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Molecular mapping of the human major histocompatibility complex by pulsed-field gel electrophoresis

(HLA/gene map/tumor necrosis factor/cosmid cloning)

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ABSTRACT **Pulsed-field gel electrophoresis and "cosmid walking" have been used to establish a molecular map of the human major histocompatibility complex (MHC). We have isolated ≈230 kilobases (kb) of genomic DNA in overlapping cosmid clones covering the genes for the second and fourth components of complement (C2 and C4, respectively), factor B, and steroid 21-hydroxylase, and ≈82 kb of genomic DNA surrounding the genes for the tumor necrosis factors α and β. Single-copy hybridization probes isolated from these cosmid clusters and probes for the known MHC gene loci were hybridized to Southern blots of genomic DNA that had been digested with infrequently cutting restriction endonucleases and separated on pulsed-field gels. The data obtained allowed the construction of a long-range genomic restriction map and indicated that the MHC spans 3800 kb. This map orients the MHC class III gene cluster with respect to the DR subregion; the C2 gene is on the telomeric side of the 21-hydroxylase B gene. In addition we have defined the positions of the genes for the tumor necrosis factors α and β in the human MHC. Genes for the α chain of DR and 21-hydroxylase B are separated by at least 300 kb, while the distance between the genes for C2 and tumor necrosis factor α is 390 kb. The HLA-B locus lies ≈250 kb on the telomeric side of the tumor necrosis factor genes.**

The human major histocompatibility complex (MHC) is located on the short arm of chromosome 6 in the distal portion of the 6p21.3 band (1). It consists of three major linked gene clusters. The class I and class II regions each encode highly polymorphic families of cell-surface glycoproteins involved in immune regulation. The class I loci consist of at least 17 highly related genes (2) that include those encoding the classical transplantation antigens (HLA-A, -B, and -C). The class II region (HLA-D) is arranged into four subregions DP, DZ/DO, DQ, and DR, each containing at least one α- and β-chain pair of genes (3). The class III region contains the genes encoding the serum complement proteins factor B and the second and fourth components of complement (C2 and C4, respectively), as well as two copies of the steroid 21-hydroxylase (21-OHase) genes that are closely linked to the two C4 loci, *C4A* and *C4B* (4, 5). Analysis of recombinant HLA haplotypes in family studies has established that the class I loci are telomeric to the class II genes. Within the class II region the DP subregion maps on the centromeric side of DQ and DR. Located between the *HLA-DR* and *HLA-B* loci are the class III genes, but the orientation and distance of these genes relative to the class I and class II genes have not been determined. The possibility that a number of other genes may reside within the MHC was exemplified by the finding that the genes for the tumor necrosis factors α and β (TNFα and TNFβ, respectively) are linked to the HLA genes (6) and map close to the *H-2D* region in mouse (7).

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Clusters of overlapping cosmid clones have been isolated from the subregions of the class II loci (3) and from the class III region (4) but have not yet been linked. The technique of pulsed-field gel electrophoresis (PFGE) (8, 9), which allows the resolution of DNA fragments >2 megabases, in combination with restriction enzymes that cut rarely in the mammalian genome, has been used for long-range restriction-site mapping of the MHC in man (10–12) and in mouse (13). Using PFGE, Hardy *et al.* (10) determined a map for the human class II region and established the order of the HLA-D subregions. Physical linkage between the human class II and class III loci has also been demonstrated using this technique (11, 12). In mouse the orientation and molecular map position of the complement gene cluster have been established (13).

We have used PFGE, restriction enzymes that cut genomic DNA infrequently, and Southern blotting to derive a map of ≈4000 kb of DNA encompassing the human MHC. Making use of single-copy hybridization probes generated by "cosmid walking," we have orientated the class III loci with respect to the gene for the α chain of DR (DRα), the C2 gene being on the telomeric side of the 21-OHase B gene. In addition, we have defined the position of the TNFα and, by inference, TNFβ genes in the MHC. Our mapping indicates that the DRα and 21-OHase B genes are separated by at least 300 kb, whereas the distance between the C2 gene and the TNFα gene is 390 kb. In addition the HLA-B locus lies 250 kb from the telomeric side of the TNF genes.

