Am. J. Hum. Genet. 48:745–756, 1991

Pulsed-Field Electrophoresis Screening for Immunoglobulin Heavy-Chain Constant-Region (IGHC) Multigene Deletions and Duplications

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Summary

Genome regions containing multiple copies of homologous genes, such as the immunoglobulin (Ig) heavy-chain constant-region (IGHC) locus, are often unstable and give rise to duplicated and deleted haplotypes. Analysis of such processes is fundamental to understanding the mechanisms of evolution of multigene families. In the IGHC region, a number of single and multiple gene deletions, derived from either unequal crossing-over or looping-out excision, have been described. To study these haplotypes at the population level, a simple and efficient method for preparing large numbers of DNA samples suitable for pulsed-field gel electrophoresis (PFGE) analysis was set up, and a sample of 110 blood donors was screened. Deletions were found to be frequent, as expected on the basis of previous serological surveys for homozygotes. Furthermore, a number of multigene duplications, never identified before, were detected. The total frequency of individuals bearing rearranged IGHC haplotypes was 10%. The genes involved in these deletions and duplications were assessed by densitometric analysis of standard Southern blots hybridized with several IGHC probes; two types of deletion and two types of duplication could thus be characterized. These data provide further evidence of the instability of the IGHC locus and demonstrate that unequal crossing-over is the most likely origin of rearranged IGHC haplotypes; they also suggest that such recombination events may be relatively frequent. Moreover, the simplicity and effectiveness of the large-scale PFGE screening approach will be of great help in the study of multigene families and of other loci involved in aberrant recombinations.

Introduction

Multigene families evolve through duplication and deletion, leading to the formation of complex genome regions composed of several linked genes with high levels of sequence homology. Such regions are often unstable and continuously give rise to new duplicated and deleted haplotypes (for discussion, see Hood et al. 1975; Maeda and Smithies 1986; Hunkapiller and Hood 1989). One typical example is the human immunoglobulin (Ig) heavy-chain constant-region gene locus (IGHC locus). This is composed of 11 linked genes, arranged in the order M–D–G3–G1–EP–A1–GP–G2–G4–E–A2 (Ravetch et al. 1981; Flanagan and Rabbitts 1982; Lefranc et al. 1982), on the subtelomeric region of the long arm of chromosome 14 (q32.3) (Kirsch et al. 1982).

The nine types of deletions and the one duplication characterized so far in this region involve from one to six genes (Lefranc et al. 1982, 1983*b;* Migone et al. 1984; Bech-Hansen and Cox 1986; Chaabani et al. 1986; Bottaro et al. 1989*a,* and in press; Hendriks et al. 1989; Smith et al. 1989). Both unequal crossing-over and looping-out—excision models have been proposed to explain their origin (Lefranc et al. 1982, 1983*b;* Migone et al. 1984; Carbonara et al. 1986).

Pulsed-field gel electrophoresis (PFGE) has been widely used for the study of large-scale genome rearrangements (e.g., see DenDunnen et al. 1987; Dun-

Received September 17, 1990; revision received November 26, 1990.

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0002-9297/91/4804-0014$02.00

ham et al. 1989*b*). PFGE analysis of IGHC deletions has been used to complete the physical map of the locus (Bottaro et al. 1989*c*). However, the usefulness of this technique is hindered by the complex procedure required to prepare DNA samples. Standard protocols (Poustka et al. 1987; Smith and Cantor 1987), in fact, enable only a small number of samples to be processed at one time. This has limited the application of PFGE to selected cases, even if in relatively large numbers (as in DenDunnen et al. 1987). To our knowledge, PFGE screening of large random samples has not yet been reported.

Here we describe a method allowing the preparation of PFGE DNA samples from as many as 25 individuals at one time, starting from 1-ml peripheral blood samples. Enough material to perform three or four digestions per individual is easily obtained. This method could be used to screen large normal population samples for rearrangements in any specific locus. Its use in the search for IGHC deletions and duplications in 110 subjects resulted in the identification of several unusual haplotypes. The most notable result was the detection of large, previously unobserved, multigene duplications. This finding supports the view that unequal crossing-over is the most likely mechanism for the rearrangement process.

Material and Methods

Preparation of PFGE DNA Samples

DNA samples were prepared from 110 healthy blood donors, starting from 10 ml peripheral blood collected in EDTA. One milliliter was used for PFGE DNA samples, and the rest was kept frozen for successive standard DNA extraction. The red blood cells were lysed in 5-ml Falcon tubes by adding 3 vol (3 ml) 115 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4 at 4°C, according to a method described by Baas et al. (1984). White blood cells were centrifuged (10 min at 160 *g*, 4°C), and, if necessary, a further lysis step was performed. Finally, the white blood cells were washed in PBS and were resuspended in 150 μl of the same buffer.

In most cases, the concentration of cells at this stage was 10–30 × 10^3/μl. DNA samples were prepared from this cell suspension by the method of Barlow and Lehrach (Poustka et al. 1987). In brief, equal volumes of cell suspension and 1% low-melting-temperature agarose in PBS were mixed, and three 80-μl blocks/subject were prepared in a plastic slot former. Blocks were allowed to solidify and were treated for 36 h at 50°C in 2-ml Eppendorf tubes with 0.5 ml of 0.5 M EDTA pH 9, 1% Sarkosyl, 2 mg proteinase K/ml (Boehringer Mannheim). They were then washed twice in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA), incubated in TE plus 40 μg phenylmethylsulfonylfluoride/ml for 30 min at 50°C and then again in TE (30 min at 50°C); finally, they were stored in 0.5 M EDTA pH 9 at 4°C.

