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Isolation and Characterization of a cDNA Clone Encoding an IgE-Binding Protein from Kentucky Bluegrass (*Poa pratensis*) Pollen

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Abstract. We reported previously on the isolation and characterization of several allergens from Kentucky bluegrass (KBG) (*Poa pratensis* L.) pollen with the aid of the corresponding murine monoclonal antibodies (Mabs). In the present study, (1) an analysis of various tissues of this grass revealed that the allergenic components recognized by these Mabs were confined to the pollen; (2) intact translatable mRNA was isolated from the KBG pollen, and (3) a cDNA library was constructed with this mRNA in the λ gt11 expression vector. Screening of this library with a pool of six sera from KBG-allergic patients, in combination with enzyme-labeled antibodies to human IgE, led to the isolation of a cDNA clone, referred to as KBG7.2. The nick-translated cDNA probe of KBG7.2 hybridized to a 1.5-kbp RNA transcript from KBG pollen. Moreover, transcripts corresponding to KBG7.2 were found in pollens of eight other grasses, indicating that the proteins similar to the one encoded by this cDNA may be present in these grasses. The nucleotide sequence of KBG7.2 was determined; interestingly, the corresponding derived amino acid sequence did not match any other sequence recorded in the protein data banks. The peptide encoded by KBG7.2 was expressed as a fusion protein utilizing the plasmid vector pWR590.1. Whereas none of the above allergen-specific Mabs bound to the fusion protein, all the 15 individual sera from grass pollen allergic patients recognized the fusion protein. It is, therefore, concluded that the cDNA clone KBG7.2 encodes a polypeptide of a major IgE-binding protein of KBG pollen and that the murine Mabs used in this study do not recognize the epitopes responsible for induction of human IgE antibodies.

Introduction

Previous studies on the aqueous extract of Kentucky bluegrass (KBG) (*Poa pratensis* L.) pollen, which is one of the main factors leading to respiratory allergies in the Canadian prairies, other parts of North America, and Europe, revealed a multiplicity of antigenic and allergenic components with a wide range of molecular sizes and charges [1-3]. Moreover, a number of murine monoclonal antibodies (Mabs) [4, 5] had been developed against several allergens of grass pollens which include group I allergens [6].

Some of the allergens were isolated directly from the crude aqueous extracts of the grass pollens [7, 8] by the use of physicochemical methods and reverse immunosorbents, consisting of immobilized Mabs specific to pollen constituents. However, one of the main drawbacks of these extremely labour-intensive

purification methods is the minute yield of pure allergens which are insufficient for structure-function relationship studies. On the other hand, recent developments in recombinant DNA (rDNA) technology have paved the way for the synthesis of allergenic proteins in a consistently pure state and in large amounts. Furthermore, by the application of this technology, it is possible to determine the molecular properties of allergens which are responsible for eliciting immediate-type hypersensitivity diseases in humans. It is also important to note that identification of the epitopes of allergens may provide new insights into the pathogenic and immunochemical mechanisms of allergic diseases and may lead to the development of novel therapeutic derivatives capable of specifically suppressing the culprit IgE molecules responsible for common forms of allergy in man.

In the present study, we have shown that intact

mRNA suitable for constructing libraries of cDNA clones can be isolated from commercially available KBG pollen. The isolation and analysis of a cDNA clone, that was identified upon screening the library with a pool of sera from 6 grass pollen allergic patients, are described in detail.

Materials and Methods

Pollens

Pollens of ten grasses, i.e., KBG (*P. pratensis*), ryegrass (*Lolium perenne*), cultivated barley (*Hordeum vulgare*), Canary grass (*Phalaris arundinaceae*), colonial bent grass (*Agrostis maritima*), Canada bluegrass (*Poa* spp.), redtop grass (*Agrostis alba*), smooth brome grass (*Bromus* sp.), tall oat grass (*Avena* spp.), and quack grass (*Agropyron repens*), were purchased in the dehydrated state from Hollister-Stier, Spokane, Wash., USA. Root, leaf, and meristematic tissues of KBG were obtained following germination or cultivation of KBG seeds.

