Volume 279, number 2, 210–215 FEBS 09407 February 1991

ADONIS 001457939100095R

Cloning and sequencing of *Lol p*I, the major allergenic protein of rye-grass pollen

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Received 5 December 1990

We have isolated a full length cDNA clone encoding the major glycoprotein allergen *Lol pI*. The clone was selected using a combination of immunological screening of a cDNA expression library and PCR amplification of *Lol pI*-specific transcripts. *Lol pI* expressed in bacteria as a fusion protein shows recognition by specific IgE antibodies present in sera of grass pollen-allergic subjects. Northern analysis has shown that the *Lol pI* transcripts are expressed only in pollen of rye-grass. Molecular cloning of *Lol pI* provides a molecular genetic approach to study the structure–function relationship of allergens.

Lolium perenne; Allergen; cDNA cloning; Nucleotide sequence; Amino acid sequence

1. INTRODUCTION

Hayfever and seasonal allergic asthma due to grass pollen allergens are environmental diseases that afflict up to 25% of the population in cool temperate climates [1–3]. Although a degree of cross-reactivity has been described among antigens from different grass species [4–6], it is well established that rye-grass antigens are the most reactive allergens [7,8]. The most prominent allergen in rye-grass pollen is group I or *Lol pI* (IUIS nomenclature [9]). This has been shown to be the major IgE-binding protein [7,8]. Elevated levels of *Lol pI*-specific IgE have been detected in sera of up to 95% of grass pollen-allergic individuals, and thus of major clinical significance [7,8]. Other clinically significant allergens of rye-grass include *Lol p*II, III, IV [9] and the newly identified *Lol p*Ib [10].

Lol pI is an abundant acidic glycoprotein of M_r 35 kDa [8] and *p*I 5.5 [10], located in the cytosol of the pollen [10,11]. The amino acid sequence of this allergen has not been reported, although short sequences from an N-terminal and an internal peptide fragment have been determined [12,13]. In this paper, we report the cloning, expression and sequencing of a full-length cDNA clone encoding *Lol pI*.

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2. MATERIALS AND METHODS

2.1. *Plant materials and antibody probes*

Pollen of *Lolium perenne* was collected from plants flowering in Melbourne, and stored in liquid nitrogen until used. MAbs 40.1, 21.3 and 3.2 are specific for *Lol pI*, and their preparation and characterization have been described elsewhere [4,10,14–16]. IgE antibodies were from sera of grass pollen-allergic individuals, as determined by skin prick test and/or RAST (Radioallergosorbent test).

2.2. *Isolation of pollen proteins and immunoblotting*

Soluble proteins were extracted from rye-grass pollen by vigorous shaking in PBS (150 mM, pH 7.2) containing 1 mM PMSF, on ice for 3 h. Conditions for electrophoresis and immunoblotting with MAbs were essentially as described [16]. For IgE antibody binding, blots were incubated in allergic sera collected from at least 4 patients with high RAST scores for grass pollen, pooled and used diluted 1:5 in TBS/0.5% BSA. The bound IgE was detected using ^{125}I-labelled anti-human IgE (Kallestad, USA) essentially as described by Ford and Baldo [8].

2.3. *Purification of* Lol p*Ia for N-terminal sequencing*

Lol pI was isolated using preparative isoelectric focussing (Rotofor, BioRad, Richmond, CA) followed by SDS-PAGE (A. Avjioglu and M.B. Singh, unpublished data). The allergen was recovered from SDS-PAGE gels by electroftransfer (90 V/2 h; 4°C) onto polyvinylidene difluoride (PVDF) membrane, (Millipore, Bedford, MA) [17]. Proteins were visualized by staining with Coomassie brilliant blue R-250, washed extensively with deionized water [17] and subjected to sequence analysis [17,18]. Amino acid residues are given using the one letter notation.

