DIRECT OBSERVATION OF THE GENE ORGANIZATION OF THE COMPLEMENT C4 AND 21-HYDROXYLASE LOCI BY PULSED FIELD GEL ELECTROPHORESIS

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The class III region of the human MHC between HLA-B and HLA-DR includes genes encoding the complement components C2, Factor B, C4A and C4B, and the cytochrome P450 steroid 21-hydroxylase (21-OHase) (1–3). The C2 and Factor B genes are separated by 421 bp (4) and lie 30 kb from the C4A locus, which in turn is ~10 kb from the C4B locus (5). One of the two copies of the 21-OHase gene, 21-OHase A and 21-OHase B, lies 3 kb downstream of each C4 gene (6). Recently, a gene that has been called RD, due to the presence of an unusual periodic structure, has been mapped between the factor B and C4A genes (7).

Although the two C4 isotypes, C4A and C4B, are highly homologous and differ by <1% in their derived amino acid sequence, C4 is exceptionally polymorphic (8). The sequences of four C4A and five C4B cDNA and genomic clones have established the pattern of polymorphism in the C4d fragment of the α chain of C4 (9) and have provided a structural basis for the observed functional (10, 11) and serological differences (12, 13) between the isotypes. There is also heterogeneity in C4 gene size (9, 14, 15). All C4A genes studied at locus I are 22 kb in size (long C4 gene). C4B genes, however, may be either 22 or 16 kb (short C4 gene), due to the presence or absence of a 6–7-kb intron ~2.5 kb from the 5' end of the gene. In addition, variation in the number of copies of C4 genes present on individual chromosomes has been observed. Duplication of the C4B locus resulting in the presence of three C4 genes has been observed on the extended haplotype B14 C4A2 C4B1,2 C2C BIS DR1, and the gene frequency has been estimated at 1–2% (16–18). Null alleles at either locus are much more common and gene frequencies of 5–15% for C4AQ0 alleles and 10–20% for C4BQ0 alleles have been estimated (19–21). Investigation of the molecular basis of null alleles is particularly important as they occur with increased frequency in some HLA-associated diseases, such as SLE (22–24). It has been estimated that about half the null alleles are due to deletion of the C4 gene together with the flanking 21-OHase gene (14).
A number of RFLPs are available to follow the differences at the human \( C4 \) loci in the population (see reference 25 for summary, and 26, 27). In particular, the \( Taq \) I polymorphism at the 5' end of the genes and the \( Nla \) IV and \( EcoO \) 109 polymorphic patterns can give information about the nature and characteristics of the two \( C4 \) loci. These include whether the 6-7-kb intron is present, whether they express the \( C4A \) or \( C4B \) isotypes, and whether they would express the \( Rg1 \) or \( Ch1 \) antigenic determinants. However, it is not easy to deduce the gene copy number as it is necessary to compare the intensities of the bands revealed by autoradiography and comparison between different samples can be difficult. Second, family studies are necessary to imply which genes lie on the same chromosome.

We have recently constructed a physical linkage map of the human MHC (2) using the powerful technique of pulsed field gel electrophoresis (PFGE)\(^1\), which allows the separation of large DNA fragments (28, 29). These studies have been extended to compare the DNA organization of different HLA haplotypes using PFGE and homozygous typing lymphoblastoid cell lines. By utilizing large DNA fragment RFLPs, we have been able to observe directly the variations in size of the \( C4 \) loci in different haplotypes. Specifically, the size of the diagnostic \( BstH \) II or \( Sac \) II restriction fragments observed with a \( C4 \) - or \( 21 \)-OHase-specific DNA probe indicates both the number of \( C4 \) genes present on a chromosome and their size (\( C4 \) long or short). This technique can be applied to PBMC DNA, and together with the previously established RFLPs (14, 25), allows a complete definition of the \( C4 \) gene organization of an individual.

**Materials and Methods**

**Preparation of DNA.** The HLA and complement types of the cell lines studied are listed in Table I. The method for preparing high molecular weight DNA in agarose blocks from tissue culture cells has been described previously (2), except that cells were finally resuspended in 1% agarose at \( 2 \times 10^7 \) cells/ml.

