

The structural basis of allergenicity: recombinant DNA-based strategies for the study of allergens

B. A. BALDO & G. R. DONOVAN

Kolling Institute of Medical Research, Royal North Shore Hospital of Sydney, St. Leonards, N. S. W. Australia and CSIRO Wheat Research Unit, North Ryde, N. S. W. Australia.

Key words: allergenic determinants; allergens; allergen structure; antigenic determinants; cloning; recombinant DNA.

1. Protein antigens

Potentially antigenic proteins are widely distributed and abundant throughout the biosphere. Proteins to which the immune system can respond may be of vertebrate, invertebrate, plant or microbial origin and thus constitute an ever-present pool of molecules with the capacity to stimulate antibody and cellular immune responses.

While the importance of protein antigens is self-evident to the immunologist, the molecular explanations that underlie the relationships between antigenicity, immunogenicity and the interaction between antigen and antibody have been undergoing a reappraisal in recent years. The reasons for, and the essential details of this reappraisal, the implications this has for our understanding and interpretation of allergenic structure, and strategies utilizing recombinant DNA technology for applying and extending the understanding of protein antigens to the field of immediate hypersensitivity will be examined in this article.

(1) *The nature of antigenic determinants.* The term "antigenic determinant" refers to an accessible region on a protein that is both immunogenic and antigenic, that is it has the capacity to both elicit antibody formation and to react with them. In the 1960s, Sela (59) showed that antigenic determinants could be either sequential or conformational (see below). Factors identified

as influencing antigenicity of a protein include size, conformational freedom, hydrophilic and hydrophobic properties and sequence flexibility but overlap between these may occur (8, 20, 37, 52, 53, 58, 59).

From the time of the earliest investigations on the production of antibodies and the empirical studies on the antigenicity of proteins and their interaction with antibodies, a widely held view has been that a few sites, more or less independent of the rest of the protein molecule, determine the entire antigenicity of a protein (5, 8, 22, 23). This notion that antigenic determinants are discontinuous and relatively few in number appeared to provide, superficially at least, a satisfying view of a protein antigen and one that seemed to accord with the molecular complementarity that exists between discrete hapten determinants and the antibody combining site in hapten-carrier - antibody interactions. In addition, only limited portions of a protein seemed to be immunogenic and since determinants are often destroyed by proteolysis or denaturation, this was interpreted as indicating that determinants are conformational or discontinuous or, what would now be called assembled topographical determinants. Other determinants are called sequential determinants although some investigators believe that the division into conformational and sequential types creates an artificial distinction (67). Sequential refers to a determinant which is composed of a continuous linear sequence of

amino acids, whereas a conformational determinant is one assembled from residues not normally adjacent to each other in the primary sequence of the protein but brought together by the folding of the protein into its native three dimensional conformation. The general conclusion seems to have been that about one antigenic site exists for each 5000 dalton of protein (22). For some proteins, and in particular for serum albumin, results of many studies over an extended period of time (reviewed in 19) support the view that multiple, distinct but occasionally overlapping antigenic determinates exist. Compelling evidence for the few site view of antigenicity is said to be the definitions of the precise determinants of the entire antigenic structures of myoglobin and hen egg white lysozyme (5, 6). However, results demonstrating the existence of more antigenic sites on myoglobin and lysozyme (reviewed in 45) than previously claimed raises doubts about the classical picture of antigenic determinants as short, discrete peptide sequences of inherent antigenicity.

A vital omission from any consideration of immunogenicity and antigenic sites is the failure to define these sites in terms of host responses. Recognition that the sites can only be defined with respect to a particular responding individual leads logically to the realization that the total sum of all the sites recognized by a large but unknown number of responding individuals and species constitutes the antigenicity of a protein. With this in mind, the view of a protein antigen as a continuum of potential, and perhaps overlapping, antigenic sites covering all or most of the accessible surface of the protein embodies what has been termed the "multideterminant-regulatory model" of the nature of protein antigenicity (19). The multideterminant theory is supported by observations of the immunogenicity of small, free peptides, the existence of multiple overlapping domains on synthetic peptides and overlapping recognition patterns demonstrated by the combining sites of monoclonal antibodies (19, 20, 45). In findings inconsistent with those of Atassi (5, 6), the existence of multiple surface domains is also supported by the change in cross-reac-

tivity between myoglobins from different species as a result of evolutionary sequence divergence of amino acids on the surface and the differing affinities of different myoglobins for the same antibody population despite the presence of identical known determinants (reviewed in 45 and 67).

(2) *Identification of antigenic determinants.* Although the primary structures of many proteins are known, the tertiary and quaternary structures are available for a much smaller number. Hence, it is to be expected that there has been a concentration of effort on sequential determinants. Identification of determinants is usually based on one or more of a few standard procedures, the most important of which include fragmentation of the native protein; generation of anti-peptide antibodies and assessment of their reactions with native protein (8, 19, 45, 67); employment of antibody probes of known specificity (45); immunochemical studies of cross-reactivity between related proteins possessing known substitutions (72); sequential synthesis of overlapping sets of peptides (32) and crystallographic study of antigen-antibody complexes (3). In addition, there have been many attempts to devise empirical or theoretically based methods for predicting the positions of antigenic determinants on proteins. Some success has been achieved with some of the predictive procedures and, in particular, with hydrophilicity profiling of the polypeptide chain (38, 60).

2. Allergens

(1) *The nature of allergens and their sources.* The words "allergy" and "allergen" tend to be reserved for use in the context of immediate allergic reactions and that association will be maintained here. Hence, the term allergen used here will mean an antigen that can induce an immediate hypersensitivity (that is, an IgE-mediated), and not a delayed, allergic reaction in man. Sources of allergens are widely distributed in man's environment and include epithelia, saliva, semen and urine from higher animals, venoms, hairs, scales and faeces from

insects, arachnids, marine invertebrates, pollens, fruits, leaves and gums from plants, fungi, bacteria and probably viruses (12, 41, 49). In addition, components from some of these and other sources may provoke allergic reactions after being ingested as foods, for example, meat, milk, shellfish and other marine animals, cereals and vegetables from plants, mushrooms from micro-organisms and so on (10, 14, 66). Even "small" organic molecules, either naturally occurring or synthesized in the laboratory and taken by man as medicaments, may act as allergens (11, 25). Most allergen sources are complex mixtures and contain more than one allergen (12, 13, 41, 49).

(2) *Allergenic determinants*. In general, allergens are proteins or glycoproteins with MWs in the range approximately 5000–70,000 (41). Apart from the requirement that allergens be at least antigenically divalent and capable of inducing and reacting with IgE antibodies, little is known of the physicochemical basis of allergenicity. Too few allergens have been purified and chemically characterized and even fewer allergenic determinant structures have been identified to allow us to understand the physicochemical basis of allergenicity. Many studies in immunology have focussed on the analysis of antigenic determinants in proteins. However, the characterization of the allergenic, or IgE-binding determinants, has proceeded more slowly and few comparisons of allergenic and antigenic determinants have been made. Allergenic determinants for a small number of allergens have been elucidated and details of the findings are summarized.

Codfish allergen M (Gad c I): This is the major allergenic protein of codfish. Initially, synthetic and native peptide fragments were used to locate the immunoglobulin binding sites at residues 33–44, 49–64, 65–74 and 88–96 in the native protein (for a review, see 27). The binding locus for the immunoglobulin molecule in the region 49–64 on the cod protein was characterised by Elsayed et al. (29) as comprising two tetrapeptide units of asp-glu-asp-lys and asp-glu-leu-lys, separated by a 6-amino acid spacer sequence. This was achieved following the sequencing of the protein

and synthesis of the hexadecapeptide corresponding to residues 49–64. Three homologous tetrapeptides in the region 41–64 were eventually shown to be responsible for the full immunological activity of the protein. The third tetrapeptide (residues 41–44) was located using four synthetic peptides from the region 39–54 to probe cod-allergic sera and in inhibition studies with Gad c I. Because Gad c I retains its allergenicity despite denaturation with heat, acid, alkali and salt, it has been suggested that its allergenic determinants are sequential rather than conformational.

Ragweed allergen Ra3 (Amb a III): Atassi & Atassi (7) reported the localisation of the full profile of continuous antigenic determinants in this molecule. Ten consecutive peptides, each 15 residues long (except the final one) and overlapping the adjacent peptide by 5 residues were used to localise four continuous antigenic sites at residues 8–17, 28–38, 53–63 and 73–83. These sites coincided with the allergenic sites.

