

# Nucleotide sequence analysis of a complementary DNA coding for a *Blomia tropicalis* allergen

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*Blomia tropicalis* is a mite of allergenic importance in tropical and subtropical areas. A clone (Bt11a) from a *B. tropicalis* complementary DNA library was expressed in lambda phage and analyzed by plaque radioimmunoassay. The recombinant allergen produced by this clone was bound by IgE in 16 of 32 sera from individuals with asthma with a positive RAST response and none of 3 control sera from healthy individuals with negative RAST response to *B. tropicalis*. The cDNA insert was amplified by polymerase chain reaction with use of universal primers. A 582-base-pair (bp) fragment was cloned into a pCR II vector. The complete sequence of both strands was determined by using T7, SP6, and internal primers. The sequence shows a 432 bp reading frame with a 34 bp 5' untranslated region and a 116 bp 3' untranslated region with a poly A tail. Analysis of the sequence suggests that it encodes a putative signal peptide of 20 residues and a 124-residue mature protein allergen of 14,206 Da. The nucleotide and the inferred amino acid sequences did not show homology to any known sequence. No potential N-linked glycosylation site was found. The recombinant protein appears to represent a major allergen of the mite *B. tropicalis*. (*J Allergy Clin Immunol* 1996;98:932-7.)

**Key words:** *Blomia tropicalis*, recombinant allergens, cDNA sequence, mite allergens

*Blomia tropicalis*, a domestic mite of the family Glycyphagidae, is commonly found in house dust samples in tropical and subtropical areas of the world.<sup>1-6</sup> In the United States, it is the fourth most common mite species in house dust and is most common in the southern subtropical states.<sup>7,8</sup> The role of this mite as an inducer of allergic asthma and allergic rhinitis has been well documented.<sup>2,3,9-11</sup>

The prevalence of IgE antibody responsiveness to *B. tropicalis* allergens has been investigated by

## Abbreviations used

|       |                                    |
|-------|------------------------------------|
| bp:   | Base pair                          |
| BSA:  | Bovine serum albumin               |
| cDNA: | Complementary DNA                  |
| LB:   | Luria-Bertani medium               |
| mRNA: | Messenger RNA                      |
| PBS:  | Phosphate-buffered saline solution |
| PCR:  | Polymerase chain reaction          |

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RAST and immunoblots using sera from allergic individuals. Of the 25 IgE-binding components detected in such sera, at least 4 were reported to be important on the basis of their frequency of binding to IgE.<sup>10</sup> By using crossed radioimmuno-electrophoresis, 21 different IgE-binding antigens from *B. tropicalis*, most of them species specific, have been detected.<sup>12</sup> *B. tropicalis* has little cross-reactivity with other mite species such as *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*.<sup>2,9,12</sup> Several allergens from these two species of Pyroglyphidae have been characterized by complementary DNA (cDNA) cloning, monoclonal anti-

bodies, and immunoblotting.<sup>13-16</sup> However, the allergens from *B. tropicalis* remain less well characterized.

Most sera from allergic individuals recognize five to nine allergens in *B. tropicalis* extracts.<sup>10</sup> To assess their relative importance, it is necessary to characterize these allergens and quantitate their reactivity with specific IgE and T cells. A cDNA library of *B. tropicalis* has been constructed to characterize these allergens and to provide reagents for epitope analysis.<sup>17</sup> We report the nucleotide sequence of the recombinant allergen produced by a clone isolated from that library and show that it reacts with a 50% frequency to specific IgE in the sera of patients allergic to *B. tropicalis*.

## METHODS

### Serum sources

Sera collected from 32 individuals with asthma were used to establish the IgE-binding frequency of the recombinant allergen by plaque immunoassay. The sera were obtained from adults in Tampa, Florida, who had positive skin test and RAST responses to *B. tropicalis*. Three sera from healthy donors with negative skin test and RAST responses to house dust mites and with no history of respiratory allergies were selected as controls. The sera were preadsorbed with the lysate of *Escherichia coli* Y1090 to remove IgE directed against those proteins that would interfere with the plaque radioimmunoassay.

