Chapter 95
New methods for human allotyping

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Gm: a highly polymorphic system for genetic analyses

The family of human immunoglobulin (Ig) molecules comprises a highly polymorphic system that has yet to be fully exploited in population and medical studies. Serologically defined variants have been identified in the heavy chain constant regions of three of the four IgG subclasses (IgG1, IgG2, and IgG3), in one of the two IgA subclasses (IgA2), in the IgE subclass, and in one of the light chain families (κ). Given the number of different alleles found within the γ subclasses, the Gm system is second only to HLA in genetic complexity. Genetic drift alone is unlikely to be responsible for this degree of genetic variation. Selective pressures have probably played a major role in increasing the diversity of the Ig heavy chain constant region (IgCh) loci since these genes code for structures that determine the effector functions of antibody molecules. Several studies have shown an association between Gm type and specific antibody responses [1–5]. Moreover, since the variable and constant region genes are linked [6–7], selection favouring extensive variation in the combining site may concomitantly increase variation in the constant region as well.

The Ig polymorphisms have long been considered excellent markers for population studies and are especially useful for evaluation of admixture between populations. In contrast to other polymorphic systems, differences between ethnic groups are qualitative in that some Gm haplotypes or alleles are absent from or present only in certain populations. Each of the major ethnic groups is characterized by a set of haplotypes that is unique to that group [8]. Quantitative variation also exists. Among ethnic groups, the greatest difference in gene frequencies for a single polymorphic system is found with these markers. Even populations that are geographically close, such as northern and southern Chinese or northern and southern Melanesians [8], exhibit great differences in the prevalence of certain Gm alleles.

The Gm system has also become increasingly important in clinical and medical genetic studies because of the growing number of reported associations between Gm markers and certain diseases [9–24]. These diseases, which usually display an autoimmune aetiology, are thought to be multifactorial, with disease development and progression under the influence of multiple genes. In some cases, there is a combined or interactive association involving both Gm and HLA [12,16,20]. Gm associations have been reported for multiple sclerosis [13–15], auto-immune thyroid disease [9], systemic lupus erythematosis [11], chronic active hepatitis [12], Graves' disease [16], Hashimoto's disease [11], myasthenia gravis [10], juvenile onset diabetes [18], Alzheimer's disease [21], gluten-sensitive enteropathy [22,23], and several cancers, including lung cancer [17], primary hepatoma [17], neuroblastoma [19], and Kaposi's sarcoma [24]. An association with the Km markers has been reported for a category of squamous cell carcinomas [25].

Although the Gm system has much to offer, some difficulties inherent to the current typing system have precluded full exploitation of this richly polymorphic region as a tool for genetic studies. Good typing reagents (antisera) are not commercially available and are difficult to produce. The principal source of antisera comprises fortuitously immunized human donors such as recipients of multiple transfusions, multiparous women alloimmunized against paternal Gm determinants, or rheumatoid arthritis patients who spontaneously produce antiglobulins (rheumatoid factors). In addition, considerable processing, including multiple absorptions, have often proved necessary to yield monospecific reagents required for Gm typing. Finally, antisera defining particular Gm

specificities are often limited in supply. Particular sera may be impossible to replace once exhausted, for two individuals rarely make the same subset of antibodies; in some instances [26] Gm specificities were functionally lost when the original defining antiserum was depleted.

Attempts to develop typing antisera in animals have been partially successful, but the processing required to yield usable typing reagents from these sera is often more laborious than that required for antisera of human origin [27]. Furthermore, heterologous immunizations tend to yield antibodies that detect 'isoallotypic' markers which are polymorphic (allotypic) within one immunoglobulin isotype (class) but which are always present on at least one other isotype. Antisera to such 'isoallotypic' determinants, although useful for molecular studies on isolated subclasses of immunoglobulins, cannot be used in the standard haemagglutination-inhibition Gm typing assay since the isotypic determinants are present in all individuals and will obscure polymorphic variation among individuals. Therefore Gm typing has largely been restricted to a few laboratories that have the resources to produce and process human antisera detecting Gm polymorphisms.

