

Toshifumi Yuuki^a
Yasushi Okumura^a
Hirokazu Okudaira^b

^a Central Research Laboratories,
Asahi Breweries Ltd., and

^b Department of Medicine and Physical
Therapy, Faculty of Medicine,
The University of Tokyo, Japan

Genomic Organization and Polymorphisms of the Major House Dust Mite Allergen Der f 2

Key Words

Der f 2
Genomic sequence
Polymorphisms
House dust mite
Allergen

Abstract

We amplified genomic DNA encoding the major house dust mite allergen, Der f 2, from *Dermatophagoides farinae* by means of the polymerase chain reaction and cloned it into *Escherichia coli*. The nucleotide sequences of the amplified fragments were determined and compared with those of the cDNA previously reported. Four Der f 2 genomic clones were obtained, suggesting that the genomic Der f 2 gene had sequence polymorphisms like the cDNA clones. Each of the genomic clones had a single small intron. With respect to the exon sequences, two of the four genomic clones were identical with two cDNA clones, respectively. The others were combinations of the two clones. Genomic Southern blotting suggested that the Der f 2 gene is located at one locus in the mite genome and that sequence substitutions were due to polymorphisms among individual mite genes.

Introduction

It has been proposed that house dust mites of the *Dermatophagoides* genus are sources of allergens, that cause allergic diseases such as asthma, perennial rhinitis, and eczema [1–4]. For diagnostic and therapeutic purposes, we attempted production of purified and biologically active recombinant proteins of major allergens derived from the house dust mite, *Dermatophagoides farinae*. Der f 2 is one of the major house dust mite allergens from *D. farinae*, that reacts with IgE from about 80% of patients allergic to mites. However its physiological function in the mite remains unknown. Previously we reported the isolation of the three types of cDNA clones for Der f 2 [5, 6] and a large scale production of recombinant protein was expressed in *Escherichia coli* [7].

The three cDNA clones (clone 1, clone 2 and clone 11) of Der f 2 are highly homologous. Compared with the nucleo-

tide sequences of clone 1, clone 11 possesses six-base substitutions in the coding region resulting in four amino acid exchanges. Clone 2 is a combination type of clone 1 and clone 11; it has two-base substitutions compared with clone 1 and the substituted bases are identical with those of clone 11 [6]. All three cDNA clones were expressed in *E. coli* and recombinant proteins of Der f 2 (rDer f 2) were purified. All the variants of rDer f 2 are identical to native Der f 2 in terms of allergenic activity, because they are equally recognized by human IgE antibodies from the sera of 14 patients allergic to mites [8].

Furthermore, amino acid sequencing of native Der f 2 revealed that at least two types of Der f 2 were produced in *D. farinae* [8]. However, it is not clear whether these base substitutions are due to polymorphisms in the population or to the presence of more than one copy of the Der f 2 gene in each individual. In this study, we cloned the genomic DNA and identified molecular polymorphisms in Der f 2.

KARGER

E-Mail karger@karger.ch
Fax +41 61 306 12 34
<http://www.karger.ch>

© 1997 S. Karger AG, Basel
1018–2438/97/1121–0044 \$12.00/0

This article is also accessible online at:
<http://BioMedNet.com/karger>

Correspondence to: Dr. Toshifumi Yuuki
Central Research Laboratories
Asahi Breweries, Ltd.
2-13-1, Ohmori-kita, Ohta-ku
Tokyo 143 (Japan)

Received:
March 7, 1996
Accepted after revision:
July 24, 1996

Materials and Methods

Mites

D. farinae culture supplied by Torii Pharmaceutical Co., Ltd. (Tokyo, Japan) was cultivated in a flat flask containing animal feed at 25°C and 75% humidity for 30 days. Saturated NaCl was added to the culture, and live mite bodies were separated from the feed by filtration as described previously [6].