Protein Isolation and Western Blotting

The non-dialyzable components of KBG pollen were isolated by extracting diethylether-defatted pollen with distilled water at room temperature, followed by dialysis against distilled water in Spectrapor membrane tubing with a molecular weight cutoff of 6,000–8,000 (Spectrum Medical Industries, Los Angeles, Calif., USA). The non-dialyzable components, referred to as the retentate, were stored in the lyophilized state. For isolation of proteins from other plant tissues, such as root, leaves, and meristematic tissues, these tissues were frozen in liquid nitrogen and handground by mortar and pestle in a buffer containing 50 mM Tris-HCl pH 8.0, 1 mM ethylenediaminetetra-acetic acid, and 1 mM phenylmethylsulfonyl fluoride, and the homogenate was centrifuged; the supernatant served as the source of proteins.

The proteins extracted from the above tissues were separated by 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [9] and electroblotted onto a nitrocellulose membrane which was then blocked with a solution of gelatin (1%). For the identification of allergenic proteins of KBG, the membrane was treated overnight with a cocktail of Mabs, i.e., Mab 1, Mab 12, [10], and Mab 60 [3], and the proteins were visualized with alkaline phosphatase linked goat antimouse IgG (Promega, Mississauga, Ont., Canada).

RNA Isolation and in vitro Translation

A procedure was devised for the isolation of maximum translatable RNA. Briefly, the pollen material was handground with a pestle in liquid nitrogen in a mortar. The ground material was then homogenized in guanidium isothiocyanate buffer [11] in a polytron for 30 s. This was followed by repeated extractions with a phenol-chloroform mixture (1 part of phenol and 1 part of chloroform-isoamyl alcohol) prior to centrifugation of the aqueous phase containing RNA on a cesium chloride gradient. Total RNA was affinity-purified by oligo-dT-cellulose chromatography [12] to obtain poly A⁺ RNA (i.e., putative mRNA). Both total and poly A⁺ RNA were translated in vitro, employing a rabbit reticulocyte system obtained from BRL/Gibco (Canada). The reaction mixture (total vol-

ume of 30 µl) contained, in addition to all other essential components, either 10 µg of total RNA or 1 µg of poly A⁺ RNA and 30 µCi of [³⁵S]-methionine (1,148 Ci/mmol). Following 1 h of incubation at 30°C, 2-µg aliquots of the mixture were used for determination of the incorporation of [³⁵S]-methionine by precipitation of the products with trichloroacetic acid and liquid scintillation spectrometry. Translation products containing equal radioactivity were separated by SDS-PAGE. The gels were treated with Amplify (Amersham, Canada), dried, and then exposed to Kodak XAR-5 film.

Construction and Screening of a λgt11 Library

Poly A⁺ RNA, prepared as described above, was used to construct double-stranded cDNA [13]. After methylation of *Eco*RI sites and addition of *Eco*RI linkers to the ends of the cDNAs, the resulting products were ligated to λgt11 arms at 15°C overnight. The ligation product was packaged in vitro, and the library was amplified in *Escherichia coli* strain Y1088 using the protocol described by Huynh et al. [14]. In order to identify the cDNA clones encoding IgE-binding proteins, the plated phage library was first incubated with the primary antibody, i.e., with sera from patients allergic to grass pollens. The allergen-cDNA clones were detected by subsequent treatment of the phage blot with murine monoclonal antihuman IgE (generously provided by Dr. A. Saxon, University of California at Los Angeles School of Medicine, USA) and then with goat antimouse alkaline phosphatase conjugate (Zymed). Positive phage which appeared in duplicate filters were purified to homogeneity. One of these selected clones, referred to as KBG7.2, was further studied. The insert from KBG7.2 was excised with *Eco*RI and was subcloned into the plasmid vector pT7T319U (BRL/Gibco, Canada).

