A gamma 3 hinge region probe: first specific human immunoglobulin subclass probe

S. Huck, G. Keyeux*, N. Ghanem°, M.-P. Lefranc and G. Lefranc

Laboratoire d'Immunogénétique, Université des Sciences et Techniques du Languedoc, UA CNRS 1191, 34060 Montpellier Cédex, France

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We report the first specific human immunoglobulin subclass probe which was obtained by subcloning the gamma 3 hinge region. This specific γ3 probe allowed us to identify with certainty the Cγ3 gene on Southern genomic blots, to describe the first Cγ3 restriction fragment length polymorphism (EZZ γ3 RF) and to show that an IgG3 selective deficiency, previously described serologically, was not due to a deletion of the Cγ3 gene. Such a probe should be particularly useful for screening libraries from individuals with IgG3 immunodeficiencies or presenting unusual Cγ3 genes and, consequently, for studying the Cγ gene evolution.

Immunoglobulin IgCH gene (Human) Gm allotype Restriction fragment length polymorphism Immunoglobulin G3 deficiency Hinge

1. INTRODUCTION

In the human immunoglobulin system, there are five classes (IgM, IgD, IgG, IgE and IgA) which are defined by the isotypic antigenic determinants, physico-chemical properties and biological activities of their heavy chains (μ, δ, γ, ε and α, respectively). Furthermore, subclasses are known for IgG (IgG1, IgG2, IgG3 and IgG4) and IgA (IgA1 and IgA2) [1]. The γ1, γ2, γ3, α2 and ε heavy chains can also be identified by allotypic antigenic determinants located on their constant region domains (CH) and called, respectively, G1m, G2m, G3m, A2m and Em allotypes [2,3]. The immunoglobulin heavy (H) chain locus, mapped on chromosome 14 [4] at q32 band [5], consists of four linked families of variable (VH), diversity (D), joining (JH) and constant (CH) gene segments. The order of the CH genes has been determined as 5′-μ-8 kb-δ-...-γ3-26 kb-γ1-19 kb-ψε1-13 kb-α1-...-ψγ-...-γ2-18 kb-γ4-23 kb-ε-10 kb-α2-3′ [6–10] (fig.1a).

The CH genes can be detected on Southern blots using Cμ, Cδ, Cγ, Cε and Cα probes [7,8,11–17]. The Cγ probes used until now detect all Cγ genes since they cross-hybridize with the genes for the various subclasses [8,12]. Moreover a polymorphism exists for the different Cγ genes and five to eight hybridizing bands can be detected with genomic DNAs cut with the *Bam*HI restriction enzyme [8]. In this paper, we describe a γ3 hinge region probe, the first specific human immunoglobulin subclass probe, which enables us to identify with certainty the Cγ3 gene on Southern blots and to describe the first Cγ3 restriction fragment length polymorphism as well as to show that an IgG3 selective deficiency, previously described serologically, is not due to a deletion of the Cγ3 gene.

* Permanent address: Universidad Javeriana, Facultad de Medicina, Unidad de Génética Clinica, Bogota, Colombia

° Permanent address: Université Libanaise, Faculté des Sciences Médicales, Beyrouth, Lebanon