### cDNA library

A *B. tropicalis*/lambda gt11 cDNA library was constructed previously as follows<sup>17</sup>: 1 gm of live *B. tropicalis* was frozen in liquid nitrogen, and the total RNA was extracted by using RNeasy-B solution (Qiagen/Biotech Laboratory International, Inc., Friendwood, Tex.). The messenger RNA (mRNA) was isolated by using a cellulose poly-dT column, and the cDNA synthesized by using the Directional Cloning Tool Box Kit (Pharmacia LKB Biotechnology, Piscataway, N.J.). The cDNA was ligated into the *EcoRI*/*NotI* cloning site of the lambda gt11D phage vector (Pharmacia LKB Biotechnology) and packaged in *E. coli* Y1090. The cDNA library was immunoscreened by using serum pooled from individuals allergic to *B. tropicalis*. Clones that bound IgE were stored in dimethyl sulfoxide at  $-70^{\circ}\text{C}$ . One of these clones, Bt11a, was selected for the current study.

### Frequency of IgE binding

The frequency of IgE binding was determined by the plaque radioimmunoassay method<sup>18</sup> as follows: an overnight culture of *E. coli* Y1090 was diluted 1:10 in Luria-Bertani medium (LB) containing 100  $\mu\text{g}/\text{ml}$  ampicillin and 0.4% maltose and incubated at  $37^{\circ}\text{C}$  for 1 hour. The bacteria were pelleted and resuspended in 3 ml of magnesium sulfate 10 mmol/L. For 10 cm Petri dishes, 100  $\mu\text{l}$  of *E. coli* Y1090 was incubated with 100  $\mu\text{l}$  of diluted lambda gt11/Bt11a phage for 20 minutes at

$37^{\circ}\text{C}$  and then plated on LB agar in 3.5 ml of 0.7% agarose overlay and incubated for 3 to 4 hours, after which plaques became visible. At this time a nitrocellulose filter, which had been saturated with 10 mmol/L isopropyl  $\beta$ -D-thiogalactoside and dried, was placed on top of the lawn. After overnight incubation at  $37^{\circ}\text{C}$ , the filter was removed and blocked with phosphate-buffered saline solution (PBS)/3% bovine serum albumin (BSA)/0.1% Tween-20 for 1 hour. The filter was cut into triangular strips ( $1 \times 3.5 \times 3.5$  cm) that were individually incubated overnight at room temperature with 2 ml of sera (diluted 1:4 in PBS/3% BSA) from each of 32 subjects with asthma with IgE antibodies to *B. tropicalis* and from 3 nonallergic controls. One strip was incubated in PBS/3% BSA instead of in serum. After three washes with PBS/3% BSA, each strip was incubated overnight with 2 ml of iodine 125-labeled antihuman IgE (Kallestad Diagnostics, Inc., Chaska, Minn.) diluted in PBS-3% BSA and containing 100,000 counts per minute. After three washes with PBS, the strips were exposed for 24 to 48 hours to x-ray film with intensifying screens at  $-70^{\circ}\text{C}$  and developed.

### Polymerase chain reaction amplification

A lysate of the Bt11a clone was prepared by the plate-culture lysate method.<sup>19</sup> Fifteen microliters of the lysate was used in a 100  $\mu\text{l}$  polymerase chain reaction (PCR) reaction mix containing 0.4 mmol/L of lambda gt11 forward and reverse primers, 2.5 U Taq polymerase (Promega, Madison, Wis.), 0.2 mmol/L deoxyribonucleoside triphosphates, and 10  $\mu\text{l}$  of  $10\times$  Taq polymerase buffer M1904 (Promega). The reaction was allowed to proceed for 30 cycles of amplification in a thermal cycler (Coy Temp Cycler II model 110S; Coy Corporation, Grass Lake, Mich.); each cycle consisted of  $95^{\circ}\text{C}$  for 1 minute,  $50^{\circ}\text{C}$  for 1 minute, and  $72^{\circ}\text{C}$  for 1 minute. The final extension cycle was performed at  $72^{\circ}\text{C}$  for 7 minutes.

### Subcloning of PCR product into a sequencing vector

The amplification product was ligated into the pCR II vector (Invitrogen, San Diego, Calif.). The resulting recombinant plasmid DNA was transformed into the INV $\alpha$ F' *E. coli* strain (Invitrogen) by using a standard protocol.<sup>19</sup> Color selection of transformed colonies was accomplished by plating the transformation mix on LB plates containing 50  $\mu\text{g}/\text{ml}$  ampicillin and 60  $\mu\text{g}/\text{ml}$  of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside. These recombinant clones were confirmed by restriction enzyme analysis of plasmid DNA isolated from colorless colonies and digested with *EcoRI* and *NotI*.