Whole blood was isolated from healthy individuals and PBMC were obtained by separation on Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) according to the protocol supplied by the manufacturer. The cells were washed three times in PBS containing 1 mM EDTA before counting and were finally resuspended at \( 4 \times 10^7 \) cells/ml in PBS. DNA in agarose blocks was then prepared as previously described (2).

**Restriction Enzyme Digestion and PFGE Analysis.** Agarose blocks containing \( \sim 5 \mu \)g DNA were treated before digestion as previously described (2). Restriction enzyme digestions were carried out as specified in reference 2, except that for \( BstH \) II (New England Biolabs, Beverly, MA), 10-15 U were used in a 3-h incubation, for \( Sac \) II (Northumbria Biologicals Ltd., Cramlington, Northumberland, UK), 40 U of enzyme were used for a 3-h incubation, and for \( Taq \) I (Bethesda Research Laboratories, Gaithersburg, MD), 50 U were used in an overnight incubation at 55°C. After digestion, DNA in agarose blocks was loaded directly into PFGE gels without proteinase K treatment.

The PFGE gel box was constructed in our departmental workshop according to the design of Southern et al. (30). Digested samples were subjected to electrophoresis on 1.5% agarose (type I; Sigma Chemical Co.) gels in 20 mM Tris-acetate, 1 mM EDTA, pH 8.5, at 150 V for 30 h at a 30-s switching interval or for 36 h at a 7.5-s switching interval. Markers were intact yeast chromosomes in agarose blocks (31), concatemers of \( \lambda \) c1857S7 DNA prepared in agarose blocks (32), or \( \lambda \) DNA digested with \( Hind \) III (Amersham International, Amersham, UK). Gels were stained, transferred onto Genescreen plus (DuPont Co., Stevenage, UK) membranes, and hybridized with radiolabeled DNA probes as previously described (2).

\(^1\) Abbreviation used in this paper: PFGE, pulsed field gel electrophoresis.
Taq I-restricted DNA in agarose blocks was subjected to electrophoresis on conventional 0.7% agarose submarine gels, transferred to nylon-backed nitrocellulose membranes (Hybond C extra; Amersham International), and hybridized using standard procedures (33).

Southern blots were stripped of probe and rehybridized as described by Dunham et al. (2).

**Probe.** The human C4 5' cDNA probe, PA, is a 476-bp BamH 1/Kpn I restriction fragment from the full-length C4 cDNA clone, pATF (34). The human 21-OHase probe is a 1.2-kb Cla I/PvuII II genomic DNA restriction fragment isolated from a cloned 21-OHase B gene (35). The other probes were a 1.6-kb Hind III/BamH I genomic DNA fragment located ~6 kb 5' to the C2 gene (probe K) and a 1.7-kb BamH I genomic DNA fragment lying 10 kb 5' of the C4 gene, which were both derived from cloned cosmid DNA inserts as described by Dunham et al. (2). The positions of probes relative to the complement loci are shown in Fig. 4.

All probes were labeled with α-[32P]dCTP (Amersham International) by random hexanucleotide priming (36).

**Results**

**Detection of Deletions at the C4 Loci.** To observe differences between the DNA organization in several common HLA haplotypes, high molecular weight DNA in agarose blocks was prepared from the HLA-homozygous lymphoblastoid cell lines whose HLA types are detailed in Table I. The DNA was digested with restriction enzymes that cut infrequently in the mammalian genome (see reference 31 for review) and were known to give DNA fragments of an informative size around the complement gene cluster in the HLA class III region (2). DNA from each of the cell lines digested with a single restriction enzyme was then separated by PFGE at a switching interval so as to optimally resolve the DNA fragment size of interest. The DNA was depurinated, transferred to nylon membranes, and then hybridized with genomic and cDNA probes specific for the complement gene cluster.

It is important to note that the amount of DNA loaded on a PFGE gel affects the migration of the DNA relative both to other genomic DNA samples and to the DNA size markers (37, I. Dunham, unpublished observations) and that DNA fragment sizing can be inconsistent between different gel runs and switching intervals (38). These problems can be overcome by loading equivalent amounts of DNA for each sample and by comparing the sizes of restriction fragments for different cell lines on the same gel. We also possess a number of internal size markers for our prototype cell line 1, since the exact size of certain genomic DNA fragments produced with specific enzyme and probe combinations in the class III region has been determined by comparison with the cloned cosmid DNA (Sargent, C. A., I. Dunham, and R. D. Campbell, manuscript submitted for publication).