MATERIALS AND METHODS

Preparation of DNA. Genomic DNA for PFGE analysis and for construction of cosmid libraries was prepared from a HLA-homozygous lymphoblastoid cell line, HLA and complement typed as, A2, B7, DR2, C2 C, Bf S, C4A 3, C4B QO. For PFGE, high molecular weight genomic DNA in agarose blocks was prepared essentially as described by van Ommen and Verkerk (14), except that 2% (wt/vol) low-geling temperature agarose (Sigma) that had been treated with DEAE-cellulose (15) was mixed 1:1 (vol/vol) with the cells suspended at 2×10^7 cells per ml of isotonic phosphate-buffered saline. High molecular weight genomic DNA for the construction of cosmid libraries was prepared as described by Bell *et al.* (16).

Construction of Cosmid Libraries and Cosmid Walking. Cosmid libraries were prepared according to the method of Steinmetz *et al.* (17). Insert fragments of 35–50 kb, generated from 300 μg of genomic DNA by partial digestion with *Mbo* I, were ligated into the *Bam*HI-digested cosmid vector pDVcos, and the DNA was packaged according to the method of Sternberg *et al.* (18), prior to transduction of *Escherichia coli* strain NM554. Approximately 10^6 colonies

Abbreviations: MHC, major histocompatibility complex; C2 and C4, second and fourth components of complement, respectively; 21-OHase, 21-hydroxylase; TNFα and -β, tumor necrosis factors α and β, respectively; PFGE, pulsed-field gel electrophoresis; DRα, DQα, and DPα, HLA-DR, -DQ, and -DP α chains.

were screened by hybridization according to Grosveld *et al.* (19). Cosmid DNA inserts were characterized by restriction enzyme mapping and Southern blotting (20), and regions of nonrepetitive DNA were detected by probing Southern blots with radiolabeled genomic DNA. Linkage of single-copy sequences with preexisting probes was confirmed by hybridization to common fragments on standard genomic Southern blots (20) and on Southern blots from PFGE.

Restriction Enzyme Digests and PFGE Analysis. Agarose blocks containing ≈5 μg of DNA were washed three times in 10 ml of 10 mM Tris·HCl, pH 8.0/0.1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride for 30 min at 4°C and were then equilibrated with restriction buffer (22) at 4°C for 30 min prior to digestion. Restriction enzyme digestions were carried out according to the supplier's recommendations with 20 units of enzyme for 3 hr. All buffers containing NaCl concentrations ≥50 mM also contained 5 mM spermidine. After digestion, the blocks were incubated in 0.5 M EDTA, 10 mM Tris·HCl, 1% lauroyl sarcosine (pH 9.5), and proteinase K at 1 mg/ml for 2 hr at 50°C prior to loading.

The PFGE apparatus used was provided by the MRC Mammalian Genome Unit (Edinburgh) (21). Digested samples were electrophoresed on 20 cm × 20 cm × 0.5 cm 1.5% agarose (Sigma) gels in 20 mM Tris acetate/1 mM EDTA, pH 8.5, for 22 hr at 12°C and 330 V with pulse intervals up to 70 s. Molecular weight markers were intact yeast chromosomes (*Saccharomyces cerevisiae* strain X2180-1B) prepared in agarose blocks (14) or concatemers of λ *c*I857S7 DNA (22). After ethidium bromide staining and depurination with 0.25 M HCl, DNA fragments were blotted onto nylon membranes (GeneScreen*Plus*, New England Nuclear). Hybridization and high-stringency washing (30 mM NaCl/3 mM sodium citrate/1% $NaDodSO_4$, 65°C) of filters was carried out following standard procedures (4). Autoradiography was between two intensifying screens at −70°C for 1–5 days. Filters were stripped of probe by washing twice in 2 mM Tris·HCl, pH 7.4/1 mM EDTA/1% $NaDodSO_4$ at 80°C for 1 hr.