Gm nomenclature

The current Gm notation [26] used to describe the polymorphisms of Igh constant region genes is derived from a notation system devised before the discrete subclasses of heavy chain loci had been identified. In this system, all polymorphic determinants on IgG heavy chains were designated as a single series of Gm markers. Later, when IgG subclasses (isotypes) were recognized as products of individual loci, the notation was changed so that allotypic determinants on IgG1 molecules were named G1m and a superscript letter was appended to designate the individual determinant. Alleles were then named with the locus name followed by a superscripted list of determinants ($G1m^{af}$). Finally, the letters were changed to numbers so that $G1m^{af}$ became $G1m^{1,3}$. This last change has yet to be universally accepted and both the old and the new notation are currently in use. Haplotypes are formally constructed by concatenating allele designations. These names tend to be too long for ordinary usage; therefore a shorthand that omits locus names and designates a haplotype as a list of the major allotypic determinants it contains (e.g. f,a,n,b, which is equivalent to 3,1,23,5) is commonly used.

Gm notation creates confusion by codifying a technical distinction between allotypic determinants that can be used for Gm typing in the haemagglutination-inhibition assay and genetically equivalent isoallotypic determinants that require the use of more sophisticated typing assays. These latter determinants arise when a mutation occurs at a locus coding for a determinant shared by several subclasses. Although the mutation generates a polymorphism at one locus, the original sequence remains as an invariant (isotypic) marker on the other subclasses. Thus, although they constitute valid genetic markers, they are always present in human sera (on the non-polymorphic isotype) and consequently are not suitable for use in current haemagglutination-inhibition assays where all of the Ig in a serum sample is brought into contact with the typing reagent. Therefore, according to current nomenclature, they are called 'non-markers', and Gm notation dictates placing the prefix 'non-' before the names of these iso-allotypic determinants (e.g. non-G3mb). However, because they serve as normal genetic markers (in an appropriate assay), they are treated here as simple allotypic determinants.

Problems with current Gm typing methods

Allotyping by the classical immunodiffusion (Ouchterlony) methods used widely for mice and rabbits has rarely been possible with the reagents available for humans [27]. Although radioimmunoassay (RIA) is one of the most convenient, sensitive and quantitative methods for measuring allotypes in mouse, rabbit and chicken Ig [28–30], there are few reports of human typing by RIA [31,32]. This is apparently a result of difficulties in retaining antibody activity after radiolabelling the anti-allotype antisera.

Passive haemagglutination inhibition is the most commonly used method for human allotyping. In this assay, each test system consists of an allotype-specific antiserum called an agglutinator, diluted serum or plasma to be tested, and 'indicator' red blood cells coated with the specific allotype antigen. The ability of the test sera to inhibit the standard antigen–antibody agglutination reaction is scored visually under the microscope by evaluating the extent of red blood cell agglutination. Although this read-out method is qualitative, semi-quantitative estimates of the concentration of the antigenic determinants can be obtained by assaying serial dilutions of test samples. (For a more extensive discussion of current Gm typing, see Chapter 94.)

New approaches to typing for Ig polymorphisms

During the last few years, major technical advances in both immunology and molecular biology have provided the basis for the fine structure dissection and the routine typing of complex genetic regions such as those coding for the Ig gene families. Hybridoma

technology pioneered by Kohler & Milstein [33] has made possible the production of monoclonal antibody typing reagents which eliminate many of the problems found with heterogeneous and multispecific antisera. From molecular biology, the discovery of restriction endonucleases [34] combined with methods of *in situ* transfer of nucleic acids [35] and the refinement of techniques for molecular cloning [36,37] have revolutionized strategies of genetic analysis and finally brought it directly to the level of DNA.

Monoclonal antibody reagents for Gm typing

The production of monoclonal antibodies is an application of the early discovery that one antibody-forming cell produces only one kind of antibody. Permanent cell lines secreting antibody reactive with chosen antigens are derived from the fusion of a myeloma cell capable of continuous growth in culture with a cell of the spleen of an animal immunized with the appropriate antigen. Cloning of the hybrid cells results in cell lines secreting homogeneous antibody.

Monoclonal antibody reagents have a number of inherent advantages over current anti-allotype antisera. They are homogeneous and monospecific and therefore require no additional processing after purification from ascites fluid or culture supernate. They are reproducible, essentially unlimited in supply, relatively cheap to produce and readily amenable for widespread distribution. In solid-phase radioimmunoassays, or in enzymatic assays (ELISA), these antibodies can be used at very low concentrations with very small amounts of test sera. Radioimmunoassays (RIA) are ideally suited to studies of quantitative as well as qualitative expression of immunoglobulin antigenic determinants. Furthermore, a modification of the standard solid-phase RIA (see Chapter 34) allows detection and typing of the formerly inaccessible isoallotypic determinants in samples of whole sera. This assay provides, in effect, an 'in well' purification of a particular subclass of immunoglobulin from whole, unfractionated serum samples prior to exposure to the allotypic reagents (Fig. 95.1).

