943-1948.
15. Pepin, G. A. and Oxenham, M. (1986): Zoonotic dermatophytosis (ringworm). Vet. Rec., 118, 110-111.
16. Schaller, M., Korting, H. C., Schafer, W., Bastert, J., Chen, W. and Hube, B. (1999): Secreted aspartic proteinase

(Sap) activity contributes to tissue damage in a model of human oral candidosis. Mol. Microbiol., 34, 169-180.
17. Stewart, V. and Vollmer, S. J. (1986): Molecular cloning of *nit-2*, a regulatory gene required for nitrogen metabolite repression in *Neurospora crassa*. Gene, 46, 291-295.
18. Takiuchi, I., Higuchi, D., Sei, Y. and Koga, M. (1982): Isolation of an extracellular proteinase (keratinase) from *Microsporum canis*. Sabouraudia, 20, 281-288.
19. Takiuchi, I., Sei, Y., Takagi, H. and Negi, M. (1984): Partial characterization of the extracellular keratinase from *Microsporum canis*. Sabouraudia, 22, 219-224.
20. Triglia, T., Peterson, M. G. and Kemp, D. J. (1988): A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucl. Acid Res., 16, 8186.
21. Tsuboi, R., Ko, I. J., Takamori, K. and Ogawa, H. (1989): Isolation of a keratinolytic proteinase from *Trichophyton mentagrophytes* with enzymatic activity at acidic pH. Infect. Immun., 57, 3479-3483.
22. Zaugg, C., Zepelin, M. B. V., Reichard, U., Sanglard, D. and Monod, M. (2001): Secreted aspartic proteinase family of *Candida tropicalis*. Infect. Immun., 69, 405-412.