

Cloning and Expression of cDNA Coding for the Major House Dust Mite Allergen *Der f II* in *Escherichia coli*

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A cDNA library corresponding to mite protein was screened using anti-*Der f II*, a major allergen from the house dust mite *Dermatophagoides farinae*, antibody. Three possible clones were obtained that contained cDNA fragments coding for *Der f II*, and the nucleotide sequences of the fragments were determined. There were minor differences observed affecting the deduced amino acid sequence among the three cDNA fragments. The amino acid sequence of the purified native *Der f II* protein could be analyzed to 45 residues from the N-terminus. As a result of comparison, all the three cDNA fragments code for a mature protein with a derived molecular weight of about 14,000. The amino acid sequence was not homologous to any known protein sequences and it contained six cysteine residues and no *N*-glycosylation sites.

House dust is one of the most important allergens causing atopic asthma and other atopic diseases.¹⁾ Although house dust contains various kinds of allergens such as mites, animal danders, and fungal spores, mites have been proposed as a major causative allergen in many atopic diseases.²⁾ There are many species of mite in human habitations, but two *Dermatophagoides* species, *D. farinae* and *D. pteronyssinus*, occur frequently.³⁾ Thus, many studies have been done to characterize and identify the allergen in extracts from *D. farinae* and *D. pteronyssinus*. It has been recognized that *Dermatophagoides* have two groups of major allergens, the *Der I* group (*Der p I*: P1, Dp42, Dpt12; *Der f I*: F1, Ag11, Df6) and the *Der II* group (*Der p II*: Dp X; *Der f II*: Ag19/20, DF2).⁴⁻⁶⁾

This type of allergy is mediated by IgE antibodies that react to mite components. Desensitization therapy, injecting the allergen by repeated and progressively increased dose, may be successful for that allergy. But a large

amount of purified mite allergen is necessary for therapeutic purposes as well as for the diagnostic tests to detect that allergy. However, preparation of the purified allergen on a large scale is, at present, practically impossible. Therefore, we have attempted production of the purified major allergen of *Dermatophagoides farinae*, *Der f II*, by using recombinant DNA techniques; that is, cloning and sequencing the cDNA corresponding to *Der f II*.

Materials and Methods

Mites. D. farinae was cultivated in a flat flask containing animal feed at 25°C and 75% humidity for 30 days. After cultivation, saturated NaCl was added, and live mite bodies were separated from the feed by filtration as described previously.⁷⁾

Preparation of cDNA library to D. farinae. Five grams of live mites were rapidly frozen with liquid nitrogen and 20 ml of guanidine isothiocyanate solution (RNA extraction kit, RPN. 1264; Amersham International plc.) was

added. The suspension was immediately homogenized. After the insoluble mite material was separated by centrifugation, the RNA fraction was precipitated with LiCl solution. The pelleted RNA was fractionated on an oligo(dT)cellulose column (mRNA Purification Kit, 27-9258-01; Pharmacia LKB Biotechnology AB) and bound RNA[poly(A)⁺] was isolated.

The cDNA was synthesized by the method of Gubler and Hoffman⁸⁾ using a kit (cDNA synthesizing system plus, RPN1256Y; Amersham International plc.).

The cDNA library was expressed in *Escherichia coli* MC1061 using an expression vector, pUEX1⁹⁾ (cDNA cloning system-plasmid pUEX1, RPN.1282; Amersham International plc.) using an adaptor strategy.¹⁰⁾

