

High-frequency binding of IgE to the *Der p* allergen expressed in yeast

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The production of allergens from cDNA clones will provide a clonally pure source of material for experimental and perhaps clinical studies. Attempts to produce the major mite allergen, Der p I, in a highly antigenic form in bacteria have, to date, had limited success. In this study, a high level of production of Der p I from a Cup1 gene cassette from pYELC5-13T in Saccharomyces cerevisiae is described. Although the protein was insoluble, it could be readily solubilized in a urea solution and remained in solution when it was returned to more physiologic buffers. An amount equivalent to about 1 mg/L of yeast culture could then be isolated by affinity chromatography with an immobilized monoclonal antibody. This product reacted strongly with IgE in 9/11 sera from mite-allergic patients compared to the 50% reactivity achieved for Der p I previously produced as a fusion by bacteria. Similarly, the intensity of binding and ability to absorb out Der p I specificities were much greater for the yeast, pYELC5-13T, product. Studies with monoclonal antibodies also demonstrated the yeast, Der p I, had a high degree of antigenicity, although clear differences with the native allergen were demonstrated. The high frequency of reactivity with IgE of the pYELC5-13T formally demonstrates that a single gene product of Der p I is a major allergen and demonstrates that even for Der p I, which is synthesized from a proenzyme, considerable antigenicity can be obtained by expressing the mature protein. (J ALLERGY CLIN IMMUNOL 1992;89:95-102.)

Key words: Recombinant, allergens, cDNA, yeast, IgE binding, allergenicity, Der p I, house dust mite

There has been considerable interest in the production of cDNA clones encoding allergens.¹⁻⁶ This encoding has provided valuable information for the definition and characterization of allergens and the synthesis of peptides. It also has the potential to permit the expression of clonally purified allergens from the cDNA for a wide variety of immunologic and clinical

Abbreviations used

SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
CAPS:	3-(Cyclohexylamino)-1-propanesulfonic acid
Cup1:	Metallothionein gene
MAB:	Monoclonal antibody
PBS:	Phosphate-buffered saline
EDTA:	Ethylenediaminetetraacetic acid
M _r :	Molecular weight (mass) ratio

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investigations. Fortunately, most allergens expressed from cDNA so far, *Der p* II,⁶ *Bet v* I,⁴ *Ves g* V,² and less well-defined IgE binding proteins from Kentucky bluegrass⁵ and *Dermatophagoides pteronyssinus*,³ retain considerable IgE binding reactivity. The *Der p* II expressed in *Escherichia coli*, for example, has nearly all the IgE binding capacity of native *Der p* II isolated from mite extracts⁷ and has reacted with IgE from 79 of 81 sera from patients allergic to mite.^{7, 8} The two

negative sera only had weak reactivity with mite *Der p* II.⁷ It has been more difficult to express a product with high IgE binding activity from cDNA encoding the major *Der p* I allergen. To date, the best construct has been a fusion protein with glutathione-S-transferase from the pGEX vector, which has about 50% of the IgE binding activity of native *Der p* I, although this varies with the serum tested.⁹ Correspondingly, only half the sera with anti-*Der p* I IgE antibodies elicit detectable reactions with the fusions. Attempts to produce a nonfused form in *E. coli* have been unsuccessful, apparently because of toxicity to the host cell. In this article, we describe the expression of *Der p* I cDNA in yeast from a metallothionein gene promoter and the production of recombinant allergen with high-binding activity to MAbs and the ability to react with IgE in 90% of sera from allergic patients. This finding demonstrates that the product of a single *Der p* I gene has a high frequency of reactivity with IgE as well as providing a useful source of clonally purified allergen.

MATERIAL AND METHODS

Sera and MAbs

MAbs 10H2C6, 5H8C12, 3H8G8, 5H8D8, 4C1B8, and 6A8B10D12¹⁰ were kindly provided by Dr. M. Chapman and Professor T. A. E. Platts-Mills, University of Virginia, Charlottesville. MAb Nph4¹¹ was a gift from Drs. C. Schou and H. Løwenstein, ALK Laboratories, Hørsholm, Denmark. 4C1B8 and 6A8B10D12 were raised against *Der f* I, but 4C1B8 reacts equally well with *Der p* I, whereas 6A8B10D12 is specific for *Der f* I. The other MAbs were raised against *Der p* I.