The DNA concentration per block was about 4–12 μg/ml, as judged by intensity of ethidium bromide staining. We did not observe DNA concentration to have any disturbing effect on the electrophoresis run within this range, but more sensitive techniques (e.g., field-inversion gel electrophoresis) would probably require adjustment of the final cell concentration to a more constant level (e.g., 15 × 10^3 cells/μl). Blocks prepared in this way were successfully cut with different restriction endonucleases (*Mlu*I, *Bss*HII, *Ecl*XI, and *Sal*I). This method enables a single operator to process, without any difficulty, as many as 25 different samples at one time and to prepare more than 100 samples in 1 wk.

PFGE Screening

DNA samples were digested for 4–5 h with 15–30 units of the *Mlu*I or *Ecl*XI enzymes (Boehringer Mannheim) under the conditions indicated by the manufacturer and then were electrophoresed in a 1% agarose gel by using an LKB Pulsaphor apparatus with the hexagonal electrode array and lambda cI857Sam7 phage multimers as molecular-weight markers; run conditions were adjusted to achieve optimal resolution in the desired molecular-weight range. The gel was ethidium bromide stained, photographed, depurinated for 20 min in 0.25 M HCl, denatured twice for 10 min in 0.5 M NaOH, 1.5 M NaCl, and transferred onto Genescreen nylon filters (New England Nuclear) in the same alkali solution for 36 h. Filters were then prehybridized and hybridized according to a method described by Church and Gilbert (1984). The probe was the pHγ1 plasmid clone (a gift from T. Honjo), which is described in detail in the next following subsection.

Standard Southern Analysis

Eleven subjects with anomalous fragments at PFGE and 10 subjects carrying normal fragments were further analyzed by standard Southern blots. By standard phenol/choloroform technique, DNA was extracted

from the frozen blood samples left from PFGE DNA preparation (see above), and 8 μg DNA were digested with the restriction enzymes *Bam*HI, *Hin*dIII, *Pst*I, and *Sac*I (Boehringer Mannheim). Agarose-gel electrophoresis, Southern blotting onto nitrocellulose filters, and hybridization also followed standard protocols.

The following probes (schematically represented in fig. 1) were used to look for alterations in the IGHC region:

G probe, a 8.0-kb *Hin*dIII fragment containing the entire G1 gene and its 3′ region (clone pHγ1, a gift from T. Honjo, Kyoto University) (Takahashi et al. 1982). All five IGHG genes are able to cross-hybridize (Lefranc et al. 1982).

A probe, a 1.95-kb *Pst*I fragment containing the A2 gene (clone pHα2-1.95, a gift from N. Migone, University of Turin), subcloned from the phage clone Ch4A-H-Igα25 (also from T. Honjo) (Hisajima et al. 1983). Both IGHA genes are able to cross-hybridize (Lefranc et al. 1982).

AT probe, a 1.0-kb *Bam*HI/*Eco*RI fragment from the same phage, subcloned by N. Migone (clone pHα2-8), mapping 3.5 kb 3′ of A2, and recognizing the corresponding region 3′ of A1 (Bottaro et al. 1989*d*).

PG probe, a 0.8-kb *Bam*HI fragment located 1.5 kb 5′ of SG4, subcloned (clone pSPG4) from the Ch4A-H-Igγ4-2 phage (also from T. Honjo). It recognizes (*a*) regions located 5′ of the four functional IGHG genes and (*b*) a fifth region 5′ of IGHD (Bottaro et al. 1989*d;* authors' unpublished observations).

SG probe, a 3.8-kb *Hin*dIII fragment, subcloned (clone pSSG4) from the same phage. It contains the entire SG4 region and detects the S regions of the four functional IGHG genes (Bottaro et al. 1989*d*).

E probe, a 2.6-kb *Sac*I fragment containing the E gene and its 3′ flanking sequences, eluted from the λTOUεα clone (a gift from M. P. Lefranc, University of Languedoc, Montpellier) (Lefranc and Rabbitts 1984). It detects the two IGHE genes (IGHEP1 and IGHE) in the IGHC region and sequences 3′ of them, and it weakly cross-hybridizes with an E-like processed pseudogene (IGHEP2) on chromosome 9 (Battey et al. 1982).

SA probe, a 4.65-kb *Sac*I fragment, eluted from the same phage and containing the 5′ portion of the SA2 region. It also detects the SA1 and SM regions (Migone et al. 1983; Keyeux et al. 1989*b*).

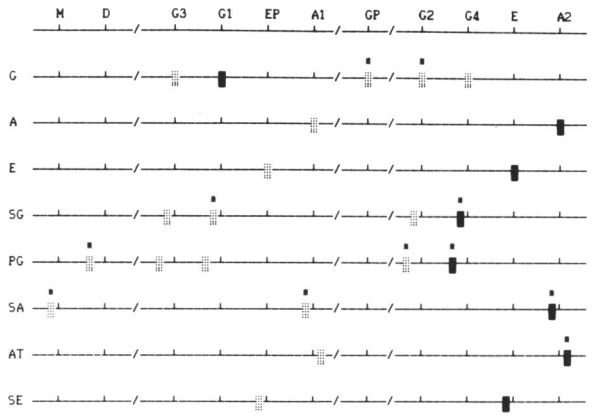


Figure 1 Schematic representation of probes used to characterize deleted and duplicated haplotypes in the present work. A black box (■) indicates the region from which the probe fragment was derived; dotted box (:::) indicates the cross-hybridizing regions; and an asterisk (*) indicates the restriction fragment polymorphism.