It is also interesting to note that the restriction fragment sizes observed with the PFGE apparatus used here were generally ~10% smaller than our previously published data using the orthogonal field alternation gel electrophoresis apparatus and were found to match more accurately with the sizes predicted from the cloned DNA.

The results of hybridization of Mlu I-digested DNA from each of the cell lines with the 21-OHase probe are shown in Fig. 1 and Table I. In cell lines 1-4, a 200-kb Mlu I fragment was seen, in cell lines 5 and 8, a 225-kb Mlu I fragment was seen, while cell lines 6 and 7 gave a 230-kb Mlu I fragment. Thus, there appears to be a 25-30-kb difference in the size of the Mlu I fragment that encompasses the complement genes between cell lines 1-4 and 5-8. Analysis of the cell line DNAs for the Taq I polymorphism (14, 25) on conventional agarose gels using the probe at the 5' end of the C4 cDNA, PA, and the 21-OHase probe suggests the basis for the
### Table I

**Restriction Fragment Sizes (in Kilobases) Observed in Cell Line DNA**

| Cell line | HLA type   | Mlu I 210H/PA | 210H/BamH I | 1.7-kb BamH I | Probe K | Sac II* 210H/PA | 1.7-kb BamH I | Probe K | Tag I PA | 210H | C4 Genotype     |
|-----------|------------|---------------|--------------|---------------|---------|-----------------|---------------|---------|---------|------|----------------|
| 1         | B7 BfS C4A3 C4BQ0 DR2 | 200 80 | 12 | 55 40 | 12 | 55 (25) | 7.0 3.7 | Long locus I |
| 2         | B8 BfS C4AQ0 C4B1 DR3 | 200 70 | 12 | 55 33 | 12 | 55 (25) | 6.4 3.7 | Short locus I/II recombinant |
| 3         | B8 BfS C4AQ0 C4B1 DR3 | 200 70 | 12 | 55 33 | 12 | 55 (25) | 6.4 3.7 | Short locus I/II recombinant |
| 4         | B18 Bf1 C4A3 C4BQ0 DR3 | 200 80 | 12 | 55 40 | 12 | 55 (25) | 7.0 3.7 | Long locus I |
| 5         | B18 BfS C4A4 C4B2 DR2 | 225 105 | 12 | 55 65 | 12 | 55 (25) | 7.0 3.7 | Long locus I + short locus II |
| 6         | B44 BfS C4A3 C4BQ0 DR4 | 230 115 | 12 | 55 70 | 12 | 55 (25) | 7.0 3.7 | Long locus I + long locus II |
| 7         | B35 BfS C4A3 C4B1 DR5 | 230 115 | 12 | 55 70 | 12 | 55 (25) | 7.0 3.7 | Long locus I + long locus II |
| 8         | B55 BfS C4A4 C4B5 DR6 | 225 105 | 12 | 55 65 | 12 | 55 (25) | 7.0 3.7 | Long locus I + short locus II |

