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(polyA+) was isolated.

The cDNA was synthesized by the method of Gubler and Hoffman\(^8\) using a kit (cDNA synthesizing system plus, RPN1256Y; Amersham International plc.).

The cDNA library was expressed in Escherichia coli MC1061 using an expression vector, pUEX1\(^9\) (cDNA cloning system-plasmid pUEX1, RPN1282; 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 antisemur 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 overlayed 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 DNasel (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 serum\(^11\) was kindly supplied by Dr. Yasueda (National Sagamihara 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 was used as a 4–20% gradient gel. After electrophoresis, 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-l-naphthol and H2O2.

Sequence 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 pUC19\(^13\) for sequence analysis. The cDNA insert was excised by BamH I. BamH I-cDNA fragments were digested with various restriction endonucleases such as Neo1 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 M13K07. Single-strand DNAs were extracted from the resulting phage particles. DNA sequencing was done by the method of Sanger\(^14\) using a 7-DEAZA sequencing kit (Takara). The sequence was analyzed using the sequence analysis software DNASIS (Hitachi Software Service).

Sequence 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. x 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 into 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.

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 NcoI and Clal) were observed in all of the inserts, while inserts of pFL11 lacked the EcoRV and downstream NcoI 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 including 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. 3. Nucleotide and Deduced Amino Acid Sequences of the cDNA Fragment of pFLI.

The predicted amino acid sequence is given below the nucleotide sequence. Adapter sequences are shown by thick letters. The amino acid residues underlined show the identity with those of native Derfll. *** 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 DNA indicated by the underline in Fig. 3. 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...
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 react 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. 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. Tovey et al. have recently isolated a clone from a cDNA library of the mite *D. pteronyssinus* using human sera of mite-allergic subjects. 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 particles and its deduced amino acid sequence from a cDNA clone is similar to those of cysteine proteases. 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.

References