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Analyses of gene structures and antigen determinants
of human class II major histocompatibility antigens.

- I. Isolation and characterization of the genomic clones
of the class II antigens from DR2DQw1 haplotypes

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ABSTRACT

440kb length of overlapping cosmid clones, including DO, DX/DV, DQ and DR regions, was obtained from two DR2DQw1 haplotypes, AKIBA and PGF. and the gene structures were investigated. Differences of the restriction sites between AKIBA (Dw12) and PGF (Dw2) were most concentrated near the DQ β region, than any other class II genes. Deletion or insertion of about 5kb long region was observed upstream from the DQ β gene. DR region contained three β , one α , and two isolated β chain exon fragments. The DQ β , DR β I and DR β III genes were expressed, together with their pairing α chains, after transfection into mouse L cells. Restriction enzyme mapping of the DR β clones and reactivity of their products against monoclonal antibodies showed that the DR β I and DR β III genes encoded the nonpolymorphic and polymorphic DR β chain, respectively. This arrangement is the reverse of that observed in other haplotypes. The alignment of the HLA class II genes on the chromosome 6, however, was consistent with other haplotypes. These results suggest that the susceptibility to mutations or gene conversions responsible for genetic polymorphisms depends on the gene itself and not on its location. Furthermore, absorption experiments of anti-DR2 allosera by the DR α /DR β transfectants revealed that the so-called DR2 specificities were determined by multiple epitopes.

INTRODUCTION

Human chromosome 6 contains a cluster of genes, known as the major histocompatibility complex (MHC), HLA, that encode a number of cell surface glycoproteins involved in various immune interactions (Kaufman et al., 1984). The HLA-A, -B and -C are designated class I antigens, and HLA-DR, -DQ, and -DP antigens are designated class II antigens. Whereas the class I antigens are expressed on almost all types of cells, the class II antigens have limited tissue distribution and are expressed on antigen presenting cells, such as macrophages and dendritic cells. The class II antigens define determinants involved in central events of immunoregulation, such as stimulation of appropriate T cells in a specific manner (Nagy et al., 1981). The class II antigens are heterodimers of two glycosylated, membrane-integrated chains, α and β . The polymorphism of these molecules has been extensively studied using serological and cellular techniques (Termijtelen et al., 1982), two-dimensional polyacrylamide gel electrophoresis (Nepom and Holbeck, 1984a), and molecular biological techniques at the genomic level (D. Cohen et al., 1984). Polymorphic determinants of the HLA class II antigens reside mainly in the β -chain (Charron and McDevitt, 1979; Silver and Ferrone, 1979), although at least one class of α -chains, DQ α , also displays genetic variability (Auffray et al., 1983; Chang et al., 1983; Schenning et al., 1984).

An analysis and a comparison of the HLA class II region

among various haplotypes is interested because many diseases are associated with HLA class II haplotypes (Svejgaard, 1982), such as narcolepsy (Honda, 1984), myasthenia gravis (Keller, 1987) and insulin dependent diabetes mellitus (Todd, 1988). They have found that linkage disequilibrium between the various alleles of the different loci from DR to DX genes. Narcolepsy is strongly associated with DR2Dw2 (Honda et al., 1984; Inoko et al., 1986), especially linked restriction fragment length polymorphisms of DQ β gene (Inoko et al., 1986). Todd et al. (1990) reports suggested the association of insulin dependent diabetes mellitus with DQ gene structure. These disease associations suggest that some genetic background which affect the development of these diseases locate in the HLA class II region.

The HLA class II gene region encodes at least three expressed products, HLA-DP, DQ and DR with two different DR products being expressed in most haplotypes (Trowsdale et al., 1985). A genetic approach, however, proved evidence for several other α and β genes. There are cloned six genes involved in the class II genes, DP, DO, DX, DV, DQ, and DR. Some of the class II genes, particularly in the DR and DQ regions, are in strong linkage disequilibrium. Therefore, it is quite difficult to dissect out their individual functions. Although numbers of genes involved in each subregion is constant, that of the DR β genes vary in various haplotypes. There are usually three or four DR β genes, named DR β I, DR β II, DR β III, and DR β IV in the centromere

to telomere direction (Auffray et al., 1985; Sorretino et al., 1985; Böhme et al., 1985) One or two of the DR β genes are pseudogenes (Spies et al., 1985; Rollini et al., 1985, 1987; Larhammar et al., 1985;) In the DR3 and DR4 haplotypes the DR β I gene encodes the polymorphic serological specificity (DR3 or DR4) and the DR β III or DR β IV gene encodes the supertypic or nonpolymorphic one (DRw52 or DRw53) (Spies et al., 1985; Rollini et al., 1985) Two DR β genes are also known to be expressed in the DR2 haplotype, leading to the formation of two DR products, on the basis of biochemical and cDNA sequencing studies (Lee et al., 1987; Nakatsuji et al., 1986; Nepom et al., 1984b; Wu et al., 1987) One of these expressed genes encodes a relatively nonpolymorphic protein which is believed to correspond to the supertypic DR allospecificity in other haplotypes such as DRw52 and DRw53, in DR3 or DR4 haplotypes, respectively. In the DR2 serotype such a supertypic antigen has not yet been serologically defined. The other encodes a protein that is polymorphic among the Dw2, Dw12 and Dw21 subtypes of DR2, and is believed to correspond to the DR β I locus-encoding molecule.

In this study we have cloned DO, DX, DV, DQ, and DR genes from the two series of genomic gene libraries on overlapping cosmid clones, in order to analyze and compare their gene structure among different Dw haplotype, Dw2 and Dw12, but the same DR2DQw1 haplotype. Furthermore, DQ β and two DR β genes were transfected separately, together with their pairing α chain

gene, into mouse L cells. DR antigen determinants were also investigated by absorption of haplotype specific sera with DR antigen-expressing transfectants.

MATERIALS AND METHODS

Isolation and characterization of genomic clones.

Genomic gene libraries were constructed from the two consanguineous homozygous B lymphoid cell lines (LCL), PGF and AKIBA whose haplotypes are DR2,Dw2,DQw1,DPw4 and DR2,Dw12,DQw1,Cp63, respectively. Briefly. DNAs from LCLs were partially digested with Sau3AI and fractionated on sucrose gradients. Fractions containing DNA fragments 35-45 kilobases (kb) long were collected, ligated with the arms of the cosmid vector, pJB8 (Ish-Horowicz and Burke, 1981) and transfected into Escherichia coli 1046 using in vitro packaging extracts. A total of 6,900,000 cosmid colonies were spread, replicated, and screened by colony filter hybridization, as described by Maniatis et al. (Maniatis et al., 1982) Single colonies containing desired HLA-class II cosmids were isolated through two or three times hybridization. Libraries were screened with cDNA and genomic DNA fragments labeled with [³²P]CTP. Colony hybridization was carried out in 5x SSPE (1x SSPE is 0.15M NaCl/ 10mM NaH₂PO₄/ 1mM EDTA)/ 5x Denhardt's solution (1x Denhardt's solution is 0.02% bovine serum albumin/ 0.02% ficoll/ 0.02% polyvinylpyrrolidone)/ 50%

formamide/ 0.1% SDS/ sonicated salmon sperm DNA (100 μ l/ml) for 16hr at 37°C. Filters were washed in 3x SSC (1x SSC is 0.15M NaCl/ 0.015M NaCl/ Cit)/ 0.1% SDS/ at room temperature for 5 min, 2x SSC/ 0.1% SDS at room temperature for 5 min, 1x SSC/ 0.1% SDS at room temperature for 5 min, 1x SSC/ 0.1% SDS at 65°C for 15 min, and 0.2x SSC/ 0.1% SDS at 65°C for 15 min, and were exposed to Kodak XRP-5 films. Clones hybridizing to the specific probes were grown and their DNAs were prepared by the miniprep method. Isolated cosmid clones were mapped by standard single and double digestions with restriction endonucleases followed by Southern blot hybridization using specific cDNA fragments, under stringent conditions. Cosmid clones designated pAK or pA, and pP are derived from the libraries of AKIBA and PGF cells, respectively.

Probes

The probes used for screening and characterization of class II genes were isolated by agarose or polyacrylamide gel electrophoresis after restriction enzyme digestion, electroeluted into dialysis tubes, and radioactively labeled with [³²P]CTP by random primed DNA labeling kit (Boehringer). The fragments used are as follows (see Fig. 1). The probes for the DR β gene were the 160bp PstI-SacI and 290bp PstI fragments and the entire sequence of the DR β cDNA clone, pDR β 102 (Inoko et al., 1985b) which encode the 5' untranslated (5'UT) region and the 3' untranslated

Fig.

(3'UT) region, respectively. The 220bp PstI fragment of the DR β cDNA clone, pDR β 101 (Inoko et al., 1985b), a part of the β_1 domain; The 700bp PstI fragment and the entire sequence from the DR α cDNA clone, pDRH2 (Lee et al., 1982), was used, which encodes a part of the α_2 domain, the whole transmembrane-cytoplasmic region (TM/CY) and the 3'UT region. 1220bp DQ β cDNA, pDC β 101, all of the coding sequence for DQ β gene (Inoko et al., 1985b). The 300bp PstI-DdeI fragment, which encode a part of α_2 , TM, CY and 3'UT, and the entire 980bp of the DQ α cDNA, pDC α 107, respectively. The 490bp DO α cDNA, pDO α 20, a part of α_2 domain, TM, CY AND 3'UT region. The 2.0kb PstI fragment of the DV β genomic clone, λ HMC β 19, which encode a part of DV β_2 domain. The 2.7kb EcoRI fragment of pAKQ056, which locate 18kb upstream from the DQ β gene (see Fig. 5)

Genomic Southern hybridization

EB transformed LCLs and PBLs ($1-2 \times 10^7$ cells) were lysed at 50°C for 16hr in 50mM Tris HCl (pH8.0), 20mM EDTA, 100mM NaCl, 1% SDS, and 150 μ l/ml proteinase K. Protein was removed from high molecular weight DNA by 400mM Tris saturated phenol and the DNA was precipitated in the presence of 0.3M K-acetate with 2 volumes of cold 100% ethanol. The DNA precipitated was dissolved in H₂O at a concentration of 1 μ g/ μ l. Four different restriction endonucleases, EcoRI, PstI, TaqI, and MspI (Toyobo Co., LTD.), were used for digestion. Samples of restriction enzyme cleaved

genomic DNA (7.0 μ g) were subjected to electrophoresis in 0.7% agarose and transferred to BioTrace membranes (Gelman Sciences Inc.) as described by Southern (Southern, 1975) After transfer, the filters were prehybridized (50% formamide, 5x Denhardt's solution, 5x SSPE, 1% SDS, 5% Dextran sulfate, 100 μ g/ml sonicated salmon sperm DNA) at 42°C for 16hr Radioactively labeled cDNA fragments were added at a concentration of 1×10^6 cpm/ml to the hybridization mixture and incubated with the filters at 42°C for 16hr. The filters were washed with 2x SSPE at room temperature for 5min, with 2x SSPE/ 0.5% SDS for 15min, at 65°C and with 0.5x SSPE/ 0.5% SDS for 15min at 65°C. The filters were then exposed to Kodak XRP-5 films with an intensifying screen at -70°C.