SE probe, a 0.97-kb *Hin*dIII fragment, subcloned (clone pSE-0.97-HH) from the phage Ch4A-H-Igε12 (also from T. Honjo) (Hisajima et al. 1983) and deriving from a region 5′ of the IGHE gene. It also detects the corresponding region 5′ of IGHEP1.

Filters were washed at high stringency and were exposed to Kodak XAR5 films for 15–72 h.

Densitometric Analysis

To quantify variations in Southern blot band intensities in subjects with unusual IGHC haplotypes, autoradiography films were analyzed by densitometry with the Kontron-AT-VIDAS/VIDEOPLAN image-analysis computer system, whose hardware/software configuration has been described in detail elsewhere (Putzolu et al. 1989). In brief, the transilluminated film image taken by a videocamera was converted into a digitalized picture of 512 × 512 pixels, with 256 grey levels/pixel, and densitometric profiles along autoradiography lanes were evaluated. The ratio between the intensities of pairs of bands in the same lane was calculated, and these values were then compared and normalized to corresponding values in normal controls. Thus, it was possible to quantify the relative increase or decrease in the intensity of specific bands. Several different control samples were run in various positions of each gel. Misinterpretations due either to different amounts of loaded DNA, to uneven transfer, or to uneven hybridization could be therefore generally ruled out.

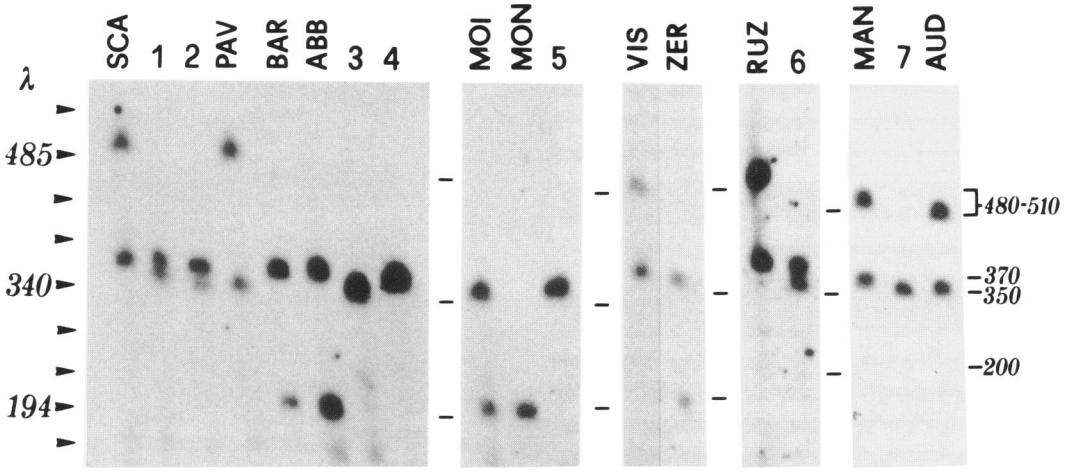


Figure 2 PFGE analysis of IGHC haplotypes (*Mlu*I enzyme, gel run 40 h at 170 V, 15°C, 45 s pulse time, filter hybridized with G probe). Two bands (350 and 370 kb) are commonly visible in the tested sample of 110 normal individuals, and subjects carrying either (e.g., lanes 3–5, and 7) or both (e.g., lanes 1, 2, and 6) bands were detected. Moreover, 11 subjects displaying very long or very short fragments were found; they are indicated by a three-letter code. Subjects VIS, SCA, PAV, MAN, AUD, and RUZ show a 350- or 370-kb band, together with a longer band of 480–510 kb; subjects MOI, ZER, BAR, and ABB show a 350/370-kb band, plus a shorter band of about 200 kb; last, subject MON only displays a 200-kb band. On the left, the positions of lambda phage multimers (monomer of 48.5 kb) are marked by arrowheads, and the lengths of three of them are indicated. The position of these three relevant markers is indicated on the left of each section of the figure, since the pictures were obtained from five different gels.

Results and Discussion

Hofker et al. (1989) have reported that digestion of normal human genomic DNA by the *Mlu*I enzyme yields a single 350-kb band containing the entire IGHC region. In our samples, two common bands were visible: one of 350 kb and one of 370 kb. Subjects displaying either or both bands were detected (fig. 2), suggesting that the two bands represent a polymorphism. This finding has been confirmed by using *Ecl*XI to digest DNA samples from subjects with various allele combinations. This enzyme is an isoschizomere of *Eag*I, which has been reported to cut the IGHC region into two fragments, one of 180 kb and one of 130 kb, containing the M–D–G3–G1–EP–A1 and the GP–G2–G4–E–A2 genes, respectively (Hofker et al. 1989). In subjects heterozygous for the *Mlu*I fragments of 350 and 370 kb, three *Ecl*XI fragments, one each of 180, 150, and 130 kb, could be identified. *Mlu*I 370-kb fragment homozygotes displayed only the 180- and 150-kb fragments, and 350-kb homozygotes displayed only the 180- and 130-kb fragments (fig. 3). Thus, a 20-kb insertion/deletion must be present in the GP–G2–G4–E–A2 region, yielding to the observed restriction-fragment differences when the *Mlu*I and *Ecl*XI enzymes are used.