Probes. The probes used were a DRα cDNA (23), a DQα-chain (DQα) cDNA (10), a DPα-chain (DPα) cDNA (3), a DRβ-chain (DRβ) cDNA (10), a DPβ-chain (DPβ) genomic probe (3), a 21-OHase genomic probe (24), a class I cDNA (25), HLA-B (B250) and HLA-C (C250)-specific genomic probes (26), factor B cDNA (4), C4 cDNA (4), and a TNFα genomic probe (a gift from Mark Bodmer) (27). Probe J, a 0.9-kb *Bam*HI–*Xho* I genomic fragment, and probe L, a 1.4-kb *Bam*HI genomic fragment, were isolated from cosmid clones as described above. All probes were labeled by random hexanucleotide priming (28).

RESULTS

Isolation of Overlapping Cosmid Clones from the Class III Region. To construct a restriction map of the MHC in man, genomic DNA from a HLA-homozygous cell line (HLA type—A2, B7, DR2, C2 C, Bf S, C4A 3, C4B QO) was used to minimize mapping problems caused by possible haplotype-specific restriction fragment length polymorphisms. The cell line used has only single C4 and 21-OHase loci on each copy of chromosome 6 (29, 30), which also serves to simplify restriction site mapping. Approximately 230 kb of genomic DNA encompassing the C2, factor B, C4, and 21-OHase B genes was isolated in a series of overlapping cosmid clones (Fig. 1*A*). This cluster of genomic clones was mapped with restriction enzymes having 6- or 8-base-pair recognition sites and containing one or two CpG dinucleotides. Since CpG is known to be underrepresented in the human genome (31), these enzymes are consequently of use in construction of