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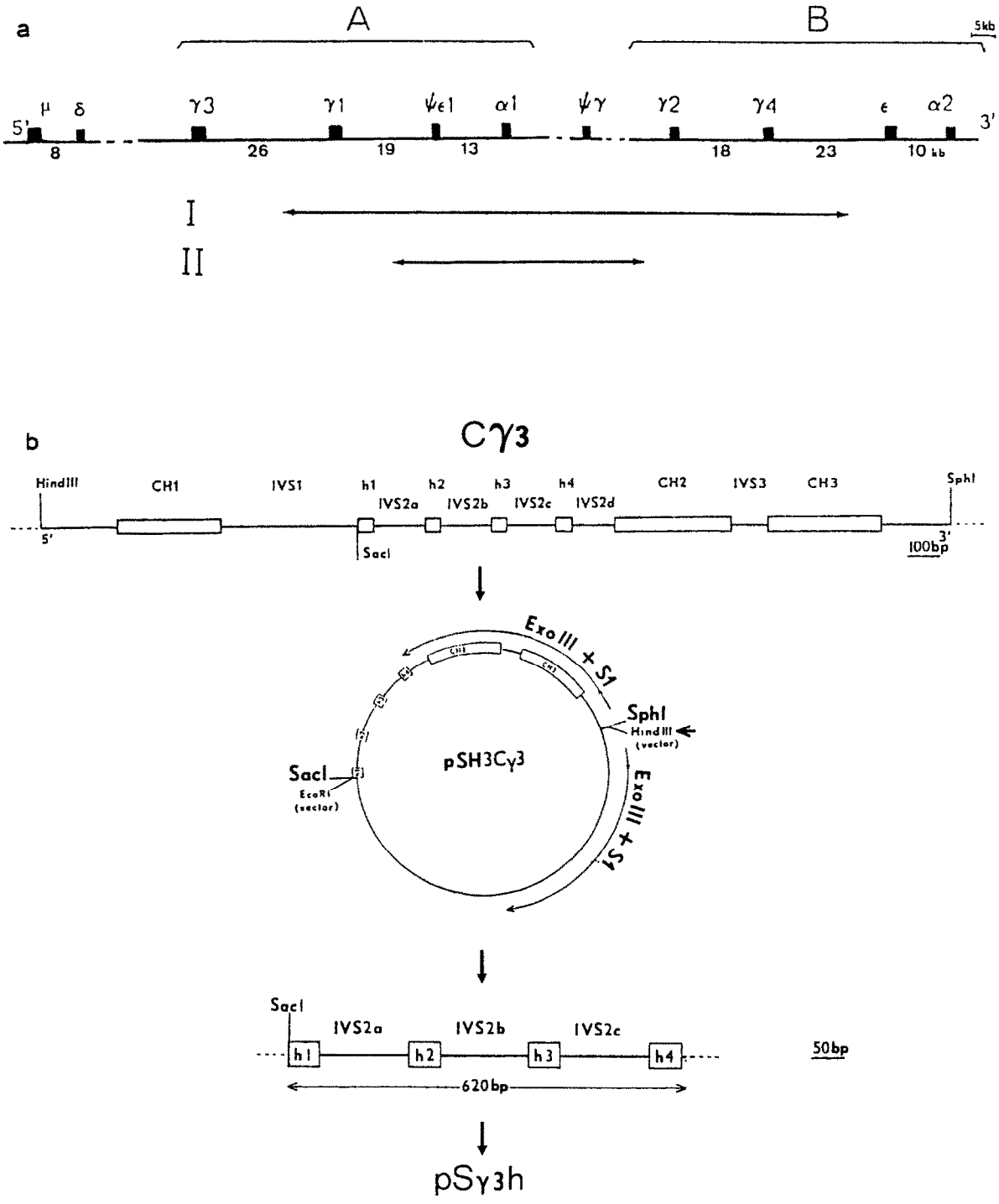


Fig.1. Organization of the human IgCH genes and cloning of a $\gamma 3$ specific probe. (a) Order of the human IgCH genes. Two groups of cosmid clones have been identified which encompass region A and region B [7]. Patterns of deletions (deletions I and II indicated by arrows) enabled us to localize region A in 5′ of region B [8] and the $\psi\gamma$ gene between $\alpha 1$ and $\gamma 2$ [9]. (b) Cloning of the pSγ3h probe. The region encompassing the human Cγ3 gene is part of a previously

2. MATERIALS AND METHODS

2.1. *Serological analysis of allotypic and isotypic markers*

Serum samples were tested by the classical haemagglutination inhibition method for the determination of the G1m, G2m, G3m and A2m allotypes and for the isotypes previously described [18].

2.2. *DNA analysis*

DNA was prepared from total peripheral blood white cells as described [19]. DNA was digested to completion with enzymes as mentioned, the digests were fractionated on 0.8% agarose gel and the DNA was transferred to cellulose nitrate filters [20]. The hybridization was carried out overnight either at 65°C in 6 × SSC or at 42°C in 5 × SSPE, 50% formamide, supplemented, in both cases, with 0.1% SDS, 0.2% Ficoll 400, 0.2% bovine serum albumin (BSA), 0.2% polyvinylpyrrolidone (PVP), 5% dextran sulfate and 50 $\mu g \cdot ml^{-1}$ sonicated, denatured, salmon sperm DNA and followed by washes in 0.1 × SSC, 0.1% SDS at 65°C before autoradiography at −70°C. Probes were nick translated to a specific activity of 10^8 cpm per μg.

2.3. *Cloning of a γ3 hinge region probe (clone pSγ3h)*

The construction and screening of a λ phage library from EZZ DNA have been described [21]. EZZ is a Tunisian (individual II-4 of the TOU family studied elsewhere [9]), whose chromosomes 14 both display a large deletion encompassing the Cγ1, ψε1, Cα1, ψγ, Cγ2 and Cγ4 genes [8,9] (fig.1a, deletion I). Therefore the only Cγ gene present is the Cγ3 one. One recombinant clone containing the Cγ3 gene, λEZZγ3 [21], was selected for shotgun subcloning.

The 2 kb *Sac*I-*Sph*I fragment (fig.1b) was first obtained into pUC 19 (clone pSH3Cγ3), this pSH3Cγ3 clone was cut by *Hin*dIII to be linearised, then shortened using the exonuclease III (Genofit) and nuclease S1 (BRL, Boehringer) method as described [22]. A 620 bp *Sac*I-blunt end fragment was selected, shown by sequencing to contain the four hinge exons (figs 1b and 2) and then subcloned into pUC 18 (clone pSγ3h).