Eleven human allergic sera were obtained from individuals who were skin test positive for *D. pteronyssinus* extract and demonstrated high positive RAST scores. Six of 11 sera were obtained from adults in Western Australia, the remaining six sera, kindly supplied by Dr. D. Hill, were from children, Royal Childrens Hospital, Melbourne. The hyper-IgE serum, a gift from Dr. N. R. Lynch, Venezuela, was from a patient with helminthic infection and did not contain anti-*Der p* I. All human sera were used at 1:2 dilution.

Radioimmunoassay and preparation of immunoblots

Dot blots were prepared by applying 5 µg of each protein onto nitrocellulose membrane with a Bio-Dot apparatus (Bio-Rad Laboratories, Richmond, Va). Western blots were prepared by electrophoretic transfer of proteins from SDS-PAGE to nitrocellulose membrane.¹² The radioimmunoassays for IgE and IgG blots were performed as previously described.⁷ Densitometry was performed by scanning autoradiographs with an Epson GT 4000 scanner (Seiko Epson Corp., Nagano, Japan), typically set at 80 dots per inch, 256 gray scale, and brightness of 2 with the Macintosh

Epson program (BIOSOFT, Cambridge, U.K.), and analyzed by the Scan Analysis Software (BIOSOFT) with an average background subtraction.

SDS-PAGE

Proteins were separated by SDS-PAGE according to the method of Laemmli¹³ and stained with Coomassie blue R-250.

Molecular cloning

The high copy number vector, pYELC5, has been described.^{14, 15} It contains a *Saccharomyces cerevisiae* *Cup1* cassette for copper-inducible expression of foreign proteins. The vector, pYEUC114, is a low copy number plasmid containing the same cassette. Because pYELC5 does not have a unique *EcoRI* site, the *EcoRI* fragment of *Der p* I-13T¹ was first cloned into the *Cup1* cassette in pYEUC114 to construct pYEUC114-13T. The *XbaI*-*BamHI* fragment of the cassette containing the *Der p* I sequence was then subcloned from pYEUC114-13T to pYELC5 to make pYELC5-13T. For cloning, these plasmids were transformed into *E. coli* MC1066¹⁵ and selected with 50 µg/ml of ampicillin. All manipulations were performed by standard procedures,¹⁶ and the constructs were checked by restriction enzyme mapping. The *Der p* I sequence in pYELC5 was verified by sequencing the entire cDNA.

S. cerevisiae strains, Fla (a *ura3 Cup^R*) and 6657-4D (a *his3 leu2 Cup^R*), were used as hosts for pYEUC114-13T and pYELC5-13T constructs, respectively. Five to 10 µg of plasmid DNA isolated by cesium chloride gradient centrifugation was used for transformation,^{17, 18} selection of pYEUC114 was for URA⁺ transformants on minimal medium,¹⁹ and selection for pYELC5-13T was for *leu*⁺ transformants on minimal medium supplemented with 20 µg/ml of histidine. Diploid strains were obtained by crossing the transformants with strain DBY 745 (*adel ura3 leu2 Cup1^R*) and then selecting on minimal medium.¹⁹

Induction of protein synthesis with copper and lysate preparation

Yeast transformants were grown in yeast minimum broth at 30°C with vigorous shaking for 16 hours. The cells were spun down, resuspended in fresh medium, and grown to midlog phase. To induce the expression of protein, copper sulfate was added to a concentration of 0.5 mmol/L, and the culture was continued for 2 hours. Cells were harvested by centrifugation at 2000 g for 5 minutes. The packed cell volume was estimated, and the yeast was resuspended in PBS buffer (150 mmol/L of NaCl, 16 mmol/L of Na₂HPO₄, and 4 mmol/L of NaH₂PO₄, pH 7.3) containing 200 U/ml of trasylol, 0.2 mmol/L of phenylmethylsulfonyl fluoride, 0.1 mol/L of EDTA, 1 µg/ml of RNase, and 0.02 µg/ml of DNase, at a ratio of 1:4 (vol/vol). The cell suspension was homogenized with 0.45 mm glass beads for 3 minutes with a Braun MSK homogenizer (Braun Instruments, Burlingame, Calif.). The lysate was centrifuged at 10,000 g for 10 minutes to separate pellet and supernatant.