### DNA sequencing

Small-scale plasmid DNA was isolated from INV $\alpha$ F' *E. coli* by using Wizard minipreps (Promega). For DNA sequencing, 4  $\mu\text{g}$  double-stranded plasmid DNA was denatured for 5 minutes at room temperature in an alkaline solution containing 2 mol/L NaOH and 2 mmol/L

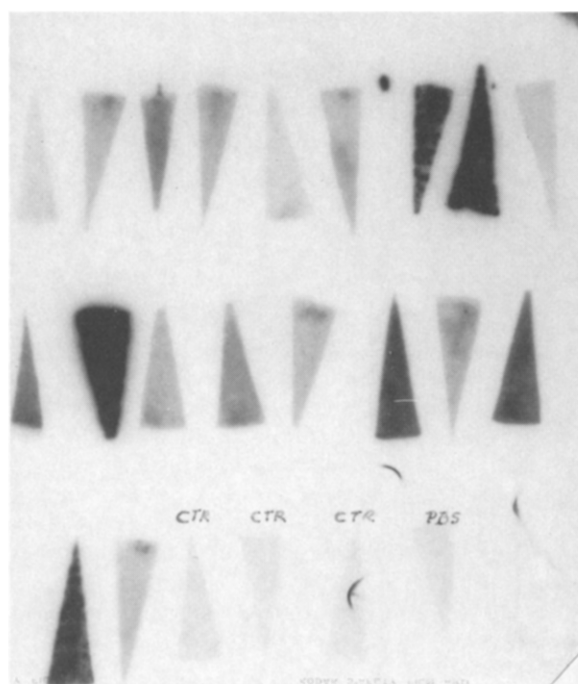


**FIG. 1.** Plaque immunoassay results showing the reactivity of some of the sera tested with the Bt11a clone. The following strips are positive (from left to right): *Upper row*—Third, seventh, and eighth; *middle row*—first, second, fourth, sixth, and eighth; *lower row*—first. CTR, Strips incubated with serum from noncontrols with no allergy; PBS, strip incubated with PBS/3% BSA.

ethylenediaminetetraacetic acid and neutralized with 8  $\mu$ L of 5 mol/L ammonium acetate, pH 7.5. Nucleotide sequence analysis was performed by the dideoxynucleotide chain termination method<sup>20</sup> with the TaqTrack Sequencing System Kit from Promega. DNA was labeled with  $\alpha$ -<sup>35</sup>S deoxyadenosine triphosphate using the manufacturer's extension and labeling protocol. Initial sequencing was performed by using T7 and SP6 primers to sequence in both directions. Two internal primers (5'-CATTCGGATGACTTGATCGT-3' and 5'-GGATCGAATTTGGTTC-CAAT-3') obtained from the DNA Sequencing Core Laboratory/Interdisciplinary Center for Biotechnology Research (Gainesville, Fla.) were designed from the initial nucleotide sequence and used to obtain the complete sequence of the Bt11a clone.

### Sequence analysis

Sequence analysis was performed with MAC Vector software (International Biotechnologies Inc., New Haven, Conn.).\* Computation for sequence homology with other nucleotide sequences and proteins on international data-

\*The nucleotide sequence of Bt11a has been submitted to the GenBank Genetic Sequence Data Bank (National Institutes of Health, Bethesda, Md.) with the accession number U27479.

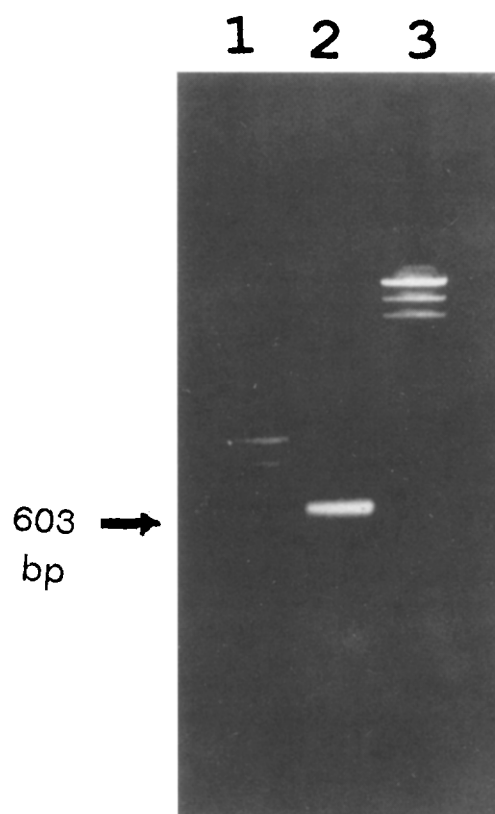


**FIG. 2.** Gel electrophoresis in 1% agarose/ethidium bromide. Lane 1,  $\Phi$ X174/HaeIII DNA marker; lane 2, PCR product; lane 3, lambda HindIII DNA marker.

bases was done with BLASTX and BLASTP,<sup>21</sup> version 1.4.7MP, and FASTA database searching programs.<sup>22</sup>

## RESULTS

### IgE binding of the Bt11a clone

With the plaque radioimmunoassay, IgE reactivity to the Bt11a clone was found in 16 (50%) of the 32 sera from subjects with asthma and allergy to *B. tropicalis*. None of the strips incubated with the sera from the 3 controls without allergy showed IgE binding (Fig. 1).