Chironomid haemoglobins (Chi t I): The haemoglobins of Chironomidae, a family of non-biting insects have been identified as potent allergens. An antigenic site, CTT IV (residues 91–101) was identified and characterised in detail by Baur et al. (18), using the 11-amino acid synthetic peptide corresponding to this region. Seven of the 11 amino acids in the determinant were polar or hydroxylated and occupied exposed positions. The side chains of the residues in this determinant were linked to neighbouring amino acid side chains by hydrogen bonds or solvent clusters. Five of the amino acids had high thermal mobility factors and were highly mobile. In contrast, the peptide CTT IV (residues 102–108) which was not cross-reactive with human antibodies to haemoglobin, contained no polar amino acids and had low thermal mobility, supporting the involvement of hydrophilicity and mobility in antigenicity.

(3) *Clinical observations suggesting structural similarities between allergens from different sources*. Although few laboratory results are available, a number of clinical observations suggest that IgE antibodies in the sera of some allergic patients may cross-react with allergens obtained from

quite unrelated sources. For example, up to 70% of patients allergic to tree pollens of the Betulaceae family, for example, birch (*Betula pendula* (verrucosa)), alder (*Alnus* spp.) and hazel (*Corylus avellana*) may be sensitive to a variety of raw fruits, vegetables and nuts (26, 30, 34, 47). Other apparent associations or clusters reported include plant proteases with grass and birch pollens on the one hand and cereal endosperm proteins on the other (17). Allergic cross-reactions between buckwheat, some vegetables and pollens and hymenoptera venom makes up another curious cluster (1). In addition, melon and banana sensitivities coincident with immediate allergic reactions to the dicot pollen from ragweeds (*Ambrosia* sp) are known (4). Recently, cross-allergenicity between celery and mugwort pollen has been investigated and an immunological relationship between these different allergen sources has been suggested (76). In the course of routine investigations on the allergenicity of various synthetic peptides derived from Gad c I it was not noted that peptides 49–64 and 88–103 inhibited the binding of specific IgE antibodies to birch pollen allergosorbent (28, 29). This, together with clinical evidence of cross-reactivity between birch pollen and cod, has led to speculation that the two are related by common allergenic determinants.

(4) *What do we still need to know about allergens?* In view of the paucity of structural information available on allergenic determinants, there is clearly no ready answer to the question: Is there a wide range of structurally diverse allergenic determinants or are there structural features common to all, or most, of the determinants? At first sight, the diverse nature of the sources and apparent functions of many allergens makes it appear unlikely that common or similar structural features responsible for the induction of hypersensitivity will be found. However, too little data exist at present to be sure one way or the other. It is also unclear whether the diversity of IgE-binding proteins from a single allergen source such as one species of pollen, mite, fungi, etc. reflects a large number of structurally distinct determinants on otherwise different proteins or, if different proteins from the same source share the

same allergenic determinants. An equally fundamental and interesting question is: Are antigenic and allergenic sites coincident on allergenic proteins? In a few cases reported so far, IgE and IgG binding sites on allergens were shown to be the same (see for example, 7, 27). We recently found that two monoclonal antibodies recognized the same antigenic determinant on cod Gad c I and that the determinant was present on several other protein components in an extract of cod muscle. When used in conjunction with sera from cod-allergic subjects, common antigenic and common allergenic determinants were detected on cod proteins but no cross-reactivity was detected between the determinants complementary to the monoclonal antibodies and those complementary to the IgE antibodies from the allergic subjects (35). These results show that allergenic and antigenic sites on cod Gad c I can be distinct. Production and examination of more monoclonal antibodies to Gad c I and examination of more sera from cod-allergic subjects may reveal common specificities detected by the IgG and IgE antibodies. Clearly, the small number of different antibodies examined may affect the conclusion reached and, for the moment, we are left to speculate regarding the relative immunogenicity of antigenic and allergenic determinants and the size and diversity of the IgE repertoire to a single allergen such as Gad c I.

Hence, in briefly reviewing what we still don't know about allergens (apart from the structures of most allergenic determinants), we immediately observed that experimental data is not yet available to show, for a wide range of different allergens, whether (i) the same determinant(s) occurs, and is being recognized (by complementary IgE antibodies), on different allergenic proteins from different sources; (ii) the same determinant(s) occurs, and is being recognized, on different allergenic proteins from the same source and (iii) the same determinant(s) on a single allergen is being recognized by IgE antibodies in sera from different subjects allergic to the allergen.

(5) *How should we view, and define, allergenicity?* It has been estimated that the vertebrate immune system is able to generate about 10^7 different

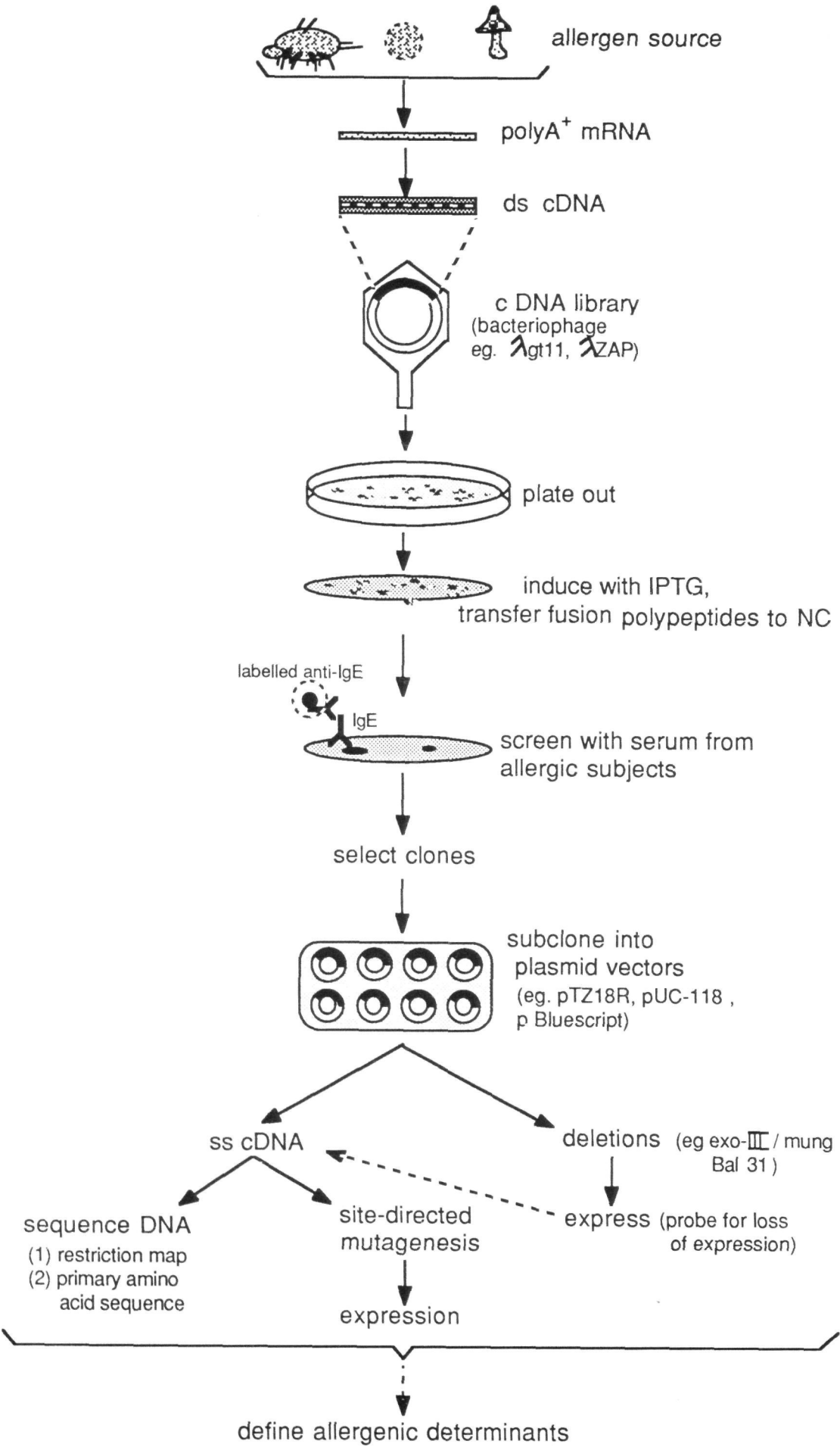
antibody combining sites (40) one or more of which may show a sufficient degree of complementarity to be selected by a protein antigenic determinant. The degree of complementarity, or fit, may vary by a factor of as much as 10^6 (65) and since the combining sites on some antibodies may recognize more than one determinant, the combining sites selected may sometimes bind more strongly than the sites on the antibody induced by the determinant on the antigen used for immunization (2).