Denaturation and renaturation of recombinant protein

The protein pellet obtained from the lysate of a 500 ml culture was washed three times with 10 ml of guanidine-triton solution (1.75 mol/L of guanidinium, 1 mol/L of NaCl, 1% Triton-X 100) and then resuspended in 3 ml of a solubilization solution of 8 mol/L of urea, 50 mmol/L of NaCl, 1 mmol/L of EDTA, and 2% 2-mercaptoethanol. The suspension was homogenized with 0.45 mm glass beads for 10 minutes with a Braun homogenizer and rotated at room temperature for 1 hour, followed by 1 hour of heat treatment at 60° C. One milliliter of CAPS buffer (50 mmol/L of CAPS [Sigma Chemical Co., St. Louis, Mo.], pH 10.7, 50 mmol/L of NaCl, and 1 mmol/L of EDTA) was added, and the mixture was rotated at room temperature for 30 minutes. Insoluble debris was removed by centrifugation at 10,000 *g* for 10 minutes, and the supernatant was dialyzed against CAPS buffer, pH 10.7 for 16 hours. Finally, the solution was dialyzed extensively against Tris buffer 50 mmol/L of Tris HCl, pH 8.0, 50 mmol/L of NaCl, and 1 mmol/L of EDTA) and then PBS.

Affinity purification of recombinant protein

Der p I from the renaturation reaction in PBS buffer was purified with an anti-*Der p* I MAbs. MAbs 4C1B8 (50 mg) was coupled to 5 ml of Sepharose 4B (Pharmacia, Piscataway, N.J.) with a rapid cyanogen bromide-activation method.²⁰ The *Der p* I solutions were applied at 10 ml/hr, and the column was washed successively in 100 ml of PBS, 100 ml of 0.1 mol/L borate, 0.5 mol/L of NaCl, 0.05% Tween 20, pH 8.5, and 100 ml of PBS. The final wash was continued until the effluent had an optical density 280 of 0.01. The column was then eluted with water to which ammonium hydroxide had been added to reach a pH of 11. The optical density of the eluate was read immediately after elution, and fractions were dialyzed against PBS.

RESULTS

Expression of *Der p* I from pYEUC114-13T and pYELC5-13T

S. cerevisiae containing the low copy number plasmid pYEUC114-13T or the high copy number pYELC5 was grown in broth culture and the *CupI* cassette induced with Cu⁺⁺. The cells were pelleted, and the lysates were examined for *Der p* I by SDS-PAGE and Western blotting with rabbit anti-*Der p* I. A band could be detected in pYEUC114-13T lysates by Western blotting, but it was of low intensity. No corresponding band could be detected by Coomassie staining of SDS-PAGE. In contrast, Western blotting of pYELC5-13T lysates demonstrated intense reactivity, and a dominant band was detected by SDS-PAGE. Thus, a large band was observed at *M_r* 30 kd, (Fig. 1, third lane from left), which, by comparing the staining intensity of electrophoresed dilutions with the

