

Isolation and preliminary characterization of cDNA encoding American cockroach allergens

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Background: Two prominent proteins of 78 and 72 kd in Cr-PI have been found to be the major allergens of American cockroach (*Periplaneta americana*).

Methods: A λ gt22A cDNA library generated from messenger RNA of American cockroach was packaged into *Escherichia coli* Y1090(r⁻) and initially screened with rabbit polyclonal antiserum raised to crude extract of American cockroach (CRA-A).

Results: Twenty-nine anti-CRA-A-positive clones were isolated, and 11 clones were recognized by rabbit anti-Cr-PI and reactive with IgE antibodies of atopic serum pool. Among these 11 clones, eight were recognized by murine anti-Cr-PI monoclonal antibodies. Four clones (C7, C8, C12, and C29) were found to contain inserts of 2.6 kilobases (kb), and clones C5 and C20 were found to contain inserts of 2.4 kb. The remaining clones (C13, C23, C25, C28, and C35) were found to contain inserts of 1.8, 1.6, 2.5, 1.7, and 0.9 kb, respectively. Clones C12, C20, C13, and C28 were selected, subcloned into the expression pET vectors, and used to transform *E. coli* BL21(DE3). Immunoblot analyses of clones C12, C20, C13, and C28 with anti-Cr-PI monoclonal antibodies revealed fusion proteins with molecular weights of 78 and 50 kd, 72 and 43 kd, 54 kd, and 46 kd, respectively. However, among those fusion proteins only those with molecular weights of 78, 72, 54, and 46 kd were able to bind human specific IgE antibodies.

Conclusions: The cDNA clones are expected to code for the major and principal allergens of American cockroach, and recombinant allergens may therefore be valuable for diagnostic and therapeutic purposes. (*J ALLERGY CLIN IMMUNOL* 1995;96:352-9.)

Key words: cDNA cloning, American cockroach, Cr-PI allergens

Cockroaches are recognized as one of the major causes of inhalant allergy and asthma, and a high prevalence of cockroach hypersensitivity in atopic and asthmatic populations has been reported.¹⁻¹¹ The significant allergens of American cockroach with molecular weights (MWs) ranging from 6 to 120 kd have been identified by various immunochemical methods in different laboratories.^{8, 12-18}

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Abbreviations used

bp:	Base pairs
CRA-A:	Crude extract of American cockroach
mAbs:	Monoclonal antibodies
MWs:	Molecular weights
IPTG:	Isopropylthiogalactoside
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TBS:	Tris-buffer saline
TBST:	TBS containing 0.05% Tween-20

In our previous studies, two partially purified allergenic fractions, Cr-PI and Cr-P II, from crude extract of American cockroach (CRA-A) were isolated.¹² Two prominent proteins of 78 and 72 kd in Cr-PI that bound 100% of the atopic sera tested have been identified as the major allergens of American cockroach,¹² and monoclonal antibodies

(mAbs) against these two allergens have been generated.¹⁹ Recently, biologically active mRNA specific for proteins corresponding to Cr-PI has been isolated from whole bodies of American cockroaches in our laboratory.²⁰ Analysis of in vitro translation products with polyclonal antibodies and mAbs revealed that synthesized proteins with MWs of 90, 78, 72, 49, 45, and 26 kd corresponded with previously identified principal and major allergens of American cockroach.¹²

The impact of recombinant DNA technology on immunochemical structure and properties of allergens is best illustrated by the fact that several allergens have recently been cloned and sequenced.²¹ Manipulation of allergen cDNA clones can provide information on size, structure, and allergenicity. Thus well-defined recombinant allergens will improve the accuracy and reliability of diagnostic tests, and structurally modified allergens can be used to develop new forms of immunotherapy. The present study describes the isolation and preliminary characterization of cDNA encoding American cockroach allergens.