Screening library with immunoassay. The clones were screened by clony immunoassay using rabbit anti-*Der f* II antiserum and immunoscreening system (Super Screen immunoscreening system, RPN1281Z; Amersham International plc.). *E. coli* clones with recombinant DNA were grown on the LB-agar plates at 30°C. A dry nitrocellulose filter was overlaid on each plate, transferred to the new LB-agar plate with the colony side up, and incubated at 42°C for 2 hr. The filters were exposed to chloroform vapor in a desiccator for 25 min and treated in TBS buffer [10 mM Tris-HCl (pH 7.9), 150 mM NaCl] containing lysozyme (0.4 mg/ml) and DNaseI (10 µg/ml) for 1 hr. Blocking was done with 2% (w/v) bovine serum albumin for 1 hr. Then the filters were incubated in TBS buffer plus rabbit anti-*Der f* II antisera for 1 hr at room temperature. The filters were washed and incubated with peroxidase-linked anti-rabbit IgG antibody for 1 hr. Finally, the filters were incubated in peroxidase substrate solution (3,3'-diaminobenzidine tetrahydrochloride; 0.4 mg/ml, H₂O₂; 0.01%). Colonies corresponding to strongly brown spots were picked up from the first agar plate and replated.

Anti-*Der f* II rabbit serum¹¹⁾ was kindly supplied by Dr. Yasueda (National Sagami Hospital, Kanagawa, Japan).

Identification of the antigenicity of the fused *Der f* II. Each transformant was cultured in L-broth with 50 µg/ml of ampicillin. The incubation was done at 30°C until the optical density at 660 nm of the culture reached 0.6. Then the cultivation temperature was raised to 42°C and the culture was incubated for 2 hr more. The cells were harvested by centrifugation. Samples from the cells were prepared by boiling at 100°C for 5 min in a buffer containing 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 3% (w/v) sodium dodecyl sulfate (SDS), and 0.02 M Tris-HCl (pH 6.8). The samples were electrophoresed on an SDS-polyacrylamide gel. The gel used was a 4–20% gradient gel. After electrophoresis, part of the gel was stained by Coomassie Brilliant Blue and the rest was Western blotted.¹²⁾ The proteins transferred onto a PVDF membrane (Immobilon, Millipore) were reacted with rabbit anti-*Der f* II antibody, washed, and were detected

by peroxidase-labeled anti-rabbit IgG antibody, 4-chloro-1-naphthol and H₂O₂.

Sequencing of cDNA. Plasmid DNA was prepared from the selected anti-*Der f* II binding plasmid clone and characterized using restriction endonuclease. The subcloning was done using the plasmids pUC118 and pUC119¹³⁾ for sequence analysis. The cDNA insert was excised by *Bam*HI. *Bam*HI-cDNA fragments were digested with various restriction endonucleases such as *Nco*I and subcloned into the appropriate sites of the vector. After transformation of the *E. coli* JM105, the isolated transformed colonies were infected by the helper phage M13KO7. Single-strand DNAs were extracted from the resulting phage particles. DNA sequencing was done by the method of Sanger¹⁴⁾ using a 7-DEAZA sequencing kit (Takara). The sequence was analyzed using the sequence analysis software DNASIS (Hitachi Software Service).

Sequencing of amino acid of native *Der f* II protein. *Der f* II protein from *D. farinae* was purified by column chromatography as reported previously.¹⁵⁾ In brief, *Der f* II was isolated by CM-52 ion exchange chromatography, (NH₄)₂SO₄ precipitation, and gel filtration. The final purification was by reversed-phase HPLC (Model 130A, Applied Biosystems Co.; Column, C₈ 300 Å (2.1 i.d. × 30 mm); buffer A, 0.1% trifluoroacetic acid (TFA) in H₂O; buffer B, 0.1% TFA in 70% acetonitrile; gradient, 0% buffer B to 100% buffer B at 2.2% per minute; flow rate, 200 µl/min). The amino acid sequencing of the purified *Der f* II was done with a gas-phase protein sequencer (Model 471A, Applied Biosystems Co.).

Results

*Selection of the clone containing *Der f* II gene*

cDNA prepared from *D. farinae* was inserted to an expression vector pUEX1, and then *E. coli* MC1061 was transformed by the recombinant plasmids. Among 1,600 transformants, three clones, No. 1, No. 2, and No. 11, appeared to be the desired clones using a colony immunoassay with anti-*Der f* II antibody. Each plasmid was recovered from the clones and analyzed by agarose gel electrophoresis. All plasmids had approximately 500 base-pair insertions at the end of the β -galactosidase gene. Therefore, the foreign proteins were fused to β -galactosidase, and the plasmids of the clones were named pFL1, pFL2, and pFL11, respectively.