Preparation of Genomic DNA from Mites

Genomic mite DNA was extracted as described by Lis et al. [9]. Five hundred milligrams of live mites were rapidly frozen in liquid nitrogen, then 6.25 ml of extraction buffer consisting of 0.2 M sucrose, 0.1 M Tris-HCl (pH 9.2), 50 mM EDTA, 0.5% SDS were added. The suspension was immediately homogenized in a Potter homogenizer, then an additional 18.75 ml of buffer were added. After heating at 70°C for 10 min, 3.75 ml of 8 M potassium acetate (pH 9.5) were added followed by an incubation on ice for 30 min. After centrifugation, 50 ml of cold ethanol were added to the supernatant, and the nucleic acid pellet was collected. After RNase treatment and phenol/chloroform extraction, the DNA was collected by ethanol precipitation. Finally about 10 mg genomic DNA were obtained.

Oligodeoxynucleotide Synthesis

Two oligonucleotide primers were made on an Applied Biosystems 381A DNA synthesizer based on the Der f2 cDNA sequence. Primer FOR, 5'-CATAACCGAAATGATTTC, corresponds to the N-terminal region of Der f2. Primer REV, 5'-TTCGATGTTGGT-GAAAA, corresponds to a sequence downstream of the termination codon of it (fig. 2). Both sequences are identical among the cDNA clones.

Isolation of Genomic Der f2 Gene

Polymerase chain reaction (PCR) was performed with 1 µg each of primer FOR and REV, 1 µg of genomic mite DNA, 2.5 units of Taq polymerase (Toyobo, Japan) in 100 µl of PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.01% (w/v) gelatin, 200 µM each dNTP] for 35 cycles (2 min, 94°C; 1 min, 55°C; 1 min, 72°C) in a programmable incubator (Astech). Aliquots of 10 µl of the PCR products were resolved by 6% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. After removing unreacted dNTPs by ultrafiltration, the PCR products were treated with T4 DNA polymerase (DNA Blunting Kit; TAKARA) to create blunt ends and ligated with the vector pUC118 cut with *Sma*I. *E. coli* JM109 cells were transformed by the ligation mixture and positive clones were selected as white colonies after plating in LB agar plates containing ampicillin (100 µg/ml) and X-Gal (50 mg/ml). Recombinant plasmids were purified and digested with *Bam*HI/*Eco*RI to determine the size of the inserts.

DNA Sequencing

DNA was sequenced by means of an Applied Biosystems 371A fluorescent sequencer using DyeDeoxy terminators in sequencing reactions. The sequence was analyzed using the software DNASIS (Hitachi Software Service).

Southern Blotting Analysis

Genomic DNA from mites was digested with restriction enzymes that cut the Der f2 gene (*Cla*I, *Eco*T22I or *Hinc*II) with a unique site in the coding region. Five micrograms of the DNA were used for each

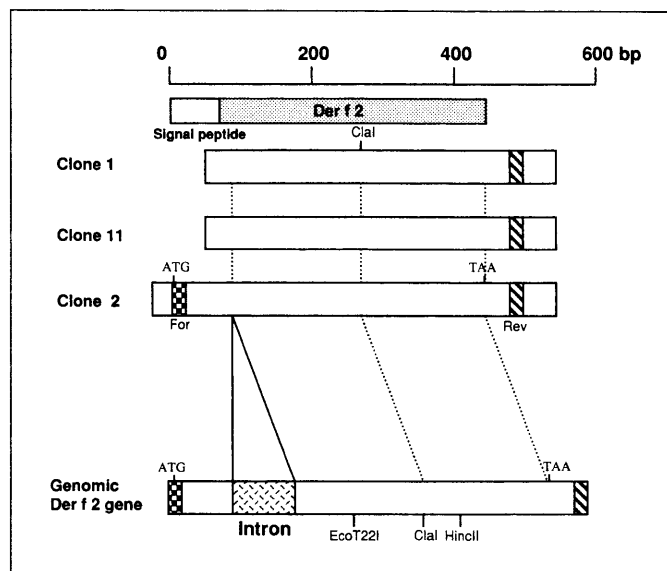


Fig. 1. Structure of the genomic gene of Der f2 and the previously cloned cDNAs [6]. The cloned fragments are shown as white bars. Restriction sites used for the Southern blotting are represented below the genomic gene. The intron is represented by stippling. Forward and reverse primers (FOR and REV) were used to amplify genomic Der f2 genes.