METHODS

Bacterial and phage strains

Escherichia coli strain Y1090(r⁻)²² has been described previously. The cloning vector, λ gt22A (Gibco BRL/Life Technologies, Gaithersburg, Md.), is a modification of λ gt22, which is derived by a series of constructions from λ gt11.²³

Antisera

Polyclonal antibodies against CRA-A and Cr-PI were raised in rabbit,¹² and Cr-PI mAbs were generated from fusion between immunized spleen cells of BALB/c mice and NS-1 cells.¹⁹ Rabbit anti-Cr-PI and mAbs were purified by sodium sulfate precipitation and protein A-agarose affinity chromatography,¹⁹ respectively. Human serum samples were obtained from cockroach-sensitive patients attending the Allergy Clinic at Taichung Veterans General Hospital and were assayed for cockroach-specific IgE antibodies by fluoroallergosorbent test.¹¹ Equal volumes of A-2 and E-4 mAbs¹⁹ or equal volumes of sera of 10 patients with an American cockroach-specific fluoroallergosorbent test result of class III or above were pooled to constitute a pool of mAbs and an atopic serum pool, respectively.

Isolation of mRNA and construction of cDNA library

Total RNA was extracted from whole bodies of American cockroaches with chaotropic salt guanidine isothiocyanate in the presence of 2-mercaptoethanol, and polyadenylated mRNA was isolated by oligothymidylic acid chromatography as described by Scheiner.²¹ Super

Script Lambda System (Gibco BRL/Life Technologies) for cDNA synthesis and a lambda cloning kit were used to construct cDNA libraries. The initiation of first-strand synthesis depends on the hybridization of *NotI* primer at the polyadenylate tail of mRNA. After first- and second-strand synthesis, *SalI* adapters were added to the cDNA before digestion with *NotI*, which placed the same *SalI* 5'-extension at both ends of the cDNA. Size fractionation of cDNA was performed with column chromatography after adapter addition and digestion with *NotI*. Size-fractionated cDNA was then ligated into the λ gt22A vector and packaged into *E. coli* Y1090(r⁻) with a Packagene Lambda DNA Packaging System (Promega, Madison, Wis.) according to the supplier's instructions.

Screening of cDNA library

The library was plated according to standard procedures²⁴ on 100 mm Petri dishes and initially immunoscreened according to the Promega Protoblot with polyclonal anti-Cr-PI (1.0 μ g/ml) preabsorbed with *E. coli* lysate. Phages from immunopositive plaques were amplified by the plate lysate method.²⁴ Amplified immunopositive clones were dot-screened by 10 mmol/L isopropylthiogalactoside saturated nitrocellulose filter and probed with either a pool of protein A affinity-purified monoclonal mouse anti-Cr-PI antibodies (1.0 μ g/ml) or human atopic serum pool (1:30), and pre-immunized rabbit or BALB/c mouse serum (1.0 μ g/ml) was used as a control. Peroxidase-conjugated goat anti-rabbit IgG (1:1000; Sigma Chemical Co., St. Louis, Mo.), rabbit anti-mouse IgG (1:8000; Bio-Rad, Richmond, Calif.), or iodine 125-labeled anti-human IgE (Pharmacia, Piscataway, N.J.) was used as second antibody. Detection of bound second antibody was performed by the 4-chloro-1-naphthol substrate system (Bio-Rad) or by autoradiography.^{12, 19}