Transfection of HLA class II genes into mouse L cells

The cosmid clones encoding HLA class II α and β genes were cotransfected with the plasmid, pSV2neo, containing the neo gene, into mouse L cells using the modified calcium phosphate technique (Chen and Okayama, 1987) to obtain cell lines expressing HLA class II antigen on their cell surfaces. The transfected cosmids are as follows, pAKQ4116 and pAKQ056, AKIBA DQ α / β genes; pPQ364 and pPQ362, PGF DQ α / β genes; pPR541 and pAKR4643, DR α / β I genes; pPR541 and pPR241, DR α / β III genes (see Fig.5 and 8) Transfectants were grown in Dulbecco's modified eagle medium supplemented with 10% fetal calf serum,

penicillin and streptomycin, and selected using 400 μ g/ml Geneticin (Gibco, Grand Island, New York) Geneticin-resistant colonies resulting from each transfection were cloned, stained with the anti-DQ MoAb, Leu10 (Becton Dickinson) and the anti-DR MoAb, L243 (Becton Dickinson) for flow microfluorimetric analysis and further cloned by limiting dilution.

Flow microfluorimetric analysis

1×10^6 cells were washed with cold PBS and incubated with 30 μ l of antibody at the concentration enough to bind LCLs (data not shown) for 30 min on ice. After three more washes in PBS, the cells were resuspended in 50 μ l of fluoresceine isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulin (Cappel) and incubated for a further 30 min at room temperature. Following three final washes, the cells were analyzed on the FACS. The specificities of the MoAbs used are as follows, L243, HLA-DR; Hu30, HLA-DR1 and DR2 (provided by Dr. Aizawa) (Nishimura et al., 1984); NDS15.38, HLA-DR2 (Makgoba et al., 1983); AB8.25, DR2+5+shortDR3 (Colombani et al., 1983) (distributed for the Tenth International Histocompatibility Workshop studies:10th IHWS); Leu10, HLA-DQ; B7/21, HLA-DP (Becton Dickinson).

Alloantisera absorption assay by DR gene-transfected L cells

The specificities of alloantisera used were as follows, 1030, 1026 and 1029;DR2 (these three were selected from the

best alloantisera submitted to the 10th IHWS); Ex107, DR4; Ex115, DR1,2,4 and 8; Ex99, DR1 and DR2; JR21-224, DR2. The specificities of the last four alloantisera were determined at the department of Tokai Univ. school of medicine. For absorption, alloantisera were incubated (30 min at 37°C and 90 min at 4°C) with 7×10^6 cells prior to the test. 3×10^3 LCLs or 5×10^3 B cell-enriched fraction from human peripheral blood lymphocytes (PBLs) were used for cytotoxicity assay. Briefly, cells were incubated for 1hr at 37°C with absorbed or unabsorbed alloantisera ($1 \mu\text{l}$), and followed by incubation with $5 \mu\text{l}$ rabbit complement for 2hr at room temperature. The percentage of dead cells was scored after eosin staining.

RESULTS

Cloning of class II genes

Two set of cosmid gene libraries were constructed from 30-45kb long high molecular weight genomic DNA of the consanguineous HLA-homozygous lymphoid cell lines, AKIBA (DR2, Dw12, DQw1) and PGF (DR2, Dw2, DQw1). By screening these libraries with HLA class II cDNA clones as probes, a set of cosmid clones for $DO \alpha$, $DX \alpha$, $DX \beta$, $DV \beta$, $DQ \alpha$, $DR \beta$, and $DR \alpha$ genes were isolated. Cosmid screening was performed through two steps (Fig. 2) 30,000 Fig. 2 colonies were spread on each nitrocellulose filter, replicated and screened by HLA class II probes (primary screening) The

positive clones were picked up, spread on small filters and hybridized with the same probe (secondary screening), resulting single colonies were isolated. Colony hybridization was done under the stringent condition to suppress cross hybridization to other subregion genes.

Isolation and analysis of the DO α , DQ, and DX/DV genes

Three continuous cosmid clones representing a length of about 40kb in the DO α subregion, identifying one DO α gene, were obtained from AKIBA by colony hybridization with DO α cDNA clone, pDO α 20 (Inoko et al., 1985a), as a probe. Restriction map of DO α cosmid clones and the autoradiogram using DO α cDNA probe were shown in Fig. 3. Restriction map of about 20kb region from Fig. 3 DO α were identical to that of the previous report (Inoko et al., 1985a).

DX genes have high homology to the DQ genes at nucleotide sequence (Auffray et al., 1984, 1987; Okada et al., 1985; Jonsson et al., 1987), resulting successful isolation of the DX genes from AKIBA using the DQ α and β cDNA as probe. Southern blotting probed by DQ β cDNA were shown (Fig. 4B).

Fig. 4

DV β is a class II light chain pseudogene, found by Ando et al. (1986a), which has highest homology to the DQ β gene among class II β genes. Two cosmid clones, pA412 and pPKV5123-2, were obtained from AKIBA and PGF, respectively. using 2.0kb PstI fragment of λ HMC β 19 encoding the DV β gene. High homology of

the DV β with DQ β gene was confirmed by Southern blot analysis of the DV β clones isolated here (Fig. 4C). A comparison of the restriction map between pA412 and pAX523, encoding the DV β and the DX α region, respectively, suggested that these two clones overlapped about 8kb long. Southern blot analysis using a short fragment in the overlapping region as probe confirmed that pA412 and pAX523 shared the same locus (Data not shown), indicating that the DV β gene located 15kb upstream from the DX α gene (Fig. 4A).

Isolation of the DQ α and β genes

At first, I expected to obtain DQ α cosmid clones using entire DQ α cDNA (pDC α 107) as probe, I obtained, however, no DQ α cosmid clone, but many DP α and DX α genes from the libraries of AKIBA because of cross hybridization. Similarly in the case of screening of the DQ β gene using entire DQ β cDNA as probe, much more DP β and DX β genes were isolated than DQ β cosmid clones. Genomic DNA sequences including the DQ α and β genes might inhibit the growth of the host E. coli resulting in low number of clones containing DQ α and β genes. Then I chose 300bp PstI-DdeI fragment of pDC α 107 as probe because of low homology in 3'UT region between DQ and other genes (Auffray et al., 1987) (see Fig. 1), resulting that a cosmid clone, pAKQ4116, including DQ α gene, was obtained (Fig. 5)

Fig. 5

Several clones for the DQ β gene were isolated. Moreover, I

tried to isolate clones covering upstream region, or intervening region between DQ β and DV β , by chromosomal walking method. Two clones, pAKQ5017 and pPKQ5027-1 were obtained from AKIBA and PGF respectively using 2.7kb EcoRI fragment of pAKQ056 about 25kb upstream from the DQ β gene (Fig. 5)

In all a region of about 100kb in length including DQ α and β genes were cloned. DQ gene region restriction maps of AKIBA (DR2, DQw1, Dw12) and PGF (DR2, DQw1, Dw2) were like each other, however, some differences were observed (Fig. 6) and those polymorphisms concentrated near the DQ β gene. It suggests a possibility to determine the Dw specificities by investigation of the structure of DQ β region. It is described in my accompanying paper (Kawai, 1990).

The restriction maps of the region about 50kb upstream from the DQ β gene of AKIBA were remarkably differed from that of PGF suggested a dynamic change of gene structure among various Dw types in this region. Then, two fragments from the cosmid clones derived from AKIBA were used as probes to analyze gene structure upstream from DQ β gene. Although 2.7kb EcoRI fragment of pAKQ5017 hybridized to the 1.6kb EcoRI fragment of pAKQ5027 located on the same position as AKIBA, 0.85kb EcoRI fragment of pAKQ5017 hybridized to 1.0KB EcoRI fragment of pPKQ5027 located about 5kb upstream from the corresponding position in AKIBA (Fig. 6B), suggested insertion or deletion of 5kb length might Fig-6 have occurred. This deletion/insertion event suggested other many

deletion/insertion events in HLA class II gene region. It might explain the different distance between DV and DQ genes (Inoko et al., 1988)

I also confirmed that cosmid clones included the DQ gene by mAb reactivity against cell surface products on DQ cosmid-transfectants. Two clones of the transfectants designated, L/A.DQ8-25 and L/P.DQA.3, were obtained. L/A.DQ8-25 was transfected with the AKIBA DQ α (pAKQ4116) and DQ β (pAKQ056) genes and L/P.DQA.3 with the PGF DQ α (pPQ364) and DQ β (pPQ362) genes. Analysis of the L/A.DQ8-25 and L/P.DQA.3 transfectants using flow microfluorimetry and anti-HLA class II specific MoAbs clearly indicated expression of the DQ gene products on the cell surface. They reacted with the anti-DQ MoAb, Leu10 but not with Fig.7 anti-DR MoAbs, L243 and Hu30 (Data not shown), confirming that the cosmid clones cloned here encode the DQ gene (Fig. 7) It is not clear that weak binding of Leu10 against L/P.DQA.3 transfectant is due to low amount or aberrant conformation of expressed DQ antigen. it was reported that the transformant class II molecules differed from "normal" class II molecules in their carbohydrate moiety (Tanigaki et al., 1987; Tosi et al., 1986)

Structure of the DR subregion

Two sets of cosmid clones including three DR β genes spanning about 140kb, and one DR α gene spanning about 70kb were isolated. They were mapped using exon-specific cDNA fragment

probes, although the TM/CY exons could not be distinguished precisely (Fig. 8). Three β genes were designated β I, β II and β III, following their orientations of transcription. An additional region homologous to the DR β leader sequence was found about 50kb downstream from the β III gene. Spies et al. (1985) also reported that the genetic region between the DR β and DR α genes contained four repeats of the DR β leader sequence in DR4 haplotype.

One DR α gene was cloned (Fig. 8) and the β_1 domain exon downstream from the DR α gene described previously (Meunier et al., 1986) was also identified at the same position in the cosmid, pPR541. The restriction map near the DR α gene in the DR2 haplotype was almost identical to those of other haplotypes (Spies et al., 1985; Meunier et al., 1986; Das et al., 1983), although in the 5' upstream flanking region it differed remarkably from others. Homology of the restriction map in the DR α gene region among the haplotypes seemed to be confined to about 30kb around the DR α gene.

In the course of this restriction mapping, the DR β II gene failed to hybridize to the probe specific for the 5'UT region of the DR β cDNA even under less stringent conditions (Fig. 9), suggesting that it might have been a pseudogene. Additionally, no transfectant was obtained expressing a product detected by anti-HLA-DR MoAb, L243, after the DR β II and the DR α gene-cotransfection to mouse L cells (Data not shown). This structure

of the DR β II gene differed from those of other haplotypes, DR3 and DR4 in that it contained all of the exon except for the first (Spies et al., 1985; Rollini et al., 1985, 1987; Larhammar et al., 1985). In contrast to the DR β II gene, both the DR β I and the DR β III genes contained the 5'UT, β_1 , β_2 and 3'UT exon necessary to express the HLA class II antigen, implying that these two DR β genes should be expressed, which was confirmed by transfection experiments as described later

The number of HLA DR β genes

Although every DR haplotype (DR1-9) has one DR α gene, DR β genes were reported to vary in number between different DR specificities. Böhme et al. (1985) described three DR β genes in DR2, 3, 4, 6 and 7, two genes in DR1, one gene in DR8 and two or three genes in DR5 and 9. Andersson et al. (1987) obtained four DR β genes in genomic clones from the haplotype, DR4Dw4. Additional isolated exon for the DR β chain observed between the DR α and DR β genes (Andersson et al., 1987; Larhammar et al., 1985; Spies et al., 1985; Rollini et al 1985; Meunier et al., 1986) Then, Southern blotting of genomic DNA extracted from AKIBA and PGF were carried out using each DR β domain specific probe under the stringent condition to estimate the number of the DR β gene exon in DR2 (Fig.10). High molecular weight DNA of Fig.10 7 μ g after digestion with restriction enzymes, EcoRI, BamHI and PstI, were electrophoresed, transferred to nylon membranes, and

hybridized by the exon specific probes. The cross-hybridizations to other subregion genes was limited under the conditions used. Each probe gave two or three bands except for the result using the DR β_1 domain specific probe. Some polymorphisms were observed between DR2Dw2 (PGF) and DR2Dw12 (AKIBA) using the leader sequence specific probe. The results are summarized in Table I. No difference in the number of hybridizing bands between AKIBA and PGF, for example three bands using EcoRI and 3'UT probe, suggested that AKIBA and PGF have the same number of the DR β gene exons. All hybridizing bands in AKIBA corresponded to the exon-containing region shown in the restriction maps of the DR β region (Fig.8), and no additional band observed suggested no other DR β gene exon than those cloned here. Then, I concluded that all of the DR β genes was isolated.