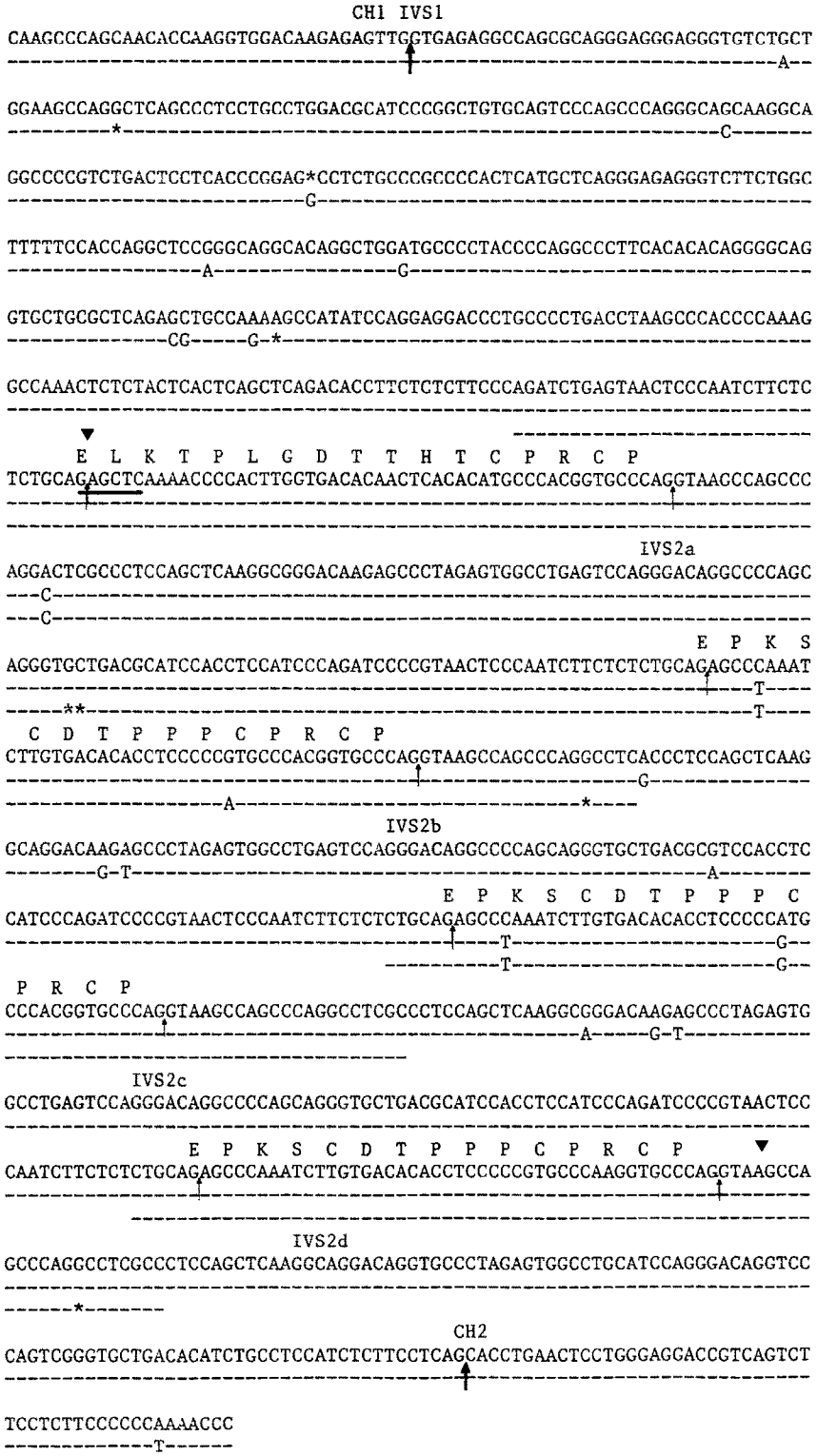
3. RESULTS AND DISCUSSION

3.1. *Clone pSγ3h: a specific γ3 subclass probe*

Southern filter hybridizations by the usual Cγ probes used until now (such as clone p3.6RH4.2 which contains a 3.6 kb *Eco*RI-*Hin*dIII fragment encompassing the whole γ3 gene coding region [12] or clone pBRH4.1 which contains an analogous fragment encompassing the γ4 gene [12] as well as clone pSH3Cγ3 with the 2 kb *Sac*I-*Sph*I Cγ3 insert (fig.1b)) detect usually between five to eight hybridizing *Bam*HI fragments as exemplified by genomic DNAs from different individuals [8] (fig.3a). Sequence of the human Cγ3 gene and its comparison with the other Cγ genes showed a high degree of homology in the coding regions (95.3–97%) as well as in the intervening sequences (93.3–95%) [21]. The only area displaying some divergence was the hinge. Pairwise comparison of the Cγ hinge exons showed that they are only 70 to 85% homologous [21]. The γ3 hinge coding region is made of four distinct exons separated from each other by short introns (IVS2a,b,c) (fig.1b) whereas the other Cγ hinges display a unique exon. A 620 bp clone, pSγ3h was isolated which starts at the *Sac*I site, specific of the γ3 hinge, and encompasses the four hinge exons (h1–4) as well as the hinge introns (IVS2a,b,c) (fig.1b). This probe does not include the IVS1 and IVS2d introns whose percentage of homology is quite high (92–95%) [21]. The sequence of the EZZγ3 hinge region from which the pSγ3h clone was derived is compared with two other γ3 hinge region sequences in fig.2.

The *Sac*I site located at the beginning of the first hinge exon (h1) and used for the subcloning is

described recombinant clone (λEZZγ3) [21]. The γ3 hinge coding region is made of four distinct exons (h1-51 bp, h2-45 bp, h3-45 bp and h4-45 bp) separated from each other by short introns of 143 bp (IVS2a,b,c) and followed by a 118 bp intron (IVS2d) whereas the other Cγ hinges display a unique exon (36–45 bp) followed by a 118 bp intron (IVS2) [21]. The clone pSH3Cγ3 containing the 2 kb *Sac*I-*Sph*I fragment in pUC 19 was cut by *Hin*dIII to be linearised, then shortened using the exonuclease III and nuclease S1 method as described [22]. A 620 bp *Sac*I-blunt end fragment containing the four hinge exons was then subcloned into pUC 18 (clone pSγ3h).


CH1 IVS1
IVS2a
IVS2b
IVS2c
IVS2d
CH2