None of 10 subjects displaying only the 350/370-kb bands showed abnormal intensities of IGHC gene bands in standard Southern blot analysis (not shown), suggesting that single gene deletions/duplications are not the primary cause of this polymorphism, as discussed in detail below.

Other subjects showed more dramatic changes of their restriction pattern, displaying either very long (480–510-kb) or very short (200-kb) fragments (fig. 2); one of them (subject MON) possessed only a 200-kb band, with no normal bands (fig. 2). From our previous experience of PFGE analysis of the IGHC region (Bottaro et al. 1989*c*), we deduced that subjects with bands of about 200 kb were probably carriers of IGHC multigene deletions; the longer bands had not been observed before.

The 11 subjects carrying unusual fragments were thus analyzed by standard Southern blots. Several probes were used to look for genomic alterations at 25 different regions in the IGHC locus (fig. 1)—in particular in the G3–A2 region, where all IGHC deletions so far described have been found. Some of the restriction patterns are shown in figure 4 and are discussed in the legend to that figure).

Densitometric scanning demonstrated that the

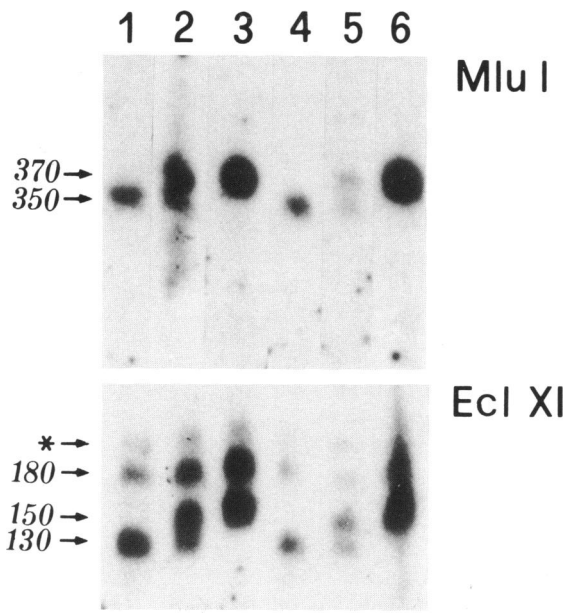


Figure 3 PFGE analysis of six subjects carrying different combinations of 350- and 370-kb bands (350-kb homozygotes in lanes 1 and 4, 350/370-kb heterozygotes in lanes 2 and 5, 370-kb homozygotes in lanes 3 and 6). These subjects do not correspond to subjects 1–6 shown in fig. 2. *Above, Mlu*I restriction patterns (run conditions are as in fig. 1). *Below,* Subjects' DNA digested with *Ecl*XI enzyme (gel run 40 h at 155 V, 15°C, 50 s pulse time), which cuts IGHC locus into two fragments, one of 180 kb and one of 130 kb, containing 5′ and 3′ portion (from the GP gene on) of region, respectively (Hofker et al. 1989). Fainter bands of higher molecular weight (indicated by an asterisk [*]) and probably derived from partial digestions were also observed in all subjects. Subjects homozygous for the *Mlu*I 370-kb band also displayed a longer, 150-kb *Ecl*XI band, instead of the 130-kb band which is the only one present in homozygotes for the *Mlu*I 350-kb allele; 350/370-kb heterozygotes show both 130-150-kb bands. Therefore, the 20-kb difference in the bands obtained with the two enzymes must be due to an insertion/deletion of DNA sequences in the region 3′ of the GP gene.