Analysis of cDNA inserts

DNA was prepared from liquid lysates of plate lysate-amplified immunopositive λ gt22A clones by using polyethylene glycol precipitation²⁴ in a rapid small-scale procedure.²⁵ Briefly, phage lysate was mixed with an equal volume of 20% polyethylene glycol containing 2.0 mol/L NaCl in SM buffer and incubated for 1 hour on ice. After incubation, the mixture was centrifuged at 25000 g for 20 minutes, and the pellet was resuspended in Luria broth. The mixture was then mixed with an equal volume of diethylaminoethyl cellulose (DE52; Whatman, Clifton, N.J.) in Luria broth and mixed gently by inversion 20 to 30 times. After centrifugation, proteinase K and sodium dodecylsulfate (SDS) were added to the supernatant for a final concentration of 17 μ g/ml and 4.2 μ g/ml, respectively, and incubated at room temperature for 5 minutes. Potassium acetate was added, incubated at 88° C for 20 minutes, cooled on ice for 10 minutes, and then centrifuged. The supernatant was mixed with an equal volume of cold isopropanol and

cooled to -70°C for 10 minutes; the pellet was then dried and resuspended in TE buffer, pH 8.0. Restriction endonuclease digestion was performed on miniprep DNA with the enzymes *EcoRI* and *NotI* or *Sall* and *NotI*, and gel electrophoresis was performed in 0.8% agarose to determine the size of cDNA inserts.

Target gene expression

Plaque-purified DNA of inserts (C12, C20, C13, and C28) were ligated into the multiple cloning sites of pET System (Novagen, Inc., Madison, Wis.) and used to transform *E. coli* BL21(DE3). Recombinant antigens were prepared from *E. coli* BL21(DE3)²⁶⁻²⁸ transformed with the resulting expression vectors pET-21a(+) (C12 and C20) or pET-21b(+) (C13 and C28). Briefly, pET-21a(+) or pET-21b(+) transformed *E. coli* BL21(DE3) was grown in Luria-Bertani medium containing appropriate antibiotics at 37°C overnight. Cells of the stationary culture were inoculated into fresh Luria-Bertani medium and incubated at 37°C until the optical density at 600 nm reached 0.6 to 1.0. Then isopropylthiogalactoside was added to a final concentration of 1.0 mmol/L and incubated at 37°C with aeration. Cells were harvested at 4 hours after induction and resuspended in SDS sample buffer and boiled for 5 minutes before sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.²⁴

Detection of recombinant proteins by SDS-PAGE and immunoblotting

Proteins were subjected to SDS-PAGE under denatured conditions with a discontinuous buffer according to the method of Laemmli²⁹ as previously described.¹⁹ After SDS-PAGE, protein bands were transferred onto 0.45 μm polyvinylidene fluoride membrane (Immobilon P, Pharmacia) by the methods of Towbin et al.³⁰ as described.¹⁹ Polyvinylidene fluoride membranes were washed in 10% methanol in Tris-buffered saline (TBS) (20 mmol/L Tris, 0.5 mol/L NaCl, pH 7.5) with rocking, and then incubated for 2 hours with 3% gelatin in TBS. After three washes with TBS containing 0.05% Tween-20 (TBST), the blots were incubated overnight at 4°C with purified anti-Cr-PI mAb (15 $\mu\text{g}/\text{ml}$)¹⁹ diluted in TBST containing 0.5% gelatin or sera of atopic subjects (diluted 1 in 30 with 0.05% Tween-20 and 0.1% sodium azide in TBS). Polyvinylidene fluoride strips were then washed with TBST twice in 1 hour with rocking and then incubated either with peroxidase-labeled anti-mouse IgG (Sigma, 1:30,000) for 2 hours at room temperature or with ¹²⁵I-labeled anti-human IgE (Pharmacia) overnight at room temperature. After extensive washing, air-dried strips were either immersed in a substrate solution of 0.05% 4-chloro-1-naphthol and 0.03% H_2O_2 in TBS or autoradiographed on Hyperfilm-MP (Amersham International plc., Amersham, U.K.) for 5 days at -70°C .