* The Sac II fragment of 25 kb (in parentheses) detected by probe K is due to partial digestion.
Figure 1. Mlu I restriction fragment patterns of haplotypes with one or two C4 loci. (a) The EtBr-stained PFGE gel showing separation of Mlu I-digested cell line DNA at a 30-s switching interval. The markers are yeast chromosomes (Y) and λ concatemers (λ). (b) The autoradiograph of the Southern blot after hybridization with the 21-OHase probe showing the presence of a single Mlu I fragment of either 200, 225, or 230 kb, dependent upon the number of C4 genes present and their size (i.e., long or short). Numbers above tracks refer to the cell lines described in Table I.
differences in size observed using Mlu I (Fig. 2 and Table I). Cell lines 1 and 4 had a single 7.0-kb Taq I fragment with the PA probe and a 3.7-kb fragment with the 21-OHase probe. Thus, these cell lines have a single long C4 gene (which is C4A from the complotyping) and a single 21-OHase B gene on each haplotype. The Taq I results for cell lines 2 and 3 indicate that they possess a single short C4 gene and a 21-OHase B gene. However, cell lines 5 and 8 gave two Taq I fragments of 7 and 5.4 kb with PA, and 3.2- and 3.7-kb fragments with the 21-OHase probe. This suggests that cell lines 5 and 8 have one long C4 gene, one short C4 gene, and a copy of both 21-OHase A and 21-OHase B genes. Cell lines 6 and 7 had Taq I fragments characteristic of two long C4 genes, and a copy of each 21-OHase gene. Since the size of the DNA segment containing single C4 and 21-OHase genes is ~25 kb when the C4 gene is short and ~30 kb when there is a long C4 gene, the observed difference in size of the Mlu I restriction fragment appears to be due to the possession of two C4 genes in cell lines 5-8, one of which is deleted in cell lines 1-4. Indeed, the Mlu I fragment in cell lines 6 and 7 was ~5 kb larger than for cell lines 5 and 8. This corresponds to the presence of the 6-7-kb intron at the second C4 locus in cell lines 6 and 7, but not in cell lines 5 and 8, allowing for the limits of the size resolution of this PFGE gel. However, the equivalent difference due to the presence or absence of this intron could not be detected between cell lines 1 and 4 and 2 and 3. Hybridization of other probes to the same Southern blot of Mlu I-digested genomic DNA (results not shown) suggested that the mobility of the DNA fragments for cell lines 2 and 3 was slightly retarded in the PFGE gel, and hence, the size difference due to the 6-7-kb intron has been obscured.

Pattern of the BssH II and Sac II Polymorphisms. To confirm these observations, high molecular weight DNA from each of the cell lines was digested with BssH II (Fig. 3 and Table I) and Sac II (Table I). The digested DNA was separated by PFGE at
a switching interval of 7.5 s to obtain optimal resolution in the 10–200-kb size range. Fig. 3 shows the results of hybridization of probes from the complement gene cluster of a Southern blot of BssH II-digested DNA. The positions of the probes are indicated in Fig. 4. Hybridization of probe K (1.6-kb Hind III/BamH I fragment from a region telomeric of the C2 gene) to the BssH II-digested cell line DNA revealed a common 55-kb fragment for all the cell lines. The size of this fragment is different from what had been observed previously (2), where only a larger 150-kb fragment was observed due to partial digestion.

Hybridization of the same blot with a 1.7-kb BamH I fragment lying ~10 kb upstream of the C4A gene identified a 12-kb BssH II fragment also in all the cell lines. Hence, there appears to be no difference between these cell lines in ~67 kb of DNA containing the C2 and factor B genes. Hybridization of these probes to Sac II-digested DNA confirmed this result (Table I). However, when either the 21-OHase or Pα probes were hybridized to the Southern blot of BssH II-digested DNA, the sizes of the fragments observed were different between the cell lines depending on the organization of the C4 loci. Cell lines 2 and 3, which possess a single short C4B gene and a 21-OHase B gene, gave a 70-kb BssH II fragment with the Pα probe. For cell lines 1 and 4, which instead have a long C4A gene, the Pα probe hybridized to an 80-kb BssH II fragment. Thus, by using PFGE at this resolution, the difference in size of the C4 genes due to the 6–7-kb intron can be readily detected. Similarly, in the cell lines with one long and one short C4 gene (cell lines 5 and 8), the BssH II fragment was ~10 kb shorter than in those with two long C4 genes (cell lines 6 and 7). In addition, the difference between the BssH II fragment sizes for haplotypes with one C4 gene or two C4 genes corresponded to the size of a C4-21-OHase unit that is deleted or present. Therefore, the size of the BssH II fragment that hybridizes to the Pα or 21-OHase probes is directly related to the amount of DNA that is present at the C4 and 21-OHase loci.

It is also apparent that the size differences between these haplotypes that were observed in the Mlu I fragment can be completely accounted for by the difference in the size of the BssH II fragment that contains the C4 genes. In these haplotypes, at least, it seems that there are no other changes in the amount of DNA present in the region surrounding the C4 genes as defined by the Mlu I fragment. The results using BssH II were completely reflected using Sac II (Table I). With Pα or the 21-OHase probe, a smaller fragment with Sac II was observed (70 kb for two long C4 genes) than with BssH II, but the differences in size of the Sac II fragments between haplotypes were again fully accounted for by the number and type of C4 genes present. Therefore, the extent of the differences between these cell lines due to deletion of C4 and 21-OHase genes is completely defined to be within the limit of the Sac II fragment that hybridizes to the Pα or 21-OHase probes.