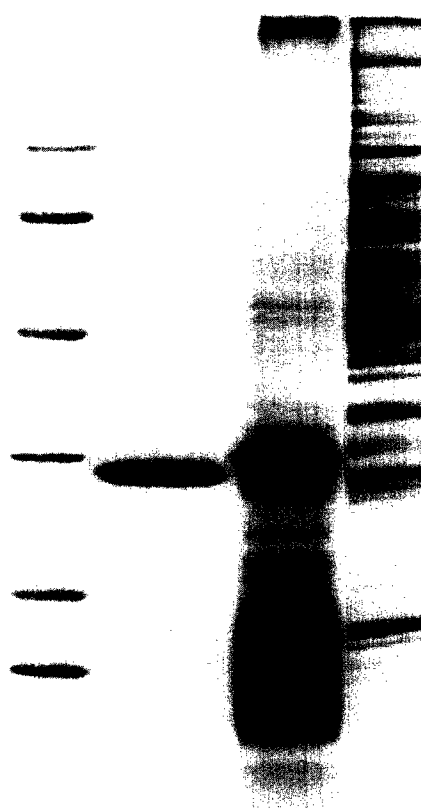


FIG. 1. Coomassie-stained SDS-PAGE of pYELC5-13T lysate. Lanes, left to right: molecular standards of 98, 66, 45, 30, 21, and 14 kd; affinity-purified *Der p* I from mites; lysate of yeast with pYELC5-13T; and lysate of vector control, respectively. Dark band at *M_r* 30 kd in third lane from right corresponds to recombinant *Der p* I.

standards, was estimated to be produced at 40 mg/L. On Western blotting, strong reactivity was found at *M_r* 30 kd with weaker reactivity at about *M_r* 27 kd and lower molecular weight bands. The difference in the size of the recombinant and native *Der p* I is expected because the recombinant *Der p* I contains an additional 28 amino acid residues, 23 from the *Der p* I proenzyme sequence¹ and five from the vector.¹⁴ The lower *M_r* bands were less intense than the *M_r* 30 kd and are interpreted as degradation. Centrifugation at 10,000 *g* for 15 minutes removed the reactivity to anti-*Der p* I in the lysate, demonstrating that the recombinant *Der p* I was insoluble.

Solubilization and purification of pYELC5-13T *Der p* I

To solubilize and purify the recombinant *Der p* I, the pYELC5-13T lysate was centrifuged, and the



FIG. 2. Western blotting of pYELCL5-13T lysate. Samples were electrophoresed in an 8% to 18% SDS-PAGE, blotted, and reacted by radioimmune assay with rabbit anti-*Der p* I; *Lane 1*, affinity-purified *Der p* I; *lane 2*, pYELC5-13T lysate; *lane 3*, pellet pYELC5-13T lysate; *lane 4*, supernatant of pYELC5-13T lysate.

pellet was washed in 1.75 mol/L of guanidine, 1 mol/L of NaCl solution and then suspended in a solution containing 8 mol/L of urea and 2-mercaptoethanol, as described in MATERIAL AND METHODS. This was then homogenized with glass beads and rotated for 1 hour at room temperature and 1 hour at 60° C, and the insoluble fraction was removed by centrifugation. As judged by SDS-PAGE staining, at least 50% of the pellet was solubilized.

No significant precipitation occurred when urea and 2-mercaptoethanol were removed by extensive dialysis. Assays with dot blots for IgE reactivity did not reveal improved binding; therefore, affinity chromatography was attempted with the MAb 4C1B8. Approximately 5% of the total soluble recombinant *Der p* I was bound and eluted from a column coupled with

this MAb. When the affinity-purified protein was checked on SDS-PAGE, a single M_r 30 kd protein band was visible in the stained gel. The final yield was 1 mg/L of yeast culture.

Antigenicity of the pYELC5-13T *Der p* I

The reactivity of IgE from the serum of patients with mite allergy was tested by radioimmune dot blot. Five micrograms of native and recombinant *Der p* I were used in each blot. The same amount of ovalbumin was included as a negative control. Sera from six allergic children and five allergic adults were tested. All the adult sera demonstrated strong IgE binding to both forms of allergen, as illustrated for the example in Fig. 3, A. The results obtained with the children's sera were heterogenous. Two of six sera reacted strongly to native *Der p* I but very weakly to the recombinant allergen (Fig. 3, B). The remaining four sera elicited similar results to the adult sera, demonstrating strong binding to both forms (Fig. 3, C). The control hyper-IgE serum did not reveal any IgE binding (Fig. 3, D). A comparison of the IgE reactivity of native *Der p* I, *Der p* I produced, in bacteria as a fusion with glutathione transferase and yeast, pYELC5-13T. *Der p* I is illustrated in Fig. 4. With a direct dot-blot radioimmune assay with 5 µg of antigen, IgE from serum of allergic patients did not bind to the *Der p* I pGEX fusion, but the binding to pYELC5-13T was similar to that of mite *Der p* I. The absorption study in Fig. 4 illustrates that native *Der p* I and pYELC5-13T *Der p* I removed most reactivity to native *Der p* I when it was added to a dot-blot assay at 67.5 µg/ml, whereas the serum absorbed with the bacterial pGEX-*Der p* I 13T fusion still retains 50% of the reactivity.