RNA Hybridization

For northern analysis 10 µg of total RNA or 1.0 µg of poly A⁺ RNA was electrophoresed in 1.5% agarose gel under denaturing conditions and blotted onto Hybond-N nylon membrane (Amersham, UK). Hybridization of the nick-translated cDNA probe (see below) to the northern blot was allowed to proceed for 20 h at 65°C in 6× SSC (15 mM NaCl, 1.5 mM Na citrate, pH 7.5), 5× Denhardt's solution (0.1% w/v of each bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 100 µg/ml heat-denatured sheared *E. coli* chromosomal DNA, and 0.1% SDS. After hybridization, the membranes were washed for 15 min at 65°C in 2× SSC and 0.1% SDS and for 15 min at 65°C in 0.2× SSC and 0.1% SDS, followed by autoradiography for 24 h at -75°C with an intensifying screen.

For RNA dot-blot hybridization, 5 µg of total RNA isolated according to the procedure described earlier [15] was dotted in triplicate onto a dry nitrocellulose filter which had been pretreated by soaking in 20× SSC. Hybridization and autoradiography were carried out as described above, and the radioactivity of RNA dots was quantified by liquid scintillation spectrometry.

Nick Translation

For nick translation, plasmid DNA was prepared [16] and digested with *Eco*RI and the cDNA inserts were purified. Nick translation was carried out by radiolabelling with [α -³²P]-dCTP as described previously [17]. Nucleotides which had not been incorporated were removed by passing the reaction mixture through Sephadex G-50.

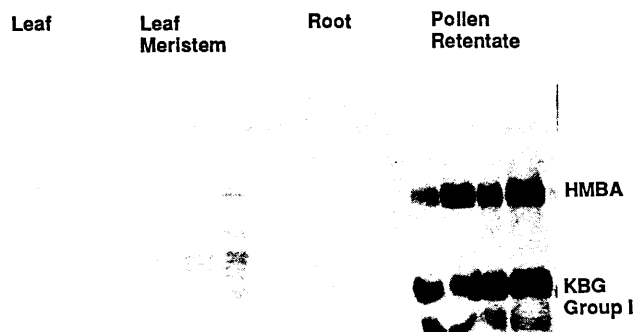


Fig. 1. Immunological detection of specific allergens in KBG plant extracts. About 50 μ g of protein extracts from leaf, leaf meristem, root, and of the pollen retentate was separated by 15% SDS-PAGE, transferred onto nitrocellulose, and the blot was incubated with a cocktail of Mabs consisting of Mabs 1, 12, and 60. HMBA represents high molecular weight basic allergen of about 57 kD and group I proteins of about 33 and 35.8 kD [3].

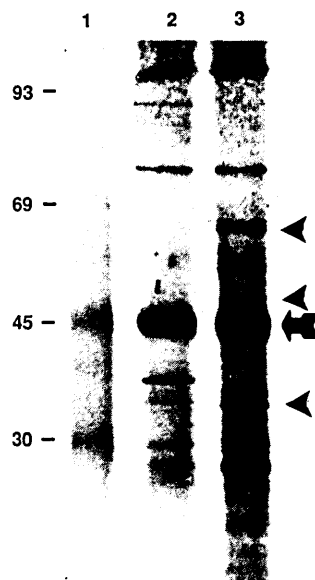


Fig. 2. Fluorograph of in vitro translation products of KBG pollen RNAs. Standard size markers (lane 1), total RNA (lane 2), and poly A⁺ RNA (lane 3) were translated in a rabbit reticulocyte lysate. The products labeled with [³⁵S]-methionine were separated by SDS-PAGE and fluorographed. The arrow shows the translation product from RNA endogenous to the lysate system; the arrowheads indicate possible translation products corresponding to allergen mRNAs.

DNA Sequencing

Nucleotide sequence of the cDNA insert KBG7.2 was determined by both the dideoxy sequencing procedure [18] and the chemical cleavage method [19].