The question of how many allergenic determinants occur on any single, purified allergen cannot yet be definitely answered since any results will always depend upon the number of allergic sera examined and, at a more fundamental level, upon a host of other variable factors including the genetic composition of allergic groups, geographical location and local varieties of allergen sources, and presence in the environment of cross-reacting allergens (19, 20). Unless the mechanisms for IgE formation and selection are far more restricted than appears to be the case for IgG antibodies, it is difficult to support the belief that a single allergen possesses allergenic determinants all of the same structure and that these determinants react only with IgE antibodies with combining sites all of exactly the same specificity. Combined cellular and immunochemical studies may prove this one way or the other but, in the meantime, it seems reasonable to assume that mechanisms for antibody formation and selection will not be significantly different for IgG and IgE.

In the light of the multideterminant-regulatory model of the nature of protein antigenicity, the question of the model's relevance to allergens naturally arises. Even with so few allergenic determinants identified so far, it is already clear that a purified allergen may contain more than one determinant. Hence, if there are no fundamental differences between the type and distribution of determinants detected by IgG and IgE antibodies and if large enough numbers of sera from appropriate allergic subjects are examined, antigens that bind IgE, like those that bind IgG, can be viewed as a continuum of overlapping anti-

body-binding sites distributed over the accessible surface of the protein. This conclusion has perhaps the dubious merit of presenting a unified view of antigenic and allergenic determinants but, unless one is prepared to accept that allergenic (IgE-binding) and antigenic (IgG-binding) determinants show some fundamental differences, it seems logical to extend the multideterminant-regulatory model of antigenicity to cover allergens. At the same time, however, there is, as yet, too little data to discard a "few site" view of allergenicity and some doubts remain which question the immediate and wholehearted acceptance of the multideterminant model. Even though little information on allergen structure, and in particular on allergenic determinants, is available yet, some features, namely stability to heat, proteolytic enzymes and pH variation (41) do seem common to allergens from widely differing sources. This stability may indicate that allergenicity resides in a particular sequence of amino acids rather than topological features of an intact protein. We are still not in a position, however, to say whether IgE antibodies are selected only by determinants with certain, as yet unknown, features.

(6) *Studying the structural basis of allergenicity - where do we go from here?* The discussion so far has emphasized the gaps in our knowledge concerning the chemical compositions of allergenic determinants, the number of allergenic sites on an allergen, the antigenic relationship between allergenic determinants on proteins from the same and from different sources and the relationship between IgE and IgG binding determinants, and it is clear that with all the major questions a great deal of careful investigation will be needed before we can feel confident about answers. In our work, we have concentrated initially on identifying allergens so that, within any one extract, we can be sure about the number and importance of all of the component allergens. As with antigenic determinants, the "full" complement of allergenic determinants on a protein can only be defined in terms of the number of subjects who recognize and respond with IgE antibodies to the protein. It



must be remembered that in the allergic patients, IgE antibody responses occur “naturally”, that is, following inhalation, ingestion or injection of the allergen. The number of individuals with IgE antibodies to the allergen is potentially very large. With allergen sources such as house dust mites and common grass pollens such as ryegrass (*Lolium perenne*), it is not difficult to accumulate many hundreds or even thousands of sera for examination. With the advent of immunoblotting and its utilization for allergen detection, the rapid identification of the “full” spectra of allergens in complex extracts has become possible.

Contribution of immunoblotting to identification of allergens and determination of their frequencies of recognition

Antigenic determinants that are probably conformational in nature usually do not give detectable reactions by immunoblotting. Hence, immunoblotting is likely to be of little value for the identification of IgE-binding determinants that may be denatured by the dissociating conditions frequently employed. However, immunoblotting is ideally suited for the recognition of sequential determinants, and such determinants are often revealed when polypeptides are unfolded by sodium dodecylsulphate. Extensive application of immunoblotting under dissociating conditions to the identification of a wide variety of allergens (13, 68) has indicated that the vast majority of IgE-binding determinants are sequential rather than conformational in nature. Loss of IgE-binding capacity under the dissociating conditions used was almost never a problem (13), and with every source studied so far, we have been able to identify all of the allergens already described and, in most cases, some other allergenic proteins that have not been reported (13, 31). Hence, if all, most, or even many allergenic determinants are

sequential, immunoblotting opens the way to study allergenic and antigenic cross-reactions between individual proteins from the same, and from different allergen sources. By elution of IgE or IgG antibodies from individual separated and blotted proteins and rebinding of the antibodies to other separated proteins, the presence or absence of common determinants can be established. This approach becomes even more powerful if monoclonal antibodies to allergenic and antigenic determinants are employed concurrently with IgE antibodies from allergic subjects. When using this approach to investigate the allergenicity of Gad c I and other proteins from codfish, the binding profiles of the monoclonal antibodies and IgE antibodies suggested that common antigenic determinants exist on Gad c I and on other protein components in cod extract (see section 2 (4)) (35). The results thus appear to provide evidence to support the hypothesis that, in codfish at least, the apparent diversity of allergens arises from the presence of cross-reacting allergenic determinants on different proteins rather than from structurally distinct allergenic determinants.

The value of protein blotting as an important part of the methodology employed in allergen identification and characterization can be enhanced by the application of high resolution two-dimensional electrophoresis and direct sequence analysis of proteins transferred to membranes (69, 71).

Although this review emphasises the value of immunoblotting for the detection of IgE-binding determinants, the value of immunoelectrophoretic methods should not be overlooked, particularly for the detection of topographical allergenic determinants. The applicability of these elegant methods to the allergen field has been well established (46, 74) although the need to employ a heterologous antiserum can sometimes be a disadvantage (13, 46).

Despite the usefulness and analytical power

Fig. 1. Overall strategy for isolation and characterization of DNA sequences corresponding to protein allergens. Messenger RNA from an allergen source is used to prepare double stranded cDNA which is ligated into a bacteriophage vector to generate a cDNA library. The library is induced to express fusion polypeptides which are screened with serum from allergic individuals. Positive cDNA clones are sub-cloned into plasmids for DNA sequencing and mutagenesis. For an explanation of the generation of deletions in a plasmid and site-directed oligonucleotide mutagenesis see text and Figs. 2 and 3.

of monoclonal antibodies and the techniques for protein identification and characterization currently employed for allergen studies, we still have a limited capacity to rapidly obtain unequivocal answers to some of the most important questions about allergens and structural information on a large number of allergens from diverse sources. We believe that many of the uncertainties and restrictions in studying allergens can be overcome by employing recombinant DNA approaches of the sort outlined below.

3. *The use of recombinant DNA techniques for the isolation and characterization of allergens and allergenic determinants*

(1) *Background and cDNA cloning.* The advent of molecular cloning has had a major impact not only on the study of gene structure and regulation but also on the characterization of the polypeptides expressed by those genes. The techniques of DNA sequencing have become so efficient and rapid that it is possible to obtain the whole primary sequence of a protein in a few days. In addition, the use of various forms of *in vitro* mutagenesis of cloned DNA has enabled the controlling regions of a large number of genes to be characterized (73).