Western blot analysis of fused *Der f* II protein

It was possible to make the inclusion bodies visible using phase-contrast light microscopy in the recombinant *E. coli*. To examine the nature of proteins produced by recombinant *E. coli*, the transformants were grown in broth

culture and the proteins were detected by Western blotting using anti-*Der f* II antibody, as described under Materials and Methods. As shown in Fig. 1, clones No. 1 (pFL1) and No. 11 (pFL11) did not produce 116-kilodalton β -galactosidase bands but instead produced antibody-binding components with molecular weights about 130,000. They were consistent with fusion proteins with *Der f* II contributing a 14-kilodalton moiety. On the other hand, clone No. 2 (pFL2) did not produce such a protein.

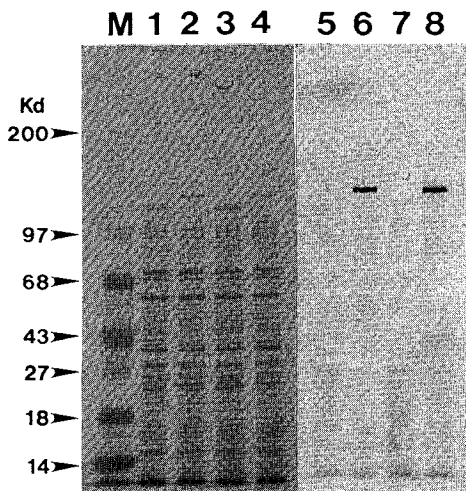


Fig. 1. Western Blotting Analysis of Recombinant *Der f* II Protein.

Total cellular proteins of *E. coli* JM105 transformed by recombinant plasmids pFL1 (lanes 2 and 6), pFL2 (lanes 3 and 7), and pFL11 (lanes 4 and 8) were analyzed on SDS-polyacrylamide gel. *E. coli* containing vector plasmids, pUEX1⁹ was analyzed in the same manner (lanes 1 and 5). Molecular weight markers (BRL) were run in lane M. Proteins blotted on the membrane were stained with Coomassie Brilliant Blue (lanes M, 1, 2, 3, and 4) or by immunostaining (lanes 5, 6, 7, and 8).

Sequencing of cDNA

The cDNA inserts of the three clones were about the same length. To confirm their identity, the cDNA inserts of the clones were characterized using restriction endonucleases (Fig. 2). The common restriction sites (upstream *Nco*I and *Cla*I) were observed in all of the inserts, while inserts of pFL11 lacked the *Eco*RV and downstream *Nco*I sites. The nucleotide sequence analyses were done on these clones by the strategy shown in Fig. 2.

The cDNA sequence and the deduced amino acid sequence from the cDNA insert of pFL1 is shown in Fig. 3. It consists of 501 nucleotides included a 17-nucleotide-long poly(A) tail. A putative polyadenylation signal, AATAAC, was located at position 474-479, 15 bp upstream from the poly(A) tail. An open reading frame consisting of 416 nucleotides was

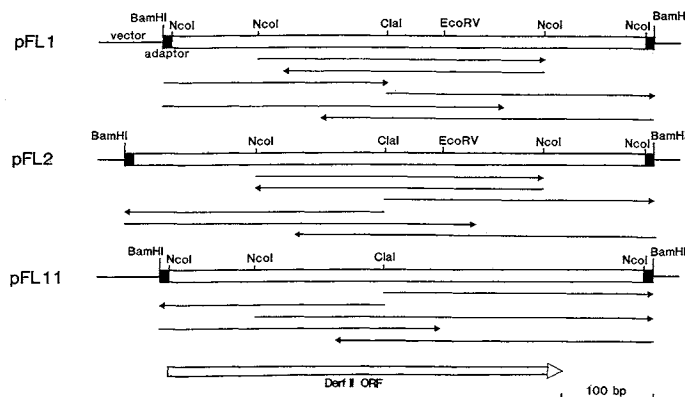


Fig. 2. Restriction Map and Sequencing Strategy for the Three cDNAs Encoding *Der f* II.