IgE-Binding Assay for Recombinant 7.2 Polypeptide

The KBG7.2 cDNA insert was excised from the purified phage and recloned into the plasmid vector pWR 590-1 [20] which was subsequently transformed into *E. coli* strain JM 105. The transformants were immunoscreened with a serum pool from grass pollen allergic patients in an IgE-detecting system. The IgE-binding protein was produced by growing a selected colony in Luria broth containing 100 μ g Amp/ml to stationary phase. The collected cells were suspended in 7 M guanidine-HCl (pH 7.5) and 1 mM phenylmethylsulfonyl fluoride and sonicated for 30 s. After centrifugation at 30,000 g for 10 min, the immunoreactivity of the soluble protein was examined by membrane dot-blot immunoassay. Briefly, the proteins were dotted onto nitrocellulose which, after blocking by 1% gelatin in Tris-buffered saline, was sequentially reacted with sera from allergic patients and alkaline phosphatase conjugated goat antihuman IgE antibodies (Tago). The IgE-containing sera and the conjugate were diluted in Tris-buffered saline with Tween and 0.1% gelatin, and the membrane was washed three times with Tris-buffered saline containing Tween between each incubation step. The color reaction was based on the NBT/BCIP system.

Results

In order to determine if tissues other than pollen grains contain IgE-binding constituents, proteins were extracted from leaves, leaf meristems, and roots of KBG and subjected to western blot analysis using the Mabs raised against some of the major allergens of KBG (fig. 1). Mab 1 and Mab 12 had been shown to recognize the high molecular weight basic allergen [10], and Mab 60 recognizes group I allergens in pollen extracts [3]. The immunoblot shown in figure 1 demonstrates that the allergenic proteins, detected by a cocktail of Mabs which recognize allergens, are abundant in pollen extracts and are essentially absent in root and mature leaf tissues. Some proteins that reacted with the Mabs were detected at very low levels in leaf meristematic tissue. Thus, plant tissues other than pollen did not contain allergenic proteins in sufficient amount to be considered suitable for the isolation of mRNA.

Grinding of mature KBG pollen in small amounts with pestle, in a chilled mortar, yielded about 1 mg of total RNA per gram of pollen. The recovered RNA (in the form of total RNA or poly A⁺ RNA) was translated in an in vitro rabbit reticulocyte system, and the pattern of labelled proteins (fig. 2) closely resembled

the pattern of extractable proteins from KBG pollen [3]. The translation products ranged in size from 11 to 100 kD. Several major polypeptides in the range of 30–60 kD were detected which correspond to the major allergens in grass pollen [1–3].

The cDNA library constructed in λ gt11 from the KBG pollen poly A⁺ RNA contained about 300,000 phages, of which 90% were recombinants, i.e., contained inserts. This cDNA library was amplified once in order to facilitate screening with antibodies. The library was screened with a pool of sera from 6 grass pollen allergic patients. Several positive clones were detected; one was designated as KBG7.2 and was further analyzed.

The 558-bp cDNA insert from KBG7.2 was excised by *Eco*RI digestion and subcloned for further analysis. The size of the RNA transcript corresponding to this cDNA was determined by northern blot hybridization. From the autoradiograph shown in

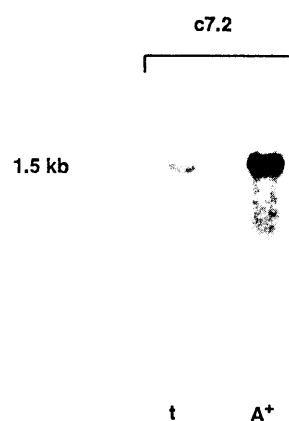


Fig. 3. Northern blot hybridization: 10 μ g of total RNA from KBG pollen and 1 μ g of poly A⁺ RNA were separated by electrophoresis, blotted onto nitrocellulose, and hybridized with nick-translated cDNA insert. The number on the left indicates the apparent molecular weight of the transcript as estimated from the migration of the RNA ladder (BRL/Gibco, Canada).