In addition to the IgE binding studies, the antigenicity of the recombinant and native *Der p* I was compared with a panel of MAbs by dot-blot immunoassay. There was a degree of heterogeneity in the binding of the MAbs to the recombinant antigen. MAbs 10H2C6 and Nph4 bound strongly to native *Der p* I but had significantly weaker binding to recombinant material. Binding by 5H8D8, 3H8G8, and 5H8C12D9 was similar with both forms. The MAb 6A8B10D12 (*Der f* I specific) bound weakly with both recombinant and native *Der p* I. This differential binding by the MAbs was further investigated in a dose-response manner with MAbs 10H2C6 and 5H8D8 (Fig. 5). Reactivity of 10H2C6 to native *Der p* I was higher than the reactivity of 5H8D8 at both low and plateau concentrations. The reactivity of 10H2C6 with the recombinant *Der p* I, however, only achieved half its binding to native protein. In contrast, although 5H8D8 had lower reactivity to mite *Der p* I, it retained much of the activity when it was expressed by pYELC5-13T.

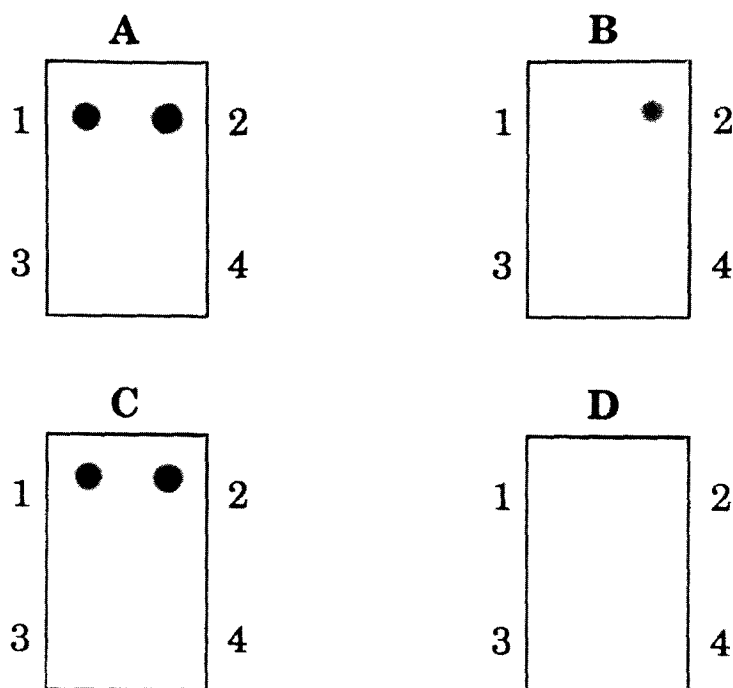


FIG. 3. Reactivity of IgE from sera of mite-allergic subjects with pYELC5-13T *Der p* I. Examples of radioimmune dot blots with ^{125}I -labeled monoclonal anti-IgE are illustrated. **A**, Assay of serum from an allergic adult demonstrating *position 1*, strong reactivity to pYELC5-13T *Der p* I; 2, native *Der p* I; 3, no reactivity to yeast lysate; 4, no activity to ovalbumin. **B**, Example of one of the 2/6 children with no reactivity to pYELC5-13T *Der p* I. **C**, Child with reactivity to recombinant and native. **D**, Assay with serum from subject with hyper-IgE without anti-*Der p* I activity at 500 IU of IgE per milliliter.