The polymorphic DR antigen is encoded by the DR β III gene.

Two DR β cDNA sequences were reported previously (Lee et al., 1987) and two DR β chains were detected in two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis (Nakatsuji et al., 1986; Nepom et al., 1984b; Sone et al., 1985). An acidic one is a polymorphic chain possibly encoding the Dw specificity, which has the variable amino acid sequences unique to the different Dw subtypes of DR2 (Dw2, Dw12 and Dw21) and the other basic one is a nonpolymorphic chain which has a more conserved amino acid sequence as well as isoelectric point. To distinguish

the polymorphic from the nonpolymorphic gene, each cosmid containing the DR β I, β II or β III gene was digested with a restriction endonuclease, BssHII, which recognizes the sequence "GCGCGC", and should cut in exon 2 (β_1 domain) of the polymorphic, not of the nonpolymorphic DR β gene at the position of nucleotide 213 as predicted from the previously reported nucleotide sequence of the cDNA clones (Fig. 11A), and subjected Fig. 11 to Southern hybridization with the β_1 domain-specific probe (Fig. 11B and C). Fig. 11 showed that neither the DR β I nor the DR β II gene contained a BssHII site, but unexpectedly, the 3.2kb fragment encoding the β_1 domain of the DR β III gene in pAKR4617-1 and pPR241 was cleaved to produce a 2.3kb fragment, establishing that the DR β III gene had a BssHII site. Therefore, I can conclude that the polymorphic and nonpolymorphic DR2 antigens are specified by the DR β III and DR β I genes, respectively, which is the reverse of the situation in other haplotypes such as DR3, DR4 and DR6 (Spies et al., 1985; Rollini et al., 1985; Gorski et al., 1985; Andersson et al., 1987; Tosi et al., 1986).

Arrangement of the three DR β genes on chromosome 6

Inoko et al. (1988) constructed a large-scale genetic map of the HLA class II region in the DR2Dw12 haplotype (AKIBA) by pulsed field gel electrophoresis and found only two SfiI sites in the DR-DQ/DX subregions. One site was located between the DR α

and β genes, and another between $DX\alpha$ and $DV\beta$ genes, giving the 330kb *Sfi*I fragment shared by $DV\beta$, $DQ\beta$, $DQ\alpha$, $DR\beta$ I, $DR\beta$ II and $DR\beta$ III genes. Therefore, I searched for an *Sfi*I site in my DX - $DV\beta$ and $DR\beta$ cosmid clones, both *Sfi*I sites, one was located 25kb downstream from the $DR\beta$ III gene and another 6kb upstream from the $DV\beta$ gene were found (Fig.12). Thus, *Sfi*I cleaved 10kb fragment of pA412 encoding DX - DV subregion and 6.2kb of pAKR5414 encoding downstream flanking region from $DR\beta$ III gene (Fig.12B). These results clearly indicated that the order of the class II genes in the DR2 haplotype was $DX\beta$ - $DX\alpha$ - $DV\beta$ - $DQ\beta$ - $DQ\alpha$ - $DR\beta$ I- β II- β III- $DR\alpha$ from the chromosomal centromere. This gene order and orientation of transcription in the DR subregion of the DR2 haplotype are identical to those of DR3 and DR4 as previously described (Spies et al., 1985; Rollini et al., 1985; Andersson et al., 1987).

Transfection and expression of DR genes in mouse L cells.

I obtained two clones of the transfectants designated, L/ $DR\alpha + \beta$ I and L/ $DR\alpha + \beta$ III, after separately cotransfecting pAKR4643 ($DR\beta$ I) and pPR241 ($DR\beta$ III) together with pPR541 ($DR\alpha$), respectively. Analysis of the L/ $DR\alpha + \beta$ I and L/ $DR\alpha + \beta$ III transfectants using flow microfluorimetry and a panel of anti-HLA class II specific MoAbs clearly showed successful transfection expression of the HLA-DR products on the cell surface. They reacted with the anti-DR MoAbs, L243 and Hu4 (Koide et al., 1982)

(data not shown) but not with Leu10(anti-DQ) or B7/21(anti-DP) (Fig.13). Binding of the MoAb Hu30 to L/DR $\alpha + \beta$ I, but not to L/DR $\alpha + \beta$ III is consistent with the result of 2D-PAGE studies showing that Hu30, which recognizes both DR1 and DR2 cells, is specific for the nonpolymorphic and supertypic DR2 antigen (Nishimura et al., 1984). Weak binding to the L/DR $\alpha + \beta$ I transfectant by the AB8.25 MoAb suggested that a common determinant recognized by the anti-DR2, DR5 and short DR3 MoAb, AB8.25, was shared by the nonpolymorphic DR2 (DR α /DR β I), DR5 and short DR3 molecules. Neither of the two transfectants was found to react with the MoAb NDS15.38 which was previously described to be anti-DR2 (Makgoba et al., 1983).

Absorption of DR2 alloantisera by the DR gene-transfectants.

To investigate which of the DR β I and DR β III genes determines the so-called DR2 specificity for serological characterization of their products, the absorption assays was adopted using specific alloantisera. After absorption by the transfectants alloantisera were tested for cytotoxic ability against LCLs and B cell-enriched fractions from PBL as target cells. The alloantisera used in Fig.14, 1030, 1026 and 1029 were selected from the best sera against DR2 submitted to the 10th IHWS. Ex107, used as a negative control, is an anti-DR4 serum. As shown in Fig.14, L/DR $\alpha + \beta$ I and/or L/DR $\alpha + \beta$ III significantly absorbed the DR2 alloantibodies of 1030, 1026 and 1029, but never

of Ex107. L cells did not reduce the cytotoxic titer of these alloantisera. These results imply that the DR gene products expressed on the cell surface of the transfectants adopt a normal conformation. The alloantibodies, 1030 and 1026 were absorbed by both L/DR $\alpha + \beta$ I and L/DR $\alpha + \beta$ III transfectants. On the other hand, 1029 was absorbed only by L/DR $\alpha + \beta$ I, suggesting that the DR2 determinants were comprised of at least two epitopes, and one was recognized by 1030 and 1026 residing on both the DR2 (DR α /DR β I) and DR2 (DR α /DR β III) molecules, the other by 1029 only on the polymorphic DR2 molecule, the DR α /DR β III heterodimer

Four local alloantisera were also used in the transfectant absorption assay (Table II). All of the DR2 alloantibodies tested were absorbed by both or either transfectants. Ex115 seemed to contain at least two alloantibodies, one against DR2 and the other against DR1 and/or DR4. A determinant recognized by Ex115 was located both on the nonpolymorphic and polymorphic DR antigens. Ex99 could detect the common epitope expressed both on the DR1 and nonpolymorphic DR2 (DR α /DR β I) molecule. JR21-224, like 1029, recognized the DR2 epitope specific for the polymorphic DR2 (DR α /DR β III) molecule. These results suggested that the DR antigens in DR2 had at least three allo-determinants defined by the alloantisera used here. One of them was the public or supertypic e.g., the common determinant both on the DR1 and DR2 antigens recognized by Ex99. The remaining two were the DR2

private determinants, one both on the DR2(DR α /DR β I) and DR2(DR α /DR β III) heterodimers recognized by 1026, 1030 and Ex115, and the other one on the DR α /DR β III heterodimer recognized by 1029 and JR21-224.

DISCUSSION

By screening the human genomic libraries constructed from HLA-homozygous B cell lines, AKIBA (DR2, Dw12, DQw1) and PGF (DR2, Dw2, DQw1), with the cDNA clones and successive gene-walking procedures, about 440kb DNA from the HLA-D region has been cloned and characterized.

Determination of encoding gene by isolated clones.

Nucleotide sequences of HLA class II genes have conserved homologies, so that cross hybridization were observed both in the colony and in the Southern hybridization, Hybridization have been proceeded under the stringent condition to eliminate cross hybridization as possible. Moreover, short fragments of cDNAs, encoding the subregions which were low homology among whole HLA class II genes, were used as a probe, resulting successive isolation of genomic clones. Regions encoded by each isolated cosmid were determined using following strategies. (1) Homology between these cosmid clones and well-characterized cDNA clones using southern hybridization. (2) Comparison of restriction maps

of isolated clones with previously published class II genes. (3) Some cosmid clones including class II genes were transfected into mouse L cells, and expressed products on their cell surfaces were detected by class II antigen-specific mAb.

HLA class II genes and their organization

I have cloned DO α , DX α , β , DV β , DQ α , β , and DR α , β genes from AKIBA and PGF. Exon-intron organization of DX α , β , and DQ α , β genes are same to those of other haplotypes, (Okada et al., 1985; Jonsson et al., 1987), however, that of DR gene of DR2 haplotype has unique structure in comparison of other haplotypes.

DO α was reported to have less polymorphism. The restriction map around AKIBA DO α was identical to that of the DO α gene isolated from the genomic library of Maniatis (Inoko et al., 1985a). Localization of other class II gene α and β suggest that a couple of α and β genes encoding class II antigen were found to be close each other, about 20kb. I could not, however, find β chain gene encoding β chain expected to associate with DO α chain. β chain gene associating with DO α is remained unclear.

DX α and β genes have high homology with DQ α and β , respectively. at levels of nucleotide sequence, exon-intron gene organization, gene size and direction of transcription (Okada et al., 1985) No expression of DX gene, however, was reported until now. DX α and β genes have less polymorphisms among various

haplotypes and Southern blotting pattern suggested no difference between the map of AKIBA (DR2,Dw12,DQw1) and of PGF (DR2,Dw2,DQw1) (Data not shown).

DV β cosmid clones were obtained from the libraries of AKIBA and PGF (Fig. 4A). Restriction map analysis and Southern blotting using locus specific probes revealed that DV β gene located 15 kb upstream from the DX α gene. In addition to the string sequence homology of DV β with DQ β (Ando et al., 1986a; and confirmed in Fig. 4), mapping of the DV β gene between the DX and DQ subregion strongly suggests that DV β gene has been generated by gene duplication from the DX β or DQ β gene or their common ancestral gene in the process of the class II gene evolution.

I cloned more than 90kb long region around DQ α and β genes from two DR2DQw1 haplotypes, AKIBA (Dw12) and PGF (Dw2), and compared their gene organization, indicating the two characteristic differences. They are restriction site map polymorphisms around the DQ β gene and the deletion/insertion event upstream from the DQ β gene. Restriction site differences between these haplotypes which were concentrated near the DQ β gene, suggested the possibility of Dw typing by analysis of the DQ β gene region, and it was studied in accompanying paper (Kawai, 1990)

Dw specificities are determined by MLR, in which alloreactive T cells respond to proliferate against allogeneic class II antigens. Two reasons why DQ gene restriction map

associates with Dw specificities. One is that Dw specificities are determined only by DR genes and restriction map polymorphisms are linked to the DR gene. The other is that DQ gene could present alloantigen to T cells. It was reported that HLA DQ antigen expressed on mouse L cells by gene transfection stimulate MLR responses (Nakatsuji, 1987) and DQ gene could control antigen specific immune response (Nishimura et al., 1990, Hirayama et al., 1987)

I have cloned three DR β genes by overlapping cosmid clones encompassing 140kb of continuous genomic DNA, and also one DR α gene from the DR2 genomic libraries (Fig. 8) These clones include three DR β genes, one DR α gene, and isolated two exons (one leader sequence and one β 1 exon) Böhme et al. (1985) suggested that three DR β genes existed in the DR2 haplotype. Indeed, digestion with EcoRI, BamHI and PstI followed by hybridization with the 3'UT specific probe revealed the presence of three bands in genomic DNAs isolated from AKIBA (DR2, Dw12) and PGF (DR2, Dw2), corresponding to each of the three DR β genes cloned here (Fig. 10 and Table II), suggesting that these cosmid clones spanned the entire DR β subregion.