2.4. *cDNA library and immunological screening*

Poly (A^+) mRNA was isolated essentially as described [19]. cDNA was synthesized [20] and cloned into the *Eco*RI site of the vector lambda-gt 11. Immunological screening was done by plating the

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cDNA library, and screening duplicate filters with MAb 40.1, using peroxidase-labelled anti-mouse Ig as secondary antibody. The plaques that were antibody-positive on both of the duplicate filters were picked off, purified, then replated and tested for binding to other MAbs as well as IgE antibodies.

2.5. *Nucleotide sequencing*

The cDNA clone 13R was isolated from the phage, and subcloned into pGEM-3Z vectors (Promega). DNA sequence was determined by double-stranded sequencing carried out by the dideoxy chain termination method [21], using T7 DNA polymerase (Pharmacia). PCR amplified DNA was cloned into M13 vector for dideoxy sequencing using sequenase (US Biochemicals).

2.6. *Oligonucleotides*

Oligonucleotides used in this study are shown in Table I. Anchor Primer (AP), Anchor Linker (AL) and Anchor Template (AT) have been described previously [22]. LpA-3 and LpA-5 were derived from the sequence of the *Lol p*I cDNA clone 13R. Sequencing primers were derived from the internal sequence of PCR-generated clones. All oligomers were purchased from Research Genetics (Huntsville, AL, USA).

2.7. *Polymerase chain reaction (PCR) cloning*

Full length clones encoding *Lol p*I were obtained using a modification [22] of the Anchored PCR [23,24]. Double stranded cDNA was synthesized from 1 μg of total RNA with the cDNA Synthesis System (Life Technologies, Inc., Gaithersberg, MD, USA) using oligo dT as a primer, blunt ended with T4 polymerase and blunt end ligated to self-annealed AT and AL primers. Linkered cDNA (3 μl from a 20 μl reaction) was mixed with 100 pmol each of AP and LpA-5 primers, 10 μl of 10X reaction buffer (GeneAmp kit, US Biochemicals, Cleveland, OH, USA) and 0.5 μl of *T. aquaticus* polymerase. The mixture was brought to 100 μl with deionized water and amplified for 25 cycles as described [22]. Briefly, the first 5 cycles consisted of denaturation at 94°C for 1 min, annealing at 45°C for 1.5 min, and polymerization at 70°C for 1.5 min. The last 20 cycles were as stated, except that annealing was at 55°C. 0.01 volume of the primary PCR was then reamplified with oligomers AP and LpA-3 as above. LpA-3 is nested (internal) relative to oligomer LpA-5 used in the primary PCR. DNA from the secondary PCR was recovered by sequential chloroform, phenol and chloroform extractions, precipitated with 0.5 vol. 7.5 M AmoAc and 1.5 vol. isopropanol. DNA from the pellet was digested with *Xba* I and *Pst* I, run on a preparative low melt gel, and ligated into *Xba* I/*Pst* I-digested M13.

3. RESULTS

3.1. *Identification of* Lol p*I by immunoblotting*

The recombinant clone (13R) was isolated using *Lol p*I-specific MAbs, which provides evidence of its relationship to the native allergen. On Western blots of pollen proteins, MAbs 3.2, 21.3 and 40.1 show strong affinity for a 35-kDa protein (Fig. 1). This protein showed high specificity for IgE antibodies from pooled sera of grass pollen-allergic individuals (Fig. 1). All the MAbs and IgE used in this study bound strongly to the *Lol p*I reference standard provided by NIAID (National Institutes of Health, Bethesda, MD; data not shown).