A gene is expressed by being transcribed in the form of messenger RNA (mRNA). mRNA after transcription undergoes some additional processing in eukaryotes, in particular, the addition of a polyadenylic acid (polyA) tail to the 3' end of the transcript, an addition which has been exploited to facilitate its separation from the other RNA species (9). This mRNA represents only 1–2% of the total RNA, the bulk being ribosomal RNA. The mRNA transcript after processing is translated to produce a polypeptide (73). mRNA can be used to prime the synthesis of single stranded complementary DNA (cDNA) using the enzyme reverse transcriptase. Single stranded cDNA can then be converted into double stranded cDNA (Fig. 1) in a number of simple steps (33), and ligated into the DNA of a suitable vector, that is, a self-replicating piece of DNA such as a bacteriophage or a plasmid specially modified to

accept the foreign DNA and form a recombinant molecule without losing its replicating function. In an appropriate host, the recombinant bacteriophage, or plasmid, transforms the host cell and the vector replicates itself in high copy number. With the appropriate multiplicity of infection for bacteriophage or an appropriate plasmid/host cell ratio, individual plaques or colonies representing different cDNA clones can be selected. A useful and often used expression bacteriophage vector is lambda gt11 (39). Although a number of useful plasmid expression vectors are available (e.g. pKK223-3 from Pharmacia), the bacteriophage vectors have some significant advantages. These include more efficient transformation providing a larger number of clones in the primary library, and a simple method of carrying out primary screening (48). The site for insertion of foreign DNA into lambda gt11 is a unique *EcoRI* site located within the *lacZ* gene, 53 bases upstream from the beta-galactosidase translation termination codon. Because the site for insertion is within the structural gene for beta-galactosidase, foreign DNA sequences have the potential to be expressed as fusion proteins of the enzyme. Adequate levels of expression of the foreign DNA are obtained by plating the phage in a lawn of *lon* protease-deficient *E. coli* cells to aid the stability of the fusion proteins. The problem of production of potentially toxic proteins to the host cells is minimized by using cells producing large amounts of *lac* operon repressor to prevent *lacZ*-directed expression of the fusion protein during the initial hours of plaque formation. When the number of infected cells surrounding the plaque is sufficiently large, *lacZ*-directed expression is induced by inactivating the repressor with IPTG (isopropyl- β -D-thiogalactopyranoside). In this way, detectable amounts of foreign antigen can be produced even if the antigen is toxic to the cells (39). Although lambda gt11 is often suitable for the primary screening of a cDNA library for the presence of sequences that express as fusion polypeptides, the large size of the vector relative to the size of the inserts usually precludes its use when manipulations upon the inserted DNA need to be undertaken. Once a cDNA clone expressing a

polypeptide has been identified by, for example, reaction with an antibody probe, and purified, the insert is usually sub-cloned into a plasmid vector where restriction site analysis and *in vitro* mutagenesis are easier to carry out. Of choice is a plasmid vector capable of expression and one where the site of the insert DNA is adjacent to the *lacZ* promoter and in the correct orientation to the direction of transcription from the promoter (e.g. pBluescript (Stratagene), pTZ18, pUC-118). Deletions and site-specific *in vitro* mutagenesis can be coupled with expression to identify, for example, the precise location of regions of the insert DNA essential for its expressed polypeptide to function biologically.

(2) *A cloning strategy for allergens.* For allergen studies, cDNA clones corresponding to allergenic polypeptides can be identified by using IgE antibodies from the sera of allergic subjects. The allergen-reactive antibodies together with a labelled second antibody are used to screen polypeptides expressed by clones from a library derived originally from a heterogeneous m-RNA population isolated from the allergen source. A strategy for the isolation of DNA sequences corresponding to allergen determinant structures is summarized diagrammatically in Fig. 1. Wherever it is possible to define the source of an allergen(s), and providing that the allergenic determinants are peptide in nature, there should be no major difficulty in preparing a mRNA fraction. cDNA can then be prepared from the mRNA and ligated into an appropriate expression vector to form a cDNA library.

Once the clones have been identified, several options for gaining insights into the nature of an allergenic structure exist. Firstly, the piece of cDNA can, after appropriate sub-cloning into a plasmid or M13 phage strain, be sequenced by one of several methods (21, 50, 51, 57) to generate the nucleotide base sequence and thus the putative amino acid primary sequence of the protein. From the base sequence and using one of a number of available computer systems, all the restriction sites of the piece of cDNA can be deduced (54–56, 63, 64; IBI/Pustell Sequence Analysis System; Bio-Rad Gene Master). It may also be useful to isolate a smaller

piece of DNA or to modify the piece of DNA which can be induced to express fusion polypeptides and then probe the clones again with serum. It is in this area that many of the methods of *in vitro* mutagenesis can be elegantly exploited to elucidate the important sequences that are essential for a protein to behave as an allergen.

(3) *Identification of allergenic sequences.* The term mutagenesis here refers to a wide range of procedures for modifying the cDNA insert in the vector (73). These procedures can include the large scale deletions of part of that DNA between characterized restriction sites using restriction enzymes. Deletions may also be made from either end of the cDNA insert, from a point within the insert cDNA or from one end only using a unidirectional deletion system (36, 43). It can also include the introduction of short DNA sequences (insertions) into the cDNA (15, 16), the alteration of single or double bases (42, 61) to generate amino acid changes in the primary amino acid sequence in expressed polypeptides, or the introduction of new restriction enzyme sites by single base mutations to enable precise fragment sizes to be excised or deletions to be made. By such means, the region of the protein which contains the allergenic determinant(s) can be deduced and, most importantly, the nature of allergenic determinants elucidated. The place of *in vitro* mutagenesis in the overall strategy for the characterization of DNA sequences corresponding to allergenic determinants is summarized in Fig. 1 while important aspects of the mutagenic procedures will now be outlined.

Preparation of a family of unidirectional deletions allows the sequence to be determined from the overlapping regions of the family of “nested” deletions.

Unidirectional deletions

These can be introduced from one end of the cDNA insert by use of the exonuclease III (exoIII)/mung bean nuclease system (36, 43). This system relies upon the requirement of exoIII for double stranded DNA and its

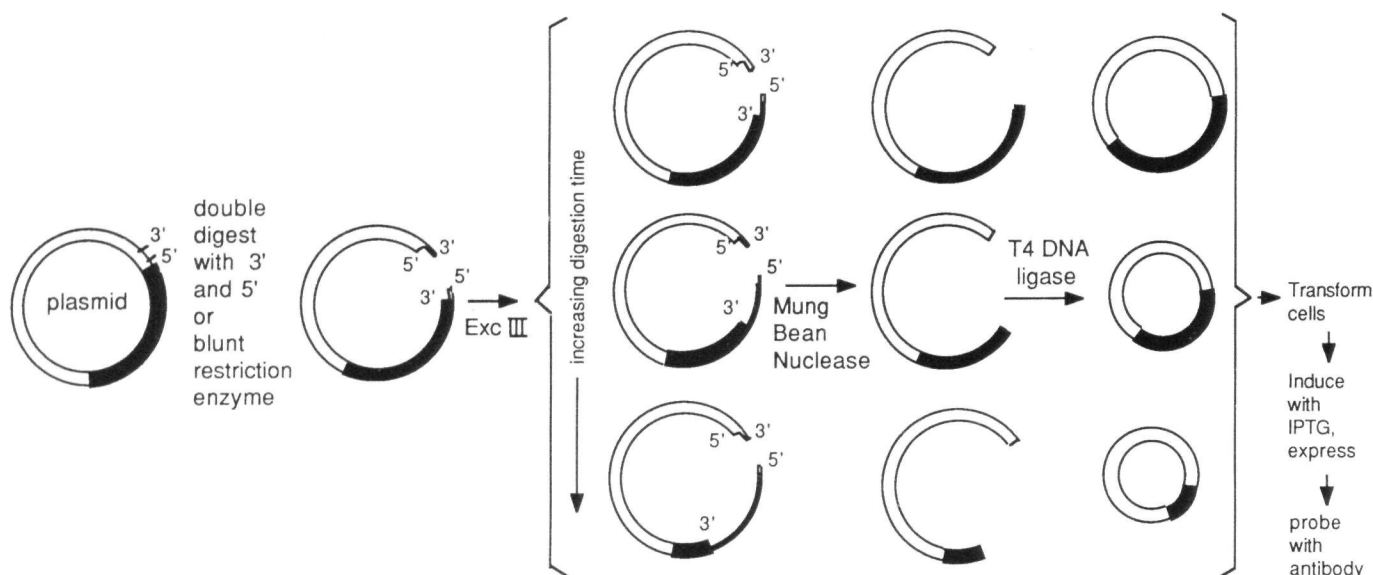


Fig. 2. Generation of unidirectional deletions in a plasmid (e.g. pBluescript, pTZ18R, pUC-118) using the exonuclease III/mung bean nuclease system. The plasmid is double digested with a 3' overhang and a 5' overhang or blunt restriction enzyme. Exonuclease III digests 3' ends from blunt or 5' overhangs. Mung bean nuclease digests the single strand overhangs to give blunt ends. T4 DNA ligase ligates the blunt ends to re-circularize the plasmid. The deletion mutants are then used to transform *E. coli*.

capacity to digest 3' ends from 5' overhangs or blunt ends. To generate deletions in the insert but not in the plasmid vector, use is made of unique 3' restriction sites on the polylinker and blunt and 5' restriction sites (e.g. pBluescript, pUC-118, pTZ18). The plasmid cDNA clone is simply double digested with a 3' and a 5' or blunt restriction enzymes followed by the exoIII/mung treatment (Fig. 2).