### PCR amplification and subcloning

The size of the amplification product, as determined by electrophoretic separation in 1% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide, was approximately 620 bp (Fig. 2). Restriction analysis of the recombinant plasmid pCR II from several transformed colonies revealed the expected *EcoRI/NotI* 620-bp insert.

### Sequence of Bt11a

The complete nucleotide sequence of the Bt11a clone consists of 582 bp. The sequence has an ATG

|                                                             |                         |     |
|-------------------------------------------------------------|-------------------------|-----|
| GGCACGAGATAGTAACTTACAAATAGAAAAAAG                           | ATG AAA TCT GTT TTG ATT | 52  |
|                                                             | Met Lys Ser Val Leu Ile |     |
| TTT TTG GTT GCT ATT GCC TTA TTC TCG GCT AAC ATT GTT TCA GCT |                         | 97  |
| Phe Leu Val Ala Ile Ala Leu Phe Ser Ala Asn Ile Val         | <u>Ser Ala</u>          |     |
| GAT GAG CAA ACC ACT CGC GGT CGA CAC ACC GAA CCC GAT GAT CAT |                         | 142 |
| Asp Glu Gln Thr Thr Arg Gly Arg His Thr Glu Pro Asp Asp His |                         |     |
| CAC GAA AAG CCA ACG ACC CAG TGC ACC CAT GAG GAG ACA ACA TCA |                         | 187 |
| His Glu Lys Pro Thr Thr Gln Cys Thr His Glu Glu Thr Thr Ser |                         |     |
| ACT CAA CAT CAT CAT GAA GAA GTA GTA ACC ACT CAA ACT CCT CAT |                         | 232 |
| Thr Gln His His His Glu Glu Val Val Thr Thr Gln Thr Pro His |                         |     |
| CAT GAA GAG AAG ACC ACA ACA GAG GAA ACT CAT CAT TCC GAT GAC |                         | 277 |
| His Glu Glu Lys Thr Thr Thr Glu Glu Thr His His Ser Asp Asp |                         |     |
| TTG ATC GTC CAT GAA GGA GGA AAA ACT TAT CAT GTA GTC TGT CAC |                         | 322 |
| Leu Ile Val His Glu Gly Gly Lys Thr Tyr His Val Val Cys His |                         |     |
| GAA GAA GGA CCC ATT CAC ATC CAG GAA ATG TGC AAC AAA TAT ATA |                         | 367 |
| Glu Glu Gly Pro Ile His Ile Gln Glu Met Cys Asn Lys Tyr Ile |                         |     |
| ATA TGT TCC AAA TCT GGT TCA TTG TGG TAC ATT ACC GTA ATG CCA |                         | 412 |
| Ile Cys Ser Lys Ser Gly Ser Leu Trp Tyr Ile Thr Val Met Pro |                         |     |
| TGT TCG ATT GGA ACC AAA TTC GAT CCA ATT TCT CGA AAT TGT GTA |                         | 457 |
| Cys Ser Ile Gly Thr Lys Phe Asp Pro Ile Ser Arg Asn Cys Val |                         |     |
| CTT GAT AAT TAATTGATTAATTGTTCTATTCGATTATTTTCGTATATCGATTAAAA |                         | 514 |
| Leu Asp Asn                                                 | <u>—</u>                |     |
| GATGATTGAATAATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA    |                         | 574 |
| AAAATTCT                                                    |                         | 582 |

**FIG. 3.** Nucleotide sequence and predicted amino acid sequence of Bt11a clone. The first 20 amino acid residues are a putative leader sequence followed by a 124-residue polypeptide of 14,206 Da. The proposed cleavage site for the leader peptide, the TAA stop codon, and the polyadenylation signal are underlined.