Early work in the mouse system has demonstrated not only that many distinct anti-allotypic reagents can be generated by the hybridoma strategy, but also that the resulting monoclonal reagents are able to expand the allotypic complexity previously defined by serology, to probe immunoglobulin structure at the molecular level, and to elucidate relationships between structure and function of antibody molecules [38–41]. More recently, these reagents have been used to demonstrate significant variation between wild mouse populations [42]. It is likely that a similar increase in genetic resolution will result when this approach is applied to the human immunoglobulin system.

Using hybridoma techniques, Zelaschi *et al.* [43] have generated reagents which detect polymorphic determinants on human IgG3. One of these markers appears identical in genetic distribution to the previously described G3m b1 marker (although it has a distinct isotype distribution), while a second one detects a new marker, G3m b6i. The first determinant provides a reference point that may facilitate transition to monoclonal-based RIA assays for Gm typing. The second adds a new level of complexity among Gm haplotypes, defining a previously unrecognized split in the G3mb complex of Caucasians.

DNA restriction fragment length polymorphisms for human allotyping

The traditionally defined allotypes originate from mutations altering one or more amino acids, which supply the genetic markers that currently characterize the Gm system. A potentially more powerful method of detecting polymorphisms in the Ig complex employs cloned sequences from this region as molecular probes to detect DNA sequence differences. This method does not require antigenically recognizable amino acid changes, only changes in the DNA sequence that are revealed as fragment length differences when the DNA is digested by one of the more than 150 commercially available restriction enzymes (R.E.). Therefore insertions and deletions occurring within or between restriction sites, or base pair substitutions which either create or destroy restriction sites, are detectable.

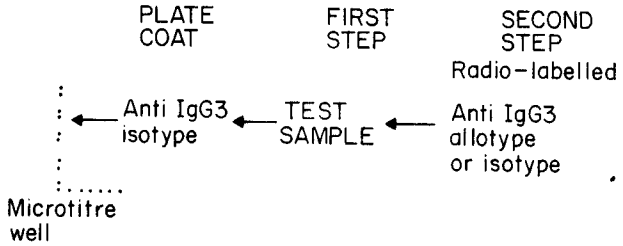

Fig. 95.1. Schematic of the RIA 'sandwich' assay for IgG3 allotypes. *In this figure the authors give an example of a determinant that is polymorphic on IgG3; therefore the first step is the 'in well' purification of IgG3 molecules present in the test serum.

whether or not they occur within coding sequences. The ability to detect a variety of new markers for the Ig region will greatly expand the existing haplotypes, exposing additional heterogeneity in what are now considered to be homozygous groups. Perhaps more importantly, new markers have now been identified in such regions as the variable heavy chain genes where no serologically defined markers exist. Screening can be done rapidly since several loci can be examined with a single probe and, as with monoclonal antibodies, supplies of the appropriate DNA probes are virtually inexhaustible.

Immunoglobulin probes

All of the human Ig heavy chain constant region genes have been cloned including IgM and IgD [44,45], the IgG genes (IgG1, IgG2, IgG3, IgG4, and pseudo IgG) [46–51], IgE, and two pseudo ε genes [51,55] and the two IgA genes (IgA12, IgA2) [51,55]. Several variable heavy chain genes representing subclasses I, II and III [56,57,57a] have also been cloned. Sequences representing both κ and λ constant regions have been isolated [58,59] as well as two κ variable genes [60]. By using these cloned segments of DNA as probes, new genetic markers can be identified in all of the immunoglobulin gene clusters.

Polymorphic studies

Initial work by Migone *et al.* [61] describing restriction fragment length polymorphisms (RFLPs) [62] in the human Ig heavy chain region demonstrates that a single probe can define polymorphisms at multiple loci. Using as a probe a cloned 12 kbp fragment containing the IgM heavy chain constant region as well as 5′ switch (S) region sequences, fourteen distinct hybridizing fragments were detected in Sst1 cleaved DNA. Thirteen of these share homology with the S segment of the probe alone. Of six polymorphic loci analysed, three were identified as being the heavy chain switch regions of IgA1, IgA2 and IgM. Altogether, a total of thirty-three haplotypes were determined (as compared to only five original Gm haplotypes); twenty-eight haplotypes could be distinguished by the Sst I restriction pattern alone (Table 95.1).