Using the nucleotide base sequence of the insert DNA to identify restriction sites, many deletions can be made using these restriction sites within the insert to digest it into fragments which can then be removed or re-ligated back into the plasmid. Using site-directed mutagenesis described below, it is also possible to produce mutant sequences in which new restriction sites have been introduced into the insert DNA allowing the mutant insert DNA to be further digested to generate additional fragments for re-ligation into the plasmid vector. The use of restriction enzymes to prepare fragments for sub-cloning into the plasmid where expression of fusion polypeptides is to be carried out requires the DNA fragment to be in the correct reading frame, remembering that the genetic code is made up of three base codons. To achieve this with restriction fragments may

require the use of appropriate oligonucleotide adapters (48).

With the exception of precisely engineered deletions using known restriction sites or those introduced in a precise manner by site-directed mutagenesis, the deletions created using exoIII/mung or Bal 31 will produce random fragment lengths many of which will not be in the correct reading frame to express a fusion polypeptide retaining the allergenic properties of the original lambda gt11 clone. However, the randomness of the process will ensure that at least one in three of the deletions using the exoIII/mung system will be in the correct reading frame to express a polypeptide retaining its allergenic properties. Thus, it should always be possible to use this technique of deletions to define a relatively small portion of the whole DNA sequence still capable of expression of fusion polypeptides retaining allergenic sequences which can be used for site-directed mutagenesis.

Site-directed mutagenesis

Site-directed mutagenesis allows single or double base changes to be introduced into the DNA insert so that single or double amino acid mutations are introduced in the expressed polypep-

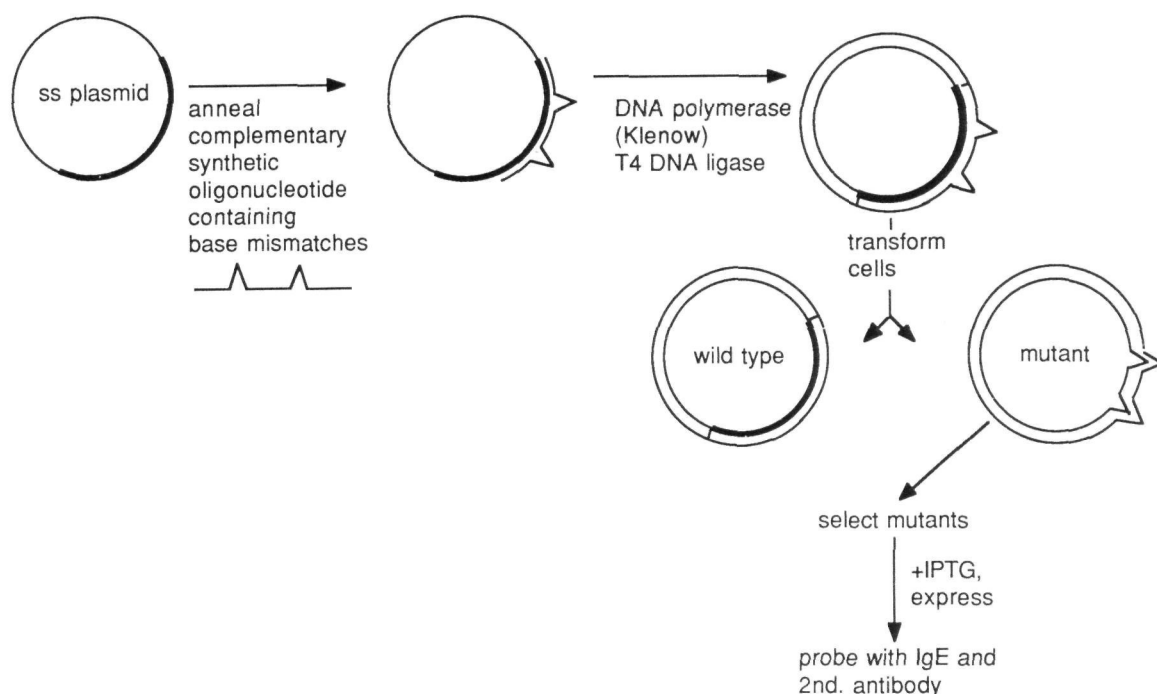
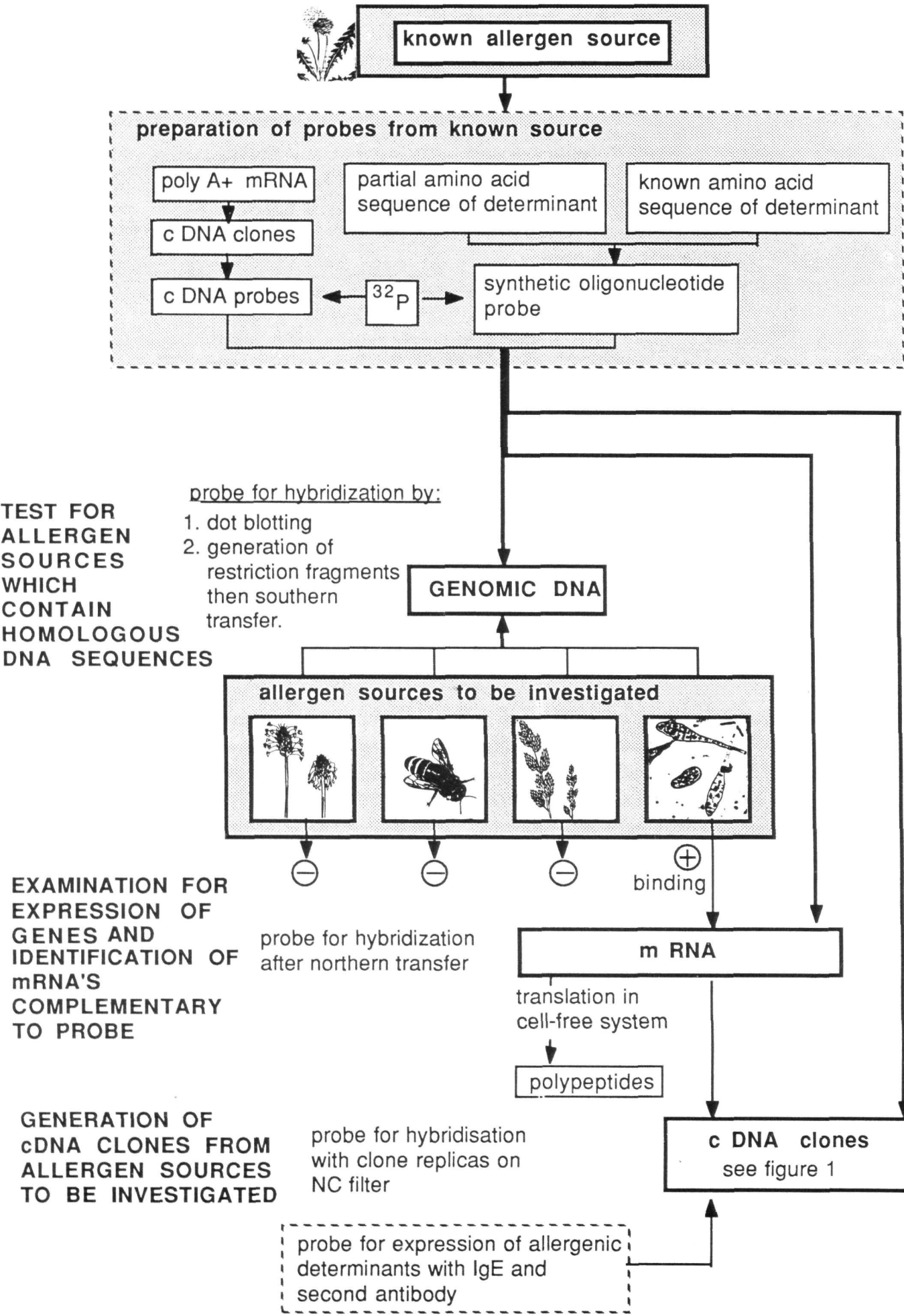