The region of genomic DNA contained in the 200-kb Mlu I fragment that hybridizes with the 21-OHase probe has been cloned in a series of overlapping cosmid clones from a cosmid library prepared using DNA from cell line 1 (2; Sargent, C. A., I. Dunham, and R. D. Campbell, manuscript submitted for publication). These cosmid clones were mapped for rare cutting restriction enzymes and the data are presented in Fig. 4. The restriction enzyme sites that are cleaved in the genomic DNA of cell line 1 were identified using a series of probes derived from the cloned cosmid inserts (Sargent, C. A., I. Dunham, and R. D. Campbell, manuscript submitted for publi-
Figure 3. BamHI restriction fragment patterns of haplotypes with differences in the number and size of the C4 loci present. (a) The EtBr-stained PFGE gel showing separation of BamHI-digested cell line DNA at a 7.5-s switching interval. (b–d) The same filter hybridized with the \( P_\lambda \) probe, the 1.7-kb BamHI fragment, and probe K (see Fig. 4 for position of probes), respectively. Numbers above each track refer to the cell lines described in Table 1. Markers are concatemers of \( \lambda \) DNA (\( \lambda \)) and a HindIII digest of \( \lambda \) DNA (M).
cation) and these data are also shown in Fig. 4. It is obvious that some sites that are present in the cell line DNA are not cleaved by the restriction enzymes in genomic DNA. This is presumably because the sites are methylated in the genomic DNA as both BssH II and Sac II are methylation sensitive (39). Taking this information together with the Mlu I, BssH II, Sac II, and Taq I data presented above, it is possible to construct genomic restriction maps for the cell lines analyzed (Fig. 4), depicting the differences in size and number of the C4 and 21-OHase genes present. It is not possible to say whether sites that are not restricted in cell line 1 genomic DNA are present in the other cell lines.

Analysis of the C4/21-OHase Genes in PBMC DNA. The results presented above suggested that it might be possible to apply the BssH II and Sac II RFLPs to the analysis of the C4 loci of different individuals. To this end, high molecular weight DNA from five healthy C4-typed and, in three cases, HLA-typed individuals was prepared in agarose blocks from PBMC. One possible pitfall of this approach might be that the pattern of methylation of restriction enzyme sites in PBMC is different from that observed for the EBV-transformed lymphoblastoid cell lines. The DNAs were digested with Taq I, BssH II, and Sac II and separated on conventional 0.7% agarose gels or PFGE gels as appropriate. Southern blots of the separated, digested
DNAs were hybridized with the PA and 21-OHase probes. The results are shown in Fig. 5 (Taq I) and Fig. 6 (BssH II and Sac II) and are summarized in Table II.

Individual A had a single BssH II fragment of 115 kb identified by hybridization with probe PA, migrating in the same position as the BssH II fragment for cell line 7, which was also subjected to electrophoresis on this gel as a standard. This fragment is characteristic of a chromosome possessing two long C4 genes and the information from the Taq I digest confirms the presence of long C4 genes at locus I and locus II (Fig. 5) and one copy of each of the 21-OHase A and B genes.

Individual B possesses a 70-kb BssH II fragment characteristic of a short C4 gene. This corresponds in size to the 70-kb fragment of cell line 2, which was also subjected to electrophoresis on this gel as a standard. In addition, this individual has a novel 135-kb BssH II fragment. Examination of the Taq I restriction enzyme data for this individual showed the presence of a 6.4-kb Taq I fragment, which is due to a short C4 gene on the SCOI compleotype (14, 15, 25). This Taq I fragment corresponds to the presence of the 70-kb BssH II fragment, which also goes with this compleotype. In addition, comparison of the band intensities in the Taq I digest hybridized with PA for individual B shows the presence of a single long C4 gene (7.0-kb Taq I fragment) and two short C4 genes (5.4-kb Taq I fragment). Since only one C4 gene lies on the 70-kb BssH II fragment, it can be deduced that the 135-kb BssH II fragment contains one long C4 gene and two short C4 genes. The size of the fragment is consistent with this.

