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Genetics

Cloning of the HLA class II region in yeast artificial chromosomes

(major histocompatibility complex/HLA-DR molecules/class III/large-scale cloning/human genome)

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ABSTRACT **Yeast artificial chromosomes (YACs) have been applied to clone the entire class II region of the human major histocompatibility complex (MHC), including its flanking regions, in a contig over 1.5 million base pairs (bp) long. The human DNA inserts in the YACs have a size between 60 and 1300 kbp and were isolated from two *Eco*RI partial digest libraries. The gaps between *DRA* and *DRB*, *DRB* and *DQA*, and *DOB* and *DPA*, which had not been cloned by other means, have been bridged with YAC clones. The contig extends through the 400 kbp of DNA between the *DRA* and *C4* genes, thus linking the class II region with the complement gene cluster in the class III region. The cloning in YACs has been supported by a conventional cosmid walk of 290 kbp in the *C4–DRA* region. Restriction enzyme sites in the YAC clones were compared to the sites in the cosmid walk, to published cosmid clones, and to the already existing physical maps, leading to a detailed characterization of a region of the human genome over 1500 kbp. The YAC clones will be valuable for functional analysis of the MHC.**

The human major histocompatibility complex (MHC) is situated on the short arm of chromosome 6 in the 6p21.3 band. It contains more than 50 genes in about 4 million base pairs (bp) of DNA (1). The organization of the genes has been determined by a combination of techniques including recombination analysis, cosmid walking, and pulsed-field gel electrophoresis (PFGE) (2–5). The telomeric end of the MHC contains the class I genes (6) and the centromeric end the class II genes (7). The class I and class II gene products play a central role in immune responses by presenting antigens to T lymphocytes. The class III region, between class I and class II, contains a large number of genes encoding proteins of diverse functions such as the complement components C2 and C4 (8), steroid 21-hydroxylase (9), tumor necrosis factors α and β, and the heat shock protein HSP70 (3, 10, 11).

The class II region is divided into the DR, DQ, and DP subregions, each of which contains at least one *A* and one *B* gene, encoding one α and one β class II protein chain, respectively. The organization of the class II genes has been analyzed in detail by PFGE, leading to the construction of physical maps (12–15). Differences in the maps between individual haplotypes have been revealed (16, 17), in particular in the segment of DNA between the *DQA* and *DRA* genes (14). Differences have also been observed in the class III region due to the number and size of *C4* genes present (18, 19). Cosmids have been isolated from each of the class II subregions, but large uncloned gaps remain. In the DR subregion different groups have isolated cosmids containing *DRA* or *DRB* genes, but it was not possible to link all *DRB* genes with each other (20) or to *DRA* (21–23). The DQ subregion has been cloned (24), but it was not linked to DR or to the *DNA* gene (25). The *DNA* gene has not been linked to the DP subregion by cosmid walking (25). Similarly, the 400 kilobase pair (kbp) region between class II and the complement gene cluster in the class III region has not been linked by this method.

There are impelling reasons to clone the whole of the class II region. Many diseases—for example, insulin-dependent diabetes mellitus and IgA deficiency—are associated with certain class II alleles, particularly in the highly polymorphic DR and DQ subregions (26–29). The explanation for this is not yet clear, but it is possible that so-far-undiscovered genes play a role in some of these diseases. Novel non-class II genes, including *RING4*, have been discovered already in the *DNA–DOB* interval, and from physical maps there is room for more genes in this region (30). In addition, loci influencing the normal expression and antigen presentation of HLA class I and class II genes may map within the class II region, or between the class II region and the *C4* genes in the class III region (30–32).

To clone and characterize the complete class II region we screened two yeast artificial chromosome (YAC) libraries constructed by partial *Eco*RI digestion of genomic DNA with probes from the entire region. In parallel we initiated cosmid walks in the *DRA* to *C4* gene interval.