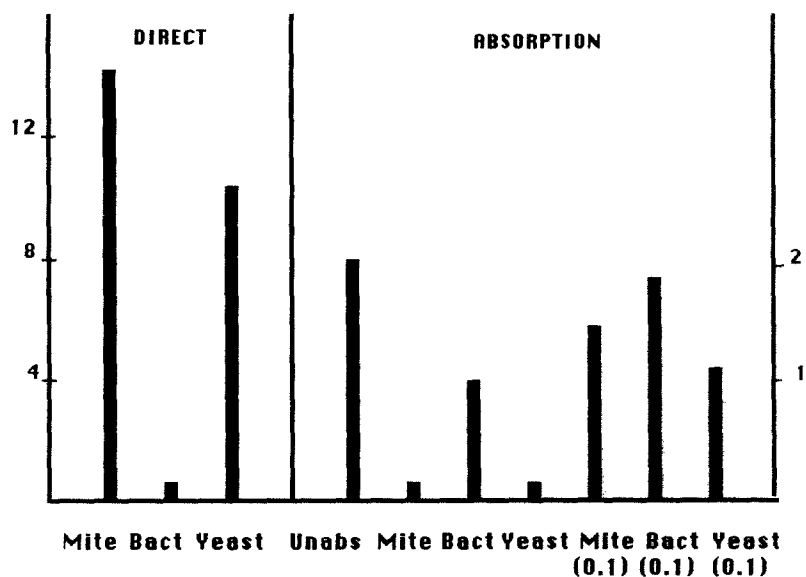


FIG. 4. Reactivity of IgE in sera of allergic subjects with pYELC5-13T *Der p* I. Direct dot-blot assay (*left panel*) was performed by applying 5 μg of *Der p* I (*mite*), pGEX-13TB fusion (*bacteria*), and pYELC5-13T *Der p* I (*yeast*). Allowance was made for the fact that bacterial fusion protein is only half *Der p* I. IgE dot-blot assay was performed and autoradiograph was measured by densitometry and illustrated here as gray scale units $\times 10^{-3}$. For the absorption assay (*right*) the antigens described above were added to the allergic serum (F.B.) to a final concentration of 67.5 $\mu\text{g}/\text{ml}$ or 6.75 $\mu\text{g}/\text{ml}$ (0.1), and a dot-blot immunoassay was performed with the serum at a dilution of 1:4. Reactivity was compared to unabsorbed serum (*unabs*).