The organization of the HLA class II genes in the DR2 haplotype was determined to be centromere-DX-DV β -DQ-DR β I- β II- β III-DR α -telomere (Fig. 12) This arrangement and the orientation of three DR β genes is similar to other haplotypes such as DR3 and DR4 (Spies et al., 1985; Rollini et al., 1985;

Andersson et al., 1987) The similarity extends to the restriction maps around the DR α gene, the existence of the isolated β_1 exon next to the DR α gene, the SfiI site downstream from the DR β III gene (Inoko et al., 1988) and of the isolated DR β leader sequence downstream of the DR β III gene (Fig. 8). This conservation of arrangement suggests that the establishment of the organization of the region preceded the evolutionary diversification of the DR haplotypes. On the other hand, the differences in the gene structure around the DR β III gene, such as the lack of the DR β leader sequence in DR2 which was identified 20kb downstream from the DR β III gene in DR3 and DR4, and the absence of the leader sequence in the DR β II gene, must reflect a degree of independent DR β gene organization in some of the DR haplotypes (Fig. 15)

Antigenicity of the two DR antigens.

Restriction enzyme digestion and transfection experiments clearly showed that the DR β I and DR β III genes encode the nonpolymorphic and polymorphic DR β chain, respectively. This DR β I gene corresponds to the nonpolymorphic gene product which reacts with the supertypic MoAb, Hu30 after association with the DR α chain. On the other hand, mRNA from the DR β III gene corresponds to the polymorphic cDNA containing the BssHII site in the β_1 exon, and its product does not react with Hu30 (Fig. 11 and Fig. 13) Although in other haplotypes such as DR4, DR3 and

DR6 (Spies et al., 1985; Rollini et al., 1985; Gorski et al., 1985), the nonpolymorphic or supertypic DR β chain, which encoding DRw52 and DR253 specificity. was demonstrated to be encoded by one of the most telomeric DR β genes (DR β III in DR3,6, and DR β IV gene in DR4), in DR2 the DR β I gene represents the nonpolymorphic DR β chain (Fig.15). These facts indicate that the susceptibility to mutations or gene conversions responsible for genetic polymorphism or diversity does not depend on the location of gene on the chromosome, but is a property of the gene itself. Furthermore this factor controlling the susceptibility might exist either within the gene or near it, because it can affect about 10kb area surrounding it.

How has the apparent inversion of DR β genes happened? Two mechanisms are possible. 1) reciprocal intergenic exchange between the DR β I and β III genes. Reciprocal genetic exchange might have frequently occurred between the two DR β genes, resulting in generation of a polymorphic DR2 allele in the DR β III gene, together with creation of the nonpolymorphic DR β I gene. Wu et al. (1986) suggested reciprocal intergenic exchange responsible for generation of Dw21 (Dw" FJO") from Dw12. 2) change of the susceptibility to gene exchange, divergence or mutation. After establishment of the DR gene structure by a succession of gene duplications, divergence and gene conversion, one gene might have changed to be variable and other gene to be conservative. In either case, these events might undergo concurrent

diversification of the DR2 haplotypes, because both AKIBA and PGF with the same DR2 but with different Dw specificities (Dw12 and Dw2, respectively) similarly had the polymorphic DR β III gene and the nonpolymorphic DR β I gene.

I described here that the single DR antigen in DR2 had multiple determinants and the so-called DR2 specificities were determined by several epitopes, some of which were expressed on both the polymorphic and nonpolymorphic DR antigens (Fig. 14 and Table II). Similarly, in other haplotypes, it has been established that some DR antigenic determinants exist on two DR molecules distantly-related to each other. For example, the nonpolymorphic DRw52 and polymorphic DR3 molecules share the common determinant recognized by the MoAb, Tr81 (Berte et al., 1988). Although Maeda and Hirata (1984) reported that the DR2 alloantiserum precipitated two DR molecules in 2D-PAGE analysis, the possibility remains that the alloserum contained polyclonal antibodies. Indeed, anti-DR1, 3, 4 and 8 antiserum, Ex115, was shown to contain polyclonal antibodies, in which anti-DR2 antibodies reacted both with polymorphic and nonpolymorphic DR2 antigens (Table II). Here, we could clearly show that both of the two DR molecules had the same allo-determinant as evidenced by the absorption assays with the transfectants expressing the single DR molecule. The fact that all DR2 sera selected here except Ex99 antiserum recognized at least the polymorphic DR molecule implies that the DR2 haplotype can be mainly defined by

the allo-determinants residing on the polymorphic DR β chain. An epitope recognized by Ex99 resides on both DR1 and nonpolymorphic DR2 antigens, indicating supertypic antigen feature correspond to DRw52 and DRw53 of other haplotypes.

Serum absorption assay suggesting the multiple determinants on the two DR molecules indicate that there are possibly three types of restriction by the HLA-DR antigens in immune responses, first restriction to the polymorphic DR molecule, second to the nonpolymorphic DR molecule, and third to both of two molecules. Wilkinson et al. (1988) clarified that M. leprae-specific T cell clones isolated from leprosy patients could use only the polymorphic DR β chain as their restriction molecule but not the nonpolymorphic DR β chain by using the DR-transfected mouse L cells.

General discussion

In this study, I analyzed gene structure and antigenicity of the HLA class II antigens in DR2. Large number of genomic clones including most of class II genes, DR, DQ, DX, DV and DO genes, were isolated. Previously, DP region have been isolated by Ando et al. (1986b) from DR2. Although DR β genes vary in number between different specificities, pulse field gel electrophoresis analysis suggested that the organization of different HLA haplotypes is apparently very similar, thus, DP, DO, DX/DV, DQ and DR (Weiss and Ziegler, 1989; Hardy, 1986) Length of clones

isolated from DR2 until now, encompasses through 500kb, which is about half of whole class II region. There is possibility that some gene(s) other than the HLA genes exist in these cloned region and that it affect the development of non-autoimmune, HLA-associated disease. Genes of the cytokines tumor necrosis factors α and β (Dunham et al. 1988; Carroll et al., 1987), the heat shock protein HSP70 (Sargent et al., 1989), and two HLA-B associated transcript genes (Banerji et al., 1990) were described in the HLA region. Gene insertion-deletion event observed in the DQ β gene upstream region might associate structure and expression of 'disease susceptibility gene'. On the other hand, in the study of HLA-associated autoimmune disease, mouse L cell transfectants expressing either DQ or DR antigen will be available. Furthermore, since antigen presenting cells, such as dendritic cells and macrophages, express all of HLA class II antigens, DR, DQ and DP on their cell surfaces, it is hard to dissociate and analyze each function of those class II antigens. It will be possible to understand the molecular basis for the immune response and MHC-disease association by use of the DR- and DQ-transfected cells described here.

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Table I Reactivity of the hybridizing bands with the DR β -exon specific probes

probe ¹	enzyme	size ²	PGF	AKIBA	expected gene-exon ³	estimated number of each exon
leader	EcoRI	17	+	+	β III-L	3
		4.9	+	+	β I-L	
		4.2		+	β' -L	
	BamHI	3.0	+			
		14		+	β' -L	
		5.7	+			
		5.0	+	+	β I-L, β III-L	
β_1	EcoRI	17	+	+	β III-L	4
		6.1	+	+	DR α -1	
		3.9		+	β' -L	
		3.8	+	+	β I-1	
		3.3	+	+	β II-1, β III-1	
	BamHI	14		+	β' -L	
		5.9	+		DR α -1	
		5.0	+	+	β II-1, β III-L	
		4.2	+	+	β I-1, β III-1	
β_2	EcoRI	6.4	+	+	β I-2	

		4.8	+	+	β II, β III-2	
	BamHI	5.0	+	+	β II-2	3
		4.3	+	+	β I-2	
		3.9	+	+	β III-2	
3' UT	EcoRI	6.4	+	+	β I-3'	
		5.1	+	+	β II-3'	
		4.6	+	+	β III-3'	3
	BamHI	7.8	+	+	β I-3'	
		3.3	+	+	β II, β III-3'	

1 Molecular size of the bands in kilobases

2 See the legend for Fig. 11

3 Abbreviations for exons are as follows; L, leader sequence; 1, β_1 domain; 2, β_2 domain; 3', 3'UT domain. DR α -1 expresses the isolated β_1 domain near the DR α gene. β '-L indicates the leader sequence down stream from the DR β III gene.

Table II Microcytotoxicity analysis of anti-DR2 alloantisera obtained after absorption by each DR gene-transfectant cell

Target cells ¹	%Cytotoxicity									
	Antisera ²		Ex99		JR21-224		-		-	
Designation	Phenotype	-3	β I ³	β III ³	-	β I	β III	-	β I	β III
Sa	DR1	100	80	80	70	10	60	10	20	30
PGF	DR2	70	0	0	80	10	80	10	20	0
SCHU	DR2	90	10	10	100	20	100	80	90	20
AKIBA	DR2	80	20	30	100	30	80	90	90	20
E418	DR2	100	0	10	100	20	90	90	80	10
DEU	DR4	80	80	100	30	0	10	0	10	10
MANN	DR7	10	0	0		N. T. ⁴			N. T. ⁴	

1 3×10^3 LCLs were used for cytotoxicity assay

2 Alloantisera specificities are as follows, Ex115, DR1,2,4,8; Ex99, DR1, 2short; JR21-224, DR2

3 Cells used for absorption, -,unabsorbed; β I,L/DR α + β I; β III,L/DR α + β III

4 Not tested

FIGURE LEGENDS

Figure 1

Restriction maps of cDNA clones for HLA class II antigens. L, leader sequence; $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$, regions coding for first and second extracellular domain of α and β chains; TM, transmembrane region; CY, intracytoplasmic region. Abbreviations for restriction enzymes are follows, H, HindIII; P, PstI; St, StuI; Sa, SacI; A, Aval R, RsaI. Open box in genomic clone, λ HMC β 19 represents the exon encoding the DV β gene.

Figure 2

Autoradiograms of colony hybridization. The replicated filters were hybridized with DO α cDNA, pDO α 20. Arrows indicate a part of positive colonies. Filters were oriented by placing a series of ink marks on their edges. (A) Primary screening of AKIBA, (B) Secondary screening; Two picked up colonies from the master plate were spread on the right and left of the nitrocellulose filter separately

Figure 3

(A) Restriction map of EcoRI for the DO α subregion. The location and orientation of the DO α gene is shown by filled box. The exons are not distinguished precisely. Three continuous cosmid clones shown below defined 50kb stretch. (B) Autoradiogram of DO α cosmid clones digested with EcoRI was shown carried out

using pDO α 20 as probe

Figure 4

An organization and restriction map of the DX-DV subregion is shown (A). The top line gives the scale in kb. The 100kb stretches of DNA are defined by overlapping cosmid clones shown below the cleavage sites of the restriction enzymes, EcoRI, BamHI, SfiI, ClaI. Down stream region from DV β gene is not yet mapped with BamHI (dotted line). The exons are shown by black boxes. L; leader, 1, 2; first and second external domains; TM; transmembrane region; 3; 3' untranslated region of the mRNA. Arrows above the map indicated the direction of transcription of each gene. Southern blot of the DX β and DV β cosmid clones, pAX523 (B) and pAK412 (C), respectively, digested with EcoRI (E) and/or PstI (P) hybridized with DQ β cDNA, pDC β 101.