characteristic of the Cγ3 gene. Whereas the first amino acid of all the γ hinge exons is well conserved (Glu encoded by the GAG triplet), the second amino acid is different from one subclass to the other (table 1). For the γ3h1 exon, this amino acid is Leu which is encoded by the CTC triplet resulting in a *Sac*I restriction site (GAGCT↓C) in the γ3 hinge sequence (fig.2; table 1).

In order to check the specificity of the pSγ3h clone, Southern filter hybridizations of this probe were carried out with *Bam*HI digested genomic DNAs using standard conditions of hybridization (see section 2). Samples from Caucasoid populations (European, Lebanese and Tunisian) were selected for the various Gm-Am haplotypes. These samples displayed with the pSγ3h probe a unique 10.8 kb *Bam*HI hybridizing fragment corresponding to the Cγ3 gene (fig.3b) whereas hybridization of the same DNAs with a Cγ probe (clones pBRH4.1, p3.6RH4.2 [12] or clone pSH3Cγ3) detected five to eight bands, depending on the samples (fig.3a). These results, obtained after 2 days exposure, show that the pSγ3h clone is a specific γ3 subclass probe since the cross hybridization with the other Cγ genes is too weak to be detected in these conditions. On longer exposures (1 week) the other Cγ genes may be detected as very faint bands compared to that of the γ3 gene.

3.2. *A Cγ3 restriction fragment length polymorphism*

We already reported that only one γ hybridizing fragment corresponding to the γ3 gene, the only γ gene present, could be detected with *Bam*HI or *Hin*dIII digested DNA of the individual EZZ above mentioned, homozygous for the large deletion encompassing the γ1, ψε1, α1, ψγ, γ2 and γ4 genes [8,9]. The EZZ hybridizing *Bam*HI γ3 fragment detected on Southern blot (fig.4a and b, lane 2) is 10.2 kb long, i.e. 600 base pairs (bp) shorter than the usual 10.8 kb *Bam*HI γ3 fragment (fig.4a and b, lane 1). Likewise the EZZ hybridizing *Hin*dIII γ3 fragment (fig.4c, lane 2) is 7.2 kb long, again 600 bp shorter than the usual 7.8 kb *Hin*dIII fragment (fig.4c, lane 1). In order to determine if these shorter sizes were a consequence of the large deletion known to have occurred on the chromosome 14 or whether they were due to an independent event, a λ clone (λEZZγ3) was isolated from the library made from EZZ DNA. This clone was shown to contain a Cγ3 gene with normal coding regions and to extend 12 kb downstream of the gene [21]. The restriction map of its

Table 1

First two amino acids and encoding triplets of the human Cγ hinge exons

Amino acids and encoding triplets		Cγ subclass
GAG	CCC	γ1, ψγ
Glu	Pro	
GAG	CGC	γ2
Glu	Arg	
*Sac*I site:		
GAG	CT↓C	γ3 (h1)
Glu	Leu	
GAG	CCC or CCT	γ3 (h2-h3-h4)
Glu	Pro	
GAG	TCC	γ4
Glu	Ser	