RESULTS

Screening of cDNA library

A $\lambda\text{gt}22\text{A}$ cDNA library derived from American cockroach mRNA was screened initially with anti-CRa-A polyclonal antibody. Twenty-nine anti-CRa-A-positive clones were isolated (data not shown). Further screening with rabbit polyclonal anti-Cr-PI yielded 11 clones expressing Cr-PI (data not shown), and they were amplified by the plate lysate method and selected for further analysis. Aliquots of amplified stock were spotted onto a lawn of *E. coli* Y1090(r⁻) and tested with either human atopic serum pool or the murine anti-Cr-PI mAbs, and preimmunized rabbit or BALB/c mouse serum was used as a control. None were found to be reactive with pre-immunized rabbit or mouse serum (data not shown). All of the 11 clones were found to express protein epitope(s) recognized by human IgE antibodies (Fig. 1, A), and eight clones were positive according to tests with mAbs (Fig. 1, B).

Determination of size of inset by restriction enzyme mapping

DNA isolated from these 11 polyclonal anti-Cr-PI-positive clones were digested with enzymes *EcoRI* and *NotI*, and the insert sizes were determined by electrophoretic analysis (Fig. 2, A and B). Clones C7, C8, C12, and C29 appear to contain an identical insert of 2.6 kb with two *Sall* sites. Clones C5 and C20 have an identical 2.4 kb insert, and clone C25 contains a 2.5 kb insert with a single *Sall* site. Clone C23 had a 1.6 kb insert containing one *EcoRI* site and one *Sall* site, whereas clone C35 contains a 0.9 kb insert with a single *EcoRI* site. The remaining clones, C13 and C28, have inserts of 1.8 and 1.7 kb that contain neither an *EcoRI* nor a *Sall* site. Results are summarized in Table I.

Expression and immunodetection of fusion proteins

Clones C12, C13, C20, and C28 recognized by both human specific IgE and murine anti-Cr-PI mAbs were selected, subcloned into pET expression vectors, and used to transform *E. coli* BL21(DE3). SDS-PAGE-separated components of recombinants clearly demonstrated fusion proteins of MWs 78, 72, 54, and 46 kd for C12, C20, C13, and C28, respectively (Fig. 3, A, lanes b-e). Fusion proteins of approximately 78 and 50 kd (Fig. 3, B, lane b), 72 and 43 kd (Fig. 3, B, lane c), 54 kd (Fig. 3, B, lane d), and 46 kd (Fig. 3, B, lane e) were identified by anti-Cr-PI mAb probed immunoblots for C12, C20, C13, and C28, respectively. Immunoblot analyses of CrA-A and Cr-PI