Figure 5

A organization and restriction map of the DQ subregion including one α and one β genes. See a legend of Fig.4 for abbreviations. The open box 20kb upstream from the DQ β gene indicates the 2.7kb EcoRI region of pAKQ056 used as the walking probe. The cleavage sites of the restriction enzymes were shown for AKIBA.

Figure 6

(A) Differences of the DQ subregion restriction sites between

DR2Dw12 (AKIBA) and DR2Dw2 (PGF) Filled boxes indicate the DQ α and β genes. different EcoRI and BamHI sites between Dw2 and Dw12 were signified by asterisks. Hatched fragment, 0.85kb and 2.7kb EcoRI fragment, were used as site specific probe, and their hybridizing regions were showed by arrows. Southern blot of the DQ β upstream cosmid clones digested with EcoRI using 2.7kb and 0.85kb EcoRI fragments of pAKQ5017 as probe (B and C, respectively).

Figure 7

Flow microfluorimetric analysis of the transfectant, L/A.DQ8-25 and L/P.DQA.3 which express DQ antigens, using anti-HLA class II MoAbs. The specificities are: L243, HLA-DR; and Leu10, HLA-DQ. 5,000 cells were analyzed in each profile and plotted as log. fluorescence intensity against cell number by solid lines. In each profile, the negative control is the transfectant cell line stained with FITC rabbit anti-mouse Ig alone shown by dotted lines.

Figure 8

(A) Molecular map of the DR subregion. The top line gives the scale in kb. The location of exons is shown by filled boxes. See the legend of Fig.4 for abbreviations. Overlapping cosmid clones were shown below the cleavage sites of the restriction enzymes.

Figure 9

Southern hybridization of the DR β cosmid clones using domain-specific cDNA fragments as probes under non-stringent conditions. DNA from the cosmids pAKR4643 (DR β I), pAKR4647 or pAKR5109 (DR β II), and pAKR4617-1 (DR β III) were digested with EcoRI and electrophoresed. The probes used were pDR β 102 160bp PstI-SacI fragment (A), pDR β 101 (Inoko et al., 1985b) 140bp PstI fragment (B), pDR β 102 PstI 520bp fragment (C) and pDR β 102 290bp PstI fragment (D), which encode the leader, β_1 , β_2 +CY/TM and 3'UT region of the DR β chain, respectively.

Figure 10

Southern blot of genomic DNA using DR β domain specific probes. High molecular weight DNA (7 μ g) from AKIBA and PGF were digested with the restriction endonucleases, EcoRI, BamHI and PstI, electrophoresed and transferred to nylon membranes. Probes used were the each domain specific fragment of pDR β 102 as follows: PstI-SacI 160bp fragment (leader sequence), PstI-RsaI 70bp (β_1 domain) (B), PstI 520bp fragment (the β_2 domain) (C) and PstI 290bp (3'UT) (D)

Figure 11

(A) Nucleotide sequences of the β_1 domain of the two DR β chains from the DR2Dw12 cell line (BGE) taken from published data (Lee et al., 1987). The polymorphic sequence is labeled "a", and the

nonpolymorphic sequence is labeled "b" Dashes in the sequence "b" indicate identity with the sequences "a" The site cleaved (a) or not cleaved (b) by the restriction endonuclease, BssHII which recognizes the sequence "GCGCGC", is boxed. Electrophoresis (B) and Southern hybridization (C) of the DR β genes singly digested by EcoRI (E) or BssHII (II), or double digested by EcoRI+BssHII (E+B) with the β_1 domain specific cDNA fragment as a probe. Each lane contained DNA from the cosmid clones pAKR4643 (DR β I), pAKR5109 (DR β II) and pAKR4617-1 (DR β III) derived from AKIBA, pPR209 (DR β II) and pPR241 (DR β III) derived from PGF.

Figure 12

(A) Two SfiI sites in DX-DR subregions. A large scale map and fragments obtained by using pulsed field gel electrophoresis analysis with SfiI digestion are illustrated (Inoko et al., 1988). The alignment and the orientation of the DX-DV genes and the three DR β genes predicted from the position of the SfiI site in my cosmid clones are shown. (B) Electrophoresis of the cosmid clones encoding the DR β and DX-DV β subregions digested by EcoRI (E) or by EcoRI+SfiI (E+Sf) Each lanes contained DNA from the cosmid clones. DNA and its coding region of each lane are; pA412, DX α -DV β ; pAKR4643, DR β I; pAKR5109, DR β II; pAKR4617-1, DR β III; and pAKR5414, downstream from DR β III.

Figure 13

Flow microfluorimetric analysis of the transfectant, L/DR $\alpha + \beta$ I and L/DR $\alpha + \beta$ III (which express the DR α /DR β I and DR α /DR β III heterodimers on the cell surface, respectively) using a panel of anti-HLA class II MoAbs. The specificities of each MoAbs are: L243, HLA-DR; Hu30, HLA-DR1 and DR2; NDS15.38, HLA-DR2; AB8.25, HLA-DR2, DR5 and short DR3; Leu10, HLA-DQ and B7/21, HLA-DP. 5,000 cells were analyzed in each profile and plotted as log. fluorescence intensity against cell number by solid lines. In each profile, the negative control is the transfectant cell line stained with FITC rabbit anti-mouse Ig alone shown by dotted lines.

Figure 14

Cytotoxic activities of various alloantisera after absorption by L cells (L), L/DR $\alpha + \beta$ I (β I) or L/DR $\alpha + \beta$ III (β III), or unabsorbed (-) against LCLs (PGF, AKIBA, SCHU and E418, DR2) and B cell-enriched fraction from PBL (JK, DR4/-) used as target cells. Specificities of alloantisera used are as follows, 1030, 1026 and 1029, DR2; Ex107, DR4.

Figure 15

Comparison of the DR gene region structure among various haplotypes. References for other haplotype than DR2 as follows; DR3 (AVL), Rollini et al. (1985); DRw6 (HHK), Gorski et al. (1987); DR4 (B. O.), Andersson et al. (1987); and DR4 (Priess), Spies et al. (1985). See the legend of Fig. 4 for abbreviations.

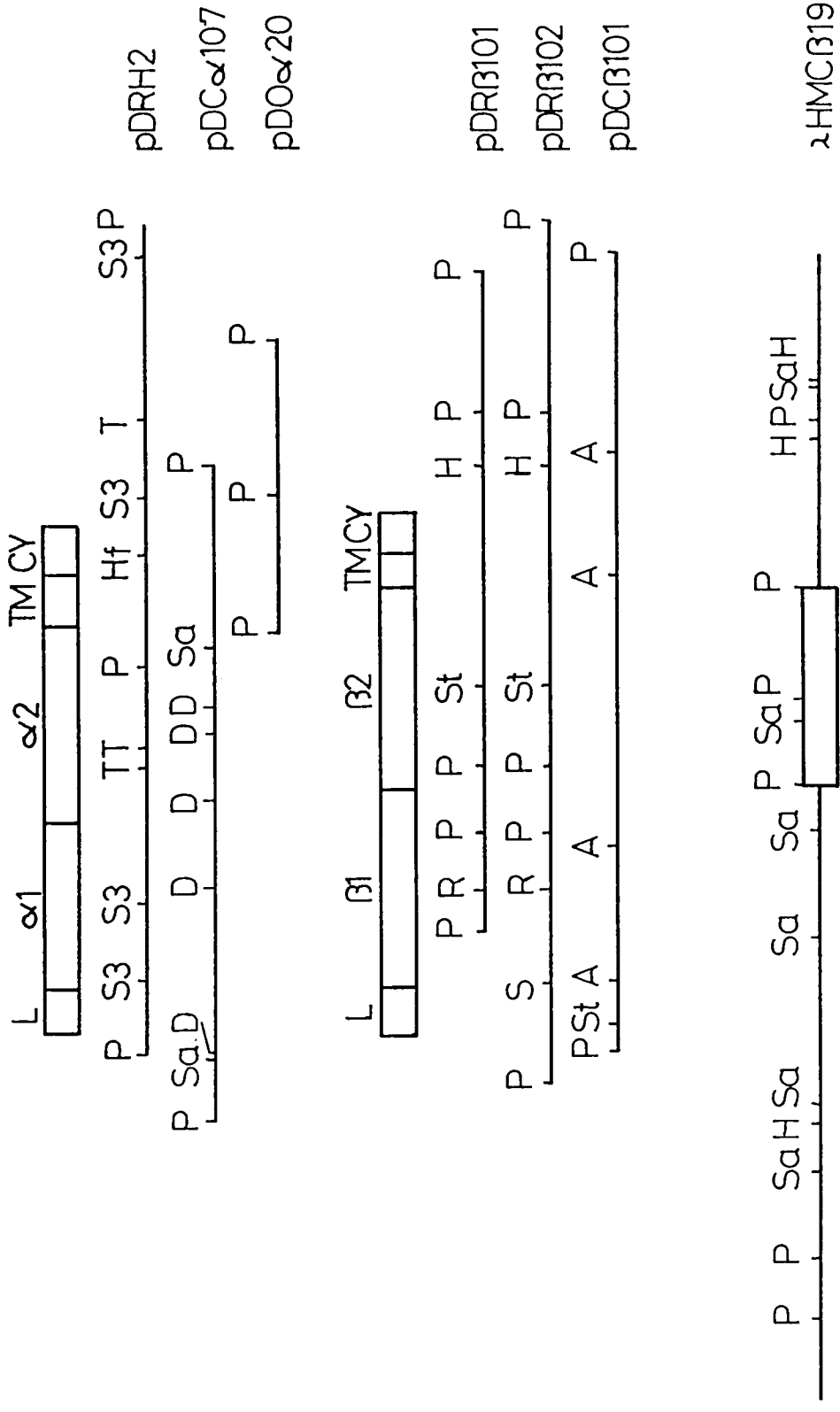
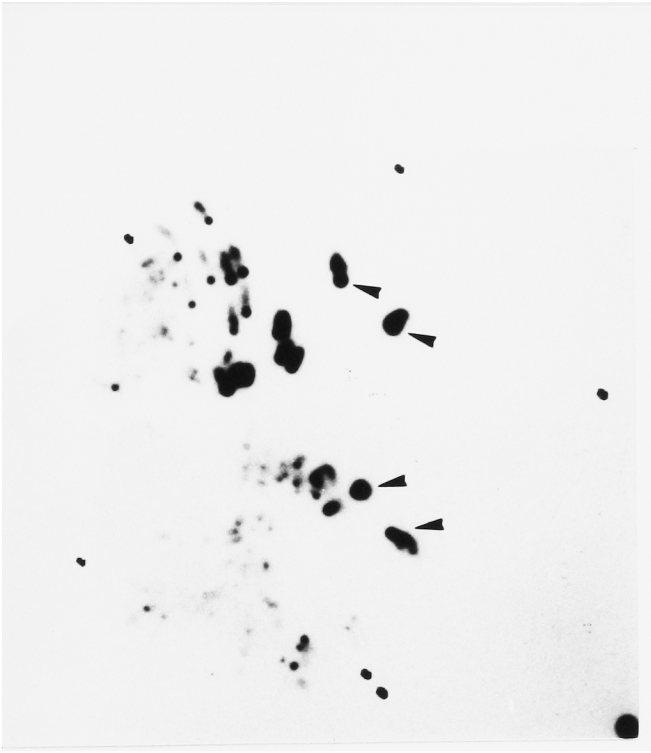