The first two amino acids and encoding triplets of the human Cγ hinge exons are indicated [12,21,28–30]. h1, h2, h3, h4 correspond respectively to the four hinge exons of the Cγ3 gene (see fig.1b). The *Sac*I site (GAGCT↓C) is only present in the first hinge exon (h1) of the Cγ3 gene

Fig.2. Sequence of the γ3 hinge region from which the pSγ3h clone was obtained and comparison with two other γ3 hinge region sequences. The pSγ3h clone encompasses the four hinge exons of the Cγ3 EZZ gene [21]. (▼) Beginning and end of the pSγ3h clone. The *Sac*I site which is characteristic of the γ3 hinge is underlined. The introns or intervening sequences are represented by IVS1 (CH1-h1 intron), IVS2a (h1-h2 intron), IVS2b (h2-h3 intron), IVS2c (h3-h4 intron) and IVS2d (h4-CH2 intron) (see fig.1b). The RNA splicing sites at the end of the CH1 exon and beginning of the CH2 exon, as well as those of the hinge exons are indicated by arrows. Deduced amino acid sequences are shown for the hinge exons (the letter is above the first nucleotide of a codon). Dashes represent identity of the γ3 EZZ hinge region sequence [21] (first line) to the other completely [28] (second line) or partially [12] (third line) known hinge region sequences. Gaps introduced to maintain sequence homology are indicated by asterisks.

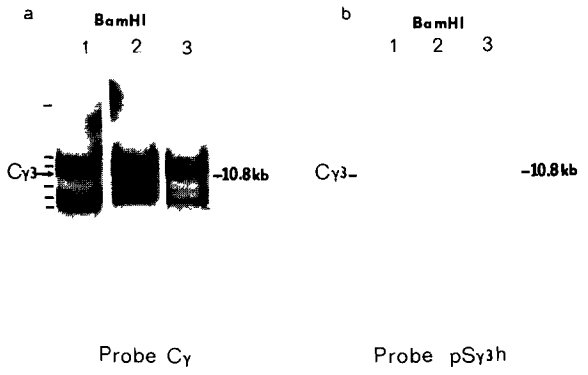


Fig.3. Southern hybridization of genomic DNA using a Cγ probe and the γ3 hinge region probe. (a) Hybridization of *Bam*HI digested genomic DNA with the Cγ probe pSH3Cγ3 (fig.1b) which detects all Cγ subclass genes. The lines on the left of the panel signify location of the most commonly observed restriction fragments in Caucasoid populations (European, Lebanese, Tunisian). The DNA samples shown in this figure were selected for their different pattern of hybridization. No clear correlation between Gm-Am haplotypes and pattern of hybridization was detected. (b) Hybridization of the same DNA samples, with the γ3 hinge region probe pSγ3h (fig.1b) which only detects the Cγ3 gene after 2 days exposure. Size of the γ3 *Bam*HI hybridizing fragment (10.8 kb), shown on the right of the panel, was estimated from the restriction map of a cloned Cγ3 gene [7] yielding a *Bam*HI fragment of equal size on a gel.

3′-flanking region is compared with that of a usual Cγ3 gene isolated from a cosmid library [7] in fig.4d. Three easily recognizable restriction sites '*Xho*I-*Bam*HI-*Hin*dIII' are noticeable in 3′ of the usual Cγ3, as of every Cγ gene [7]. These sites are also found in 3′ of the Cγ3 EZZ gene (fig.4d), but the restriction map localizes them 600 bp more upstream than for the usual Cγ3 gene, showing that a short 600 bp deletion must have occurred in the 3′-flanking region of the gene and upstream of the '*Xho*I-*Bam*HI-*Hin*dIII' sites. Downstream, the EZZ clone map is identical to that of the cosmid clone (fig.4d) confirming that the shorter *Bam*HI and *Hin*dIII EZZ fragments are not a consequence of the large deletion previously described on the chromosome 14 and, therefore, must represent a restriction fragment length polymorphism of the γ3 gene. A 10.2 kb-*Bam*HI and a 7.2 kb-*Hin*dIII hybridizing fragment (not shown) were found recently in the heterozygous state in another DNA sample studied at random confirming that they indeed represent a γ3RFLP. The simultaneous presence of these two (10.2 kb and 10.8 kb) allelic Cγ3 *Bam*HI fragments were also detected in two members of the TOU family. These individuals (TOU I-2, TOU II-3) were previously shown to have two different multigene deletions, one encompassing the γ1-ψε1-α1-ψγ-γ2-γ4 genes on one chromosome 14 and the ψε1-α1-ψγ genes on the other chromosome 14 [9] (fig.1a, deletions I and II). Hybridization of a Cγ probe (clones pBRH4.1, p3.6RH4.2 [12] or clone pSHCγ3) with *Bam*HI digested DNAs detected four bands, the unusual 10.2 kb band being stronger than the others and thought to represent two species [9] (fig.4a, lane 3). The same DNA samples hybridized with the γ3 specific probe (clone pSγ3h) showed two bands, 10.8 kb and 10.2 kb representing, respectively, the usual Cγ3 gene and the allelic γ3 EZZ gene (fig.4b, lane 3). Both bands have the same intensity confirming that the Cγ probe (not subclass-specific) was detecting two non-allelic Cγ genes in the unusual 10.2 kb-hybridizing band (one of which being the γ3 EZZ gene and the other being either a polymorphic Cγ not yet described or a Cγ(Cγ1 or Cγ2) affected by the deletion which encompasses the ψε1-α1-ψγ genes).