The open boxes indicate cloned fragments, and the closed boxes indicate the "adaptor." The sequencing strategy is indicated by arrows showing the direction and extent of DNA sequencing.

Adaptor			
1	GGT ACC ATG GGT TCA TTG TTG GTA GCA GCC	30	
1	Gly Thr Met Val Ser Leu Leu Val Ala Ala	10	
31	GTT GTT GCC GAT CAA GTC GAT GTT AAA GAT	60	
11	Val Val Ala Asp Gln Val Asp Val Lys Asp	20	
61	TGT GCC AAC AAT GAA ATC AAA AAA GTA ATG	90	
21	(Cys)Ala Asn Asn Glu Ile Lys Lys Val Met	30	
91	GTC GAT GGT TGC CAT GGT TCT GAT CCA TGC	120	
31	Val Asp Gly(Cys)His Gly Ser Asp Pro(Cys)	40	
121	ATC ATC CAT CGT GGT AAA CCA TTC ACT TTG	150	
41	Ile Ile His Arg Gly Lys Pro Phe Thr Leu	50	
151	GAA GCC TTA TTC GAT GCC AAC CAA AAC ACT	180	
51	Glu Ala Leu Phe Asp Ala Asn Gln Asn Thr	60	
181	AAA ACC GCT AAA ATT GAA ATC AAA GCC AGC	210	
61	Lys Thr Ala Lys Ile Glu Ile Lys Ala Ser	70	
211	CTC GAT GGT CTT GAA ATT GAT GTT CCC GGT	240	
71	Leu Asp Gly Leu Glu Ile Asp Val Pro Gly	80	
241	ATC GAT ACC AAT GCT TGC CAT TTT GTC AAA	270	
81	Ile Asp Thr Asn Ala Cys His Phe Val Lys	90	
271	TGT CCA TTG GTT AAA GGT CAA CAA TAT GAT	300	
91	Cys Pro Leu Val Lys Gly Gln Gln Tyr Asp	100	
301	ATC AAA TAT ACA TGG AAT GTG CCG AAA ATT	330	
101	Ile Lys Tyr Thr Trp Asn Val Pro Lys Ile	110	
331	GCA CCA AAA TCT GAA AAC GTT GTC GTT ACA	360	
111	Ala Pro Lys Ser Glu Asn Val Val Val Thr	120	
361	GTC AAA CTT ATC GGT GAT AAT GGT GTT TTG	390	
121	Val Lys Leu Ile Gly Asp Asn Gly Val Leu	130	
391	GCT TGC GCT ATT GCT ACC CAT GGT AAA ATC	420	
131	Ala Cys Ala Ile Ala Thr His Gly Lys Ile	140	
421	CGT GAT TAA AAA AAA ATA AAT AAG AAA ATT	450	
141	Arg Asp ***		
451	TTC ACC AAC ATC GAA CAA AAT TCA ATA ACC	480	
481	AAA ATT TGA ATC CAA AAA AAA AAA AAA	510	
511	ACC ATG G		
Adaptor			

Fig. 3. Nucleotide and Deduced Amino Acid Sequences of the cDNA Fragment of pFL1.

The predicted amino acid sequence is given below the nucleotide sequence. Adaptor sequences are shown by thick letters. The amino acid residues underlined show the identity with those of native *Der f II*. *** is a stop codon.

found to code for a polypeptide of 139 amino acids in the same frame as β -galactosidase.

The amino acids of native *Der f II* purified by HPLC were sequenced. Forty-five amino acid residues from the N-terminus could be identified and the sequence was completely the same as that deduced from the cDNA indicated by the underline in Fig. 3.