MATERIALS AND METHODS

Cell Lines and YAC Libraries. The 4X YAC library (average insert size 620 kbp, 10,000 clones) was constructed by using partially *Eco*RI-digested DNA from the cell line GM 1416B (Human Genetic Mutant Cell Repository, Camden, NJ) (Zoia Larin, A.M., and Hans Lehrach, unpublished results). The HLA type was determined as A1,A3; B7,B8; Cw7; DR3,DRw8,DRw52; DQw2,DQw4. The 4Y YAC library (average insert size 130 kbp, 15,000 clones) was constructed from partial *Eco*RI digests of 4Y cell line (OXEN) DNA (A.M., unpublished results).

Isolation of Positive YAC Clones. Primary filters of YAC libraries were screened by hybridization as described (33) and secondary screening of positive clones was performed as follows: The candidate clones were spread on plates lacking uracil and tryptophan and grown for 2–3 days. Colony lifts were taken on Hybond-N^+ filters (Amersham). The filters were placed colony side up on Whatman 3MM paper soaked in 1 M sorbitol/0.1 M sodium citrate, pH 5.8/10 mM EDTA/10 mM dithiothreitol and Lyticase (Sigma) at 2 units/ml and incubated for 4–12 hr at 37°C. Filters were prepared for hybridization as described in ref. 33. DNA from positive clones was prepared in agarose inserts from yeast cultures in double selection media as described (33).

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Abbreviations: YAC, yeast artificial chromosome; MHC, major histocompatibility complex; PFGE, pulsed-field gel electrophoresis; RFLP, restriction fragment length polymorphism.

Cosmid Libraries. The cosmid libraries used were in the lorist 3 vector obtained from P. Little (34) or in the pDVCOS or lorist 6 vectors as described in ref. 10.

PFGE Analysis. The Rotaphor type IV apparatus (Biometra, Kent, U.K.) was used and conditions were as described in the figure legends.

Southern Analysis. Preparation of YAC DNA and the probes for YAC vector analysis are described in ref. 35. The probe for the identification of human DNA fragments was a cloned 800-bp *Xba* I fragment (1E12) containing *Alu* and LINE repeats (J.R., unpublished results). In some experiments total human DNA was used as a probe. Probes from the HLA region were C4 and C2 (8), 17M (10), and I6 and WPF (43). FCH is a 1.5-kbp *Cla* I/*Hin*dIII fragment from cosF62, WPH is a 1.8-kbp *Hin*dIII fragment from cosG91, Hb is a 2-kbp *Hin*dIII genomic fragment derived from cosmid LE1, and LEE3 is a 1-kbp *Eco*RI fragment from the same cosmid. DRH2 is the probe for the *DRA* cDNA (20). The *DRB* cDNA (20) was used along with pRTV-I, -II, and -III (36), the *DQB* and *DQA* probes were described in ref. 3, the *RING4* probe was described in ref. 30, and *RING3* is another gene near a CpG island in the *DNA–DOB* interval (I. Hanson, personal communication), the probes for *DPA* and *DPB* are described in ref. 15; 33X1 is 87 kbp centromeric of *DPB2* and 25X1 is a 3-kbp *Xho* I genomic fragment from cosmid cosHcol.11 located 60 kbp centromeric of *DPB2* (I. Hanson, personal communication). All probes were labeled with ^{32}P according to ref. 37.

PCR Analysis. The *Alu* primers 517 and 559 (38) were used along with primer IV (39) under conditions described in the corresponding publications. The vector primers have been described elsewhere (40). For vector to *Alu* PCR the primers specific for the *ura*$^-$ and *trp*$^-$ arm were mixed in 2:1 ratio with the 517, 559, or IV primers, respectively, and amplified under conditions described earlier for 517 and 559 (38) and for primer IV (39).