Individual C was essentially the same as individual A with respect to the polymorphic fragments seen with BssH II, Sac II, and Taq I and, therefore, has the same C4 gene organization (Table II).

Individual D has a different combination of BssH II fragments revealed with probe PA. The 105-kb fragment contains one long C4 gene and one short C4 gene on one chromosome, while the 135-kb fragment again represents a chromosome with three C4 genes, one long and two short. Examination of the results from the Taq I digest

**Figure 5.** Taq I restriction patterns showing the nature of the C4 and 21-OHase loci in the five unrelated individuals summarized in Table II.
FIGURE 6. (a) BssH II and (b) Sac II patterns showing the number and type of C4 loci present in five unrelated individuals. Numbers above each track refer to the cell lines described in Table I, while letters refer to the individuals described in Table II. Markers are concatemers of λ DNA.

| Individual | HLA/complement type | Taq I | BssH II | Sac II | C4 Genotype |
|------------|---------------------|-------|---------|---------|-------------|
| A          | B7 C4A3 C4B1 DR5    | 7.0   | 3.7     | 115     | 70          | 2 x long locus I + long locus II |
|            | B44 C4A3 C4BQ0 DR6  | 6.0   | 3.2     | 115     | 70          | locus II |
| B          | B14 C4A2 C4B1,2 DR13| 7.0   | 3.7     | 135     | 90          | 1 x long locus I + short locus II + short locus III |
|            |                      |       | 6.4     | 3.2     |             | 1 x short locus I/II recombinant |
| B          | B8 C4AQ0 C4B1 DR3   | 2 x 5.4 | 70     | 33      |             | 1 x short locus I/II recombinant |
| C          | B45 C4A3 C4BQ0 DR11 | 7.0   | 3.7     | 115     | 70          | 2 x long locus I + long locus II |
|            | B18 C4A3 C4B1 DR4   | 6.0   | 3.2     | 115     | 70          | locus II |
| D          | C4A3, A4; C4B1, B2  | 2 x 7.0| 2 x 3.7 | 135     | 90          | 1 x long locus I + short locus II + short locus III |
|            |                      | 3 x 5.4| 3 x 3.2 | 105     | 65          | 1 x long locus I + short locus II |
| E          | C4A3, AQ0; C4B1, B1 | 7.0   | 2 x 3.7 | 115     | 70          | 1 x long locus I + long locus II |
|            |                      | 6.4   | 3.2     | 70      | 33          | 1 x short locus I/II recombinant |

### Table II

Restriction Fragment Sizes (in Kilobases) Observed in PBMC DNA
confirm that this is the case. With PA, the band intensities suggested the presence of two long C4 genes (7.0-kb Taq I fragment) and three copies of the short C4 genes (5.4-kb Taq I fragment). Similarly there are two 21-OHase B genes and three 21-OHase A genes. This case clearly demonstrates that since the size of the BssHII fragment is altered with the gene copy number present in an individual rather than the band intensity, it is easy to identify the number of C4 genes present. In addition, the size of the BssHII fragment is also diagnostic of the C4 gene organization on an individual chromosome.

Individual E has BssHII fragments consistent with two long C4 genes on one chromosome and one short C4 gene on the other SCOI haplotype, and this is confirmed by the Taq I digests. In all five cases the Sac II fragment sizes were entirely consistent with the organization of the C4 loci suggested by the BssHII and Taq I results (Fig. 6b, Table II).

In Fig. 6a it can also be seen that in addition to the series of strongly hybridizing bands revealed with PA, a number of less intense BssHII fragments were observed. These additional bands are probably the result of partial digestion of BssHII sites, perhaps due to partial methylation at these sites or to differences in the methylation pattern of different cell types in the PBMC population. In particular, it appears that the BssHII sites that lie in the 3' end of the 21-OHase genes (Fig. 4b) are being partially digested. This phenomenon does not affect the information obtained from the autoradiographs because the informative fragments are still the major products. Therefore, it appears that the BssHII and Sac II polymorphisms can be applied to DNA isolated from PBMC and are diagnostic of the organization of the C4 loci on each chromosome.