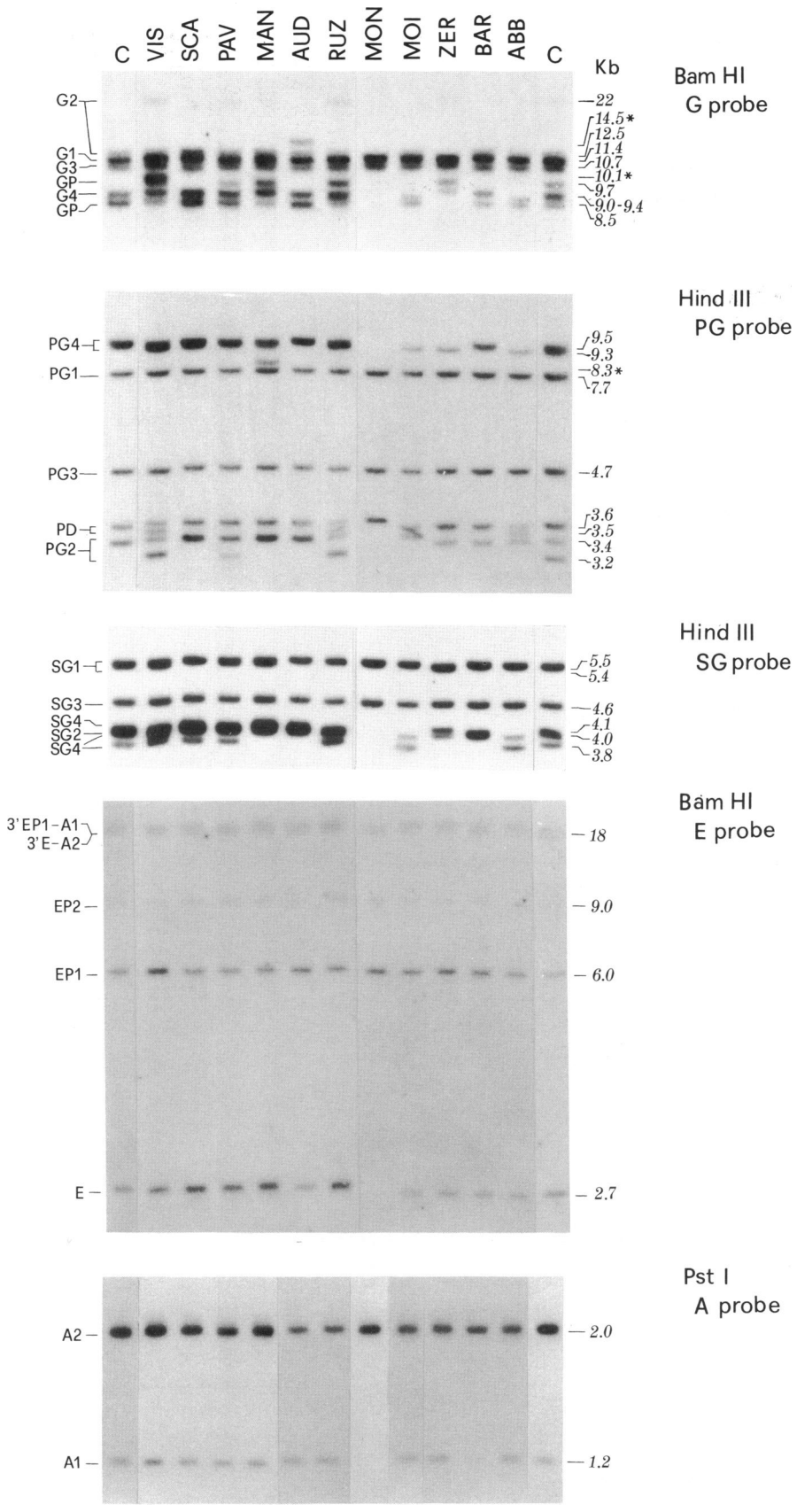
200-kb bands actually corresponded to multigene deletions. Subject MON (single 200-kb band) showed no trace of bands related to the A1, GP, G2, G4, and E genes (fig. 4), indicating that he is a carrier of a homozygous deletion of these genes. Evaluation of band intensity in subjects heterozygous for the 350/370- and 200-kb bands (table 1) led to the reconstruction of two deleted haplotypes. The first (subject BAR) involved the A1–GP–G2–G4–E region (as in the homozygous subjects described above), and the other (subjects MOI, ZER, and ABB) spanned the GP–G2–G4–E–A2 genes; both haplotypes already had been found in the Italian population (Migone et al. 1984; Bottaro et al. 1989*a*).

More interesting were the subjects showing bands of about 500 kb. All of them displayed a higher than normal intensity of specific bands (fig. 4). Densitometry helped to define the abnormal haplotypes (table 1). Two duplicated haplotypes were found; the first was the exact counterpart of one of the deletions discussed above and spanned A1–GP–G2–G4–E (subjects SCA, PAV, MAN, and RUZ), and the other included EP1–A1–GP–G2–G4 (subjects VIS and AUD). A deletion corresponding to the second duplication has also been found in an Italian subject (i.e., FRO) (Migone et al. 1984). In one case, family PFGE analysis could be performed and assessed segregation of the duplicated haplotype along three generations (fig. 5).

The finding that duplications of the same genes could yield fragments of different length (i.e., 480–510 kb; see fig. 2) is not surprising, since these latter could result from the presence in the duplicated haplotype of one or two copies of the 20-kb inserted/deleted segment originating the two common (350- and 370-kb) alleles described above. A complete list of the deleted and duplicated haplotypes found so far is shown in table 2.

Multigene duplications of the same regions commonly involved in deletions strongly suggest that the mechanism originating these haplotypes is unequal crossing-over, since the looping-out-excision model cannot account for gene duplications. Such unequal crossovers take place between homologous regions of the locus but do not usually fall within the switch (S) regions (Keyeux et al. 1989*a;* Smith and Hammarström 1989; Bottaro et al., in press; authors' unpublished observations). Two of the cases reported here showed unusual IGHSA1 restriction fragments (fig. 6); however, S regions have been shown to undergo expansion and contraction (Migone et al. 1983; Bottaro et al. 1989*d;* Keyeux et al. 1989*b*), and a multiplicity of alleles has been found, especially for the SA1 region (Migone et al. 1983; Keyeux et al. 1989*b*). Thus, it is hard to claim that these aberrant S fragments, as well as those discussed in the legend to figure 4, represent the junction fragments for the duplications/deletions. Extensive molecular analyses will be needed to verify this hypothesis.