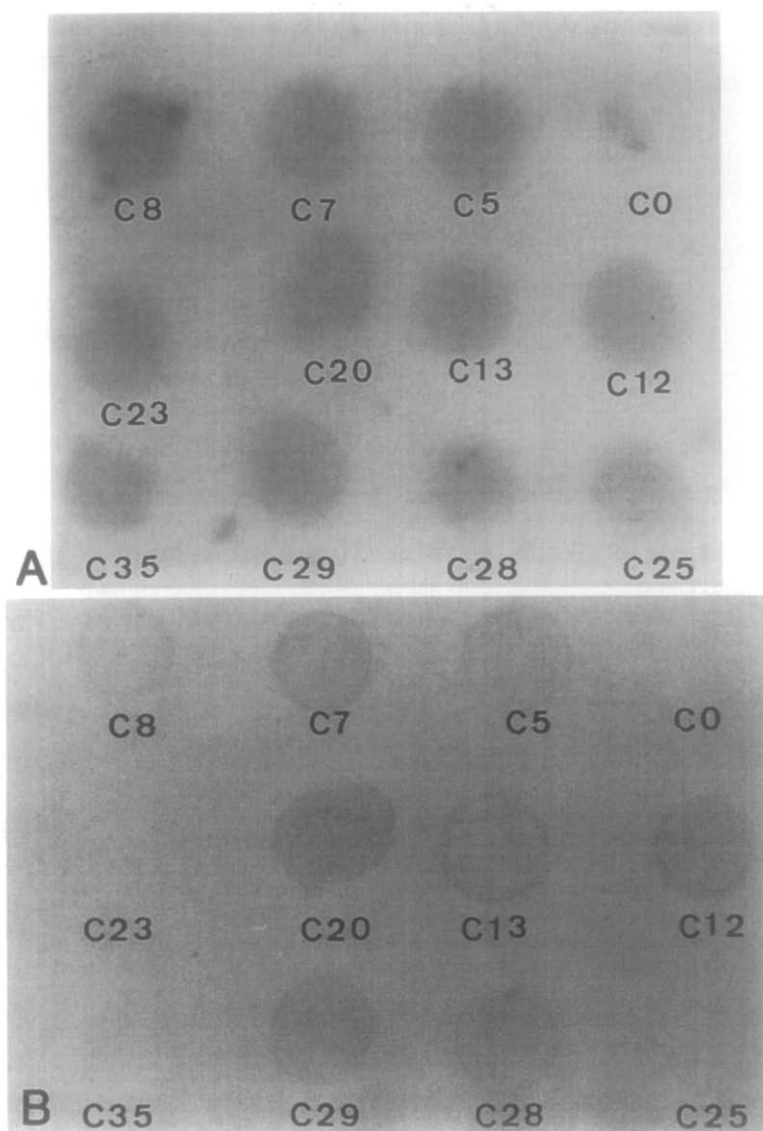


FIG. 1. Dot screening of purified λ gt22A clones on *E. coli* Y1090(r⁻). Five microliters of λ gt22A (C0) or phage stocks amplified from immunopositive clones were spotted onto a lawn of *E. coli* Y1090(r⁻) induced with a nitrocellulose filter saturated with 10 mmol/L isopropylthiogalactoside and probed with (A) IgE of human reaginic serum pool or (B) murine anti-Cr-PI mAbs.

with anti-Cr-PI mAbs revealed proteins with MWs ranging from 30 to 78 kd (Fig. 3, *B*, lanes *f* and *g*). No protein in Cr-PII was recognized by anti-Cr-PI mAbs (Fig. 3, *B*, lane *h*). Fusion proteins with MWs 78, 72, 54, and 46 kd from C12, C20, C13, and C28 were reactive with IgE antibodies in the human atopic serum pool (Fig. 3, *C*, lanes *b*, *c*, *d*, and *e*). Immunoblots of CRa-A, Cr-PI, and Cr-PII probed with human IgE revealed proteins of MWs ranging from 12 to more than 98 kd (Fig. 3, *C*, lane *f*); 78 and 72 kd (Fig. 3, *C*, lane *g*); and 32 to 78 kd (Fig. 3, *C*, lane *h*), respectively. Except for nonspecific binding of a 39 kd band, which was found in both nonrecombinants and recombinants (Fig. 3,

C), no protein from *E. coli* BL21(DE3) was recognized by both human IgE antibodies (Fig. 3, *C*, lane *a*) and anti-Cr-PI mAbs (Fig. 3, *B*, lane *a*).

DISCUSSION

The purposes of this study were the isolation and preliminary characterization of cDNA encoding allergens from the American cockroach. Eleven polyclonal anti-Cr-PI-positive clones (Fig. 1) were isolated and divided into seven groups (Table I) on the basis of size and restriction enzyme mapping (Fig. 2). Clones C12, C13, C20, and C28 were recognized by both specific IgE and mAbs antibodies and thus were selected for further characteriza-