digestion, followed by gel electrophoresis in 1% agarose gel. In order to achieve complete digestion, the DNA was incubated in 50 µl of the appropriate buffer with 50 units of an enzyme for 90 min at 37°C, and then 50 µl of buffer containing 50 units of the same enzyme were added and incubated for another 90 min at 37°C. After electrophoresis, the DNA was denatured, transferred to a nylon membrane (Hybond-N, Amersham International) and hybridized to a cloned fragment of the genomic mite Der f2 gene (No. 16). Preparation of the labeled DNA fragment and detection of the hybridized probe were carried out by means of the ECL method (Amersham International).

Results

Cloning of the Genomic Der f2 Genes

To isolate the genomic gene of Der f2, two oligonucleotide primers based on the cDNA sequences were produced and PCR was carried out using whole mite genomic DNA as the template. After the reaction, the mixture was subjected to polyacrylamide gel electrophoresis. A single band, strongly stained with ethidium bromide, was observed on the gel. The size of the fragments exceeded that predicted from the cDNA [580 vs. 490 bp; data not shown], suggesting the presence of an intron sequence within the amplified fragments. The amplified fragment was ligated into the cloning vector, pUC118. The purified plasmids were used as templates for DNA sequencing and five clones (No. 16, 35,

		10	20	30	40	50
Clone1	*****	*****	*****	***TTTCATT	GTTGGTAGCA	
Clone11	*****	*****	*****	*****	*****	
Clone2	CATAACCGAA	AATGATTTC	AAAATCTTGT	GCC-----	-----	
No16	CATAACCGAA	AATGATTTC	AAAATCTTGT	GCC-----	-----	
No35	CATAACCGAA	AATGATTTC	AAAATCTTGT	GCC-----	-----	
No40	CATAACCGAA	AATGATTTC	AAAATCTTGT	GCC-----	-----	
No42	CATAACCGAA	AATGATTTC	AAAATCTTGT	GCC-----	-----	
		60	70	80	90	100
Clone1	GCCGTTGTTG	CCGATCAAGT	CGATGTTAAA	GATTGTG***	*****	
Clone11	-----	-----	-----	-----	*****	
Clone2	-----	-----	-----	-----	*****	
No16	-----	-----	-----	-----GTA	AGTTTGTTTG	
No35	-----	-----	-----	-----GTA	AGTTTGTTTG	
No40	-----	-----	-----	-----GTA	AGTTTGTTTG	
No42	-----	-----	-----	-----GTA	AGTTTGTTTG	
		110	120	130	140	150
Clone1	*****	*****	*****	*****	*****	
Clone11	*****	*****	*****	*****	*****	
Clone2	*****	*****	*****	*****	*****	
No16	TGTGATACAT	TTTTTTTCTT	TTCATTTATT	CAACACTAAA	CACAAATTTTC	
No35	TGTGATACAT	TTTTTTTCTT	TTCATTTATT	CAACACTAAA	CACAAATTTTC	
No40	TGTGATACAT	TTTTTTTCTT	TTCATTTATT	CAACACTAAA	CACAAATTTTC	
No42	TGTGATACAT	TTTTTTTCTT	TTCATTTATT	CAACACTAAA	CACAAATTTTC	
		160	170	180	190	200
Clone1	*****	*****	***CCAACA	ATGAAATCAA	AAAAGTAAATG	
Clone11	*****	*****	***	-----	-----	
Clone2	*****	*****	***	-----	-----	
No16	TTTTTCTTGT	TTATATTCAT	ATA <u>G</u> ----	-----	-----	
No35	TTTTTCTTGT	TTATATTCAT	ATA <u>G</u> ----	-----	-----	
No40	TTTTTCTTGT	TTATATTCAT	ATA <u>G</u> ----	-----	-----	
No42	TTTTTCTTGT	TTATATTCAT	ATA <u>G</u> ----	-----	-----	
		210	220	230	240	250
Clone1	GTCGATGGTT	GCCATGGTTC	TGATCCATGC	ATCATCCATC	GTGGTAAACC	
Clone11	-----	-----	-----	-----	-----	
Clone2	-----	-----	-----	-----	-----	
No16	-----	-----	-----	-----	-----	
No35	-----	-----	-----	-----	-----	
No40	-----	-----	-----	-----	-----	
No42	-----	-----	-----	-----	-----	
		260	270	280	290	300
Clone1	ATTCACCTTG	GAAGCCTTAT	TCGATGCCAA	CCAAAACACT	AAAACCGCTA	
Clone11	-----	-----	-----	-----	-----	
Clone2	-----	-----	-----	-----	-----	
No16	-----	-----	-----	-----	-----	
No35	-----	-----	-----	-----	-----	
No40	-----	-----	-----	-----	-----	
No42	-----	-----	-----	-----	-----	