Other inferences can also be drawn from the analysis of many polymorphic loci. Even in the absence of familial analysis, the allele set associated with each deleted/duplicated haplotype can be tentatively re-


C
VIS
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MAN
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G3
GP
G4
GP
22
14.5*
12.5
11.4
10.7
10.1*
9.7
9.0-9.4
8.5
Hind III
PG probe
PG4
PG1
PG3
PD
PG2
9.5
9.3
8.3*
7.7
4.7
3.6
3.5
3.4
3.2
Hind III
SG probe
SG1
SG3
SG4
SG2
SG4
5.5
5.4
4.6
4.1
4.0
3.8
Bam HI
E probe
3'EP1-A1
3'E-A2
EP2
EP1
E
18
9.0
6.0
2.7
Pst I
A probe
A2
A1
2.0
1.2

Table 1

Densitometric Analysis of Deleted/Duplicated Haplotypes

Subject	Relative Band Intensity of IGHC Regions[a]										
	PD	PG3	PG1	EP	A1	GP	PG2	PG4	G4	E	A2
VIS	1	.87[b]	.81[b]	1.45	1.90	1.46	1.29[b]	1.33[b]	1.46[c]	1	1
SCA	1	.78[b]	.87[b]	1	1.27	1.32	1.30[b]	1.33[b]	1.31	1.22	1
PAV	1	.75[b]	.88[b]	1	1.54	1.50	1.40[b]	1.35[b]	1.30	1.74	1
MAN	1	.80[b]	.81[b]	1	1.44	1.78	1.37[b]	1.30[b,c]	1.30	1.41	1
AUD	1	.92	1.10	1.36	1.32	1.28	1.56	1.40	1.74[c]	1	1
RUZ	1	.98	1.15	1	1.32	1.64	1.73	1.56	1.44	1.45	1
MON	1	1.03	1.07	1	...	...	...	...	...	...	1
MOI	1	1.04	1.18	1	1	.52	.64	.39	.52	.47	.60
ZER	1	.86	1.12	1	1	.62	.58	.39	.50	.61	.56
BAR	1	.97	1.04	1	.59	.48	.57	.67	.66	.58	1
ABB	1	1.02	1.02	1	1	.44	.57	.37	.44	.62	.56

[a] Values calculated as described in Material and Methods. Thus, values around 1 indicate a normal gene dosage, values around 1.5 indicate a 50% increase in dosage (i.e., presence of three copies of the gene), and values around 0.5 indicate a 50% decrease in dosage (i.e., heterozygous deletion). Values most likely corresponding to deleted/duplicated regions are underlined. G2 values could not be correctly estimated because of both the low intensity of the *Bam*HI 22-kb band and the close proximity of the 12.5-kb band to the G3 and G1 bands. For the same reason, PG3 and PG1 values are shown in place of G3 and G1, whose *Bam*HI bands could not be readily separated. The PD region, which is very unlikely to be involved in deletions and duplications (Carbonara et al. 1986), has been taken as reference for the PG1 and PG3 bands. The A1/A2 and EP/E genes served as reference to each other.

[b] Lower intensities most likely reflect a transfer or hybridization artifact.

[c] Obtained by considering the intensity of restriction fragments of unusual length (see fig. 4), which may represent (except in the case of MAN) the junction fragments of the unequal recombinations.

constructed. The two subjects with EP1–A1–GP–G2–G4 duplications (subjects VIS and AUD), for instance, clearly display different sets of alleles: VIS has a double-intense PG2 3.2-kb band and a normal 3.4-kb band (and his duplicated haplotype must therefore contain at least one 3.2-kb allele), whereas AUD shows three 3.4-kb alleles; similarly, RUZ displays three SA1 alleles (6.4, 6.6, and 7.1 kb), whereas the duplicated haplotype in SCA must contain two 7.1-kb alleles. The same reasoning can be applied for other haplotypes, and the results (table 3) show that similar deletions and duplications are often associated with different alleles. At least three different A1–GP–G2–G4–E duplications (subjects MAN, SCA, and RUZ), two EP1–A1–GP–G2–G4 duplications (subjects VIS and AUD), and two GP–G2–G4–E–A2 deletions (one in subject ZER and one in both subject ABB and subject MOI) can be distinguished. These findings suggest that haplotypes with deletions or duplications involving the same set of genes may have originated independently; however, the alternative explanation—i.e., that successive crossovers occurred between normal and rearranged haplotypes—cannot be ruled out until the breakpoints of the different haplotypes are compared.

No gene duplication or deletion could be found as-

Figure 4 Southern analysis of IGHC region in subjects who displayed unusual *Mlu*I fragments at PFGE. VIS, SCA, PAV, MAN, AUD, and RUZ showed 480–510-kb *Mlu*I fragments; and subjects MON, MOI, ZER, BAR, and ABB showed 200-kb fragments. C = Normal controls. On the right, the restriction enzymes and the probes used are indicated; fragment lengths are in kb. The assignment of fragments to specific IGHC regions is shown on the left. Differences, in relative band intensity, between lanes are clearly visible. They may occasionally reflect artifacts, which are due either to uneven transfer or hybridization or perhaps to gene conversion events between the sequences recognized by the probes (which usually show a high degree of cross-hybridization; see Material and Methods); but in the cases of subjects bearing unusual *Mlu*I fragments they may be due to differences in the number of IGHC genes. Therefore, the relative intensities of fragments have been evaluated by computer densitometry (results shown in table 1). Fragments not commonly found in normal Italian controls (Bottaro et al. 1989*d*)—and which could not therefore be assigned to specific regions—are indicated by asterisks (*) near their length value; they may represent the products of the unequal recombination events originating deleted and duplicated haplotypes, but they could also be rare normal alleles. This is probably the case for the 8.3-kb PG fragment in subject MAN, whose duplication (see table 1 for densitometric analysis) cannot have involved any of these regions. The pattern in subject MON is clearly interpretable, since he does not show any fragment relative to the A1, GP, G2, G4, and E genes.