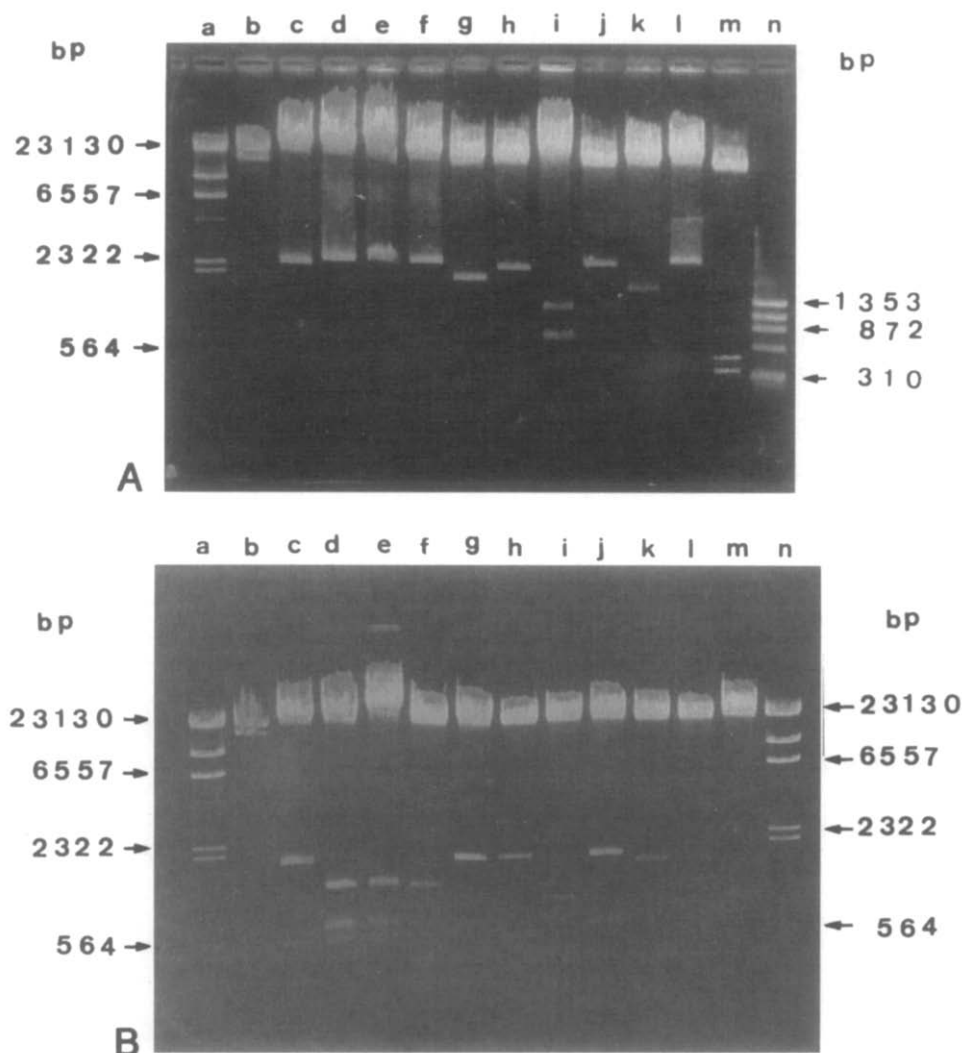


FIG. 2. Agarose gel electrophoresis of amplified and polyethyleneglycol-precipitated λ DNA digested with enzymes *EcoRI* and *NotI* (A) or enzymes *SalI* and *NotI* (B). Lane a, *HindIII* digested λ DNA; lane b, λ gt22A; lane c, clone C5; lane d, clone C7; lane e, clone C8; lane f, clone C12; lane g, clone C13; lane h, clone C20; lane i, clone C23; lane j, clone C25; lane k, clone C28; lane l, clone C29; lane m, clone C35, and lane n, *HaeIII*-digested ϕ X174 DNA. Numbers at left and right indicate size of standard DNA fragments in base pairs (bp).