Fig. 1

B



A



A

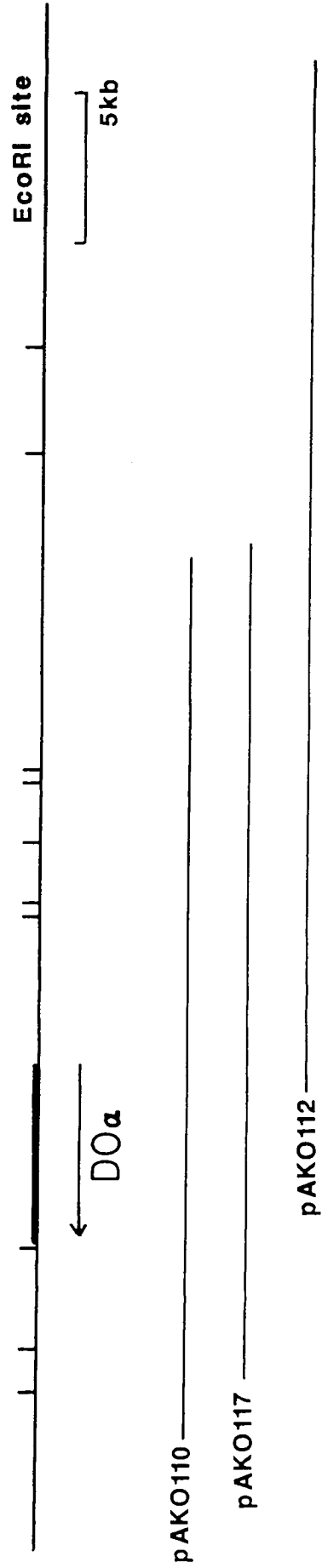
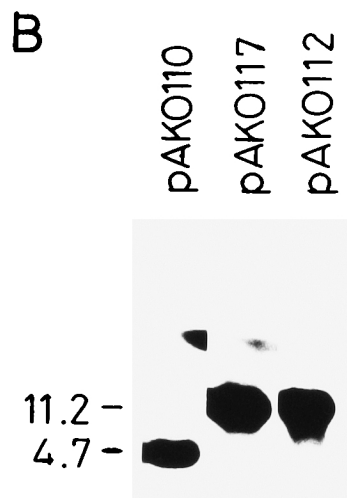


Fig. 3

Fig. 3



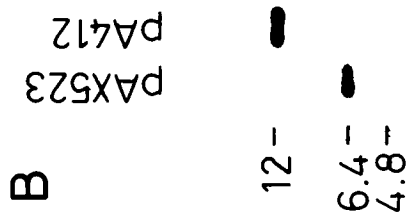
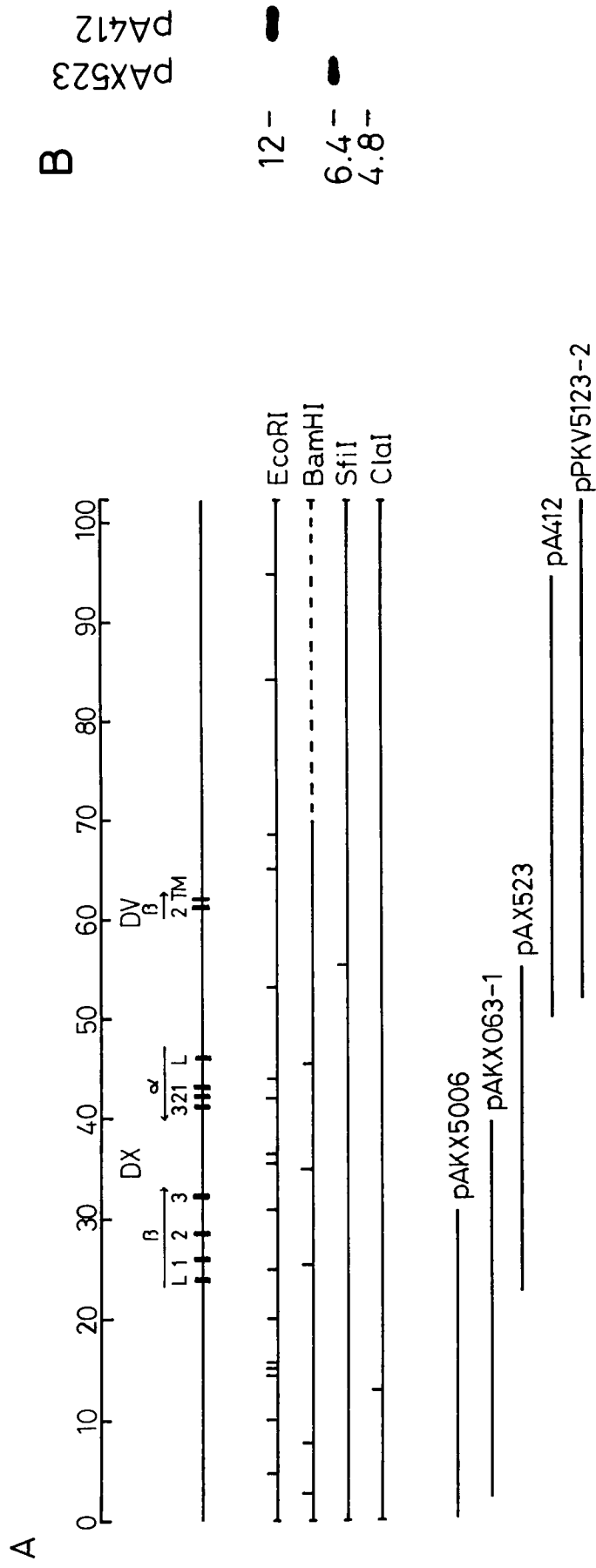


Fig. 4

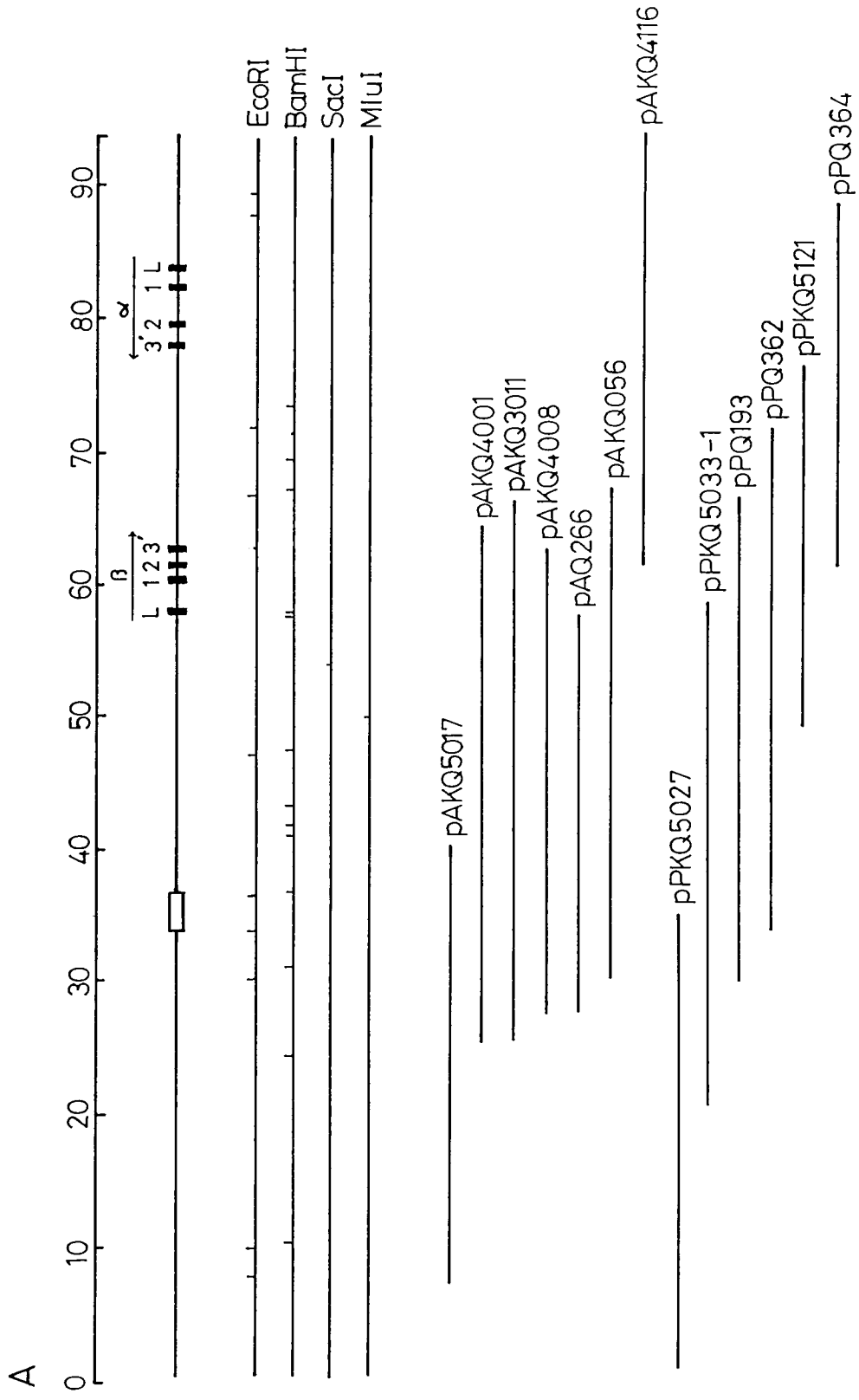


Fig. 5

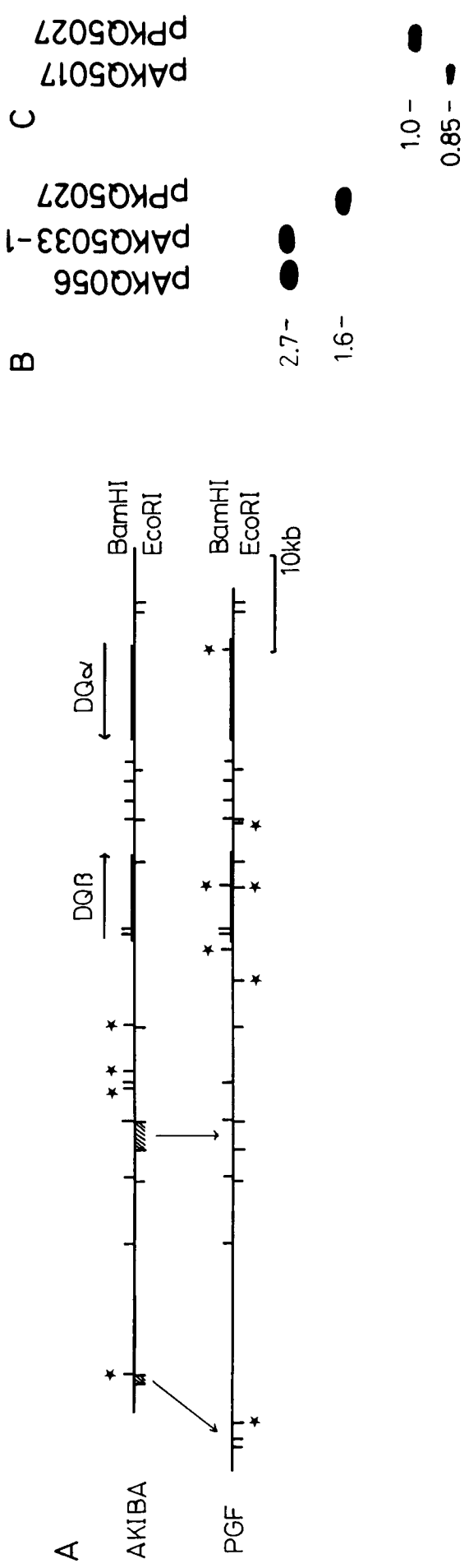
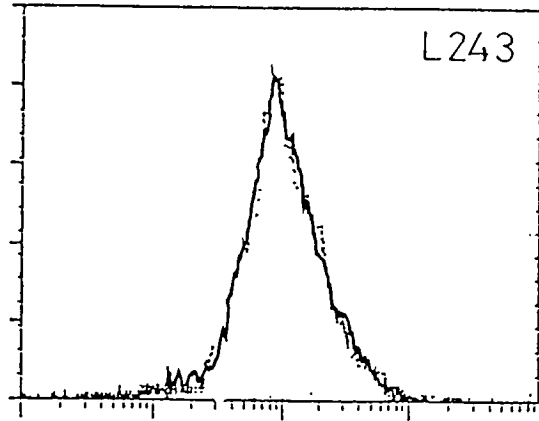
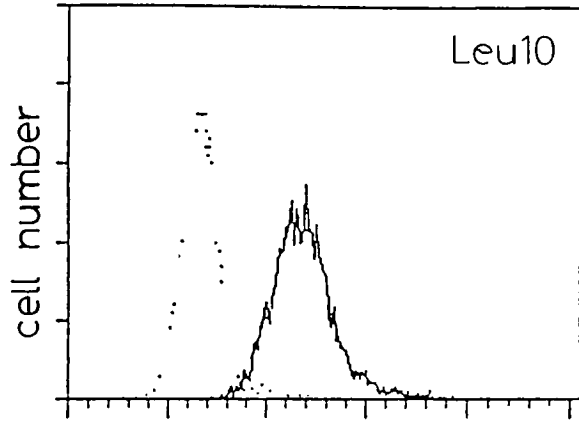