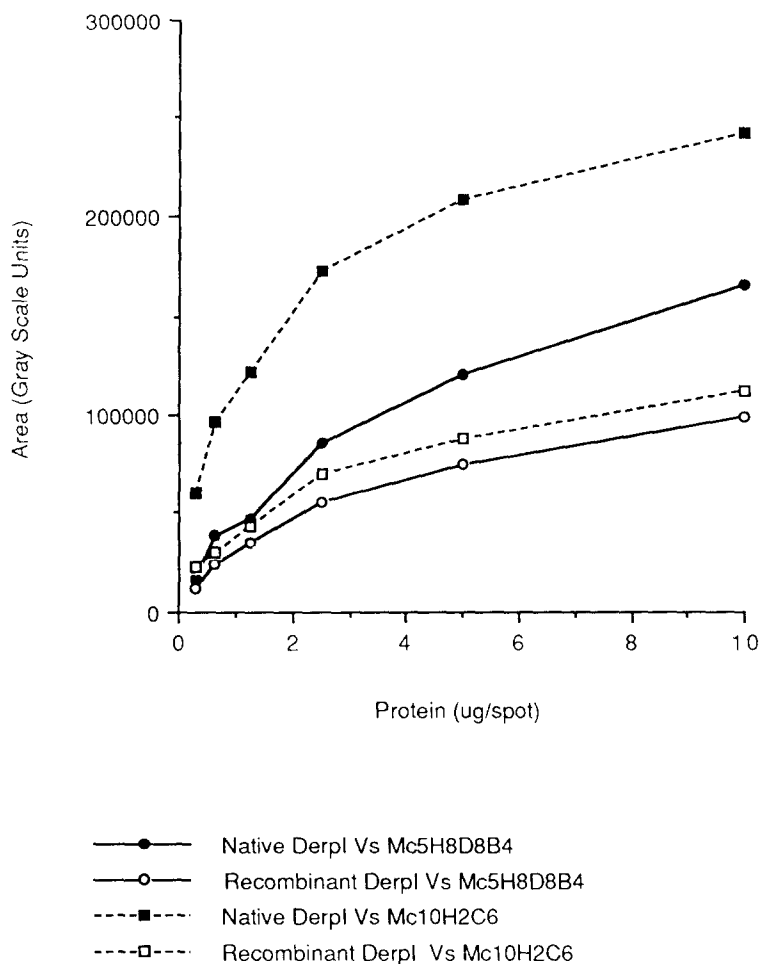


FIG. 5. Reactivity of native (■, ●) and pYELC5-13T (□, ○) *Der p* I with MAbs 10H2C6 (---, boxes) and 5H8D8 (---, circles). Results are densitometric readings of the autoradiograph. With the MAb 5H8D8, the native and pYELC5-13T *Der p* I binding was similar, whereas the yeast *Der p* I could not achieve the high reactivity with 10H2C6 with native protein. Background was 450 gray scale units.

The binding of MAbs 10H2C6 and 5H8D8 to pYELC5-13T *Der p* I was inhibited when native *Der p* I was added to 1 mg/ml (Fig. 6), demonstrating the specificity of the reaction.

DISCUSSION

The production of allergens by recombinant techniques will permit a variety of experimental and, perhaps eventually, diagnostic and therapeutic procedures. At the experimental level it provides a clonally pure source of allergen for serology, the activation of T cells, and the maintenance of T cell lines as well as a method of studying modified allergens by site-directed mutagenesis and other procedures.²¹ Unlike allergens like *Ves g* I,² *Bet v* I,⁴ and *Der p* II,⁶ the reactivity of *Der p* I produced in bacteria is suboptimal; thus, there is a need to develop better expression

systems. The group I mite allergens are interesting molecules for this procedure because they are produced with a large 100 residue proenzyme sequence, which, by analogy with other proteins, is probably involved in folding of the protein. Our previous experiments with pGEX vectors in bacteria^{7,9} have produced *Der p* I as a fusion with glutathione transferase at a yield of about 200 µg of soluble fusion protein per liter. This reacts well with IgE in about half the sera of patients with *Der p* I reactivity and, typically, by absorption analysis, has half the reactivity.⁹ Attempts to produce *Der p* I in *E. coli* in an unfused form with the pTTQ²² and pKK223-3²³ vectors produced small colonies that contained the *Der p* I sequence, as measured by DNA hybridization but which failed to grow.