Fig. 2. Comparison of nucleotide sequences of cDNAs (clone 1, clone 11 and clone 2) [6] and genomic genes of Der f2 (No. 16, 35, 40, and 42). Identical nucleotides at the same position of clone 1 of cDNA and others are indicated by bars. Asterisks * denote deletions of corresponding nucleotides. Forward and reverse primer sequences are boxed. The initiation and termination codons are underlined.

40, 42 and 60) were sequenced. Two of them (No. 42 and 60) were identical to each other, but the others had several base substitutions. Figure 1 shows a representative structure of the genomic gene of Der f2 and the cloned cDNAs.

Comparison of DNA Sequences

We compared nucleotide sequences of previously cloned cDNAs (clone 1, Clone 11 and Clone 2) [6] with those of the genomic genes. As shown in figure 2, there were no base substitutions upstream of the *Cla*I site in the cDNA sequence of Der f2. All of the genomic genes had an 87-base-pair sequence which was a nonhomologous sequence with the cDNA. From the conserved sequences at the junction

site, which start with the nucleotides GT and end with AG, this region seemed to be an intervening sequence. Therefore, the Der f2 gene had a single intron. This intron interrupted the codon at amino acid residue 9 in the mature protein, starting from nucleotide 63 of the Der f2 cDNA.

There were some base substitutions from the downstream region of the *Cla*I site of the gene. Two of the four genomic genes (No. 40 and 42) were identical with clone 1 and clone 11, respectively. The others (No. 16 and 35) were combinations of clone 1 and clone 11, but the combinations differed from that of clone 2.

	310	320	330	340	350
Clone1	AAATTGAAAT	CAAAGCCAGC	CTCGATGGTC	TTGAAATTGA	TGTTCCCGGT
Clone11	-----	-----	-----	-----	-----
Clone2	-----	-----	-----	-----	-----
No16	-----	-----	-----	-----	-----
No35	-----	-----	-----	-----	-----
No40	-----	-----	-----	-----	-----
No42	-----	-----	-----	-----	-----
	360	370	380	390	400
Clone1	ATCGATACCA	ATGCTTGCCA	TTTGTCAAA	TGTCCATTGG	TTAAAGGTCA
Clone11	-----	-----	---A-G---	-----	-----
Clone2	-----	-----	---A-G---	-----	-----
No16	-----	-----	-----	-----	-----
No35	-----	-----	-----	-----	-----
No40	-----	-----	-----	-----	-----
No42	-----	-----	---A-G---	-----	-----
	ClaI				
	410	420	430	440	450
Clone1	ACAATATGAT	ATCAAATATA	CATGGAATGT	GCCGAAATTT	GCACCAAAAT
Clone11	-----	GC-----	-----	-----	-----
Clone2	-----	-----	-----	-----	-----
No16	-----	GC-----	-----	-----	-----
No35	-----	-----	-----	-----	-----
No40	-----	-----	-----	-----	-----
No42	-----	GC-----	-----	-----	-----
	460	470	480	490	500
Clone1	CTGAAAACGT	TGTCGTTACA	GTCAAACCTA	TCGGTGATAA	TGGTGTTTTG
Clone11	-----	-----	-----G	-T-----	-----
Clone2	-----	-----	-----G	-T-----	-----
No16	-----	-----	-----G	-T-----	-----
No35	-----	-----	-----	-----	-----
No40	-----	-----	-----	-T-----	-----
No42	-----	-----	-----G	-T-----	-----
	510	520	530	540	550
Clone1	GCTTGCGCTA	TTGCTACCCA	TGGTAAATC	CGTGATTAAA	AAAAAA**TA
Clone11	-----	-----	C-C-----	-----	---AA---
Clone2	-----	-----	-----	-----	---**---
No16	-----	-----	C-C-----	-----	---AA---
No35	-----	-----	-----	-----	---**---
No40	-----	-----	-----	-----	---**---
No42	-----	-----	C-C-----	-----	---AA---
	560	570	580		
Clone1	AATAAGAAAA	TTTTCACCAA	CATCGAACAA		
Clone11	---T-----	-----	-----		
Clone2	-----	-----	-----		
No16	---T-----	-----	-----		
No35	---T-----	-----	-----		
No40	-----	-----	-----		
No42	---T-----	-----	-----		