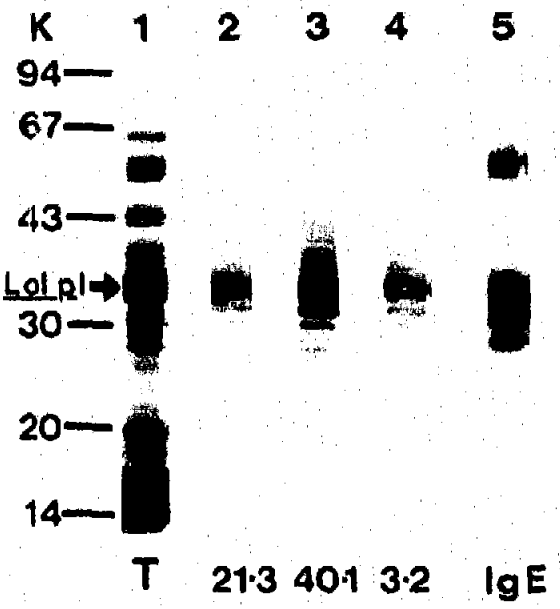

Fig. 1. Immunoblot analysis of IgE and MAb binding to *Lol p*I proteins from rye-grass pollen. M_r (kDa) is denoted on the left. 120 g of pollen proteins were loaded per lane. *Lol p*I is denoted with an arrow. Lane 1: SDS-separation of total rye-grass pollen proteins (staining is with Coomassie brilliant blue R-250); lanes 2-5: binding of MAbs or IgE on Western blots of total rye-grass pollen proteins.

3.2. *N-terminal sequence of* Lol p*I*

We purified *Lol p*I by two-dimensional gel electrophoresis involving preparative isoelectric focussing in the first dimension, followed by SDS-PAGE of the individual fractions. This procedure successfully

Table I

Oligonucleotide sequences

Oligomer	Nucleotide sequence	Strand	Nucleotides
AL	5' p-AATGATCGATGCT	------	------
AP	5' GGGTCTAGAGGTACCGTCCG	------	------
AT	5' GGGTCTAGAGGTACCGTCCGATCGATCATT	------	------
LpA-IA	5' GGAGTCGTGGGGAGCAGTC	coding	671->689
LpA-2	5' GGGAATTCCATGGCGAAGAAGGGC	coding	442->458
LpA-3	5' CCCTGCAGTCATGCTCACTTGGCCGAGTA	non-coding	810<-827
LpA-5	5' CCCTGCAGATTATTIGAGATCTTGAG	non-coding	867<-884
LpA-9	5' GTGACAGCCTCGCCGG	non-coding	351<-367
LpA-10	5' CCGTCGACGTACTTCA	non-coding	582<-607

AL and AT are synthetic linker oligonucleotides containing *Xba* I, *Kpn* I and/or *Cla* I restriction sites [22]. The AP sequence is contained within the AT oligomer [22]. LpA-IA, -2, -3, -5, -9 and -10 correspond to the coding or non-coding sequence of *Lol p*I, as indicated. LpA-2 contains an *Eco* RI restriction site, while LpA-3 and -5 contain *Pst*I restriction sites added for cloning purposes. All restriction sites are underlined.

a

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                                                        -20
CATTAAGCAGCAACCAACAAATTCAAGACAAG ATGGCGTCCTCCTCGTCGGTGCTCCTG      59
                                 M  A  S  S  S  S  V  L  L

                -10                     1
GTGGTGGCGCTGTTCGCCGTGTTCCTGGGCAGCGCGCATGGCATCGCGAAGGTACCACCG     119
 V  V  A  L  F  A  V  F  L  G  S  A  H  G  I  A  K  V  P  T

          10                    20
GGCCCCAACATCACGGCCGAGTACGGCGACAAGTGGCTGGACGCGAAGAGCACCTGGTAT     179
 G  P  N  I  T  A  E  Y  G  D  K  W  L  D  A  K  S  T  W  Y

          30                    40
GGCAAGCCGACCGGCGCCGGTCCCAAGGACAACGGCGGCGCGTGCGGGTACAAGGACGTC     239
 G  K  P  T  G  A  G  P  K  D  N  G  G  A  C  G  Y  K  D  V

          50                    60
GACAAGGCGCCGTTCAACGGCATGACCGGCTGCGGCAACACCCCCATCTTCAAGGACGGC     299
 D  K  A  P  F  N  G  M  T  G  C  G  N  T  P  I  F  K  D  G

          70                    80
CGTGGCTGCGGCTCCTGCTTCGAGATCAAGTGCACCAAGCCCGAGTCCTGCTCCGGCGAG     359
 R  G  C  G  S  C  F  E  I  K  C  T  K  P  E  S  C  S  G  E