Work with cloned γ sequences demonstrates that a single γ subclass probe cross-hybridizes to all γ subclass genes in genomic digests because of their extensive sequence homology [46]. Polymorphisms detected using the enzymes Mbo I and Bst EII [7] define new markers for both γ gene clusters, extending the amount of heterogeneity for this region by further subdividing the traditional Gm haplotypes. Using the R.E. Bam HI, Bech-Hansen *et al.* [63] have defined

Table 95.1. Distribution of 158 DNA and *Gm* haplotypes identified in 29 Caucasian families [61]

Gm haplotypes	DNA haplotypes *A*	*C*	*D*	*E*	*F*	Number observed
f;b	7.4	4.8	(*)	2.7	1.0	55
	7.4	4.8	(*)	2.2	1.0	47
	7.4	4.9	(*)	2.2	1.0	4
	7.4	4.8	(*)	2.75	1.0	2
	D2	4.8	(*)	2.2	1.0	2
	7.4	4.1	(*)	2.2	1.0	2
	7.4	5.5	(*)	2.2	1.0	1
	6.9	4.8	(*)	2.2	1.0	1
	6.8	4.8	3.7	2.2	1.0	1
az;g	6.9	4.8	(*)	2.7	1.0	6
	6.8	4.8	(*)	2.7	1.0	3
	6.8	4.8	(*)	2.2	1.0	3
	6.8	4.8	3.7	2.2	1.0	3
	6.9	4.8	(*)	2.2	1.0	2
	6.8	4.8	3.7	2.7	1.0	1
	6.9	4.8	3.7	2.2	0.9	1
	6.8	5.2	(*)	2.7	1.0	1
	7.5	4.8	(*)	2.7	1.0	1
	D4	4.8	(*)	2.2	1.0	1
azx;g	6.9	4.8	(*)	2.7	1.0	5
	6.9	4.8	(*)	2.2	1.0	2
	7.4	4.8	(*)	2.2	1.0	2
	D3	4.8	(*)	2.2	1.0	2
	6.8	4.8	(*)	2.2	1.0	1
	6.8	4.8	3.7	2.2	1.0	1
	6.9	5.5	(*)	2.2	1.0	1
	6.9	5.5	(*)	2.75	1.0	1
	D3	5.5	(*)	2.2	1.0	1
	D1	4.8	(*)	2.2	1.0	1
	6.9	4.8	(*)	2.6	1.0	1
	6.9	null	(*)	2.2	1.0	1
f;g	7.4	4.8	(*)	2.2	1.0	1
az;b	6.8	5.5	(*)	2.2	1.0	1
					Total	158

DNA fragments showing polymorphisms (polymorphic 'loci') are named from *A* to *F*. Alleles at each locus are described. *D1–D4* are duplications which can be distinguished one from the other and behave as alleles at the A locus. (*) Alleles not allocated to a specific band. The *b* symbol in both *f;b* and *az;b* haplotypes stands for the antigen complex b0,b1,b3,b4,b5.

RFLPs for three γ genes—IgG2, IgG4 and pseudo γ. Recently, Lefranc & Rabbitts [64] reported an RFLP which defines A2m allotypes. Early studies by Matthyssens & Rabbitts [57] using a V-HIII subclass clone identified a Bst EII polymorphism within this variable gene family, although their analysis only included three individuals. Bgl II and Bst EII variable region

polymorphisms have been found using a subgroup II variable heavy chain DNA probe [7] generating the first genetic markers for the variable heavy chain gene region in humans, and allowing for direct testing of linkage between the constant and variable gene clusters.

In studies involving Ig light chain genes, RFLPs were found in the human C λ locus [59], although a survey of eight individuals using a κ light chain probe and two enzymes found relatively little polymorphism in this region [60]. It should be mentioned that these latter studies were not intended to be surveys of polymorphism. They do, however, serve to indicate that variation in the immunoglobulin regions is sufficiently frequent to be found even in studies involving small sample sizes. These results illustrate the power of this technique in resolving additional genetic heterogeneity in the immunoglobulin gene clusters.