The PCR fragments were separated in 1.5–2.0% agarose gels blotted onto Hybond-N$^+$ membranes by alkaline blotting and hybridized to oligo-primer-labeled PCR products under competition conditions (41). In the case of the vector to *Alu* amplifications the vector-containing fragment was identified by hybridization to labeled pYAC4, isolated in low-melting-point agarose, and reamplified under the same conditions. A single fragment obtained thus was digested with *Eco*RI. Two fragments were visible after digestion and electrophoresis, one containing vector and the other one insert. The latter was used as the end probe of the particular YAC.

RESULTS AND DISCUSSION

The Class II–Class III Interval. We first tried to clone the class II–class III region by conventional cosmid walking. This region has a length of 390–430 kbp, depending on the haplotype (19). Starting from cosmid pAKR4705 containing the *DRA* gene (23), we accomplished two walking steps, isolating cosmids LEI and LHI (see Fig. 5). A further step was not achieved even though we screened three different cosmid libraries with an end probe from LHI. At the class III end, 16 overlapping cosmids were isolated covering 200 kbp centromeric of the *C4* gene. Similar to the experience walking from *DRA*, it was not possible to isolate additional clones further towards the class II region, and the two walks were not linked.

Since the cosmids failed to link the two regions, YAC clones were isolated, using probes derived from them. For example, YAC 313, with a length of 420 kbp, was isolated using the LEE3 probe. It was positive with all probes derived from the cosmid walks between the *DRA* and *C4* genes. Although the YAC was positive with *C4* it included neither 17M, which lies within 10 kbp telomeric of C4A, nor Bf and C2 (Fig. 1). It did not include the *DRA* gene. Therefore this YAC covered the