Table 1. Complete nucleotide sequence and the derived amino acid sequence of the cDNA clone KBG7.2

GAA	TTC	CCC	GGC	GAG	CTG	CAG	GTC	ATC	GAC	AAG	GTC	GAC	GCC	GCC	TTC	AAG	GTC	GCT
Glu	Phe	Pro	Gly	Glu	Leu	Gln	Val	Ile	Asp	Lys	Val	Asp	Ala	Ala	Phe	Lys	Val	Ala
60																		
GCC	ACC	GCC	GCG	AAC	GCC	GCC	CCC	GCC	AAC	GAC	AAG	TTC	ACC	GTC	TTC	GAG	GCC	GCC
Ala	Thr	Ala	Ala	Asn	Ala	Ala	Pro	Ala	Asn	Asp	Lys	Phe	Thr	Val	Phe	Glu	Ala	Ala
120																		
TTT	AAC	GAT	GCC	ATC	AAG	GCG	AGC	ACG	GGC	GGC	GCC	TAC	CAG	AGC	TAC	AAG	TTC	ATC
Phe	Asn	Asp	Ala	Ile	Lys	Ala	Ser	Thr	Gly	Gly	Ala	Tyr	Gln	Ser	Tyr	Lys	Phe	Ile
180																		
CCC	GCG	CTC	GAG	GCC	GCC	GTC	AAG	CAG	TCC	TAC	GCC	GCC	ACC	GTC	GCC	ACC	GCG	CCC
Pro	Ala	Leu	Glu	Ala	Ala	Val	Lys	Gln	Ser	Tyr	Ala	Ala	Thr	Val	Ala	Thr	Ala	Pro
230																		
GCG	GTC	AAG	TAC	ACT	GTC	TTT	GAG	ACC	GCG	CTG	AAG	AAG	GCC	ATC	ACC	GCC	ATG	GCA
Ala	Val	Lys	Tyr	Thr	Val	Phe	Glu	Thr	Ala	Leu	Lys	Lys	Ala	Ile	Thr	Ala	Met	Ser
290																		
CAG	GCC	CAG	AAG	GCT	GCT	AAG	CCC	GCT	GCC	GCT	GTG	ACC	GGC	ATC	GCG	ACC	AGC	GCA
Gln	Ala	Gln	Lys	Ala	Ala	Lys	Pro	Ala	Ala	Ala	Val	Thr	Gly	Ile	Ala	Thr	Ser	Ala
350																		
GTC	GGC	GCG	GCA	ACC	GGC	GCT	GCC	ACC	GCC	GCT	GCT	GGT	GGC	TAC	AAA	GCT	TGA	TCT
Val	Gly	Ala	Ala	Thr	Gly	Ala	Ala	Thr	Ala	Ala	Ala	Gly	Gly	Tyr	Lys	Val	---	

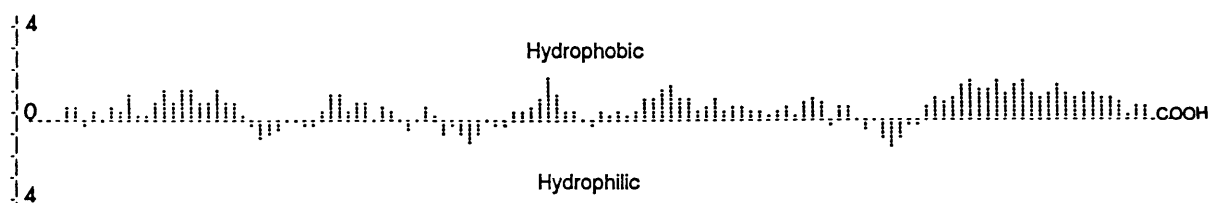


Fig. 4. Hydropathy plot derived from the amino acid sequence of KBG7.2 given in table 1.