          90                    100
GCTGTCACCGTCACAATCACCGACGACAATGAGGAGCCTATCGCACCCTACCACTTCGAC     419
 A  V  T  V  T  I  T  D  D  N  E  E  P  I  A  P  Y  H  F  D
                                                    13A
          110                   120
CTCTCGGGCCACGCATTCGGGTCCATGGCGAAGAAGGGCGAGGAGCAGAAGCTCCGCAGC     479
 L  S  C  H  A  F  G  S  M  A  K  K  G  E  E  Q  K  L  R  S

          130                   140
GCCGGCGAGCTGGAGCTCCAGTTCAGGCGGGTCAAGTGCAAGTACCCGGACGGCACCAAG     539
 A  G  E  L  E  L  Q  F  R  R  V  K  C  K  Y  P  D  G  T  K

          150                   160
CCGACATTCCACGTCGAGAAGGCTTCCAACCCCAACTACCTCGCTATTCTGGTGAAGTAC     599
 P  T  F  H  V  E  K  A  S  N  P  N  Y  L  A  I  L  V  K  Y

          170                   180
GTCGACGGCGACGGTGACGTGGTGGCGGTGGACATCAAGGAGAAGGGCAAGGATAAGTGG     659
 V  D  G  D  G  D  V  V  A  V  D  I  K  E  K  G  K  D  K  W

          190                   200
ATCGAGCTCAAGGAGTCGTGGGGGAGCAGTCTGGAGGATCGACACCCCCGATAAGCTGACG     719
 I  E  L  K  E  S  W  G  A  V  W  R  I  D  T  P  D  K  L  T

          210                   220
GGCCCATTCACCGTCCGCTACACCACCGAGGGCGGCACCAAATCCGAAGTCGAGGATGTC     779
 G  P  F  T  V  R  Y  T  T  E  G  G  T  K  S  E  V  E  D  V

          230                   240
ATCCCTGAGGGCTGGAAGGCCGACACCTCCTACTCGGCCAAGTGAGCAAGAAGTGGAGTG     839
 I  P  E  G  W  K  A  D  T  S  Y  S  A  K  *

ATCTTCTTCCAATCAGCTTAATTTTGACTCAAGATCTCAAATAATCCAGCCGCACATATA     899

TACGAGGCGGTGAGACATACAAGCTCCTCCATGAGTATATTCATTCATGCCGTATAGAGA     959

GGAGAAAGATGCCTGAATAAGAGTTTGAGGTCGACACCTTGTGAGAAGTGTATATAGGAG    1019

GAACCCAATCTGGCTCCATCTTTCTTTGCTCGCACGGTGTACTGCTAAGGTTATCTTCTA    1079

ACAGGCCAGATTAACCTACTATCTAATATATGCAACGTATGGTCATTTTCCCTAAAAAAA    1139
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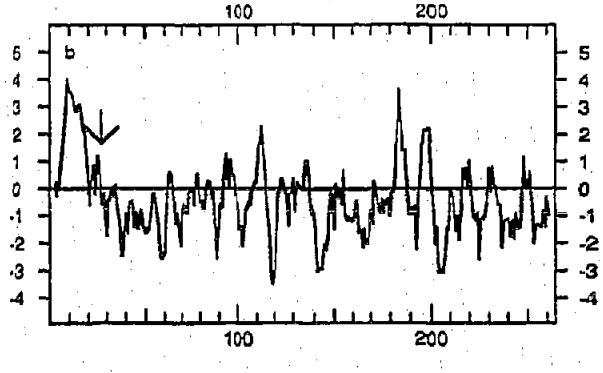
Loipl hydropathicity profile



separated *Lol pI* (*pI* 5.5) in sufficient quantities for N-terminal protein sequence analysis. The sequence obtained was as follows:

IAKVPPGPWI TAEYGDKWLD AK?T-----

This sequence is analogous to that repoted by Cottam et al. [12].