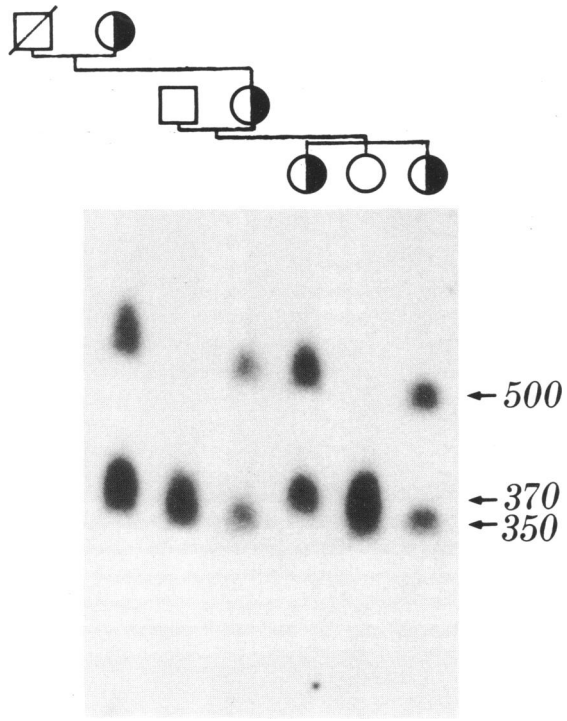

Figure 5 Segregation of duplicated haplotype (yielding *Mlu*I fragment of 500 kb) in MAN family. PFGE was run as in fig. 1. Above is the family tree, where subjects showing the duplicated fragment are indicated by half-black circles (◑). Standard Southern blot analysis (not shown) confirms that the duplicated haplotype segregated unchanged in the three generations.

sociated with the 350/370-kb bands. Indeed, no specific pattern alteration was observed in the 10 subjects bearing the 350–370-kb bands (not shown), and in those with deletions and duplications the patterns were independent of the length of the normal allele. Single gene deletions and duplications (Bech-Hansen and Cox 1986; Bottaro et al. 1989*a*, and in press; Smith et al. 1989), whose size range would be about 20 kb, have, however, been observed, although in Italy they are not as frequent as multigene deletions (Bottaro et al. 1989*b*). The 350–370-kb polymorphism could have masked their presence; thus, the frequency of rearranged IGHC haplotypes in the Italian population is probably even higher than that shown above.

Further studies will be needed to detect both the exact location of the site of the 20-kb insertion and the nature of the inserted/deleted segment. As it has been claimed for the long-range length differences in the HLA complex (Dunham et al. 1989*a*, and references therein), this insertion/deletion site might influence the localization and the frequency of recombinations in this region. It is interesting to note that both the frequency of recombination and the linkage disequilibrium values are not evenly distributed in the IGHC region, suggesting the presence of recombination hot spots (Bech-Hansen et al. 1983; Migone et al. 1985; Benger and Cox 1989; Bottaro et al. 1989*d*).

Table 2

IGHC Deletions and Duplications

Gene(s)	Subject(s)	Type of Rearrangement	Reference(s)
G1–EP–A1–GP–G2–G4	TAK and TOU	Deletion	Lefranc et al. 1982, 1983*b*
EP–A1–GP	TOU	Deletion	Lefranc et al. 1983*b*
EP–A1–GP–G2–G4	FRO	Deletion	Migone et al. 1984
	T17	Deletion	Chaabani et al. 1986
	VIS and AUD	Duplication	Present report
A1–GP–G2–G4–E	FRO and SAF	Deletion	Migone et al. 1984
	DEM, MOD, TIM, and SPA	Deletion	Bottaro et al. 1989*a*, and in press
	MON and BAR	Deletion	Present report
	SCA, PAV, MAN, and RUZ	Duplication	Present report
G2		Duplication	Bech-Hansen and Cox 1986
	MOD and TIM	Deletion	Bottaro et al. 1989*a*
GP–G2–G4–E–A2 ...	CRU	Deletion	Bottaro et al. 1989*a*, Hendriks et al. 1989
	MOI, ZER, and ABB	Deletion	Present report
G1	Ny	Deletion	Smith et al. 1989
G1–EP–A1–GP–G2	Ny	Deletion	Smith et al. 1989
G4	SPA	Deletion	Bottaro et al., in press

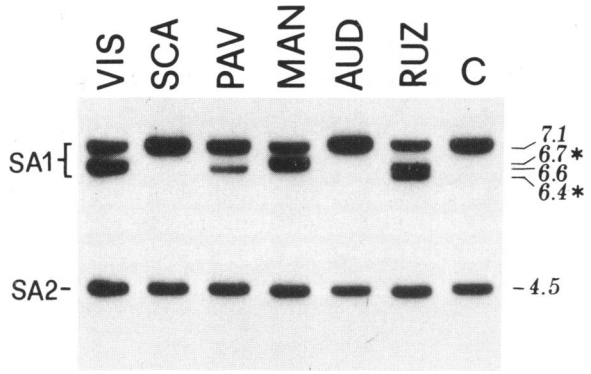

Figure 6 Southern blot analysis of SA regions in subjects with IGHC duplications (*SacI* enzyme, SA probe). Fragments of abnormal length, indicated by asterisks (*), were found in subjects MAN and RUZ. The possible involvement of switch regions in the deletion/duplication mechanism is discussed in the text.

Conclusive Remarks

In the present paper, the structure of the IGHC locus in the population has been investigated by means of PFGE screening. It has been shown that deleted/duplicated haplotypes reach a surprisingly high frequency, so that true genetic polymorphism can be claimed for the number of IGHC genes. This finding raises questions about the functional significance of both the complex organization of the human IGHC locus and the isotype differentiation.