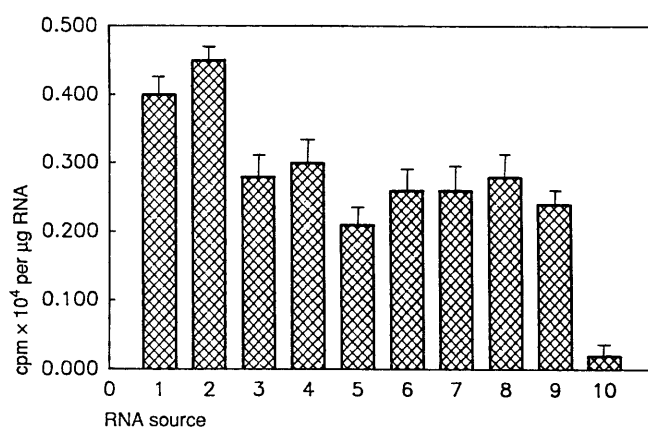


Fig. 5. Relative abundance of the RNA transcript corresponding to KBG7.2 in other grasses. RNA isolated from pollens of different grasses was dotted onto nitrocellulose and hybridized to nick-translated KBG7.2 probe (see 'Materials and Methods'). The amount of radioactivity bound to the RNA dots on the nitrocellulose was determined by liquid scintillation spectrometry. The numbers 1–10 correspond to the RNAs of pollen of KBG, ryegrass, orchard grass, colonial bent grass, redtop grass, smooth bromegrass, tall oat grass, quack grass, and leaf tissue of KBG, respectively.

figure 3, it is clear that the nick-translated KBG7.2 probe hybridized to one of the major transcript species. The abundance of the mRNA was sufficient for its detection in 10 µg of total RNA. However, a much stronger hybridization signal was observed using 1 µg of poly A⁺ RNA. Thus, it may be concluded that the RNA corresponding to KBG7.2 is present in moderately abundant amounts in KBG pollen.

The complete nucleotide sequence of the cDNA insert of clone KBG7.2 was determined (table 1). The insert is GC rich, and the deduced amino acid sequence is very rich in alanine (30%), threonine (9%), lysine (9%), and valine (8%). A hydropathy plot of the above peptide is presented in figure 4. It is clearly seen that the peptide consists of predominantly hydrophobic residues interspersed by small stretches of hydrophilic residues. The derived amino acid se-

quence of KBG7.2 does not match with any other protein sequence recorded in the data banks.

To examine if the allergenic protein encoded by KBG7.2 is unique to KBG, RNAs isolated from eight different grasses were hybridized with the nick-translated cDNA insert of KBG7.2 using quantitative RNA slot-blot hybridization. From the results presented in figure 5, it is evident that all grass pollens contain mRNA hybridizable to the KBG7.2 probe, although the amount of RNA differs from one grass pollen to another. No hybridization was seen with leaf mRNA, indicating that the hybridization was specific to pollen mRNA. Furthermore, both KBG and ryegrass pollen RNA showed a higher degree of hybridization with the KBG7.2 probe in comparison with the RNA from other grass pollens. It is, therefore, inferred that analogues of the protein which correspond to KBG7.2 may be present in all grass pollens tested.

Sufficient amounts of the peptide encoded by KBG7.2 were obtained by its expression in *E. coli* as a fusion protein in association with β-galactosidase. In order to establish its immunological reactivity, the fusion polypeptide was reacted with a pool of sera from 6 grass pollen allergic patients employing a membrane dot-blot procedure, i.e., the fusion protein was found to react with this pool of sera (but not with sera of non-atopic control individuals) known to contain allergen-specific IgG (data not shown), indicating that the reaction was due to IgE. The serum pool did not react with the protein extract prepared from a colony containing the self-ligated expression vector (results not shown). The observed IgE binding could be completely abrogated by preincubation of the atopic sera with the fusion protein (fig. 6A). However, atopic sera upon their absorption with the fusion protein still contained IgE which reacted with the KBG protein, but with reduced intensity. In attempts to further characterize the allergen which corresponds to KBG7.2, the fusion protein was reacted with a panel

of available Mabs for *Poa p* I (i.e., Mabs 60–62) [3] and *Poa p* IV (e.g., Mab 12). Interestingly, none of these Mabs reacted with the fusion protein (data not shown). However, all of the individual human sera (see figure 6B) contained IgE with specificity for the fusion protein. It is thus concluded that KBG7.2 encodes a portion of a major grass pollen allergen.