3.3. *Isolation of cDNA clones*

The cDNA expression library was screened for clones that expressed epitopes recognised by MAb 40.1. Screening of a total of 5×10^4 plaques yielded 18 immuno-positive clones. These clones were plaque-purified, and screened for IgE binding using sera from grass pollen-allergic subjects. Four clones were found to express proteins with affinity for IgE antibodies. Of these, the clone 13R (~ 0.8 kb in size including poly $(A)^+$ tail), bound strongly to MAbs 40.1, 3.2 and 21.3, all known to preferentially recognise *Lol pI* [10]. Thus, the clone expresses an allergen as defined by recognition of IgE from sera of allergic individuals and was designated as a putative *Lol pI* clone.

3.4. *Nucleotide sequence of* Lol pI

cDNA clone 13R, selected by expression screening, contains 764 nucleotides. There are 146 amino acids in the open reading frame (ORF) and the predicted M_r is 16.2 kDa. The translated amino acid sequence terminates with the 28-amino acid sequence described by Esch and Klapper [13]. Since this clone exhibits strong Mab and IgE binding, the sequence contains both antigenic and allergenic epitopes.

However, mature *Lol pI* has an approximate M_r of 35 kDa, so the translated reading frame is apparently not full-length. Clone 13R also lacks the NH_2-terminal sequence defined for *Lol pI* [12], confirming that it is a truncated clone. Full-length *Lol pI* clones were obtained by PCR of rye-grass pollen RNA. Primers based on synthetic linker sequences and *Lol pI*-specific sequences derived from the 3′ end of clone 13R were used to generate these clones.

The nucleotide sequence of *Lol pI* is shown in Fig. 2a. There is an open reading frame (ORF) of 789 bp starting with an ATG initiation codon at position 33 and terminating with a TGA stop codon at position 822. This ORF, which has a 64% GC content, corresponds to a polypeptide of 263 amino acids (M_r 29.1 kDa). The proposed translation initiation site and its flanking sequences (nucleotides 29–37) share 67% identity with the consensus plant sequence AACAATGGC [25]. The most critical nucleotide, a purine at position −3, is conserved. Sequence corresponding to the 13R clone starts at nucleotide 474.

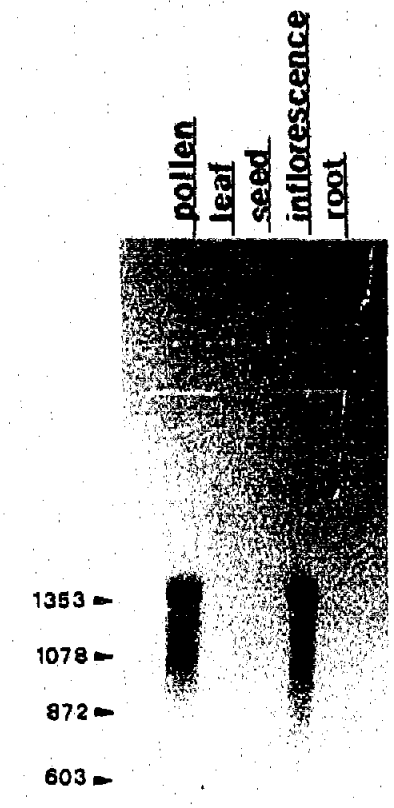


Fig. 3. Tissue-specific expression of *Lol pI* in rye-grass pollen. Northern blot analysis of total RNA from rye-grass pollen, seed, leaf, root and inflorescence using 13R cDNA as a probe.

The predicted protein sequence has a hydrophobic putative signal peptide sequence of 23 amino acids (Fig. 2 a,b). This is indicative of a mature processed protein of 240 amino acids (M_r 26.6 kDa). The amino acid composition from the deduced sequence, rich in glycine and lysine, is in complete agreement with previous data obtained by amino acid analyses of *Lol pI* protein [26,27]. There is a single potential *N*-glycosylation site having the characteristic Asn-x-Ser/Thr motif (residues 9–11) in the hydrophilic region of the mature protein (Fig. 2b).