Fig. 3. Site-directed oligonucleotide mutagenesis. A 12–15 base oligonucleotide is constructed so that it is complementary to a region of the DNA strand but with one or two mismatches. When mixed with a clone of the complementary strand, the oligonucleotide will anneal to it even though the match is not exact, the hybridization conditions are not too stringent and the mismatches are in the middle of the oligonucleotide. This segment serves as the primer for DNA polymerase. The resulting double stranded molecule is used to transform *E. coli* in which the molecule replicates to produce either the original wild type sequence or the mutant sequence. The original mismatch oligonucleotide can be used as a probe under stringent hybridization conditions to identify mutants.

tide. This approach (24, 75) provides potentially the most powerful technique for the study of the precise nature of an allergenic determinant where it has been established that the determinant comprises a linear sequence of amino acids. When the region of the nucleotide sequence expressing an allergenic fusion polypeptide in a plasmid has been identified using one of the various forms of deletion mutagenesis, site-directed mutagenesis can then be exploited to precisely identify the amino acids essential for its function as an allergen. This is carried out by introducing mutations into the amino acid sequence to effect changes in the allergenicity of the polypeptide. Plasmids capable of generating single stranded DNA (e.g. pBluescript, pTZ18 or pUC-118) can be used, and from there the procedure is the same as that used for M13 phage DNA (44). From the derived nucleotide sequence of the insert DNA, an oligonucleotide is prepared complementary to single stranded plasmid DNA but containing a single or double base mismatch. The oligonucleotide is then annealed under non-strin-

gent conditions to the complementary strand of the original plasmid. The mismatched oligonucleotide serves as the primer for DNA polymerase to synthesize the remainder of the complementary strand. The double stranded molecule is then introduced into *E. coli* to create either the wild type or the mutant sequence (Fig. 3). Theoretically 50 % of the daughter molecules will be wild type and 50 % will be mutant, however, in practice, the percentage of mutant molecules is of the order of 15 %. The mutants can be located using hybridization protocols under stringent conditions and with the mismatch oligonucleotide (^{32}P -labelled) used to generate the mutation used as the probe. If the mutation introduces a new restriction site or removes an existing site, then clones can be assayed for the presence or absence of that site to identify the mutants.

In vitro mutagenesis can also be applied to identify the important sequences in peptides isolated from allergenic proteins by conventional techniques of protein chemistry. If a peptide has been shown to demonstrate allergenic properties,



a synthetic oligonucleotide complementary to the amino acid sequence of that peptide can be prepared. This can be cloned and subjected to *in vitro* mutagenesis. The differences in the IgE-binding may then be determined and the amino acid sequences complementary to the antibody combining sites ultimately established. The employment of this approach would need to be weighed against the relative cost and ease of production of synthetic peptides generated and used to accomplish the same task.

(4) *Strategy for detection of common or cross-reacting allergenic determinants from different sources.* The observations referred to earlier in section 2 (3) and suggesting the existence of structural similarities between allergens from different sources, raises the possibility of the occurrence of families of related amino acid sequences with allergenic properties. How might one explore whether apparent cross-reactions, observed clinically or serologically, are due to the same, or similar, amino acid sequences in proteins from the different biological sources? A general strategy for doing this is set out in Fig. 4. Using the procedures outlined in the text above and summarized in Fig. 1, oligonucleotide probes that correspond to short DNA sequences that express polypeptides with allergenic properties can be prepared. After radioactive labelling the probes can be used to screen the genomic DNA from other biological sources for the presence of related sequences, especially in cases where there is evidence of cross-reactions detected by sera from hypersensitive individuals. Genomic DNA preparations from the heterologous sources can be dot blotted or digested with restriction enzymes, electrophoresed and transferred to nitrocellulose membrane using the technique known as "southern blotting" (48). The blotted DNA can then be used for hybridization with the DNA probes derived from the cDNA clones (Fig. 4). The size of the oligo-

nucleotide sequence will significantly influence the hybridization conditions to be employed but, generally, the conditions would be at least moderately stringent where exact homologies are sought. Nucleotide probes can also be derived from known amino acid sequences; however, redundancy of the genetic code may mean that a large number of possible nucleotide sequences have to be considered. Where a short allergenic amino acid sequence is derived from analysis of peptides obtained from a purified allergenic protein, it may be possible to prepare a small number of oligonucleotides corresponding to the sequence and which account for all of the possible code redundancies. Hybridization conditions for oligonucleotide probes derived from amino acid sequence data will depend on the extent of base mismatch which might arise when code redundancy presents a problem. Where it is possible to prepare probes with no redundant base sequences, hybridization conditions can be quite stringent. The size of the oligonucleotide is also an important factor since conditions chosen for hybridization will be less stringent as the size of the oligonucleotide is decreased (48). However, at lower levels of stringency, the specificity of binding will also be decreased. Where homologous sequences are identified in the genomic DNA of a related or divergent species, the most immediate and obvious question is whether the sequences are expressed. This can be tested by the procedure of "northern blotting" (48) in which mRNA from the source being investigated is separated electrophoretically in a denaturing agarose gel, transferred to nitrocellulose membrane and tested for hybridization with the DNA probes. Such an examination can provide evidence of expression and indicate the size, and possibly the number of mRNA species carrying the determinant(s). Northern blotting may also be the preferred means of screening with oligonucleotide probes where the genes carrying the

Fig. 4. Investigation of allergen sources for conserved DNA sequences. DNA probes prepared from cDNA clones or from known peptide sequences are used to probe genomic DNA from other allergen sources. Where DNA homology is demonstrated, expression of genes coding for allergens is further investigated using northern blot hybridization. More detailed investigation may then proceed after generating cDNA clones from the mRNA and examining for hybridization with the DNA probes and with IgE antibodies from allergic subjects (see Fig. 1.).

allergen are represented as single copies. Under these circumstances, oligonucleotide probes may be unsuccessful in detecting the sequences in genomic DNA. Generation of the cross-reacting allergenic polypeptides can be achieved by preparing and expressing a cDNA library from the mRNA and screening with the DNA probes prepared from the initial allergenic source and with IgE antibodies from the sera of allergic subjects (see Fig. 1). mRNAs found to hybridize with the DNA probes on the northern blot may be recovered from the gel and translated in an *in vitro* protein synthesizing system. The resultant polypeptide(s) can be electrophoresed, probed for reactivity with allergic sera and radiolabelled anti-human IgE and examined by autoradiography. The mRNA may also be used to create cDNA libraries enriched for clones coding for allergenic proteins.

(4) *A recombinant-DNA approach to the study of house dust mite allergens*

Using the strategy already referred to and outlined in Fig. 1, and the expression vectors lambda gt11 and a pUC-18-like plasmid, an allergen of MW ~15,000 has been cloned and sequenced from the house dust mite *Dermatophagoides pteronyssinus* (70, 70a). Two strategies employed in this study contributed greatly to the success of the approach used and the simple ideas and methods involved in the approaches should prove equally useful in cloning studies on other allergen sources. The first, and obvious, approach was to use IgE antibodies from mite-allergic humans for the initial screening of the cDNA library for mite proteins. The second, was the identification of the cloned allergen by using IgE eluted from blots of expressed mite protein from *E. coli* inclusion bodies and then rebinding the IgE back onto blots of extracts of native mite bodies for comparison with reactivity patterns produced by a panel of allergic sera.

(5) *Summary*

The use of recombinant DNA techniques for the study of allergenicity of proteins is a viable,

and in many ways a preferred, alternative to the traditional procedures of protein purification, digestion and analysis of peptides for both allergenicity and amino acid sequence. The process of protein purification can be difficult and in many instances workers are forced to use only partially pure fractions that make the identification of the allergenic proteins uncertain. Furthermore, the purification and sequencing of peptides and their testing for retention of allergenic properties, represents a substantial and time-consuming work load. The synthesis of families of synthetic peptides to characterize the amino acids important for allergenic properties is also expensive and time-consuming. On the other hand, the preparation of a cDNA library from an allergen source is today a relatively easy and inexpensive task. The isolation and purification of cDNA clones is comparatively trivial compared to protein purification. Using the techniques described in this text, it can be seen that the molecular biological approach, although in some respects similar in principle to those of the protein chemist to study allergens, provides the capability to study several clones at the same time, and to compare clones for the presence of conserved regions corresponding to allergenic determinants. In addition, the techniques for generating mutant sequences provides perhaps the most powerful and simple set of procedures available for defining the amino acid structures essential for proteins or peptides to behave as allergens.