The mature *Der f II* protein is coded for by a single open reading frame ending at the TAA stop codon at nucleotide position 427–429 and consists of 129 amino acids containing six cysteine residues with a derived molecular weight of 14,043. The predicted *Der f II* amino acid sequence has no potential *N*-glycosylation sites. No significant similarities were found

between this sequence and other DNA and protein sequences in the EMBL/GENBANK and NBRF/SWISSPROT data banks.

We compared nucleotide sequences of cDNA inserts of pFL1 with pFL2 and pFL11. As shown in Fig. 4, several base substitutions were found among the different clones. It was confirmed that the differences of the restriction cleavage sites resulted from base substitutions. Among them, some of the substitutions caused amino acid changes as shown in Fig. 5. These base substitutions might be due to polymorphisms among gene sequences or to artifacts during cDNA synthesis and cloning procedures.

All the sequences included 5' proximal end sequences. That is, cDNA of the *Der f II* has a pre-sequence. Inspection of the amino acid sequence at the pre-sequence suggests that *Der f II* may have a signal peptide for secretion because of the presence of a hydrophobic core region rich in valine and leucine residues and a short alanine region just before the cleavage site (Fig. 3). With respect to pFL2, the open reading frame that coded for the *Der f II* gene was different from that of β -galactosidase, but it had 45 more nucleotides at the 5' region compared with the others. There was a translation initiation ATG codon at bases 34–37 in Fig. 4, though the immediate flanking region of the ATG codon (AAAATGA) showed little similarity with the Kozak consensus sequence (ACCATGG) for the eukaryotic translation initiation site.¹⁶⁾

Discussion

We have isolated three cDNA clones coding for the complete sequence of mature protein of a major mite allergen, *Der f II*, by using molecular and immunological techniques. Forty-five amino acid residues from the N-terminus were identified, and the sequence showed no discrepancy with those predicted from the DNA sequences. With respect to pFL2, we could not detect the fusion protein which reacted with anti-*Der f II* antibody by Western blotting analysis (Fig. 1), although the

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pFL1 1      10      20      30      40      50      60
pFL2 GGTACCATGG-----TTTCA
pFL11 *****--AAAAAAAACACATAACCGAAATGATTCCAAAATCTTGTGCC*****

pFL1 61      70      80      90      100     110     120
pFL2 TTGTTGGTAGCAGCCGTTGTTGCCGATCAAGTCGATGTTAAAGATTGTGCCAACAAATGAA
pFL11 *****

pFL1 121     130     140     150     160     170     180
pFL2 ATCAAAAAGTAATGGTCGATGGTTGCCATGGTCTGATCCATGCATCATCCATCGTGGT
pFL11 *****

pFL1 181     190     200     210     220     230     240
pFL2 AAACCAITTCACITTTGGAAGCCTTATTCGATGCCAACCAAAACACTAAAACCGCTAAAATT
pFL11 *****

pFL1 241     250     260     270     280     290     300
pFL2 GAAATCAAGCCAGCCCTCGATGGTCTTGAAATTGATGTTCCGGTATCGATACCAATGCT
pFL11 *****

pFL1 301     310     320     330     340     350     360
pFL2 TGCCATTTTGTCAAATGTCCATTGGTTAAAGGTCAACAATATGATATCAATATACATGG
pFL11 *****A•G•*****GC*****

pFL1 361     370     380     390     400     410     420
pFL2 AATGTGCCGAAATATGCACCAAAATCTGAAACGTTGTCGTTACAGTCAAATATATCGGT
pFL11 *****G•T•*****

pFL1 421     430     440     450     460     470     480
pFL2 GATAATGGTCTTTTGGCTTGGCTATTGCTACCCATGGTAAATCCGTGATTAAAAAAA
pFL11 *****C•C•*****

pFL1 481     490     500     510     520     530     540
pFL2 A---TAAATAAGAAAATTTTCACCAACATCGAACAAAATTCATAACCAAAATTTGAATC
pFL11 *****T*****
pFL11 *****T*****

pFL1 541     550     560
pFL2 CAAAAA-----CCATGG
pFL11 *****

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Fig. 4. Comparison of the Nucleotide Sequences of the Three cDNA Fragments Encoding *Der f* II.