Fig. 2. cDNA, predicted amino acid sequence and hydropathicity profile of *Lol pI* from rye-grass pollen. (a) The nucleotide and deduced amino acid sequence of *Lol pI*. The deduced amino acid sequence represented by the single letter code is shown below the DNA sequence, and begins at the first potential in-frame initiation codon. The open reading frame continues for 263 amino acids and ends with the TGA stop codon denoted by an asterisk. The putative signal peptide is indicated by negative numbers. Underlined amino acids were identified by protein microsequencing (this report; and [12,13]). The arrow indicates the start of the 13R sequence. (b) Hydropathicity profile of predicted amino acid sequence based on the method of Kyte and Doolittle [35] with a window of 7 amino acids.

A search of existing data-bases showed no similarity between the deduced amino acid sequence of *Lol pI* and any other known protein. The *Lol pI* nucleotide sequence was compared with that of the newly identified *Lol pIb* [10] and no sequence homology was observed.

3.5. *Expression of gene for* Lol p*I in various rye-grass tissues*

To determine the tissue specificity of *Lol pI* gene expression, we examined the level of specific transcripts in various tissues. Northern blots probed with cDNA 13R showed hybridization to a single abundant transcript (~ 1350 bases) in pollen (Fig. 3). Hybridization was also observed to similar sized transcripts in inflorescence, but no hybridization to any transcripts in rye-grass seed, leaf or root was observed (Fig. 3).

4. DISCUSSION

In this report we describe the cloning of a cDNA sequence encoding an allergen from rye-grass pollen. Several lines of evidence confirm that clone 13R corresponds to the major glycoprotein allergen, Lol pI. First, the clone was immunoselected from the lambda-gt 11 expression library using a MAb that shows high levels of binding to *Lol pI*. Second, the polypeptide encoded by the cDNA clone was recognised by IgE antibodies from pooled sera of grass pollen-allergic individuals. Third, a segment of the deduced amino acid sequence of the clone corresponded to the 38-amino acid tryptic fragment of *Lol pI* (YTTE GGTKS EFEDV IPEGW KADTS YSAK) described by Esch and Klapper [13]. Finally, the pollen specificity of transcripts hybridizing with 13R, is consistent with tissue specificity of *Lol pI* antigens, as observed earlier by immunoblotting [10].

The N-terminal sequence of *Lol pI*, was not detected in the ORF of the 13R clone. However, full length clones obtained by PCR amplification, encoded an amino acid sequence which corresponded with the N-terminal sequence of *Lol pI* ([12]; and this paper). The longest ORF is 789 nucleotides long, which corresponds to a protein of 263 amino acids with a leader sequence of 23 amino acids. The molecular mass for the mature form of *Lol pI* (240 amino acids) was calculated to be 26.6 kDa. This would suggest that 24% of the 35 kDa molecular mass of the pollen protein is due to post-translational modifications.

The *Lol pI* signal peptide has some motifs common to other plant signal peptides, especially those of secreted plant enzymes, e.g. amylase [28]. The motifs close to the cleavage site show some similarity with those of signal peptides which direct proteins into the lumen of the ER for glycosylation, e.g. VLL...FL GSAHG[1] in *Lol pI* and VLL...LS ASLASQ[1] in alpha-amylase [28]. The -1 and -3 rule for the prediction of a leader sequence cleavage site [29] is that amino acids at these positions should be small and neutral: G and A in *Lol pI*.

The deduced amino acid sequence of the full length *Lol pI* clone contains both the N-terminal sequence [12] and tryptic peptide fragment sequence [13] previously reported for *Lol pI*, as well as the N-terminal protein sequence of *Lol pI* reported here. The N-terminal sequences ([12]; and this report) helped define the cleavage site between the leader sequence and the mature form of *Lol pI*.