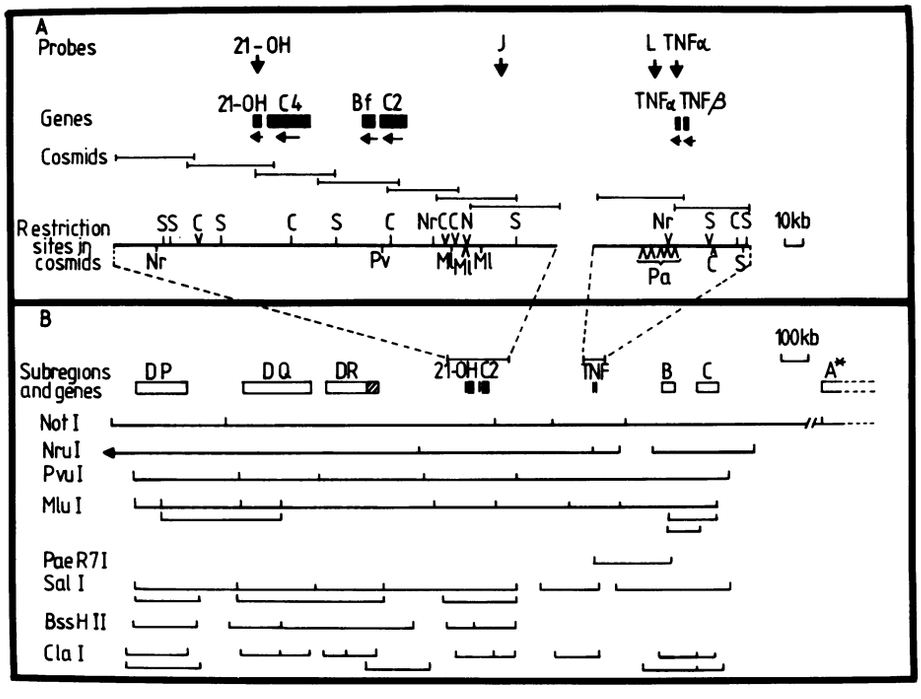

FIG. 1. Molecular map of the human MHC. (*A*) Overlapping cosmid clones from the complement/21-OHase and TNF regions. The locations of genes are shown by solid boxes (Bf, factor B; 21-OH, 21-OHase), and the 5′ → 3′ orientation is indicated by horizontal arrows. Vertical arrows indicate the positions of the probes used in PFGE analysis. The positions of infrequently cutting restriction endonuclease sites known to cut in genomic DNA are indicated by arrowheads; other sites are indicated by vertical bars. C, *Cla* I; Ml, *Mlu* I; N, *Not* I; Nr, *Nru* I; Pa, *Pae*R7I; Pv, *Pvu* I; S, *Sal* I. (*B*) Restriction map obtained by Southern blot analysis of genomic DNA separated by PFGE after single and double digests. Solid boxes, exact position of genes; open boxes, limits of the region hybridizing to the probes used; cross-hatched box, probable position of the DRα gene. For convenience the 950-kb *Not* I fragment that hybridizes with the B- and C-locus-specific probes is not to scale, and A*, the 675-kb and 400-kb *Not* I fragments that hybridize with the class I cDNA probe (see Fig. 2) and lie on the telomeric side of the C locus, are not shown. In some cases partial digests were obtained, as represented by two sets of data for some enzymes.

long-range restriction maps in conjunction with PFGE (14, 22). Examination of these restriction site data (Fig. 1*A*) reveals a striking cluster of sites for infrequently cutting enzymes (three *Mlu* I, two *Cla* I, one *Not* I, and one *Nru* I) in a 25-kb region located ≈25 kb from the transcriptional start site of the C2 gene. A single-copy genomic hybridization probe (probe J, Fig. 1*A*) was isolated from the cosmid DNA distal to this cluster of restriction sites and the C2 gene.

To establish a long-range restriction map of the human MHC, high molecular weight HLA-homozygous cell line DNA in agarose blocks was digested with a range of suitable restriction enzymes, alone or in double-digest combinations. The DNA fragments were separated by PFGE and transferred to nylon membranes. These filters were hybridized successively with a panel of ^{32}P-labeled cDNA and genomic probes specific for the class I, class II, and class III regions of the MHC (see Fig. 1).

Orientation of the Complement Genes in the Class III Region. Hybridization of *Not* I-digested DNA with DQα, DRα, and 21-OHase probes identified a common 980-kb *Not* I fragment that links the DQ and DR subregions with the complement loci (refs. 11 and 12 and Fig. 2). This fragment is digested by *Nru* I to yield a fragment of 700 kb with the DRα probe and a fragment of 280 kb with the 21-OHase probe (Fig. 2). Probe J from the class III cosmid cluster, however, hybridizes to a *Not* I fragment of 210 kb. Since the exact position of the *Not* I site is known from the cosmid map (Fig. 1*A*), these data establish the orientation of complement genes relative to the class II genes; the C2 gene is on the telomeric side of the 21-OHase B gene closest to the DR locus.

Hybridization of probes DQα, DRα, 21-OHase, and J to *Pvu* I, *Mlu* I, and *Sal* I digests is consistent with the map shown in Fig. 1*B*. In the *Sal* I digest the 21-OHase probe hybridizes to a fragment of 280 kb and a partial fragment of 490 kb (data not shown). The *Not* I/*Sal* I double digest positions a *Sal* I site 85 kb on the telomeric side of the *Not* I site, and this was confirmed by hybridization of the blot with probe J. Hybridization of the same blot with the DRα probe revealed no common fragments. Thus, the minimum distance between the 21-OHase B and the DR subregion is 300 kb.