Fig. 6

L/A.DQ8-25



L/P.DQA3

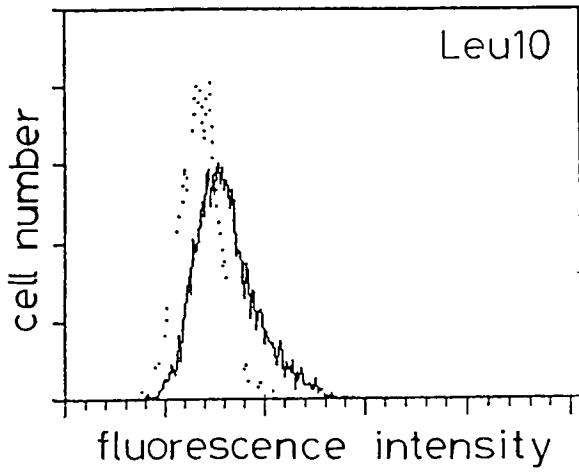


Fig. 7

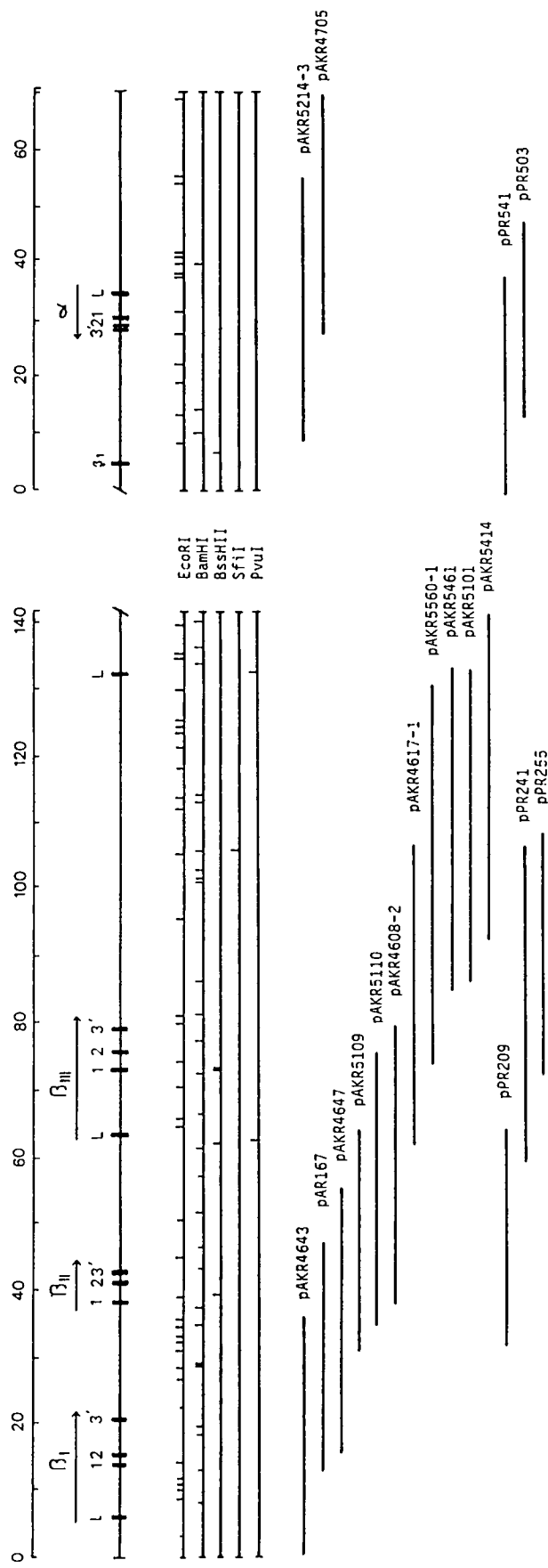


Fig 8

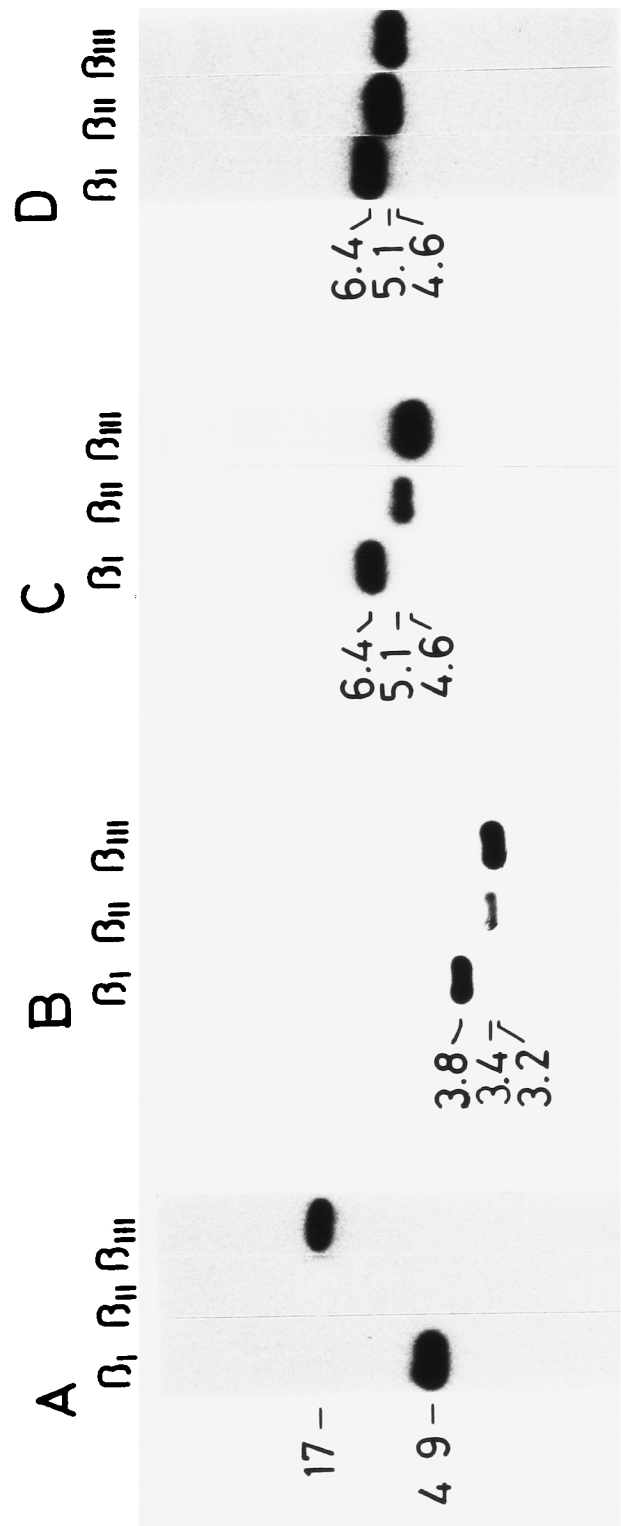


Fig. 9

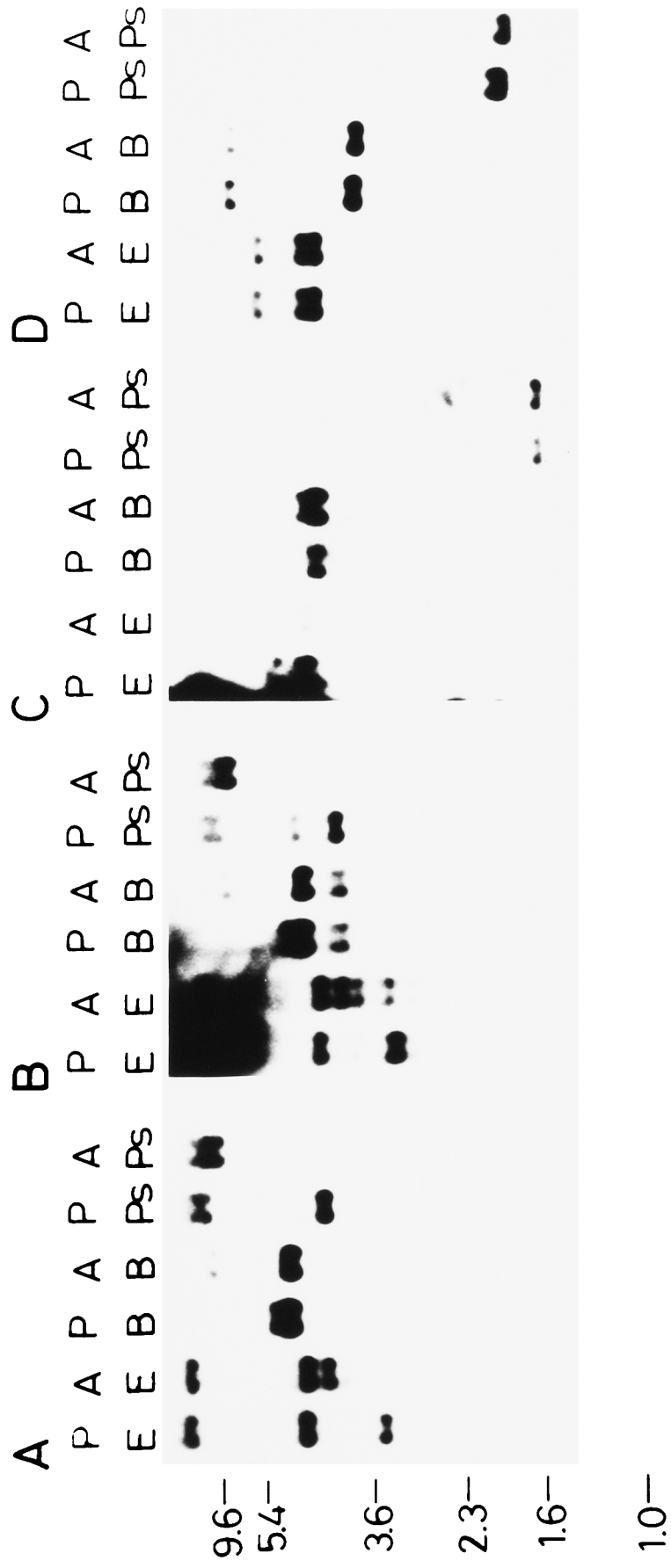


Fig. 10