The existence of an allelic Cγ3 gene (EZZ γ3 RF) due to a short deletion in the 3′-flanking region of the gene is the first one described, but our unpublished data on the human Cγ genes suggest that short deletions or insertions (of a few hundred base pairs), occurring in the 5′- or 3′-flanking regions of the Cγ genes, could be responsible for most of the Cγ polymorphism detected with *Bam*HI digested genomic DNAs.

3.3. *Familial lack of IgG3*

Previously we reported the transmission through three generations of a same family (BAR family) of three different haplotypes characterized by the absence of the allotypic markers of the γ3 chain (indicated by a dash −): $Gm^{3;23;-}A2m^{1}$, $Gm^{1,17;..;-}A2m^{2}$ and $Gm^{1,2,17;..;-}A2m^{2}$ [23,24]. These haplotypes were responsible for the absence of IgG3 in seven individuals in two related Lebanese (L) and Syrian (S) branches of this family (see the genotypes in fig.5a and table 2). *Bam*HI digested DNAs from the individual (III-6, fig.5a)

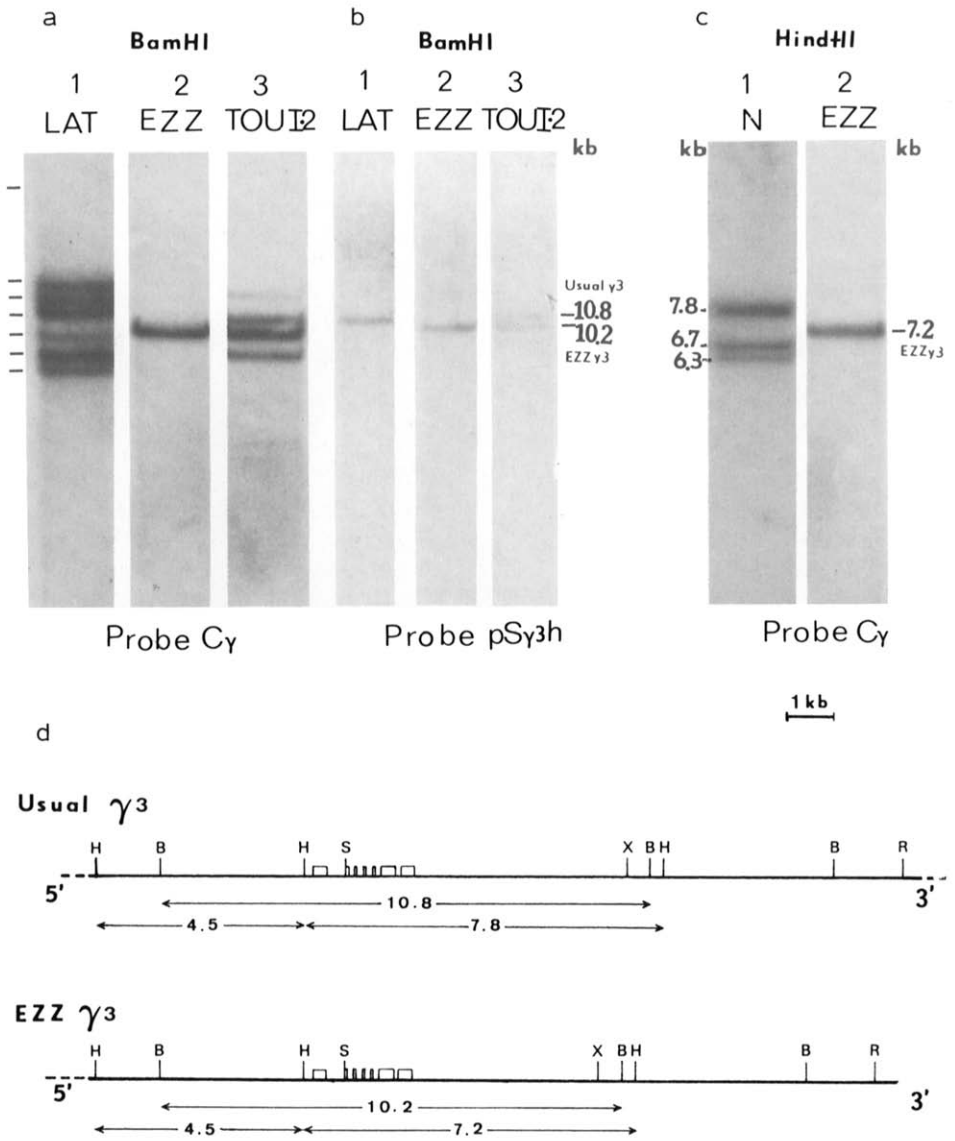


Fig.4. Southern hybridization of genomic DNAs showing a $\gamma 3$ restriction fragment length polymorphism. (a and b) Hybridization of *Bam*HI digested genomic DNAs with the Cγ pBRH4.1 probe [12] (a) and with the pSγ3h probe (b). Lanes: 1, LAT, a normal control DNA; 2, EZZ; 3, TOU I-2. The lines on the left of the panel signify location of the most commonly observed restriction fragments in Caucasoid populations. Sizes of the usual $\gamma 3$ *Bam*HI hybridizing fragment (10.8 kb) and of the EZZγ3 *Bam*HI restriction fragment (10.2 kb), shown on the right of the panel, were respectively estimated from the restriction maps of a cloned Cγ3 gene [7] and of the λEZZγ3 clone ([21] and d) yielding fragments of equal size on the gel. Previous estimations of the $\gamma 3$ EZZ *Bam*HI hybridizing fragment, made on Southern blots using λ cut with *Hin*dIII, were slightly overestimated [8,9]. (c) Hybridization of *Hin*dIII digested genomic DNAs with the Cγ probe pBRH4.1 [12]. Lanes: 1, a normal control DNA; 2, EZZ. Size estimations of the hybridizing fragments were made using λ cut with *Hin*dIII. On the basis of the restriction maps of cloned Cγ genes, the 7.8 kb band can be assigned to the $\psi\gamma$ [28], $\gamma 1$ and $\gamma 3$ [7] genes, the 6.7 and 6.3 kb respectively to the $\gamma 4$ and $\gamma 2$ genes [6,7]. The 7.2 kb EZZ band is in agreement with the λEZZγ3 restriction map ([21] and d). (d) Restriction map of the region containing a usual Cγ3 gene [7] and the EZZγ3 gene [21]. Only the *Sac*I site of the hinge is indicated. Exons are shown as boxes. B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; S, *Sac*I; X, *Xho*I.