Further single- and double-digestion data allowed the limits of the DP, DQ, and DR subregions to be defined (Fig. 1*B*). The map of the class II region shows a number of striking similarities to that published by Hardy *et al.* (10) and confirms the organization of the class II subregions. Although the order of the α and β genes within a subregion was not determined, the approximate position of the DRα gene was defined from the result of *Cla* I and *Cla* I/*Sal* I digests. A 290-kb partial *Cla* I fragment, which hybridized with the DRα probe but not the DRβ probe, was reduced by *Sal* I to 60 kb (Fig. 1*B*). This would suggest that the DRα gene lies 300–360 kb from the 21-OHase B gene.

Mapping of the TNFα Gene in the MHC. The genes for TNFα and -β have been linked to the human MHC (6), and in mouse they have been mapped ≈70 kb from the H-2D region, the murine equivalent of HLA-B (7). Hybridization of the TNFα probe to *Not* I- and *Nru* I-digested DNA failed to reveal any bands in common with the other probes (Fig. 2). However, in the *Pvu* I digest, a 780-kb fragment that also hybridized with the HLA-B and -C locus probes was identified with the TNFα probe (Fig. 3*B*).

To establish the position of the TNFα gene relative to the known MHC loci, a cluster of overlapping cosmid clones covering ≈82 kb of genomic DNA surrounding the TNFα gene was isolated and characterized (Fig. 1*A*). The position of the TNFα gene was located by restriction mapping and Southern blot analysis. The position of the TNFβ gene was inferred by comparison of the map with that of Nedospasov *et al.* (32). These cosmids were mapped for infrequently cutting restriction enzymes. A single *Nru* I site was found 2.5 kb from the 5′ end of the TNFα gene (Fig. 1*A*). A single-copy hybridization probe (probe L, Fig. 1*A*) was isolated from the cloned cosmid DNA distal to the *Nru* I site and the TNFα gene. When hybridized to *Nru* I-digested genomic DNA, this probe detected the same 640-kb *Nru* I fragment that contains the complement genes (Fig. 2). This result confirmed the linkage of the complement genes and the TNFα gene and established that the C2 gene lies 390 kb on the centromeric side of the TNFα gene. This also indicates that the TNFβ gene lies on the telomeric side of the TNFα gene.

Further single and double digests were carried out to estimate the distance between the TNFα and *HLA-B* loci. A 290-kb *Pae*R7I fragment was found to hybridize with both probes (Fig. 4*A*), but not probe L. The results from a series of double digests (Fig. 4*A*) allowed the map in Fig. 1*B* to be constructed showing that the TNFα and -β genes lie ≈250 kb on the centromeric side of the class I genes. Restriction mapping of the cosmid DNA for *Pae*R7I revealed five sites (Fig. 1*A*), one of which lies within the TNFα gene. To