Discussion

Susceptibility to a number of diseases has been shown to be associated with the possession of certain HLA haplotypes (24, 40). Having established a physical map of the HLA region (2), it was of interest to know whether there are extensive differences in the organization of the DNA in different haplotypes (deletions, insertions, or RFLPs). The existence of deletions at the C4 and 21-OHase loci has been documented (14, 41), but because of the size of the C4 genes, analysis of the extent of the deletions has required cosmid cloning studies. We set out to define whether other changes in the DNA organization exist around the complement genes in the HLA region and whether the documented differences at the C4 and 21-OHase loci could be observed in uncloned DNA.

Using PFGE, large DNA fragment RFLPs for the enzymes BssHII, Mlu I, and Sac II have been observed at the C4 loci in the HLA class III region. The sizes of the fragments hybridizing with the PA or 21-OHase probes is directly related to the number and length of the C4 genes present in the DNA samples analyzed (see Tables I and II). These RFLPs, in particular, those with BssHII and Sac II, can be used to directly observe the C4 gene organization on both chromosomes using high molecular weight DNA isolated from the whole blood of an individual. In combination with the previously described Taq I polymorphisms (14, 15, 25), these RFLPs can give a complete picture of the C4 gene organization, and by implication, the 21-OHase gene organization for an individual without the need for family studies or DNA cloning.
In addition, it has been demonstrated that in the cell lines studied, representing at least seven different haplotypes (cell lines 2 and 3 carry the same haplotype although they are derived from different sources), the only alterations in the DNA content at the ~5-kb resolution of these PFGE gels are the deletions/duplications associated with the C4 and 21-OHase loci. The extent of the differences found in the Mlu I fragment between the haplotypes studied are confined to the Sac II fragment, which contains the C4 and 21-OHase genes. This Sac II fragment starts ~10 kb 5' to the first C4 gene and ends ~8 kb from the 3' end of the last 21-OHase gene (Fig. 4). No other differences in the DNA content of the haplotypes studied over the extent of the Mlu I fragment that contains all the HLA class III complement genes were detected using PFGE.

We believe that the RFLPs described here will be invaluable in screening individuals for their C4 and 21-OHase gene organization. It is already known that C4 null alleles are associated with certain HLA-associated diseases (22–24), and this method in combination with the other available RFLPs should give a rapid screening procedure for splitting deleted from nondeleted null alleles. It is also interesting to note that of the five individuals that were analyzed (Table II), there were two chromosomes with deleted C4AQ0 alleles and two chromosomes with nondeleted C4BQ0 null alleles. In addition, there were two examples of chromosomes with three C4 genes present, both having one long C4 gene and two short C4 genes. Although there have been previous reports of three C4 genes on one chromosome (14, 18), there is little data as yet as to how frequently these duplications occur in the population. Using the RFLPs described here, it should be possible to assess the frequency of these deletions and duplications directly.

Both BssH II and Sac II give the same information as to the organization of the C4 loci. The fragments produced with Sac II are 40–45 kb smaller than those observed with BssH II using the PA or 21-OHase probes, and this may make it easier to resolve size differences between different gene organizations. Also, since there are no other Sac II sites within the observed fragments, at least from our observations of cell line 1, no smaller partial fragments are produced. However, in our experience, BssH II has given more consistent results and gives complete digestion with less units of enzyme, whereas Sac II can give very partial digestion.

Finally, it may be possible to make use of these RFLPs along with isotype and Rg/Ch-specific oligonucleotides to give a complete C4 genotype for an individual with a single PFGE gel.

Summary

Pulsed field gel electrophoresis and enzymes that cut genomic DNA infrequently have been used to define large RFLPs at the human C4 loci. With the enzymes BssH II or Sac II, and C4 or 21-hydroxylase DNA probes, it has been possible to observe directly the number of C4 genes present on a haplotype, and also whether the C4 genes are long (6–7-kb intron present) or short (6–7-kb intron absent). Haplotypes that have either two long C4 genes or one long and one short C4 gene generate BssH II fragments of ~115 or ~105 kb, respectively. Haplotypes that have either a single long or a single short C4 gene generate BssH II fragments of ~80 or ~70 kb, respectively. This technique has been used to analyze the DNA isolated from PBMC and allows
the complete definition of the C4 gene organization of an individual without the need for family studies.

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