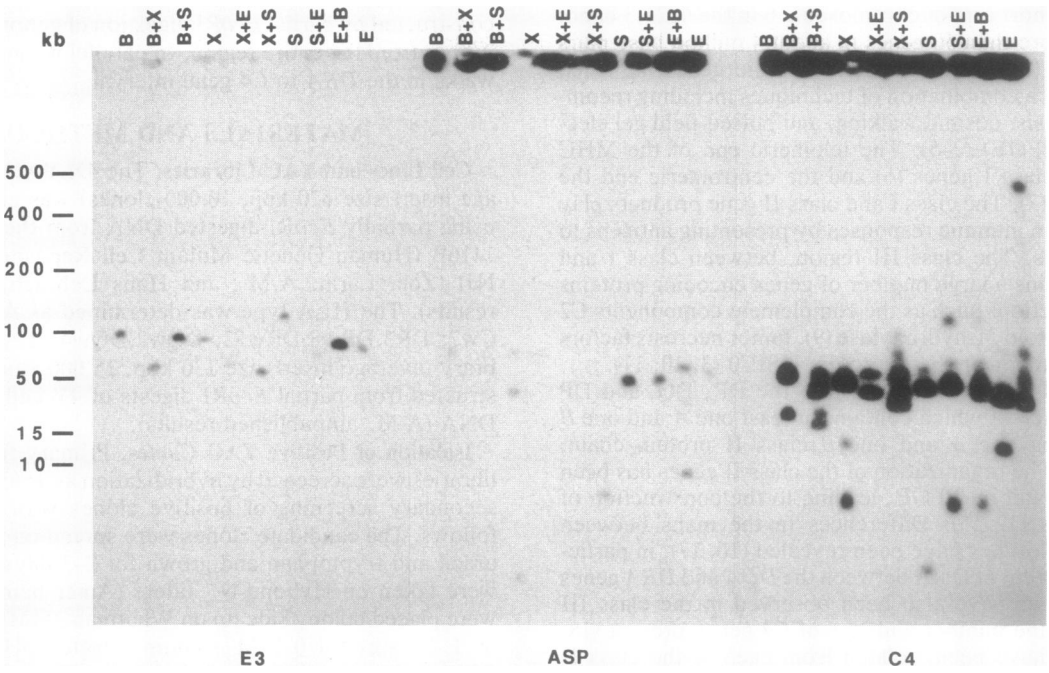

FIG. 1. YAC 313 spans the class II–class III region. DNA from YAC 313 was digested with *Bss*HII (B), *Sal* I (S), *Xho* I (X), and *Eag* I (E). The DNA was separated in the Rotaphor under the following conditions: 210 V, 30- to 3-sec pulse (linear decrease), field angle 120°–95° (linear decrease) for 16 hr at 12°C. After electrophoresis the DNA was treated and blotted onto Hybond N$^+$ membranes. The hybridization buffer was 50% (vol/vol) deionized formamide/50 mM sodium phosphate, pH 7.2/1 mM EDTA/10× Denhardt's solution/4× standard saline/citrate (SSC)/1% SDS/10% dextran sulfate containing sonicated salmon sperm DNA at 50 μg/ml for 16 hr at 42°C. The filters were washed for 5 min in 2× SSC/0.1% SDS at room temperature, 20 min in 2× SSC/0.1% SDS at 65°C, and twice for 20 min in 0.1× SSC/0.1% SDS at 65°C. Kodak X-AR films were exposed for 3–6 hr. The blot was hybridized with the probes E3 (located 35 kbp telomeric of *DRA*), Asp (WPF) (located 155 kbp centromeric of the steroid 21-hydroxylase gene), and C4. Marker sizes in kilobases (kb) are given on the left. All the probes identified fragments in the YAC 313, the sizes of which were consistent with those derived from the cosmid walks.

approximately 400 kbp between class II and the complement gene cluster (see Fig. 5). The *DRA* probe was used to isolate another class II–class III clone, YAC 15.1, with an insert of 400 kbp, extending from 20 kbp centromeric of *DRA* to 380 kbp telomeric of this gene (see Fig. 5).

Cloning of the Class II Region in YACs. Further clones were isolated to cover the whole of the class II region and to link it to the YAC clones described above. A 1300-kbp clone, YAC 16.2, included part of the complement region (*C4*) and extended over the YACs 313 and 15.1 towards the centromere. It included the *DRA* and *DRB* genes, in a 650-kbp *Mlu* I fragment, as well as the *DQA1* and *DQB1* genes, in a 180-kbp *Mlu* I fragment (Fig. 2). Since the parent cell line was heterozygous, it was important to determine which haplotype the YACs were derived from, because different haplotypes contain a different number of *DRB* genes (42). This was achieved by Southern blotting and analysis of *Taq* I restriction fragment length polymorphisms (RFLPs). Thus, YAC 16.2 was clearly derived from the *DR8* haplotype and contained only one full *DRB* gene (Fig. 3), confirming previous results (42). In contrast, YAC 11.2 (450 kbp), was clearly from the *DR3* haplotype (Fig. 3). However, this YAC covered only one complete *DRB* gene from the *DR3* haplotype. It also contained the DQ subregion, *DOB*, and the *RING4* and *RING3* genes up to the 3′ end of the *DNA* gene (data not shown, Fig. 5). Two further YACs, 12.1 and 13.1, overlapping with 11.2, extended 20 kbp farther centromeric. From Southern blotting data, these clones contained only the 5′ end of a single *DRB* gene (Figs. 3 and 4). They were also positive with *DQA*, *DQB* (not shown), *DOB*, *RING4*, and *RING3*. Clones 12.1 and 13.2 were both 450 kbp and may have resulted from the duplication of one initial colony. These two YACs overlapped with YAC Z6, which had a length of 60 kbp and contained the *DNA* gene but not *RING3*. YAC Z6 extended 45 kbp from the *DNA* gene towards *DPA1*,