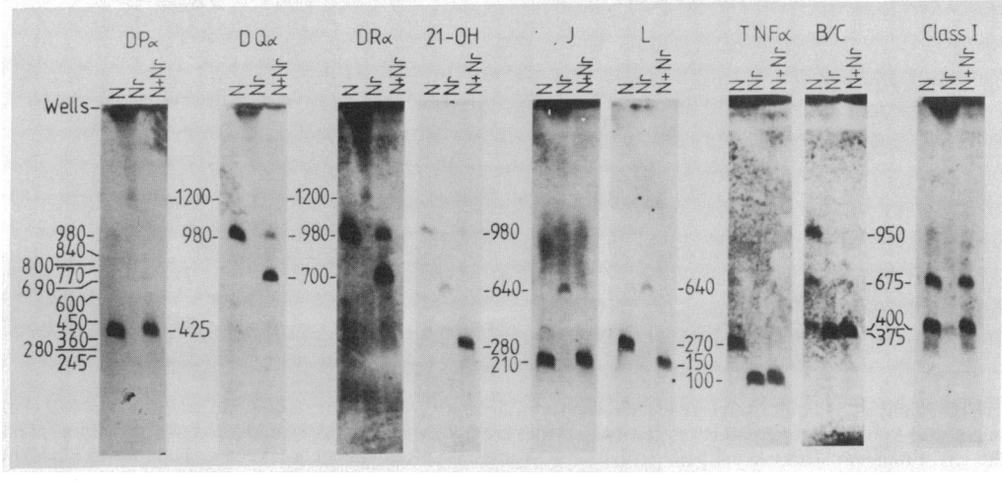

FIG. 2. Southern blot analysis of genomic DNA digests separated by PFGE. Fragments generated by *Not* I (N), *Nru* I (Nr), or *Not* I/*Nru* I (N + Nr) were separated using a 65-s pulse interval. All autoradiograms were obtained from a single filter hybridized sequentially with the probes shown. B/C indicates that the B- and C-locus-specific probes gave the same hybridization pattern, and only the B-locus-probe result is shown. Yeast chromosomes were electrophoresed as size markers and are indicated on the left. Fragment sizes are given in kb. The size of the class II-hybridizing *Nru* I fragment is approximate.

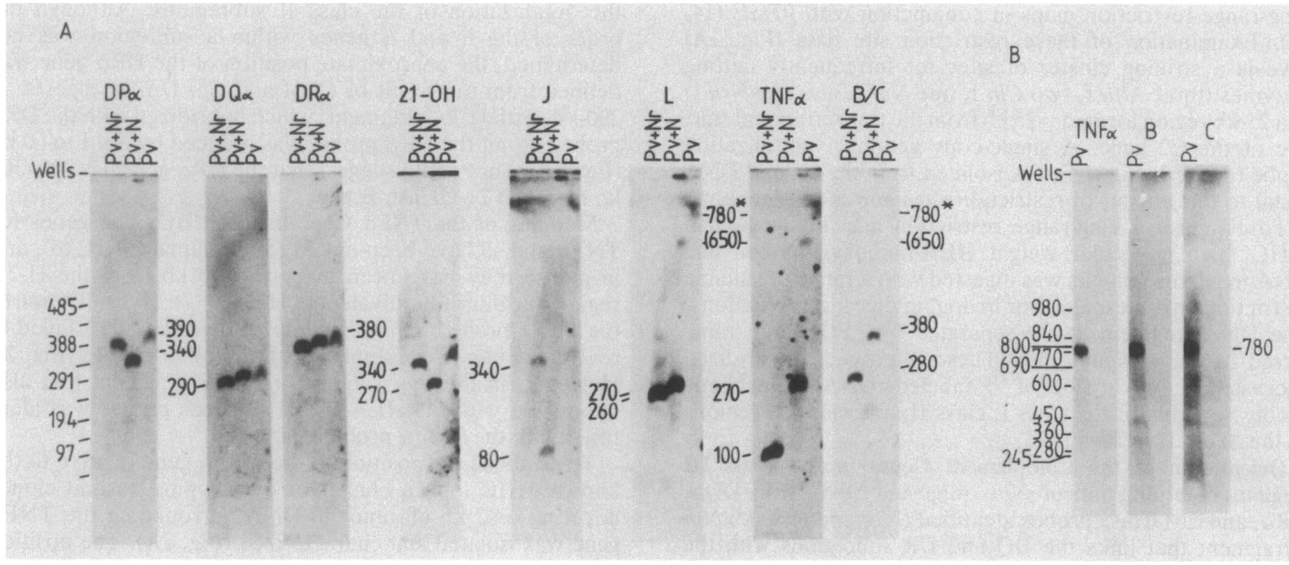

FIG. 3. Southern blot analysis of *Pvu* I-digested DNA separated by PFGE. (*A*) Sequential hybridization of the probes shown to a single Southern blot of DNA digested with *Pvu* I (Pv), *Pvu* I/*Not* I (Pv + N), or *Pvu* I/*Nru* I (Pv + Nr) and separated using a 30-s pulse interval. Concatemers of λ *c*I857S7 DNA were run as size markers and are indicated on the left. Fragment sizes are given in kb. Partial digestion products are enclosed in brackets. *, The 780-kb *Pvu* I fragment is completely resolved in *B*. (*B*) Sequential hybridization of TNFα, B-specific (lane B), and C-specific (lane C) probes to *Pvu* I-digested DNA resolved using a 60-s pulse interval. Yeast chromosomes were electrophoresed as size markers and are indicated on the left.