Fig. 2. Continued

Genomic Southern Blotting of the Der f2 Gene

We determined the copy number of Der f2 in the mite genome by means of genomic Southern blotting. Whole mite DNA was completely digested with one of the restriction enzymes, *Cla*I, *Eco*T22I or *Hinc*II, for which there were unique sites in the genomic gene then hybridized with PCR-amplified Der f2 gene (No. 16). The results are shown in figure 3. Only two labeled bands were found in each lane of the Southern blot. The results supported the notion that the Der f2 gene is located at one locus of the mite genome. Thus, the nucleotide substitutions were due to polymorphisms among individual mite genes.

Discussion

We isolated by means of PCR four genomic DNAs encoding the major mite allergen, Der f2. This report describes the first primary sequence determination of genomic DNA for the group 2 allergens of *Dermatophagoides* spp. A comparison of the gene with the cDNA [6] indicated that the Der f2 gene consisted of two exons and a single intron that interrupted the codon-specifying amino acid residue 9 of the mature protein. The sequence of the intron-exon splice junctions agreed with the GT-AT rule and the consensus sequence of Mount [10]. The intron was small (87 base pairs) and correlated well with the size (58 and 65 base

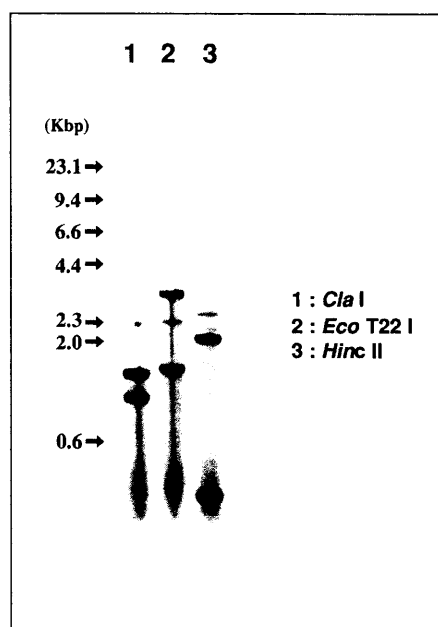


Fig. 3. Genomic Southern blot analysis of Der f2 gene. Five micrograms of mite genomic DNA were completely digested by the restriction enzymes indicated on each lane. They cut at a single site on the Der f2 gene. After electrophoresis on a 0.7% agarose gel, the separated fragments were transferred to a Hybond-N membrane and hybridized with the enzyme-labeled PCR-amplified Der f2 gene.

pairs) of the introns reported in the group 1 allergens of the house dust mite [11].