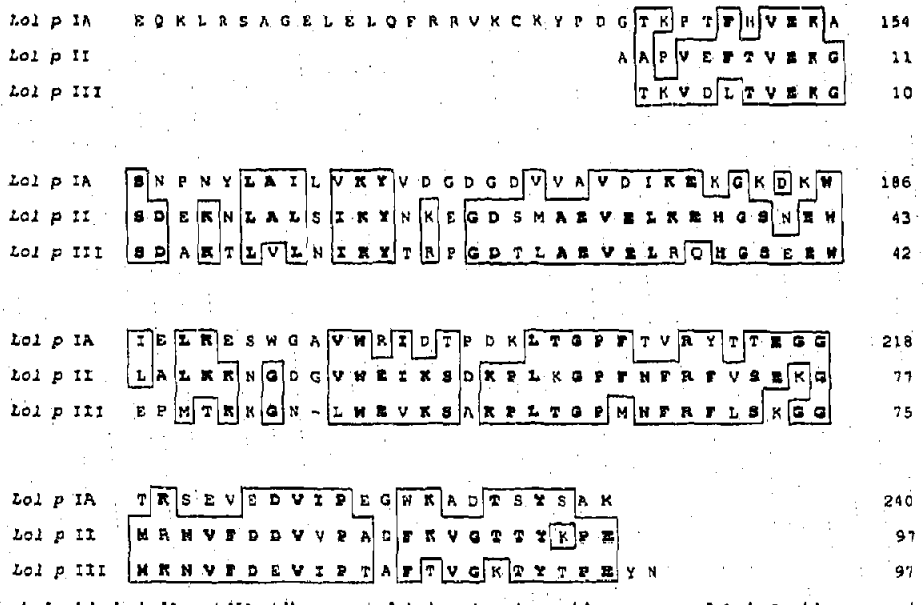


Fig. 4. Homologies of *Lol pI* with *Lol pII* and III. Alignment of deduced amino acid sequence of *Lol pI* with reported sequences of *Lol pII* [26] and *Lol pIII* [27]. Identical residues are shown in bold letters. These residues and other similar residues in the three sequences are indicated by boxes. The following residues were considered similar: A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W.

Our results show that the 28 amino acid tryptic fragment sequence YTTE GGTKS EFEDV IPEGW KADTS YSAK [13] is located at the COOH-terminus of this allergen. We also found that the entire 97 amino acid sequences of *Lol pII* [30] and *Lol pIII* [31] have strong homology with the COOH-terminal end of *Lol pI* (Fig. 4). *Lol pI* has 48% homology (33% identity) with *Lol pII* and 44% homology (26% identity) with *Lol pIII*, while *Lol PII* and *Lol pIII* have 73% homology (59% identity) which each other. However, despite strong homology between amino acids 145 to 240 of *Lol pI* with the amino acid sequences of *Lol pII* and *Lol pIII*, no serological cross-reactivity has been detected between *Lol pI* and *Lol pII* nor *Lol pIII* [32].

Immune responsiveness to rye-grass pollen allergens in the human population is significantly associated with the histocompatibility leukocyte antigen (HLA)-DR3 [33,34]. One interpretation of this finding is that HLA-DR3, or closely linked class II molecules, may be particularly effective at presenting certain allergenic determinants to T cells. Ansari et al. [30,31] predicted that the 28 amino acid tryptic fragment isolated by Esch and Klapper [13] may include an HLA-DR3 restricted T cell/Ia recognition site. Both *Lol pII* and *Lol pIII* have sequences homologous to this tryptic fragment and Ansari et al. proposed that the *Lol pI* T-cell determinant may be shared with *Lol pII* and *Lol pIII* [30,31]. This may explain the concordant responsiveness of patients to *Lol pI*, *Lol pII* and *Lol pIII* [33,34].

Now that the amino acid sequences of *Lol pI*, II and III are available it should be possible to experimentally determine the T- and B-cell epitopes of these allergens using synthetic peptides based on their sequences.

Acknowledgements: We thank the Australian National Health and Medical Research Council, Asthma Foundation of Victoria, ImmuLogic Pharmaceutical Corporation, and the Australian Government Department of Employment Education and Training for financial support for this project.

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NOTE ADDED IN PROOF

Perez et al. (J. Biol. Chem. (1990) 265, 16210–16215) have reported the cloning of two *Lol pI* isoforms. We detected the amino acid polymorphisms defined by their two clones in our PCR analysis of rye-grass pollen. In addition, we have identified other potential polymorphic residues (data not shown). This clearly demonstrates that there are multiple *Lol pI* isoforms.