ACKNOWLEDGEMENT

This work was supported by the National Health and Medical Research Council of Australia.

REFERENCES

1. Aalberse, R. C., Koshte, V. & Clemens, J. G. J.: Immunoglobulin E antibodies that crossreact with vegetable foods, pollen, and hymenoptera venom. *J. Allergy Clin. Immunol.* 68, 356-364, 1981.
2. Al Moudallal, Z., Briand, J. P. & Van Regenmortel, M. H. V.: Monoclonal antibodies as probes of the antigenic structure of tobacco mosaic virus. *EMBO J.* 1, 1005-1010, 1982.
3. Amit, A. G., Mariuzza, R. A., Phillips, S. E. V. & Poljak, R. J.: Three-dimensional structure of an anti-

- gen-antibody complex at 6Å resolution. *Nature* 313, 156-158, 1985.
4. Andersson, L. B., Dreyfuss, E. M., Logan, J., Johnstone, D. E. & Glaser, J.: Melon and banana sensitivity coincident with ragweed pollinosis. *J. Allergy* 45, 310-319, 1970.
 5. Atassi, M. Z.: Antigenic structure of myoglobin: The complete immunochemical anatomy of a protein and conclusions relating to antigenic structures of proteins. *Immunochemistry* 12, 424-438, 1975.
 6. Atassi, M. Z.: Precise determination of the entire antigenic structure of lysozyme: Molecular features of protein antigenic structures and potential of "surface-stimulation" synthesis - a powerful new concept for protein binding sites. *Immunochemistry* 15, 909-933, 1978.
 7. Atassi, H. & Atassi, M. Z.: Antibody recognition of ragweed Ra3: localization of the full profile of the continuous antigenic sites by synthetic overlapping peptides representing the entire protein chain. *Eur. J. Immunol.* 16, 229-235, 1986.
 8. Atassi, M. Z. & Young, C. R.: Discovery and implications of the immunogenicity of free small synthetic peptides: powerful tools for manipulating the immune system and for production of antibodies and T cells of preselected submolecular specificities. *CRC Crit. Rev. Immunol.* 5, 387-409, 1985.
 9. Aviv, H. & Leder, P.: Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA* 69, 1408-1412, 1972.
 10. Baldo, B. A.: Milk allergies. *Aust. J. Dairy Technol.* 39, 120-128, 1984.
 11. Baldo, B. A. & Fisher, M. M.: Anaphylaxis to muscle relaxant drugs: Cross-reactivity and molecular basis of binding of IgE antibodies detected by radioimmunoassay. *Molec. Immunol.* 20, 1393-1401, 1983.
 12. Baldo, B. A., Sutton, R. & Wrigley, C. W.: Grass allergens with particular reference to cereals. *Progr. Allergy* 30, 1-66, 1982.
 13. Baldo, B. A. & Tovey, E. R.: Protein blotting for the detection and standardization of allergens. In Baldo, B. A. & Tovey, E. R. (eds.): *Protein blotting: methodology, research and diagnostic applications*. Karger, Basel (in press).
 14. Baldo, B. A. & Wrigley, C. W.: Allergies to cereals. In Pomeranz, Y. (ed.): *Advances in cereal science and technology*, vol. 6, pp. 289-356. Am. Assoc. Cereal Chemists Inc., St. Paul, Minnesota, 1984.
 15. Barany, F.: Two codon insertion mutagenesis of plasmid genes using single stranded hexameric oligonucleotides. *Proc. Natl. Acad. Sci. USA* 82, 4202-4211, 1985.
 16. Barany, F.: Single stranded hexameric linkers: A system for in-phase insertion mutagenesis and protein engineering. *Gene* 37, 111-123, 1985.
 17. Baur, X.: Studies on the specificity of human IgE-antibodies to the plant proteases papain and bromelain. *Clin. Allergy* 9, 451-457, 1979.
 18. Baur, X., Aschauer, H., Mazur, G., Dewair, M., Prelicz, H. & Steigemann, W.: Structure, antigenic determinants of some clinically important insect allergens: Chironomid hemoglobins. *Science* 233, 351-354, 1986.
 19. Benjamin, D. C., Berzofsky, J. A., East, I. J., et al.: The antigenic structure of proteins: A reappraisal. *Ann. Rev. Immunol.* 2, 67-101, 1984.
 20. Berzofsky, J. A.: Intrinsic and extrinsic factors in protein antigenic structure. *Science* 229, 932-940, 1985.
 21. Chen, E. & Seeburg, P. H.: Supercoil sequencing: A fast and simple method for sequencing plasmid DNA. *DNA* 4, 165-170, 1985.
 22. Crumpton, M. J.: Protein antigens. The molecular basis of antigenicity and immunogenicity. In Sela, M. (ed.): *The antigens*, vol. 2, pp. 1-78. Academic Press, New York, 1974.
 23. Crumpton, M. J. & Wilkinson, J. M.: The immunological activity of some of the chymotryptic peptides of sperm-whale myoglobin. *Biochem. J.* 94, 545-556, 1965.
 24. Dalbadie-McFarland, G., Cohen, L. W., Riggs, A. D., Morin, C., Itakura, K. & Richards, J. H.: An amber suppressor tRNA gene derived by site-specific mutagenesis: Cloning and function in mammalian cells. *Proc. Natl. Acad. Sci. USA* 79, 6409-6413, 1982.
 25. De Weck, A. L.: Penicillins and cephalosporins. In De Weck, A. L. & Bundgaard, H. (eds.): *Allergic reactions to drugs*. Handbook of experimental pharmacology, vol. 63, pp. 423-482. Springer-Verlag, Berlin, 1983.
 26. Dreborg, S. & Foucard, T.: Allergy to apple carrot and potato in children with birch pollen allergy. *Allergy* 38, 167-172, 1983.
 27. Elsayed, S. & Apold, J.: Immunochemical analysis of cod fish allergen M: Locations of the immunoglobulin binding sites as demonstrated by the native and synthetic peptides. *Allergy* 38, 449-459, 1983.
 28. Elsayed, S., Ragnarsson, U., Apold, J., Florvaag, E. & Vik, H.: Allergenic synthetic peptide corresponding to the second calcium-binding loop of cod allergen M. *Scand. J. Immunol.* 14, 207-211, 1981.
 29. Elsayed, S., Titlestad, K., Apold, J. & Aas, K.: A synthetic hexadecapeptide derived from allergen M imposing allergenic and antigenic reactivity. *Scand. J. Immunol.* 12, 171-175, 1980.
 30. Eriksson, N. E., Formgren, H. & Svenonius, E.: Food hypersensitivity in patients with pollen allergy. *Allergy* 37, 437-443, 1982.
 31. Ford, S. A. & Baldo, B. A.: A re-examination of ryegrass (*Lolium perenne*) pollen allergens. *Int. Arch. Allergy Appl. Immunol.* 81, 193-203, 1986.
 32. Geyson, H. M., Rodda, S. J., Mason, T. J., Tribbick, G. & Schoofs, P. G.: Strategies for epitope analysis using peptide synthesis. *J. Immunol. Methods* 102, 259-274, 1987.
 33. Gubler, U. & Hoffman, B. J.: A simple and very efficient method for generating cDNA libraries. *Gene* 25, 263-269, 1983.
 34. Halmepuro, L., Vuontela, K., Kalimo, K. & Björkstén, F.: Cross-reactivity of IgE antibodies with allergens in birch pollen, fruits and vegetables. *Int. Arch. Allergy Appl. Immunol.* 74, 235-240, 1984.