TABLE I. Primary characteristics of cDNA clones from the American cockroach

	C7, C8, C12, C29	C5, C20	C13	C23	C25	C28	C35
Rabbit anti-Cr-PI	+	+	+	+	+	+	+
Murine anti-Cr-PI mAbs	+	+	+	—	—	+	—
Human CRa-A IgE	+	+	+	+	+	+	+
Size of insert (kb) after <i>EcoRI</i> and <i>NotI</i> digestion	2.6	2.4	1.8	1.0 and 0.6	2.5	1.7	0.6 and 0.3
Size of insert (kb) after <i>SalI</i> and <i>NotI</i> digestion	1.3, 0.7 and 0.6	1.8 and 0.6	1.8	0.9 and 0.7	1.9 and 0.6	1.7	0.9

tion. Analysis of fusion proteins by immunoblots confirmed that the immunopositive recombinants are cockroach allergen clones (Fig. 3). Two of each of the recombinant fusion proteins, 78 and 50 kd,

and 72 and 43 kd, were reactive with a mixture of anti-Cr-PI mAbs from clones C12 and C20, respectively. However, only fusion proteins of 78 and 72 kd, corresponding with previously identified major

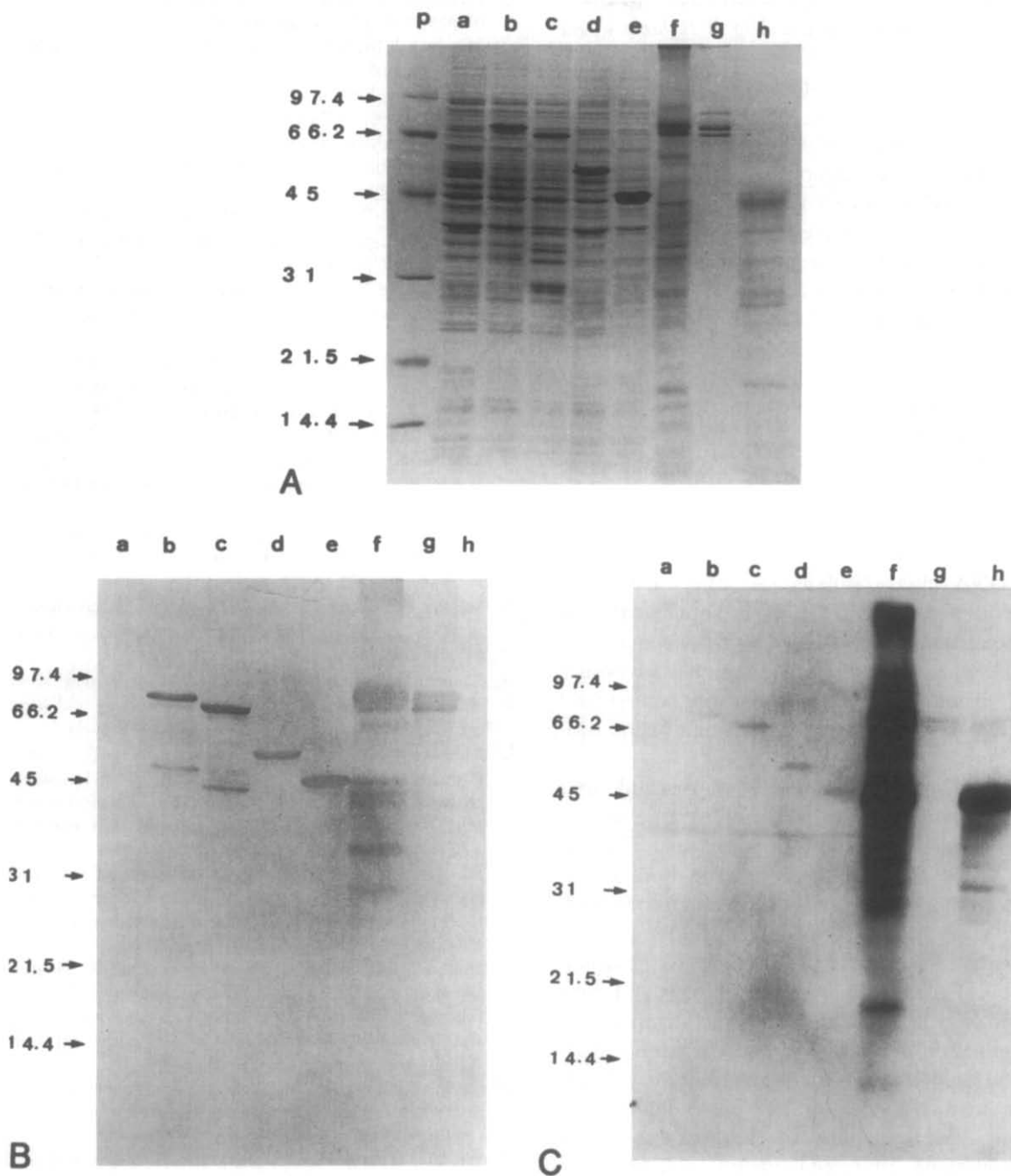


FIG. 3. Staining patterns (**A**) and immunoblots (**B**, probed with murine anti-Cr-PI mAbs and **C**, probed with IgE of human reaginic serum pool) of SDS-PAGE-separated components of *E. coli* BL21(DE3) lysate, cDNA clone lysates, CrA-A, Cr-PI, and Cr-P. Lane p, Protein markers; lane a, *E. coli* BL21(DE3); lane b, clone C12; lane c, clone C20; lane d, clone C13; lane e, clone C28; lane f, CrA-A (30 μ g); lane g, Cr-PI (10 μ g); and lane h, Cr-P. (10 μ g).

allergens of American cockroach,¹² were recognized by a human IgE serum pool. Fusion proteins of 50 and 43 kd may be the degraded products of 78 and 72 kd allergens of American cockroach. Fusion proteins of 54 and 46 kd from clones C13 and C28 recognized both anti-Cr-PI mAbs and

human specific IgE. Whether fusion proteins with MWs of 54 and 46 kd are fragments or only shared partial sequences of the 78 or 72 kd allergenic proteins remains to be determined. The 46 kd fusion protein (Fig. 3, C, lane e) and 45 kd of Cr-P. (Fig. 3, C, lane h) appear to be different

proteins because one was able to bind anti-Cr-PI mAbs and the other was not (Fig. 3, *B*, lanes *e* and *h*).