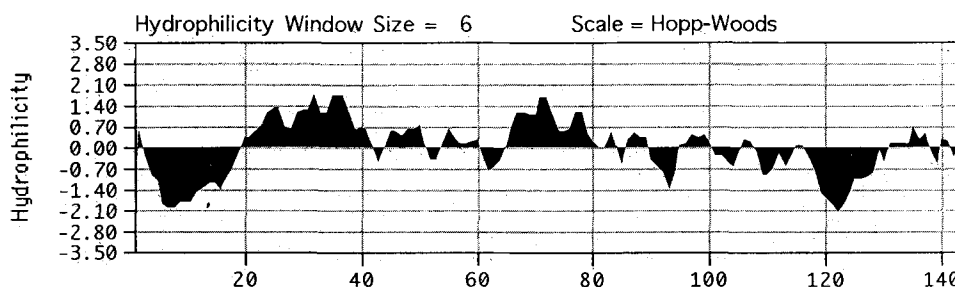


FIG. 4. Hydrophilicity plot of the inferred protein with the Hopp-Woods algorithm<sup>29</sup> computed with the Mac Vector sequence analysis software (IBI, New Haven, Conn.) and use of a six-residue window. Positive values indicate relative hydrophilicity and negative values indicate relative hydrophobicity.

start codon at position 35 to 37 and a stop codon, TAA, at position 467 to 469, which indicates a reading frame coding for a 144 amino acid residue protein. The 3' 116 bp untranslated region has a polyadenylation signal at positions 526 to 531 (Fig. 3). The inferred amino acid sequence accounted for a probable 20-residue signal peptide and a mature protein of 14,206 Da. There was no apparent *N*-glycosylation site in the predicted amino acid sequence. The nucleotide and inferred amino acid sequences did not have any homology with other known proteins.

## DISCUSSION

The 50% frequency of IgE binding of the protein expressed by Bt11a clone in *E. coli* indicates that this clone encodes an important allergen. The complete mature protein is encoded by an open reading frame terminating at the TAA stop codon at position 467 to 469. The ATG codon at nucleotide position 35 to 37 is probably the translation initiation site because the subsequent sequence codes for a peptide of 18 amino acids with predominantly hydrophobic residues, which form a classic signal peptide,<sup>23</sup> as shown in the hydrophilicity plot (Fig. 4). In addition, the sequence before ATG (AAAGATG) conforms with the Kozak consensus sequence CCACCATG for the eukaryotic translation initiation site with the crucial purine A at the -3 position.<sup>24,25</sup> This reading frame was confirmed by the finding that Bt11a was expressed in the pGEMEX-2 vector as a fusion protein of 47.5 kD, accounting for the predicted allergen peptide plus the T7-10 peptide from the vector.<sup>26</sup>

Considering that the gene sequence has an ATG start codon and a TAA stop codon followed by a poly A tail, the deduced amino acid sequence is probably the complete sequence of a *B. tropicalis* allergen. The 21 amino acids at the N-terminal end

have a positively charged lysine near the N-terminus, with the remaining amino acids being very hydrophobic, indicative of a typical leader sequence.<sup>23</sup> The putative cleavage site for the signal peptide is between amino acid residues 20 and 21, because the residues around this site are in agreement with those at the cleavage site.<sup>27,28</sup> Thus, the mature protein should have 124 amino acid residues with a molecular weight of 14,200. Although the precise molecular weight of the native mite component is not shown and the exact N-terminal sequence has not yet been identified, the data obtained strongly suggest that the cloned cDNA corresponds to the full-length mRNA. It is probable that the recombinant allergen encoded by Bt11a has its natural counterpart in the 14,400 Da allergen identified by immunoblot of the native allergenic extract of *B. tropicalis*.<sup>10</sup> This allergen demonstrated a 38.1% frequency of IgE binding against a battery of 100 sera from individuals in Cartagena who were allergic to *B. tropicalis*. The smaller sample number and the differences in the population source of sera tested with Bt11a may account for the higher frequency of IgE binding (50%) obtained with the recombinant allergen encoded by Bt11a.

The nucleotide and inferred amino acid sequences of the cDNA coding for this allergen show no homology to any known protein, including other *B. tropicalis* allergens cloned in our laboratory. The Bt-M clone codes for a peptide that shares epitopes with a 14,000 Da allergen of *B. tropicalis*.<sup>17</sup> However, there is complete lack of homology between these two sequences. Thus, the *B. tropicalis* allergen sequenced in the present study appears to be unique.

The IgE binding results showing reactivity of the Bt11a allergen with 50% of the sera from subjects with allergy indicate that this allergen may play an

important role in allergic diseases. These data support further studies to identify B and T cell epitopes and to investigate the use of recombinant allergens in desensitization programs.

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