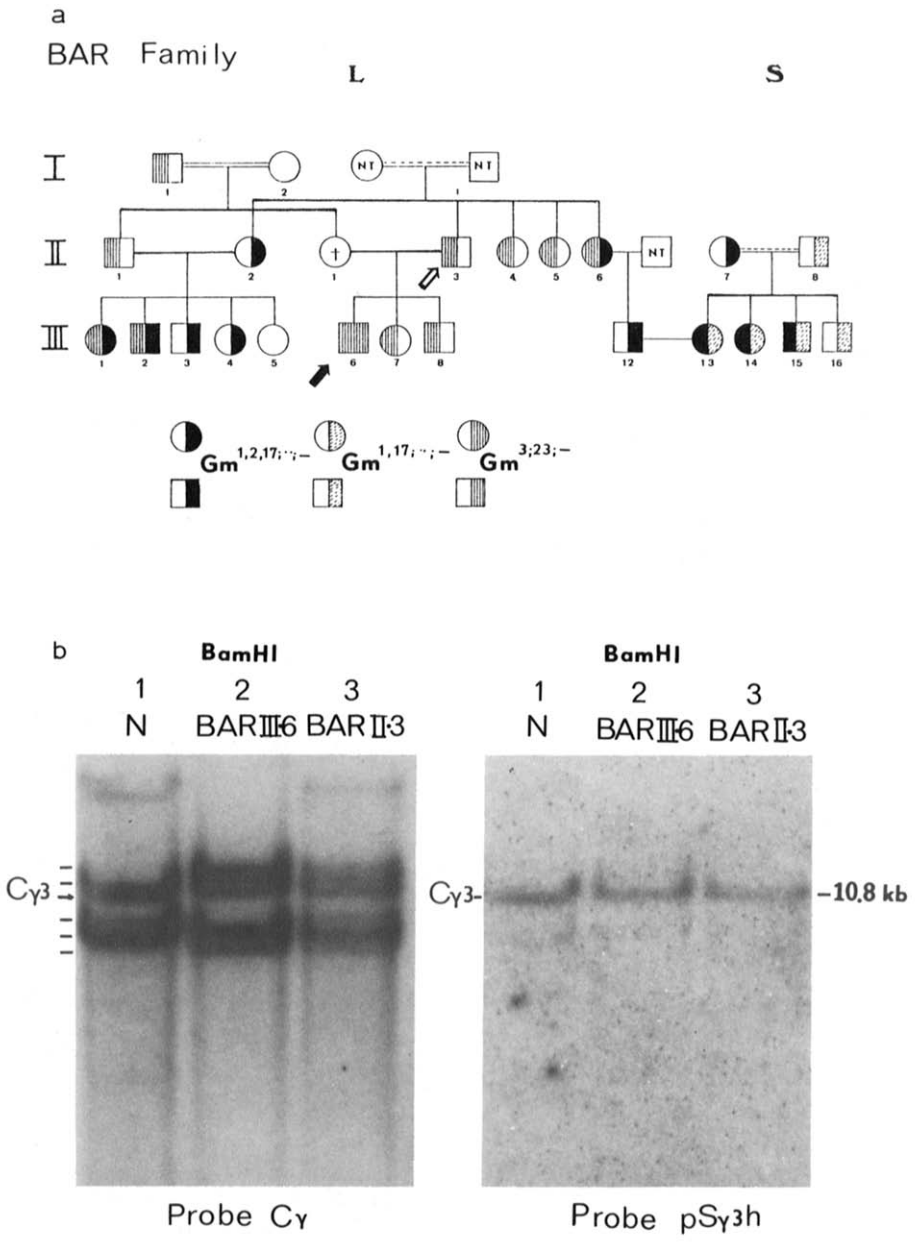


Fig.5. Southern hybridization of genomic DNA from an individual with a selective IgG3 deficiency. (a) Pedigree of the BAR family. The two Lebanese (L) and the Syrian (S) branches of the family are related. Seven individuals were shown to lack completely the IgG3 subclass (II-6, III-1, III-2, III-6, III-13, III-14 and III-15) [23,24]. Their genotypes are given in table 2. Arrows indicate the two individuals (III-6 and his father II-3) mentioned in the text. (b) Southern hybridization of the Cγ probe pBRH4.1 (panel 1) and of the γ3 hinge region probe (panel 2) to DNAs from two BAR family members, digested with *Bam*HI. Lanes: 1, a normal control DNA; 2, BAR III-6, individual homozygous for the haplotype $Gm^{3;23;-}A2m^{1}$ and lacking completely the IgG3 subclass [23,24]; 3, BAR II-3, heterozygous for the $Gm^{3;23;-}A2m^{1}$ haplotype [23,24]. Hybridization of the pSγ3h probe to BAR III-6 DNA shows that the selective absence of IgG3 is not due to a deletion of the Cγ3 gene.

Table 2

Genotypes of the members of the BAR family lacking completely the IgG3 subclass

Genotypes	No. individuals
$Gm^{3;23;-}A2m^{1}/Gm^{3;23;-}A2m^{1}$ $Gm^{f;n;-}A2m^{1}/Gm^{f;n;-}A2m^{1}$	1
$Gm^{1,2,17;..;-}A2m^{2}/Gm^{1,17;..;-}A2m^{2}$ $Gm^{z,a,x;..;-}A2m^{2}/Gm^{z,a;..;-}A2m^{2}$	3
$Gm^{1,2,17;..;-}A2m^{2}/Gm^{3;23;-}A2m^{1}$ $Gm^{z,a,x;..;-}A2m^{2}/Gm^{f;n;-}A2m^{1}$	3

Allotypic markers of γ1 (G1m), γ2 (G2m) and γ3 (G3m) heavy chains are indicated in both numerical and alphabetical nomenclatures. Only one allotype is defined on the γ2 chain and may or may not be present; 2 dots indicate the absence of this allotype. Nonetheless, the IgG2 subclass is present. The absence of G3m allotypes due to the absence of the IgG3 subclass is indicated by a dash (−)