Types of polymorphisms

In contrast to serological markers, most DNA polymorphisms detected thus far do not occur within the coding regions. The switch region variants [61] are due to insertions or deletions occurring within the multiple tandem repeat sequences in the 5′ flanking regions of each Ig heavy chain constant region gene. The Mbo I and Bst EII Ig γ polymorphisms described by Johnson & Cavalli-Sforza [7] appear to be the result of point mutations, only one of which maps to a structural gene coding region, while the IgG Bam HI polymorphisms reported by Bech-Hansen *et al.* [63] are most likely the result of insertions/deletions 5′ to the genes. The A2m(2) RFLP which can be used to distinguish this from the A2m(1) allotype, is due to a polymorphic site within C_H1 [64].

In order to increase the probability of finding polymorphisms within coding regions, one could limit these studies to enzymes which are known to cut within the genes or which have potential sites within the genes. Potential sites [65] are defined as sequences which are one base pair different from a true restriction site. In addition, probes can be shortened by subcloning, to include only the coding regions, thereby limiting hybridization to gene containing fragments. Such strategies may increase the probability of finding coding region variants with functional significance, but for the purposes of genetic analysis, polymorphisms occurring anywhere within the immunoglobulin region are useful. For purposes of linkage analysis with other chromosome 14 markers, flanking region polymorphisms may prove to be the more valuable.

Serologic vs. molecular typing for Gm

In theory, the direct analysis of the genome using recombinant DNA techniques could supplant the serologic Gm typing system. However, there are certain drawbacks to using molecular methods for routine typing. First, although DNA is easily accessible from peripheral blood tissues, processing of many hundreds of samples is very time consuming. Second, screening a large number of individuals by Southern blotting techniques is a much lengthier process than the alternative serological methods of Gm typing. Third, the cost of the restriction enzymes necessary for a genetic survey is not insignificant. Nevertheless, the greater genetic diversity revealed by molecular (DNA) analysis does much to recommend this method.

Monoclonal antibody based allotyping methods circumvent most of the difficulties in the current serological assay and should be easily implemented in most laboratories. The ability to type individuals on the basis of the allotypic determinants present in a few microlitres of serum by rapid, simple and relatively cheap methods is of prime importance for clinical, forensic and population studies involving large numbers of samples that often must be acquired from geographically isolated locations and stored for relatively long periods of time. However, adoption of these methods for routine typing awaits the generation of a suitably diverse panel of monoclonal reagents. To date only a few such reagents have been generated; but if the results in the mouse system are indicative, many additional specificities should be identified by this approach.

The combined use of molecular and serologic methods may be the key in sorting out the associations of particular diseases with the Gm markers. Most Gm associations reported to date are relatively loose, which may indicate that the susceptibility gene is outside of the Ig region but in linkage disequilibrium with particular Gm alleles. The true association may be with other Ig heavy chain genes, or with the variable region genes. Once an association is recognized, locating the position of the responsible gene(s) requires markers at as many positions as possible along the Igh chromosome. Restriction site polymorphisms thus become extremely useful in such studies. The first and currently the only variable region gene markers in the human are RFLP markers [7]. In the end, serologic typing may prove more useful for initially identifying an association, while restriction mapping may be much more valuable for localizing the genetic effect.

A similar approach should also facilitate definition of the Igh chromosomal region itself. The available serological evidence is not sufficient to definitively

localize all of the Igh loci within this region, since only those loci with known polymorphisms (allotypes) can be shown to be part of the linkage group. The identification of new RFLP alleles as well as the systematic cloning of large segments of the region [51] have clarified the Igh genomic organization. The order of four γ genes in two clusters has been established, although the two clusters have not been physically linked. A recent study [63] proposes that pseudo γ is located between the γ 1–3 and γ 2–4 gene clusters.

Methods

Monoclonal antibodies in radioimmunoassays

Hybridoma methodology for the production of monoclonal antibodies is presented in Chapter 111. Purification techniques for monoclonal antibody reagents are presented in Chapter 13. Radioimmunoassay typing methods are presented in Chapter 34.

Genomic Southern analysis

Because common techniques of recombinant DNA technology are presented elsewhere [66], only specific methods applied to human Ig typing are described here. DNA can be easily extracted from a variety of tissue types including peripheral blood. A 30 ml sample of blood is drawn into ACD (acid citrate dextrose) or heparinized tubes and the buffy coats are separated from red cells and serum by centrifugation at 2000 rev./min for 15 min. High molecular weight DNA is extracted from the buffy coats by conventional methods [67]. The yield from a 30 ml blood sample generally ranges from 500 to 1000 μg of DNA. The majority of white cells found in the buffy coat are granulocytes which are unaffected by immunoglobulin gene rearrangements that occur in B lymphocytes during differentiation. Since there are a great variety of such rearrangements, any one type will occur too infrequently to influence the DNA patterns obtained in digestions of DNA from total white blood cells. Therefore the observed pattern represents the germline configuration of DNA sequences for the immunoglobulin genes [61].