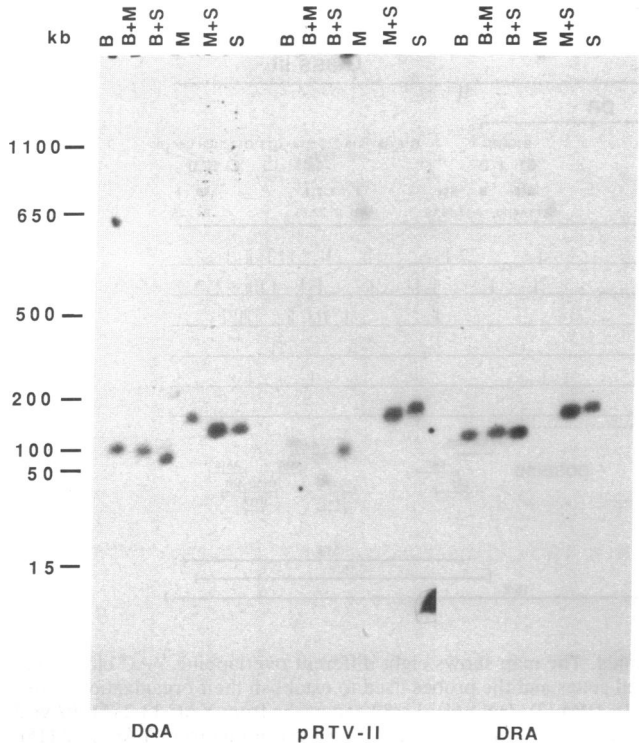

FIG. 2. YAC 16.2 links the *DRA* gene to *DQA*. DNA from YAC 16.2 digested with *Bss*HII (B), *Mlu* I (M), and *Sal* I (S) was separated under the following conditions: 200 V, 60- to 20-sec pulse linear, 110°–95° angle linear for 16 hr at 12°C. Blotting and hybridization conditions were as in Fig. 1. The probes *DQA*, pRTV-II (specific for *DRB* signal sequence and β1 domain), and *DRA* were used. The YAC contained all three genes.

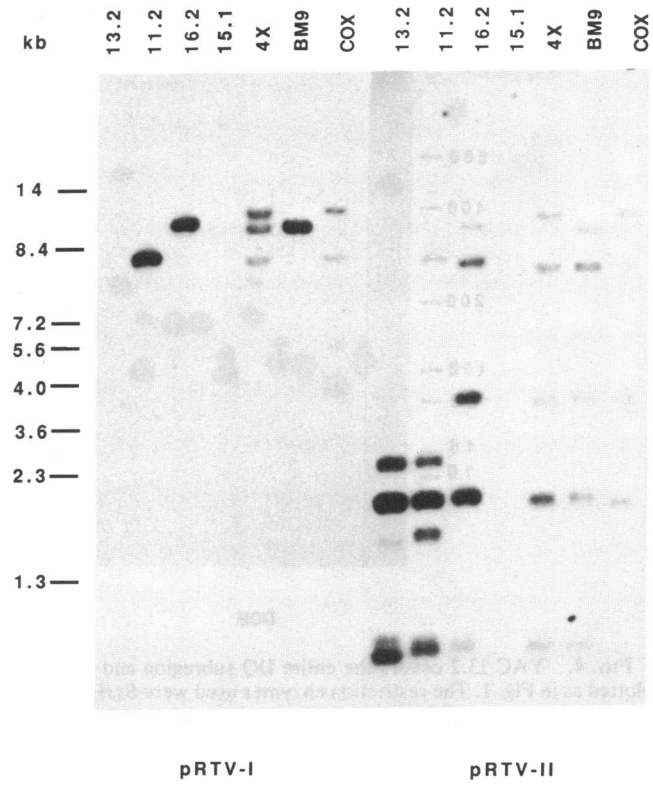

FIG. 3. Haplotype analysis of class II region YACs with *Taq* I RFLPs. Southern blot of *Taq* I-digested DNA from YACs 13.2, 11.2, 16.2, and 15.1 and from the cell lines 4X, BM9 (*DR8* homozygous), and COX (B8, *DR3* homozygous). The blot was hybridized with the pRTV-I probe, specific for the *DRB* β2 domain. Only one band is detected in YAC 16.2 and the BM9 cell line, while YAC 11.2 contains one of the three bands detected in the *DR3* haplotype (COX), proving that the YACs 11.2 and 16.2 contained one *DRB* gene. In the hybridization with pRTV-II, specific for signal sequence and the β1 *DRB* domains, YAC 13.2 was also positive. The YAC 16.2 contained all *DR8 DRB* sequences, while YAC 11.2 contained only one *DRB* gene from the *DR3* haplotype, as visible from the comparison with the 4X cell line and the control haplotypes.