homozygous for the haplotype $Gm^{3;23;-}A2m^{1}$ and from his father (II-3, fig.5a), heterozygous for this haplotype, were hybridized with the pSγ3h probe.

A 10.8 kb band representing the usual γ3 alleles was detected in both samples and with the same intensity (fig.5b). This result shows that the selective absence of IgG3 is due neither to a deletion of the Cγ3 gene nor to a major structural defect in the vicinity of the Cγ3 gene. Further analysis will be necessary to determine the molecular event responsible for the absence of IgG3; this defect is particularly intriguing since it is observed in three different genotypes (table 2) and only affects the γ3 genes.

4. CONCLUSION

In this paper, we showed that the pSγ3h clone, a specific human immunoglobulin γ3 subclass probe allowed us to identify with certainty the Cγ3 gene on Southern genomic blots, to describe a Cγ3 restriction fragment length polymorphism (EZZ γ3 RF) as well as to demonstrate the presence of the Cγ3 genes in a selective IgG3 deficiency. Unexpected and unusual sets of G3m allotypes have been described which reveal genetic events such as duplications, deletions, exchanges of CH exons, gene conversion [25–27]. Screening of genomic DNA libraries from individuals presenting either IgG3 deficiencies or such unusual Cγ3 genes and, consequently, the study of the Cγ gene evolution will be considerably facilitated by the use of such a Cγ3 specific probe.

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