establish which of these sites are cut in genomic DNA, a single *Pae*R7I digest and double digests were resolved by conventional gel electrophoresis and blotted onto nitrocellulose. Hybridization of the TNFα probe revealed a 3.9-kb *Pae*R7I fragment produced by complete cleavage at the sites within the TNFα gene and immediately adjacent (Fig. 4*B*). Hence, TNFα hybridizes to a 290-kb fragment resolvable by PFGE and a 3.9-kb fragment that can only be seen on standard agarose gels. Probe L hybridizes to a series of fragments consistent with partial cleavage at the remaining three *Pae*R7I sites (Fig. 4*B*). The double digests with *Bam*HI and *Eco*RV confirm this observation and generate the appropriate fragments predicted from the restriction map of the cloned DNA (ref. 32; C.A.S. and R.D.C., unpublished data).

B- and C-locus-specific probes hybridized to the same fragments in *Not* I, *Pvu* I, and *Sal* I digests. However, on the basis of hybridization to *Mlu* I, *Cla* I, and *Mlu* I/*Cla* I digests, the B and C loci are separated by at least 80 kb (Fig. 1*B*).

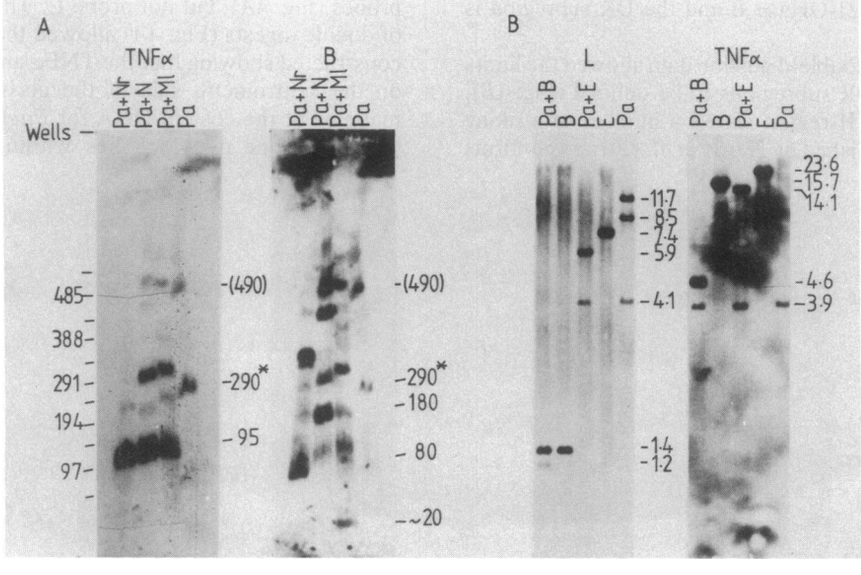

FIG. 4. Southern blot analysis of *Pae*R7I-digested DNA. (*A*) Hybridization of TNFα- and B-specific probes to *Pae*R7I (Pa) single and double digests separated using a 30-s pulse interval. Other restriction enzymes are *Not* I (N), *Nru* I (Nr), and *Mlu* I (MI). Positions of λ DNA concatemers are indicated on the left. Fragment sizes are in kb. A partial *Pae*R7I digestion product is in brackets. *, The 290-kb *Pae*R7I fragment has been sized on other blots and here shows slightly anomalous mobility. Unmarked fragments are the products of partial digestion, which have not been mapped. (*B*) Southern blot analysis of *Pae*R7I sites in genomic DNA around the TNFα gene. Genomic DNA was digested with single- and double-digest combinations of *Pae*R7I (Pa), *Eco*RV (E), and *Bam*HI (B), separated on a conventional agarose gel, and hybridized with probe L and the TNFα probe. The three bands in the *Pae*R7I lane with probe L are due to partial cleavage at two of the five *Pae*R7I sites mapped in cosmid DNA in this region (Fig. 1*A*). The TNFα probe hybridizes to a 3.9-kb *Pae*R7I fragment due to cleavage at the *Pae*R7I site within the TNFα gene and also to the 290-kb fragment that can only be resolved by PFGE (see *A*). Fragment sizes are in kb.