Cr-P II contains many low MW components of CRa-A (<50 kd, Fig. 3, *A*, lane *h*), and it may contain important allergens of American cockroach. Our recently unpublished data indicate that some components of Cr-P II, including the 45 kd protein, bound IgE in high frequency with atopic sera tested. Cr-P II is currently under investigation in our laboratory. CRa-A contains many important allergens of American cockroach; it will be crucial to determine whether these components are derived from alternatively spliced forms of the same pre-mRNA transcript or whether they are the products of different genes. We have long suspected that native 78 and 72 kd components in CRa-A may be different forms of the same protein or may share the common IgE-binding epitopes. Nevertheless, it remains to be established whether the cDNA clones isolated actually encode the major allergens in Cr-P I. It is anticipated that these questions will be solved by DNA sequencing of the clones, the raising of polyclonal antibodies to the fusion proteins and generating amino acid sequence data of native allergens and fusion proteins.

From our preliminary results, we conclude that clones C12 and C20 are predicted to code for the major allergens of American cockroach. This appears to be the first time that the isolation of cDNA coding for allergens of American cockroach has been reported. We are currently attempting to purify and raise polyclonal antibodies to the fusion proteins. The clonally purified products and their antibodies will be useful for characterization and determination of allergenicity in vitro and in vivo and will facilitate the determination of the primary amino acid sequence of the corresponding allergens. The availability of these cDNA clones is encouraging, and recombinant allergens may therefore be valuable for diagnostic and therapeutic purposes. Further characterization and sequencing of cDNA clones are also in progress in our laboratory.

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