With respect to the exon sequence, there were minor sequence variations among the four genes (fig. 2) as well as previously cloned cDNA. There were only two types of nucleotide substitutions, the positions and types being identical with

those of the cDNAs. These findings suggested that the base substitutions of cDNA were not due to artifacts during cDNA synthesis or cloning but to sequence polymorphisms of the mite and that PCR-induced mutations seemed unlikely while amplifying genomic DNA. Within the coding region, there were eight base substitutions corresponding to four amino acid codons, as well as a two-base insertion/deletion and one-base substitution at the 3' noncoding region. On comparing cDNA sequences, genomic clones No. 40 and No. 42 were completely identical with clone 1 and clone 11 of the cDNA, respectively. Clone No. 16 was a combination type of clone 1 and clone 11 like clone 2, but the combination differed. That is, three amino acid residues differed from clone 1 [6] at positions 88 (Ile→Ala), 111 (Ile→Val) and 125 (Gly→Ala). We found a new isoform of the Der f2 molecule. Clone No. 35 was also a combination type, because the 3' noncoding sequence was like clone 11, although the coding region was the same as clone 1. Other variations of the Der f2 molecule have been described by Trudinger et al. [12] and Thomas et al. [13], but we did not find these isoforms (e.g., Ser 11 or Thr52). This seemed to be due to different polymorphisms arising from the origin of the mite, for example, Australia and Japan.

Although Chua et al. [14] have also isolated five cDNA clones of Der p1, the groupe 1 allergen, derived from *Dermatophagoides pteronyssinus*, they did not clarify whether these base substitutions were due to the presence of more than one copy of the Der p1 gene in each individual, or to polymorphism in the population. As a result of genomic Southern analysis in this report, it was revealed that the Der f2 gene existed as a single gene in the mite genome. Namely, variations of the Der f2 gene are due to sequence polymorphisms within individual mites.

References

- Platts-Mills TAE, Chapman MD: Dust mites: Immunology, allergic diseases and environmental control. *J Allergy Clin Immunol* 1987; 80:755–775.
- Platts-Mills TAE, de Weck AL: Dust mite allergens and asthma – A worldwide problem. *J Allergy Clin Immunol* 1989;83:416–427.
- Chapman MD: Mite allergens and asthma. *Curr Opin Immunol* 1990;2:525–530.
- Platts-Mills TAE: Dust mite allergens and asthma: Report of a second international workshop. *J Allergy Immunol* 1992;89:1046–1060.
- Yuuki T, Okumura Y, Ando T, Yamakawa H, Suiko M, Haida M, Okudaira H: Cloning and sequencing of cDNAs corresponding to mite major allergen *Der f II*. *Jpn J Allergol* 1990;39: 557–561.
- Yuuki T, Okumura Y, Ando T, Yamakawa H, Suiko M, Haida M, Okudaira H: Cloning and expression of cDNA coding for the major house dust mite allergen *Der f II* in *Escherichia coli*. *Agric Biol Chem* 1991;55:1233–1238.
- Iwamoto N, Nishiyama C, Yasuhara T, Saito A, Yuuki T, Okumura Y, Okudaira H: Direct expression of Der f2, one of the major house dust mite allergens, in *Escherichia coli*. *Int Arch Allergy Immunol* 1996;109:356–361.
- Nishiyama C, Yuuki T, Usui Y, Iwamoto N, Okumura Y, Okudaira H: Effect of amino acid variations in recombinant *Der f II* on its human IgE and mouse IgG recognition. *Int Arch Allergy Immunol* 1994;105:62–69.
- Lis JT, Simon JA, Sutton CA: New heat shock puffs and β -galactosidase activity resulting from transformation of *Drosophila* with an *hsp 70-lacZ* hybrid gene. *Cell* 1983;35:403–410.
- Mount SM: A catalogue of splice junction sequences. *Nucleic Acids Res* 1982;10:459–472.
- Kent NA, Hill MR, Heen JN, Holland PWH, Hart BJ: Molecular characterization of group I allergen *Eur mI* from house dust mite *Euroglyphus maynei*. *Int Arch Allergy Immunol* 1992; 99:150–152.
- Trudinger M, Chua KY, Thomas WR: cDNA encoding the major mite allergen *Der fII*. *Clin Exp Allergy* 1991;21:33–37.
- Thomas WR, Chua KY, Smith WA: Molecular polymorphisms of house dust mite allergens. *Exp Appl Acarol* 1992;16:153–164.
- Chua KY, Kehal PK, Thomas WR: Sequence polymorphisms of cDNA clones encoding the mite allergen *Der pI*. *Int Arch Allergy Immunol* 1993;101:364–368.