The yeast vectors in this study contain a *Cupl*

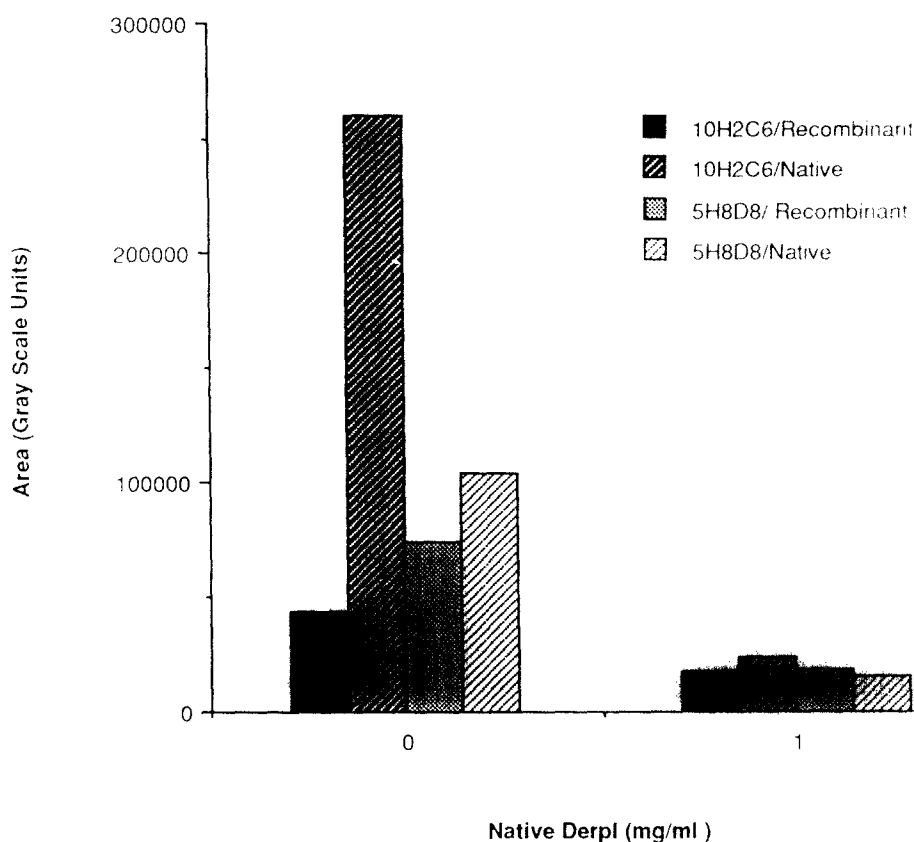


FIG. 6. Inhibition of reactivity of pYELC5-13T *Der p* I with MAbs by native *Der p* I. Results reveal densitometry of radioimmune dot blots of MAbs 10H2C6 and 5H8D8 to recombinant and native *Der p* I in the presence (1) or absence (0) of 1 mg/ml of native *Der p* I.

cassette designed to express a foreign DNA from the metallothionein promoter with only 4 to 8 additional *N* terminal amino acids provided by the metallothionein gene. The *Der p* I in these experiments therefore contained 23 residues from a truncated *Der p* I proenzyme sequence¹ and five from the vector. Although the two vectors used in this study contained the same expression cassette, the low copy number pYEUC114-13T expressed very little *Der p* I, whereas pYELC5-13T produced an estimated 40 mg/L of yeast culture, which is probably one of the highest yields of foreign protein reported for recombinant yeast. Although the pYELC5-13T *Der p* I was insoluble, this was the first time recombinant *Der p* I had been produced without a large fusion partner; therefore, procedures for solubilization in denaturing solutions and renaturation were performed. The pYELC5-13T *Der p* I could be readily dissolved in an 8 mol/L of urea buffer and remained in solution when it was dialyzed against more physiologic buffers. A portion of this protein, amounting to 1 mg/ml of yeast culture, could be isolated on a monoclonal affinity-purification column and used for further anal-

ysis. The remainder did not react with human IgE or a panel of MAbs (data not presented) but could be useful for further renaturation or T cell studies.

When the ability of the affinity-purified yeast *Der p* I to react with IgE in serum from allergic patients was examined, it was found to react strongly with 5/5 adult's sera and 4/6 children's sera, eliciting dot-blot reactions of similar intensity to the native allergen. The bacterial fusion *Der p* I pGEX-13TB does not demonstrate detectable reactivity at the concentrations used in this study, and even at high doses (50 µg per dot), reacted with only half the sera tested.⁹ Likewise, it is demonstrated in this study that the yeast *Der p* I could absorb out anti-*Der p* I IgE antibodies to a much greater degree than the fusion. The finding that 2/6 of the children reacted strongly with native, but not with yeast *Der p* I, however, demonstrates that there are still differences in the epitopes expressed.

The studies of Lind et al.¹¹ and Chapman et al.¹⁰ with MAbs have demonstrated that IgE binding can be inhibited by four non-cross-inhibiting MAbs. Our own studies with recombinant peptides demonstrate

at least five non-overlapping regions.⁹ The binding of the pYELC-13T demonstrated substantial, but not complete, reactivity to MAbs in all four groups. Also, the reactivity of the yeast *Der p I* demonstrated a correlation with the grouping of Lind et al.¹¹ and Chapman et al.¹⁰ Thus, the MAbs 10H2C6 and Nph4, which strongly cross inhibit, both react strongly to native *Der p I* and achieved about half the reactivity to the yeast molecule. The MAbs in the other groups did not react as well to native *Der p I* but had relatively high reactivity to the yeast *Der p I*. These studies, however, indicate that the renatured molecule contains much of the structure of *Der p I* and that the negative sera only recognize limited epitopes.

Although the antibody binding demonstrated by the renatured *Der p I* indicates there are differences in its structure from the native molecule, the result did demonstrate that a high degree of IgE binding reactivity can be achieved. The high frequency of binding (9/11) also formally demonstrates that a single gene product of *Der p I* is a major allergen. It is anticipated that further advances in expression will obtain a product suitable for a variety of investigative and perhaps clinical studies.

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