and its restriction map was in agreement with the map of the cosmid clones published in ref. 25 except that the YAC extends 12 kbp farther towards the *DPA1* gene. The extent of overlap of 12.1 and 13.2 with Z6 was determined by isolating a human DNA fragment at the appropriate end of 13.2 by vector to *Alu* PCR (see *Materials and Methods*) and probing on the Z6 YAC, where the end probe mapped 15 kbp away from the 3′ end of the *DNA* gene.

YAC 1.2.1 (220 kbp) extended 45 kbp telomeric of DPA and included the *DPB1*, *DPA2*, and *DPB2* genes as well as the *col11A2* gene and probes 25X1 and 33X1, which are located up to 87 kbp centromeric of *DPB2* (data not shown). The YACs 1.2.1 and Z6, isolated from the 4Y YAC library, contained only one vector arm, hindering the generation of specific end fragments. The overlap of the 1.2.1 and Z6 YACs was therefore determined by *Alu* PCR fingerprint analysis. The *Alu* PCR products of 1.2.1 and Z6 and other YAC clones were blotted and hybridized with the total labeled Z6 products under competition conditions (see *Materials and Methods*). The hybridization revealed a number of PCR products common exclusively to the two YACs, indicating an overlap. Since the distance between *DPA1* and *DNA* has been estimated to be 75 kbp by PFGE (30) the 1.2.1 and Z6 YACs overlap over 20 kbp.

One additional YAC, 4.1 (66 kbp), was isolated from the 4X library, using the *DOB* probe. It contained the region including the *DOB* and *DRB* genes. It did not include the *RING4*