DISCUSSION

PFGE, restriction enzymes that cut genomic DNA infrequently, and Southern blotting have been used to produce a long-range restriction map of the human MHC. We have established the orientation of the complement and 21-OHase gene loci relative to the class I and class II loci, the 21-OHase B gene being on the centromeric side of the C2 gene. The distance between the 21-OHase B gene and the DRα locus is 300–360 kb, while that between the C2 gene and the *HLA-B* locus is ≈650 kb. We have located precisely the genes for TNFα and -β within the MHC; the TNFα gene lies 390 kb from the C2 gene. The total size of MHC hybridizing fragments is ≈3800 kb, which represents ≈1/750 of the human genome.

Our data confirm the organization of the class II region proposed by Hardy *et al.* (10). Differences in observed fragment sizes can be explained by haplotype-specific restriction fragment length polymorphism or by insertions or deletions of DNA. There could also be methylation differences between cell lines leading to some sites not being recognized, as some enzymes with CpG in their recognition sequences appear to be sensitive to cytosine methylation (21). However, the conservation of rare-cutting restriction sites between cell lines (refs. 10–12 and this paper) in this very polymorphic region of the genome is striking and may be the product of a nonrandom distribution of nucleotides, C+G-rich regions being found in clusters. For instance, 25 kb on the telomeric side of the C2 gene is a cluster of at least seven rare-cutting sites within 25 kb. Some of these sites (two out of three *Mlu* I sites and the *Nru* I site) are not recognized in genomic DNA, possibly due to cytosine methylation, but a *Not* I and a *Mlu* I site within 1 kb of each other are both cleaved. The apparent nonrandom distribution of infrequently cutting sites in the mammalian genome has been observed (21, 33) and may represent so-called HTF Islands (*Hpa* II Tiny Fragments) (21, 31).

The organization of the human MHC is very similar to that determined in the mouse (7). The orientation of the class III genes is the same, and the proposed distances between the class III and class II loci and between class III and TNF are comparable. However, our results suggest that the TNF genes are ≈250 kb from the HLA-B region compared to ≈70 kb for the analogous genes in the murine MHC. It should be pointed out that the human and mouse MHC maps differ in that the *H-2K* locus is separated from the other class I genes at the other side of the class II region. It is possible that the difference in distance between TNF and class I in the two species is associated with the genetic event that separated the mouse class I loci. The estimated total size of the human MHC is twice as large as the murine MHC (4000 kb vs. 2000 kb), but most of this difference is made up of fragments that hybridize to general class I gene probes.

We believe that the physical linkage map of the human MHC presented here will play an important part in understanding HLA disease associations. The distances between the DRα and 21-OHase genes and between the C2 and TNFα genes are sufficiently large to accommodate a number of as yet unidentified genes. Further mapping of disease-affected haplotypes and the isolation of a complete set of overlapping cosmids covering the DRα to HLA-B region should permit the characterization of such genes. The demonstration of the precise position of the TNFα and -β genes within the human MHC is of major interest because TNFα and -β are mediators of a number of responses that may play a role in autoimmune disease. For instance, TNFα augments MHC antigen expression in some tissues (34, 35), which may lead to enhanced cytolytic damage mediated by class I antigens or presentation of autoantigens by epithelial cells aberrantly expressing class II antigens (35).

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