35. Hemmens, V. J., Baldo, B. A., Underwood, P. A. & Elsayed, S.: Common antigenic and allergenic determinants on codfish proteins detected with mouse monoclonal IgG and human IgE antibodies. *Molec. Immunol.* (in press).
36. Henikoff, S.: Unidirectional digestion with exonuclease III creates target breakpoints for DNA sequencing. *Gene* 28, 351-359, 1984.
37. Hopp, T. H.: Protein surface analysis. Methods for identifying antigenic determinants and other interaction sites. *J. Immunol. Methods* 88, 1-18, 1986.
38. Hopp, T. P. & Woods, K. R.: Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA* 78, 3824-3828, 1981.
39. Huynh, T., Young, R. A. & Davis, R. W.: In Glover, D. (ed.): *DNA cloning techniques: A practical approach*. I. R. L. Press, Oxford, 1984.
40. Kindt, T. J. & Capra, J. D.: *The antibody enigma*. Plenum, New York, 1984.
41. King, T. P.: Chemical and biological properties of some atopic allergens. *Adv. Immunol.* 23, 77-105, 1976.
42. Kramer, W., Drutsa, V., Jansen, H-W., Kramer, B., Pflugfelder, M. & Fritz, H-J.: The gapped duplex DNA approach to oligonucleotide-directed mutation construction. *Nucl. Acids Res.* 12, 9441-9456, 1984.
43. Lai, C. & Nathans, D.: Deletion mutants of Simian virus 40 generated by enzymatic excision of DNA segments from the viral genome. *J. Mol. Biol.* 89, 179-193, 1974.
44. Laski, F. A., Belagaje, R., RajBhandary, U. L. & Sharp, R. A.: An amber suppressor tRNA gene derived by site-specific mutagenesis: Cloning and function in mammalian cells. *Proc. Natl. Acad. Sci. USA* 79, 5813-5817, 1982.
45. Lerner, R. A.: Antibodies of predetermined specificity in biology and medicine. *Adv. Immunol.* 36, 1-44, 1984.
46. Löwenstein, H.: Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. *Prog. Allergy* 25, 1-62, 1978.
47. Löwenstein, H. & Eriksson, N. E.: Hypersensitivity to foods among birch pollen-allergic patients. Immunochemical inhibition studies for evaluation of possible mechanisms. *Allergy* 38, 577-587, 1983.
48. Maniatis, F., Fritsch, E. F. & Sambrook, J.: In *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory, New York, 1982.
49. Marsh, D. G.: Allergens and the genetics of allergy. In Sela, M. (ed.): *The antigens*, vol. 3, pp. 271-350. Academic Press, New York, 1975.
50. Maxam, A. M. & Gilbert, W.: Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymology*, 65, 499-560, 1980.
51. Messing, J., Crea, R. & Seeburg, P. H.: A system for shotgun DNA sequencing. *Nucl. Acids. Res.* 9, 309-321, 1981.
52. Novotny, J., Handschumacher, M., Haber, E., et al.: Antigenic determinants in proteins coincide with surface regions accessible to large probes (antibody domains). *Proc. Natl. Acad. Sci. USA* 83, 226-230, 1986.
53. Palfreyman, J. W., Aitcheson, T. C. & Taylor, P.: Guidelines for the production of polypeptide specific antisera using small synthetic oligopeptides as immunogens. *J. Immunol. Methods* 75, 383-393, 1984.
54. Pustell, J. & Kafatos, F. C.: A Convenient and adaptable package of DNA sequence analysis programs for microcomputers. *Nucl. Acids Res.* 10, 51-59, 1982.
55. Pustell, J. & Kafatos, F. C.: A high speed, high capacity homology matrix: Zooming through SV40 and polyoma. *Nucl. Acids Res.* 10, 4765-4782, 1982.
56. Pustell, J. & Kafatos, F. C.: A convenient and adaptable package of computer programs for DNA and protein sequence management, analysis and homology determination. *Nucl. Acids Res.* 12, 643-655, 1984.
57. Sanger, F., Nicklen, S. & Coulson, A. R.: DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463-5467, 1977.
58. Schulze-Gahmen, U., Klenk, H.-D. & Beyreuther, K.: Immunogenicity of loop-structured short synthetic peptides mimicking the antigenic site A of influenza virus hemagglutinin. *Eur. J. Biochem.* 159, 283-289, 1986.
59. Sela, M., Schechter, B., Schechter, I. & Borek, F.: Antibodies to sequential and conformational determinants. *Cold Spring Harbour Symp. Quant. Biol.* 32, 537-545, 1967.
60. Sette, A., Doria, G. & Adorini, L.: A microcomputer program for hydrophilicity and amphipathicity analysis of protein antigen. *Molec. Immunol.* 23, 807-810, 1986.
61. Shortle, D., DiMaio, D. & Nathans, D.: Directed mutagenesis. *Ann. Rev. Genetics* 15, 265-294, 1981.
62. Sief, I., Khoury, G. & Dhar, R.: A rapid enzymatic DNA sequencing technique: Determination of sequence alterations in early Simian virus 40 temperature sensitive and deletion mutants. *Nucl. Acids Res.* 8, 2225-2240, 1980.
63. Staden, R.: A strategy of DNA sequencing employing computer programs. *Nucl. Acids Res.* 6, 2601-2610, 1979.
64. Staden, R.: Automation of computer handling of gel reading data produced by the shotgun method of DNA sequencing. *Nucl. Acids Res.* 10, 4731-4751, 1982.
65. Steward, M. W. & Steensgaard, J.: *Antibody affinity: Thermodynamic aspects and biological significance*. CRC Press, Boca Raton, Florida, 1983.
66. Taylor, S. L.: Food allergies. *Food Technol.* February, 98-105, 1985.
67. Todd, P. E. E., East, I. J. & Leach, S. J.: The immunogenicity and antigenicity of proteins. *Trends Biochem. Sci.* 7, 212-216, 1982.
68. Tovey, E. R. & Baldo, B. A.: Characterisation of allergens by protein blotting. *Electrophoresis* 8, 452-463, 1987.
69. Tovey, E. R. & Baldo, B. A.: Specialized aspects of protein blotting. In Baldo, B. A. & Tovey, E. R. (eds.): *Protein blotting: methodology, research and diagnostic applications*. Karger, Basel (in press).
70. Tovey, E. R., Cobon, G. S., Johnson, M. C. & Baldo, B. A.: The cloning of a mite (*Dermatophagoides pteronyssinus*) recombinant protein that shares IgE-binding

- determinants with a 14–15 kD component of mite extracts. In: Molecular biology of allergens. Pergamon Press, London (in press).
- 70a. Tovey, E. R., Johnson, M. C., Roche, A. L., Baldo, B. A. & Cobon, G. S.: Cloning and sequencing of an IgE-binding recombinant protein from the mite *Der-matophagoides pteronyssinus* corresponding to a 14–15 kD protein of the mite extracts (Submitted for publication).
71. Vandekerckhove, J., Bauw, G., Puype, M., Van Damme, J. & Van Montagu, M.: Protein-blotting on polybrene-coated glass-fiber sheets. A basis of acid hydrolysis and gasphase sequencing of picomole quantities of protein previously separated on sodium dodecyl sulfate/polyacrylamide gel. *Eur. J. Biochem.* *152*, 9–19, 1985.
72. Van Regenmortel, M. H. V.: Operational aspects of the definition of epitopes in peptides and proteins. In Alitalo, K., Partanen, P. & Vaheri, A. (eds.): Synthetic peptides in biology and medicine, pp. 67–74. Elsevier, Amsterdam, 1985.
73. Watson, J. D., Tooze, J. & Kurtz, D. T.: Recombinant DNA. A short course. Scientific American Books. (W. H. Freeman), New York, 1983.
74. Weeke, B. & Löwenstein, H.: Allergens identified in crossed radioimmuno-electrophoresis. *Scand. J. Immunol.* *2*, Suppl. 1, 149–153, 1973.
75. Winter, G., Fersht, A. R., Wilkinson, A. J., Zoller, M. & Smith, M.: Redesigning enzyme structure by site-directed mutagenesis: Tyrosyl tRNA synthetase and ATP binding. *Nature* *299*, 756–758, 1982.
76. Wuthrich, B. & Dietschi, R.: Das “Sellerie-Karotten-Beifuss-Gewurz-Syndrom”: Hauttest und RAST-Ergebnisse. *Schweiz. Med. Wochenschr.* *115*, 258–364, 1985.
- Address:
Dr. B. A. Baldo
Kolling Institute of Medical Research
Royal North Shore Hospital
St. Leonards N. S. W. 2065
Australia

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.