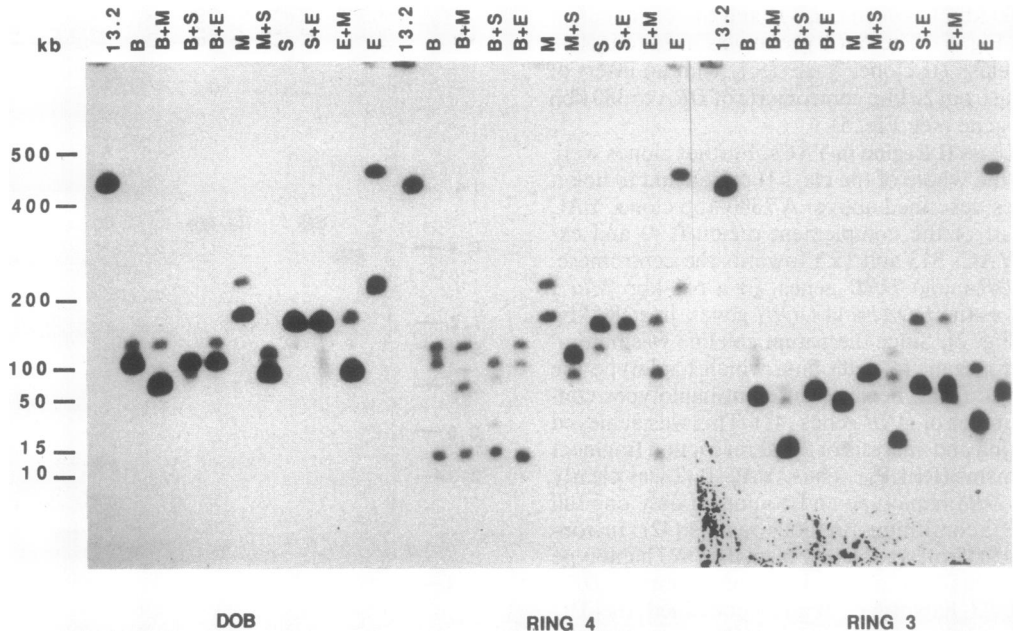

FIG. 4. YAC 13.2 covers the entire DQ subregion and includes the *RING3* gene. Southern blot with DNA from YAC 13.2 separated and blotted as in Fig. 1. The restriction enzymes used were *Bss*HII (B), *Mlu* I (M), *Sal* I (S), and *Eag* I (E). The blot was hybridized to *DOB*, *RING4*, and *RING3* probes. It included *DRB* signal sequences, the DQ genes, *RING4*, and *RING3* in a stretch of 450 kbp.

and *RING3* genes, and we concluded that it was the product of a coligation.

Comparison of *DR3* and *DR8*. The combination of the cosmid and YAC cloning data permitted us to construct the map in Fig. 5. The region between the *DRA* and *DQA1* genes was measured as 400 kbp in the *DR8* haplotype in YAC 16.2. In contrast, this region was about 250 kbp in the *DR3* haplotype, from PFGE, even though it covers three *DRB* genes. Our data