For restriction analysis, approximately 5 μg of DNA is digested to completion with the selected restriction endonuclease using 5 units/μg of total DNA and at least 6 h incubation at the recommended temperature. Samples are then precipitated with 95% ethanol, stored for between 4 h and overnight at -20 °C, resuspended in 10 mM-Tris (pH 7.5)/1 mM-EDTA and electrophoresed in a horizontal agarose gel at approximately 1.5 V/cm for 16–20 h. Following this, gels are stained in 0.5 μg/ml of ethidium bromide for 30 min and fluorescence photographed on a DNA transilluminator. Gels are then prepared for blotting by washing in alkaline denaturing solution (1.5 M-Tris/0.5 M-NaOH) twice for 20 min each, and neutralizing solution (1 M-Tris/1.5 M-NaCl) twice for 30 min each. The DNA is then transferred overnight in 20× SSPE (1× SSPE = 0.18 M-NaCl, 10 mM-NaH_2PO_4, 1 mM-Na_2EDTA, pH 7.0) to nitrocellulose paper [35]. Following the transfer, filters are rinsed in 2× SSPE, air dried for at least 1 h, and baked under vacuum for 2 h at 80 °C.

^{32}P dNTPs (usually dCTP and dATP) are incorporated into the DNA probes by standard nick-translation techniques to a specific activity of $1–3 \times 10^8$ c.p.m./μg [68]. Generally, 500 ng of DNA is nick-translated in a total volume of 25 μl. Hybridization can be carried out under a variety of conditions, depending on the degree of base mismatch between the probe and the target sequences. For fairly stringent conditions, hybridizations are done in a solution containing 5× SSPE, 50% formamide and 1–10 ng labelled probe/ml at 42 °C for 48 h, followed by several washes. The first of these is 1× SSPE, 0.1% SDS, twice—20 min each at 46 °C. Then more stringent washes of 0.1× SSPE, 0.1% SDS are done for a total of 1 h at 65 °C. Autoradiography is for 5–10 days at -70 °C using Kodak XAR-5 film and intensifying screens.

Detection of restriction fragment length polymorphism

For genetic studies, DNA isolated from a panel of random individuals should be initially screened to assess the amount of genetic heterogeneity revealed by a given restriction enzyme. After identifying a group of enzymes which reveal polymorphisms in high frequency, parental DNA can be screened for the presence of the polymorphism. Finally, DNA from children of informative matings can be examined in order to determine segregation of the polymorphic fragments. With RFLPs, all polymorphic bands can be considered as co-dominant markers. Therefore informative matings are those in which the restriction patterns seen in the two parents differ from one another. Although segregation analysis of the polymorphic bands in pedigrees will reveal which DNA fragments behave as alleles at a single locus, molecular mapping is necessary to identify the particular locus involved. Once the different loci and alleles are determined, computer programs such as LIPED (Linkage Pedigree) [69] can be used to test for linkage of the new DNA markers with the standard GM allotypes or with other markers on the chromosome. With the heavy chain markers, the information can then be used to expand the existing Gm haplotypes to

include the DNA markers and to subdivide what were previously considered homogeneous Ig haplotypes into more refined subgroups.

Assignment of RFLP variants to IgG loci

In most cases, the sequence homology between Ig heavy chain genes results in cross-hybridization between ancestrally duplicated loci. For example, any IgG DNA probe will also hybridize to sequences encoding the other IgG subclasses because of their high (>90%) nucleotide sequence homology [46]. Similarly, a probe containing the switch region 5′ to the IgM constant region segment hybridizes to at least seven and possibly thirteen distinct loci in the human genome [61]. Loci within the variable region have also been shown to cross-hybridize within but not between subclasses [56,60]. Thus, while DNA polymorphisms can be detected at multiple loci with a single probe, making screening relatively rapid, this cross-hybridization makes it more difficult to assign variants to particular loci.