The 2.7% (6/220) frequency of deleted haplotypes found in the present survey is not much different from the 1.8% calculated on the basis of the frequency of Ig-deficient homozygotes when Hardy-Weinberg equilibrium is assumed (Bottaro et al. 1989*b*), a fact suggesting a selective neutrality for IGHC deletions. Homozygotes for multigene deletions have been shown to usually compensate their immune deficiency both qualitatively (using other Ig isotypes for the same function, e.g., the subclass-restricted response to particular antigens) and quantitatively (increasing the concentration of the residual isotypes) (Lefranc et al. 1983*a*; Hammarström et al. 1987). It would be of interest to test Ig production in subjects with multigene duplications, to assess a possible gene position effect on the specificity and effectiveness of the isotype switch mechanism. This, of course, would be more easily performed on IGHC duplication homozygotes, which have not yet been identified.

Regarding the recombination mechanisms, the structure of the recombinant haplotypes indicates that a common process—namely, unequal crossing-over between homologous regions of the locus—must be the origin of both deletions and duplications. It is not clear whether preferential crossing-over sites exist within the locus, but there is increasing evidence of recombination hot spots in the IGHC region. Detailed molecular analysis of the breakpoints of several types of deletions alone can shed light on the underlying molecular mechanisms. Furthermore, the high frequency, the diversity, and the heterogeneity of deletions and duplications argue against the hypothesis that their common occurrence in the Italian population is only due to genetic drift and inbreeding. However, inbreeding has been invoked to explain the com-

Table 3

Heterogeneity of Alleles Associated with IGHC Deletions and Duplications

Haplotype and Subject(s)	Polymorphic Locus[a] for			
	SG1	SA1	PG2	SG4
EP1–A1–GP–G4 duplication:				
VIS		6.6/6.6/7.1	3.2/3.2/3.4	3.8/3.8/4.0
AUD		7.1/7.1/7.1	3.4/3.4/3.4	4.0/4.0/4.0
A1–GP–G2–G4–E duplication:				
MAN		6.6/6.7/7.1	3.4/3.4/3.4	4.0/4.0/4.0
SCA		7.1/7.1/7.1	3.4/3.4/3.4	3.8/4.0/4.0
RUZ		6.4/6.6/7.1	3.2/3.2/3.4	3.8/3.8/4.0
GP–G2–G4–E–A2 deletion:				
ZER	5.4/5.4			
ABB and MOI	5.5/5.5			

[a] Only relevant loci are shown. The possible allele combinations on the duplicated haplotypes exclude the possibility that either VIS and AUD or MAN, SCA, and RUZ carry the same forms of duplication. The same is evident for the deletion of ZER compared with those of ABB and MOI.

mon finding of deletion homozygotes in the Tunisian population (Lefranc et al. 1983*b*); extensive population analyses similar to those applied here may test this hypothesis.

Nevertheless, we favor the idea that the abundance of IGHC deletions and duplications is due to a relatively high frequency of unequal recombination events. Also, this hypothesis could be confirmed or ruled out by testing the frequency and types of deletions in other populations; recent data (Hendriks et al. 1989; Smith et al. 1989) suggest that northern European populations (Dutch and Swedish) also display IGHC-deleted haplotypes.

Finally, we have also shown that PFGE screening of relatively large population samples is feasible and effective. Thanks to the ability of PFGE to identify deletions in heterozygotes, several deleted haplotypes could be observed in as few as 110 individuals, whereas their detection by serological screening for Ig isotype–deficient homozygotes, as described by Bottaro et al. (1989*b*), would require a sample of more than 10,000 subjects. Moreover, PFGE clearly identifies duplicated haplotypes. These can be phenotypically detected only on very rare occasions, e.g., when unusual family segregation of serological markers is observed (van Loghem et al. 1980). Direct screening on standard Southern blots by densitometric evaluation is not likely to yield clear-cut results, and only the concordance of results from PFGE and Southern analysis is able to shed light on doubtful cases.

This method can be applied for any locus in which long-range genomic rearrangements are known or suspected to exist. For instance, screenings could be performed for deletions/duplications of the dystrophin gene, tumor-suppressor genes, factor VIII and IX genes, and a number of multigene families (α- and β-globins, HLA, color vision genes, and so on). Testing each subject with three restriction enzymes and testing each filter with a number of probes should allow a detailed analysis of complex rearrangements. The identification and characterization of increasing numbers of rearrangements may be expected to provide new insights into the molecular mechanisms of recombination.

Acknowledgments

We are grateful to Dr. T. Honjo for the pHγ1, Ch4A-Igα 25, Ch4A-H-Igγ 4-2, and Ch4A-H-Igε12 genomic clones, to Dr. M. P. Lefranc for the λTOUεα clone, and to Dr. N. Migone for the pHα2-1.95 and pHα2-8 clones and critical reading of the manuscript. We are also indebted to Drs. J.T. Den Dunnen and G. J. B. van Ommen, for suggestions and help with technical problems and for the hospitality in their lab in Leiden, and to Drs. P. Putzolu, M. Rabino, and A. Fasolo, for invaluable support in densitometric analysis. We also wish to thank Dr. M. G. Guerra of the Banca del Sangue di Torino, for help in blood donor collection, and C. Boccazzi, for skillful technical assistance. This work was supported by grants MPI 60% Torino and Siena and CNR Progetto Finalizzato "Ingegneria genetica—Malattie ereditarie."

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