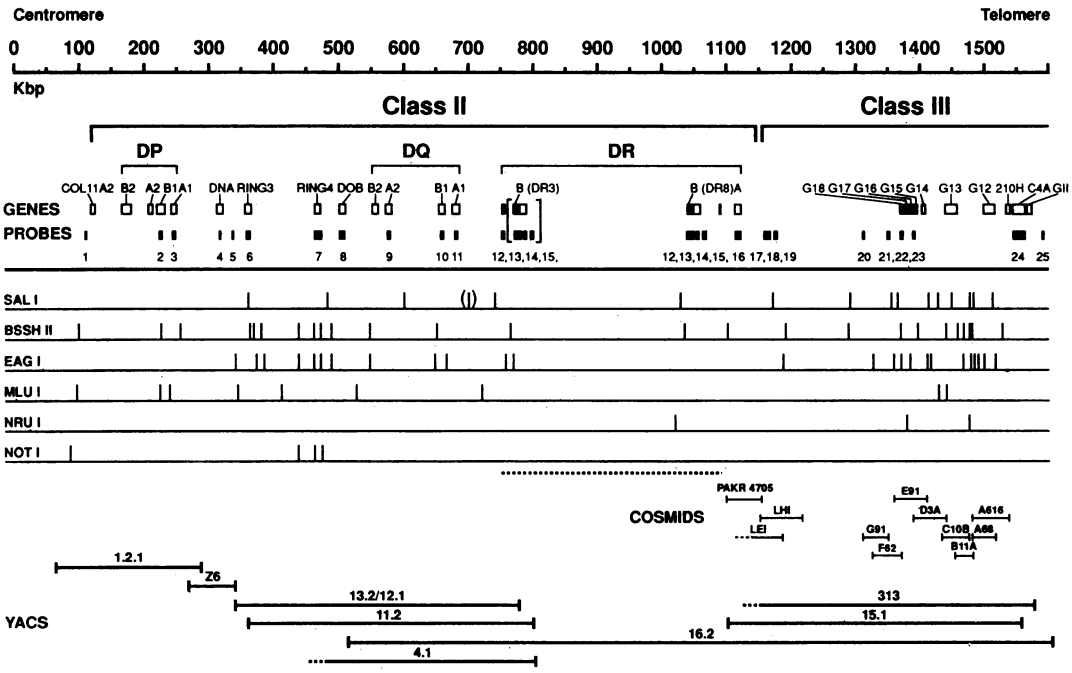

FIG. 5. The 1.5-Mbp map of the HLA class II region in YACs and cosmids. The map shows eight different overlapping YAC clones and cosmids in the class II region and the class II–class III interval. The identified genes and the probes used to establish their organization in this study are included. The numbers under the probes stand for 25X1 (1), *DPB* (2), *DPA* (3), *DNA* (4), 13/89 end probe from YAC 13.2 (5), *RING3* (6), *RING4* (7), *DOB* (8), *DQA2* (9), *DQB1* (10), *DQA1* (11), pRTV-I (12), pRTV-II (13), pRTV-III (14), 11/89 end probe from YAC 11.2 (15), *DRA* (16), E3 (17), *LEE3* (18), Hb (19), WPH (20), FCH (21), WPF (22), I6 (23), C4 (24), and 17M (25). Genes G12 to G18 are taken from ref. 43. The restriction enzyme sites were as detected in the YACs, and all of these sites are also detected in genomic DNA. The restriction site in parentheses represents a site in the *DR3* haplotype seen only in some experiments. The map was completed from PFGE data and cosmid walks as described in the text. The dashed line in the DR subregion indicates that the length and organization of this region, including the number of *DRB* genes, can vary between haplotypes. The two indicated *DRB* genes, one from the *DR3* haplotype (square brackets) and one from the *DR8* haplotype, mark the two extreme positions of *DRB* genes towards the *DQA1* gene and the *DRA* gene. The number of steroid 21-hydroxylase and *C4* genes varies also between haplotypes. Dotted lines in the clones indicate extra DNA as possible coligation events. The fidelity of YAC clone 16.2 telomeric of the *C4* gene was not investigated.

suggest that the *DR8* haplotype contains extra DNA between *DQA1* and *DRB* compared with the *DR3* haplotype. This would be consistent with all of the mapping data but an alternative explanation is that YAC 16.2 contains an insert of extra DNA in the *DRB* region. PFGE analysis of the parent cell line (*DR3/DR8*) showed two *Mlu* I fragments of approximately 600 and 800 kbp with probes around *DRA*. It is necessary to confirm the DQ–*DRB* distances with further YAC clones, but the region that contains the *DRB* genes can vary considerably between haplotypes (14). This can be explained only in part by the different number of *DRB* genes. The *DR4* haplotype has four *DRB* genes and contains an extra 110 kbp of DNA compared to the *DR3* haplotype, for example (14).

The distance between *DOB* and *DPA1* was 200 kbp and was marked with a cluster of three *Not* I sites 25 kbp centromeric of *DOB*. This region contains the newly discovered genes *RING3* and *RING4*, both of which are in CpG islands (30, 44). Other CpG islands that marked genes were detected centromeric of C4 (43).

Molecular Map of the Class II to Class III Region. The entire class II region has been cloned in YACs that cover 1500 kbp. The clones connect the class II region to the complement gene cluster in the class III region as well as all the uncloned gaps existing within the class II region. The cloned region extends to the center of the class III region at the one end and 150 kbp past *DP* at the other end. This region contains 29 genes at present, and we have unpublished evidence for several more genes. The 400-kbp segment of DNA telomeric of the *C2* gene contains a gene every 20 kbp on average, so it is possible that many more genes remain to be found in the class II region also. This map represents the most detailed analysis of a region of the human genome to date. The YAC clones proved to be suitable for closing gaps unbridgable by other cloning systems.

Some areas of the map appear to be devoid of CpG islands—for example, the 150 kbp of DNA telomeric of *DRA*. Ikemura *et al.* (45, 46) identified a boundary between A+T-rich regions of class II and the G+C-rich class III and class I regions. The distribution of restriction enzyme sites containing CpG sequences in the YAC clones and in PFGE (14) suggests that the A+T–G+C boundary lies about 150 kbp telomeric of *DRA*, overlapping with the region that was not present in cosmid libraries. Using YAC 16.2, we will be able to identify the precise A+T–G+C boundary and study its sequence. It will then be of interest to perform *in situ* studies to see how the A+T-rich and G+C-rich regions correspond to Giemsa chromosomal bands. Finally, the YAC clones can be used to study functions of genes, by transfection into cells and possibly into transgenic mice (47, 48).

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