Identical nucleotides at the same position among cDNA of pFL1 and others are indicated by (*). Bars in the nucleotide sequences denote the deletion of corresponding nucleotides. The termination codon is underlined. Adaptor sequences are shown by thick letters.

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Clone No.1 10      20      30      40      50      60
Clone No.2 DQVDV KDCAN NEIKK VMVDG CHGSD PCIIH RGKPF TLEAL FDANQ NTKTA KIEIK ASLDG
Clone No.11 -----

Clone No.1 70      80      90      100     110     120
Clone No.2 LEIDV PGIDT NACHF VKCPL VKGQQ YDIKY TWNVP KIAPK SENNV VTVKL IGDNG VLACA
Clone No.11 -----M-----A-----V-----

Clone No.1 129
Clone No.2 IATHG KIRD
Clone No.11 -----A-----

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Fig. 5. Comparison of the Deduced Primary Structures (Single Letter Code) of Mature *Der f* II.

Identical amino acids at the same position among clone No. 1 and others are indicated by (—).

clone had been isolated by colony immunoassay. The deduced amino acid sequence of mature *Der f* II of pFL2 is in a different translational reading frame from that for the β -galactosidase. It is unknown why clone No. 2 could be isolated by colony immunoassay. A small amount of *Der f* II protein may be expressed through the upstream β -galactosidase reading frame, a phenomenon called

'translation coupling'.¹⁷⁾

There were minor differences observed among the three genes (Fig. 4 and Fig. 5), but gene products of pFL1 and pFL11 reacted similarly with anti-*Der f* II antibody (Fig. 1). Therefore, the minor differences in the amino acid sequence seem not to be very important for their antigenicity. Furthermore, it was suggested that *Der f* II protein does not consist

of a single molecule but a mixture of different molecules in the mite culture. It will be important to clarify this point in the future.

Heymann *et al.* clarified the amino acid sequences of *Der p* II and *Der f* II from the N-terminus to the 35th amino acid residue. The partial sequences were very similar.¹⁸⁾ The amino acid sequence of *Der f* II based on the cDNA sequence agreed well with their results. The comparison of the total amino acid sequences of *Der p* II and *Der f* II is very interesting. Therefore, the cloning of *Der p* II gene may be necessary for further study.

The cDNA coding for *Der p* I was cloned and sequenced, but the recombinant protein did not bind IgE antibodies from mite-allergic subjects.^{19,20)} Tovey *et al.* have recently isolated a clone from a cDNA library of the mite *D. pteronyssinus* using human sera of mite-allergic subjects.²¹⁾ The clone produced an IgE-binding recombinant protein of molecular weight about 14,000. Both nucleotide and deduced amino acid sequences of the clone were quite different from those of our cloned *Der f* II genes. It is interesting to examine whether the recombinant *Der f* II proteins react with human serum IgE.

It has been suggested that *Der I* allergen might be derived from a digestive protease secreted from the gastrointestinal tract of the mite, since it is closely related to fecal particles²²⁾ and its deduced amino acid sequence from a cDNA clone is similar to those of cysteine proteases.²⁰⁾ The deduced amino acid sequence of *Der f* II showed no significant homology with *Der p* I and any other proteins in databases, although *Der f* II has six cysteine residues. It is interesting that *Der f* II also has a pre-sequence like signal peptide as well as *Der p* I.

Since it has become possible to produce *Der f* II allergen by the genes coding for with *Der f* II, the technique may be used for the production of *Der f* II in a large quantity. If the desensitization therapy as well as diagnosis by standardized and purified mite major allergen is available for various atopic diseases caused by the house dust mites, it will be of

great advantage for the clinicians as well as for those working on basic research in allergy.

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