One method of determining the specific locus involved in the polymorphism utilizes myeloma cell lines which produce a single Ig subclass protein. It is thought that the class switch mechanism involves the deletion of all Ig constant heavy chain genes between the joining region and the gene encoding the subclass gene being expressed. Deletion of the unexpressed chromosome is also common [70–74]. By using myeloma cell lines which produce IgG, the order of three clusters of Ig contant genes on chromosome 14 in humans has been established as: 5′ JH–M–D——G3–G1–E–A1——G2–G4–E1–A2 3′ [51]. Therefore IgG1 gene fragments derived from the active chromosome will not be seen in hybridization to myeloma DNA from an IgG2- or IgG4-producing cell line, just as IgG2-associated fragments will be absent on the active chromosome of an IgG4-producing cell line. By digesting DNA from a panel of different IgG-producing myeloma cell lines with the enzyme of interest and hybridizing with a γ probe, one can hope to identify fragments belonging to a specific gene, depending on when that fragment is lost. Myeloma data is, however, often ambiguous since the extent of deletions on both the active and inactive chromosome is unclear.

An alternative method is to construct restriction maps of each of the γ gene clones with the R.E. defining the polymorphisms and to determine the linear arrangement of the fragments in that clone (i.e. allele). Molecular weights of fragments in the clones should correspond in size to a particular genomic fragment and indicate to which locus a fragment belongs. Once such maps are constructed, fragments generated by the same enzyme giving the polymor-

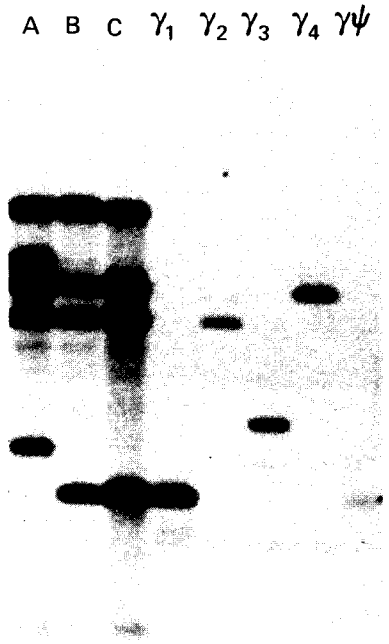

Fig. 95.2. Assignment of various hybridizing fragments to a particular Ig gamma's locus. Lanes A–C contain 5 μg of Mbo I digested genomic DNA isolated from individuals homozygous for three different Mbo I haplotypes: A, 3.6–1.9 kbp haplotype; B, 4.3 (doublet)–1.6 kbp haplotype; C, doublet 1.6–1.6 kbp haplotype. The adjoining lanes each contain 20 pg of Mbo I digested DNA from one of the five Ig γ subclones. Molecular weights of the hybridizing fragments can be used to identify two of the non-polymorphic fragments (3.4 and 3.0 kbp) as belonging to γ 4 and γ 2 respectively. One of the polymorphic fragments (1·6 kbp) is identified as belonging to both γ 1 and pseudo γ. This finding explains the 1.6 kbp doublet in haplotype C as being due to variation in two different γ genes. The γ 3 subclone used in this experiment did not correspond in molecular weight to any specific genomic fragment. Further analysis revealed that this particular subclone is too short to contain the 3′ Mbo I site; however, an additional γ 3 subclone (gift of L. Kirsh, Naval Hospital, Bethesda) was used to identify the γ 3 fragment as the 4.3 kbp fragment (data not shown).

phism can be isolated from the original clone. These can be used as probes on genomic digests of DNA from individuals homozygous for the previously established haplotypes. In this way, the overall pattern can be simplified into polymorphisms of the 5′ region, the structural gene and the 3′ flanking region groups rather than the twenty or so bands usually seen with a larger (>5 kpb) probe. Fig. 95.2 illustrates this technique. Hybridization patterns combined with molecular maps of the clones should enable the unambiguous assignment of all fragments to a particular locus. In some cases it may be necessary to further refine probes so that they will recognize only their corresponding locus rather than cross-hybridizing to a defined gene region. This can best be achieved using hybridization and washing conditions wherein precise homology is required, along with a probe representing the most divergent sequence between cross-hybridizing genes [75,76]. With the Ig gammas, the hinge regions are the most divergent and may be useful as locus specific probes under correct hybridization conditions. In general, RFLPs can be expected to generate a tremendous number of new markers for all of the immunoglobulin genes.

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