Discussion

In spite of the obvious principle that only the component(s) to which a given individual is allergic ought to be used for immunotherapy, this goal has not been achieved because of the unavailability of purified allergens. Classical physicochemical and immunochemical isolation methods are laborious and yield, at best, only limited amounts of pure allergens. Therefore, progress for the isolation and molecular characterization of the repertoire of pollen allergens has been slow. On the other hand, the rDNA methodologies are well suited for production of well defined allergens and for their structural studies, as is also supported by studies with the house dust mite allergen *Der p* 1 [21] and honeybee venom allergen, antigen 5 [22].

In the present study gene cloning procedures were used for the identification of one of the allergens present in KBG pollen and other grass pollens. To our knowledge, this is the first study directed toward the exploration of grass tissues other than pollens for the presence of allergenic proteins. The results of this study demonstrate that allergenic proteins are present predominantly in pollen and that even commercially available dry KBG pollen contains translatable mRNA coding for allergenic proteins. We have exploited this fact for (1) constructing a cDNA library in an expression vector and (2) screening the library with sera of allergic patients for identification of the appropriate cDNA clones. Thus, several cDNA clones encoding allergens were identified; this report is confined to the characterization of the KBG7.2 cDNA clone which appears to correspond to one of the principal allergenic constituents of KBG pollen.

The availability of this cDNA clone has facilitated the determination of the primary amino acid sequence of the corresponding allergen. Although the deduced amino acid sequence encodes one or more allergenic epitopes, for a complete mapping of the epitopes of this allergen, full-length cDNA clones have

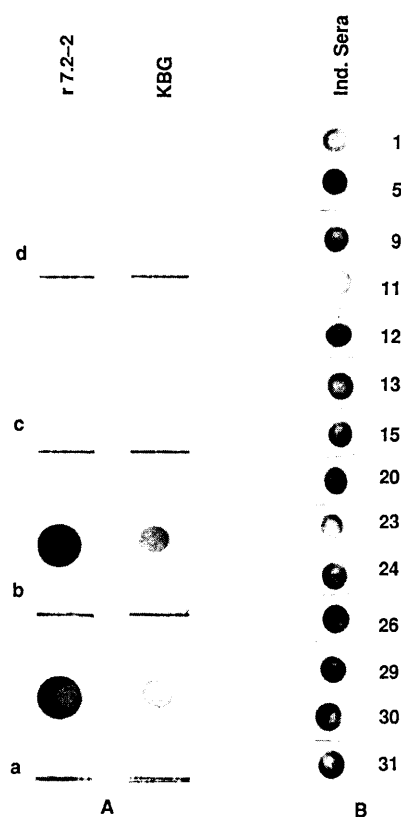


Fig. 6. Reactivity of human IgE with KBG7.2-encoded polypeptide by dot-immunoblotting. **A** Fusion protein isolated from one of the transformants, i.e., r7.2-2 (30 μ g) and KBG (10 μ g), was dotted onto nitrocellulose. The individual dots from the above were treated with a pool of sera from allergic patients which was diluted in Tris-buffered saline at 1:20 (a) and 1:10 (b), after absorption of sera used in 'b' with protein from r7.2-2 (c), and with Tris-buffered saline (d). The IgE reactivity was detected employing the procedures described in 'Materials and Methods'. **B** Protein isolated from r7.2-2 was dotted onto nitrocellulose and was treated with sera from individual allergic patients; the reactivity with IgE antibodies was detected as described above.

to be isolated and analyzed. To this end, we have recently screened our cDNA library with a probe consisting of a 5' fragment of KBG7.2, and about 150 positive plaques were identified; the analysis of these clones is currently in progress.

In view of the occurrence of the protein encoded by KBG7.2 in several grasses and the evidence that the polypeptide-encoded KBG7.2 contains epitopes which are recognized by IgE antibodies of most grass

pollen allergic patients, it can be concluded that this protein is a major allergen of grass pollens. Studies are underway to define the epitopes of the KBG7.2 polypeptide and, in particular, the immunodominant residue(s) that govern the production of IgE antibodies; clearly, this (these) residue(s) would play a major role in the pathogenesis and immunotherapy of the corresponding allergies.

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