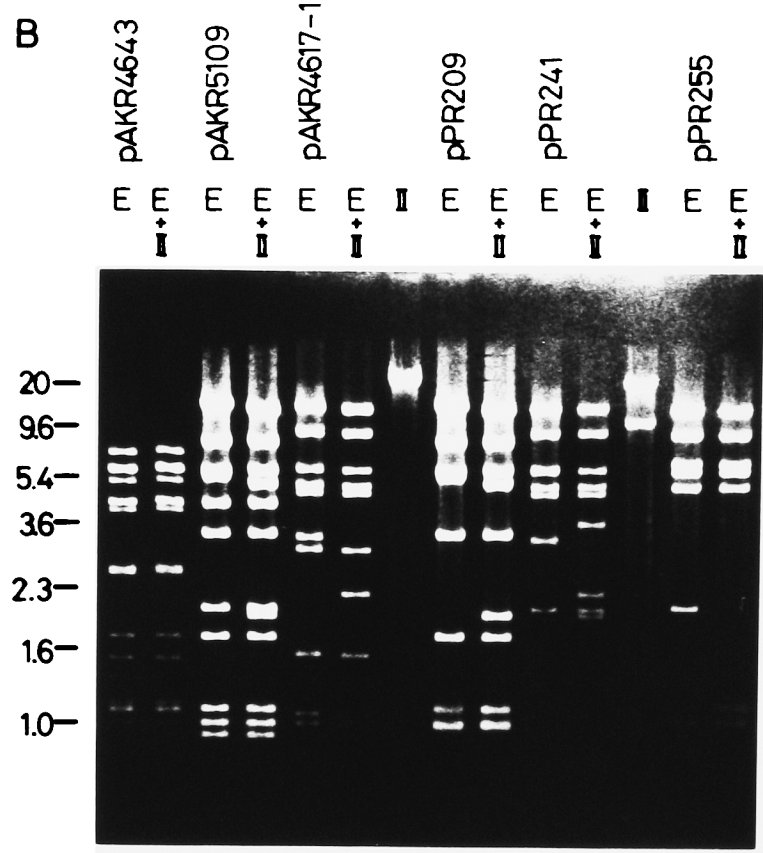


Fig 11

A

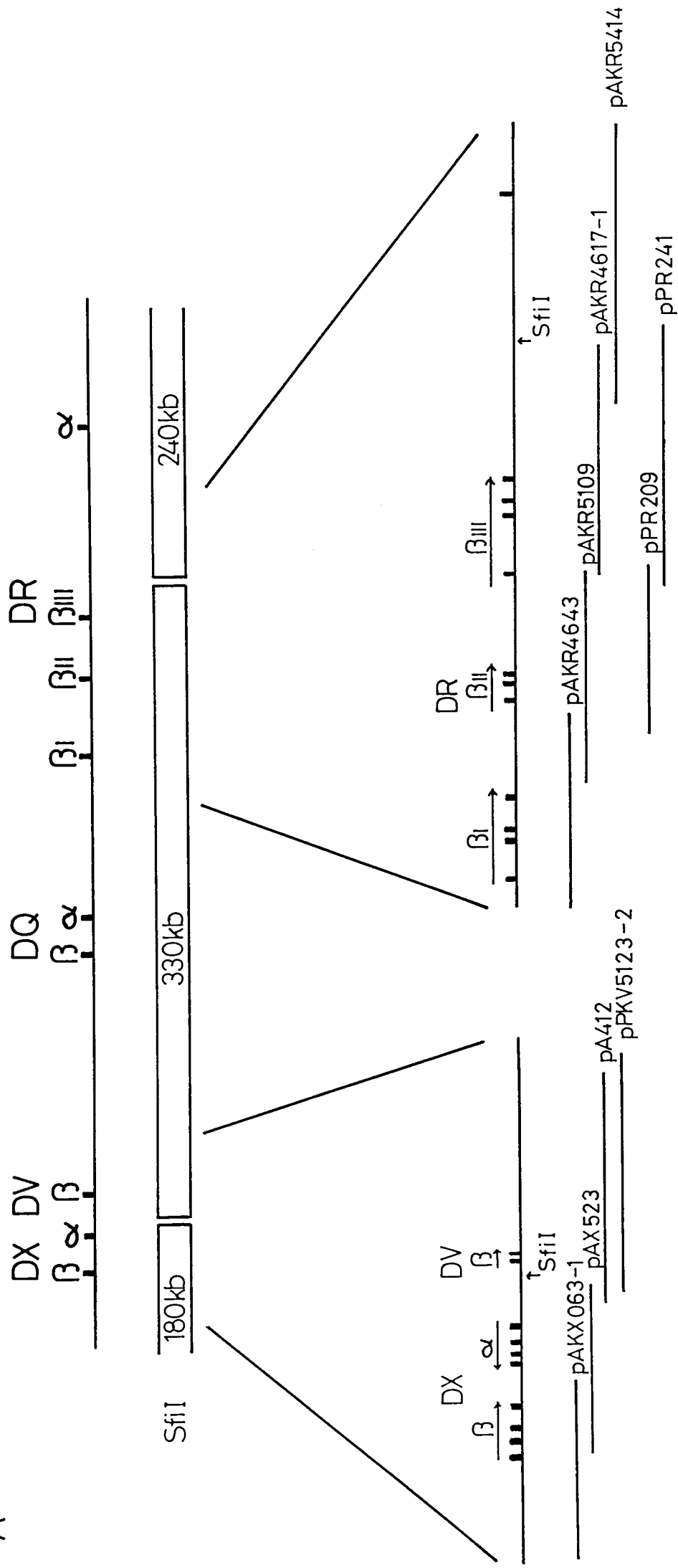


Fig. 12

B

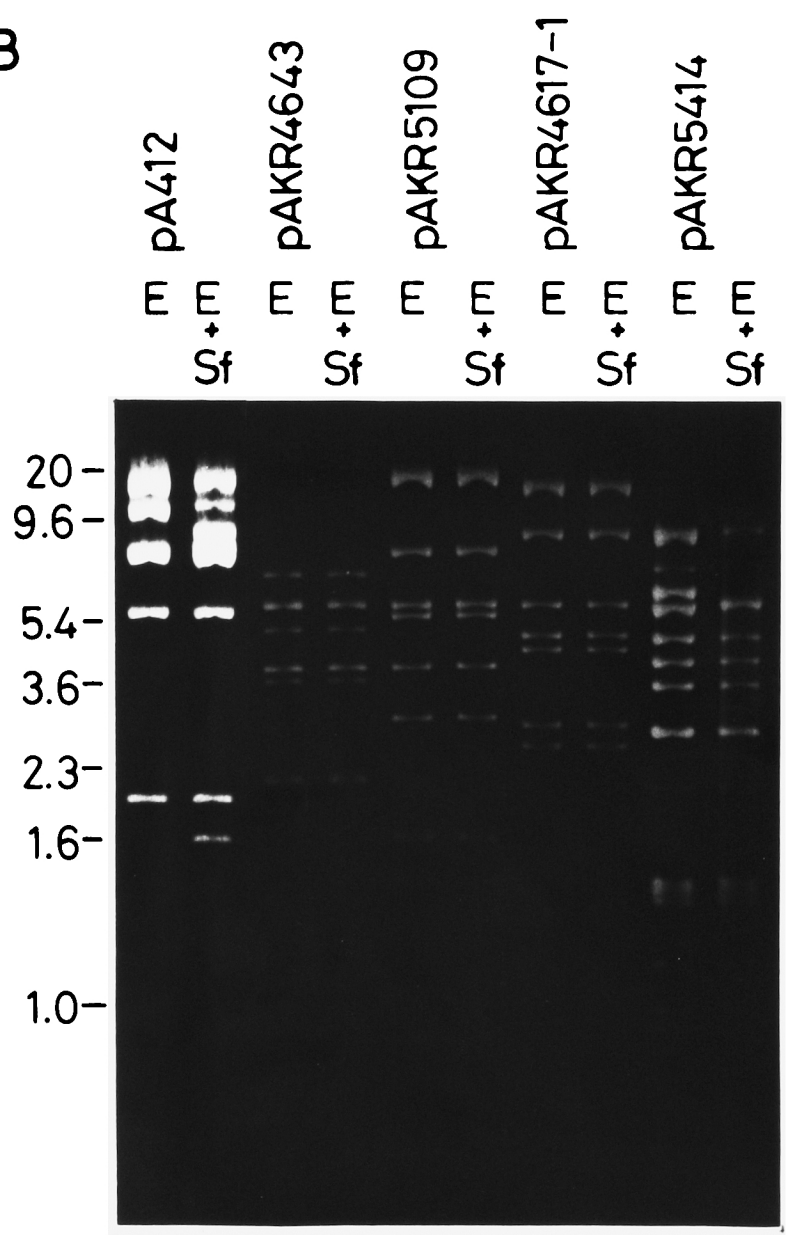
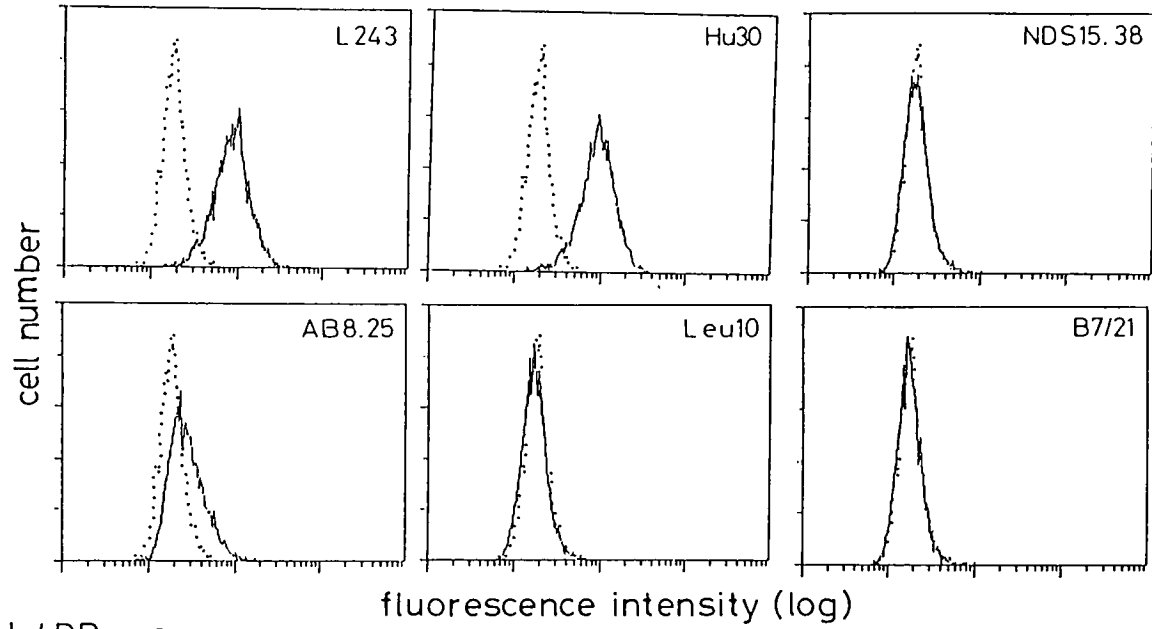


Fig. 12

L / DR α + β I



L / DR α + β III

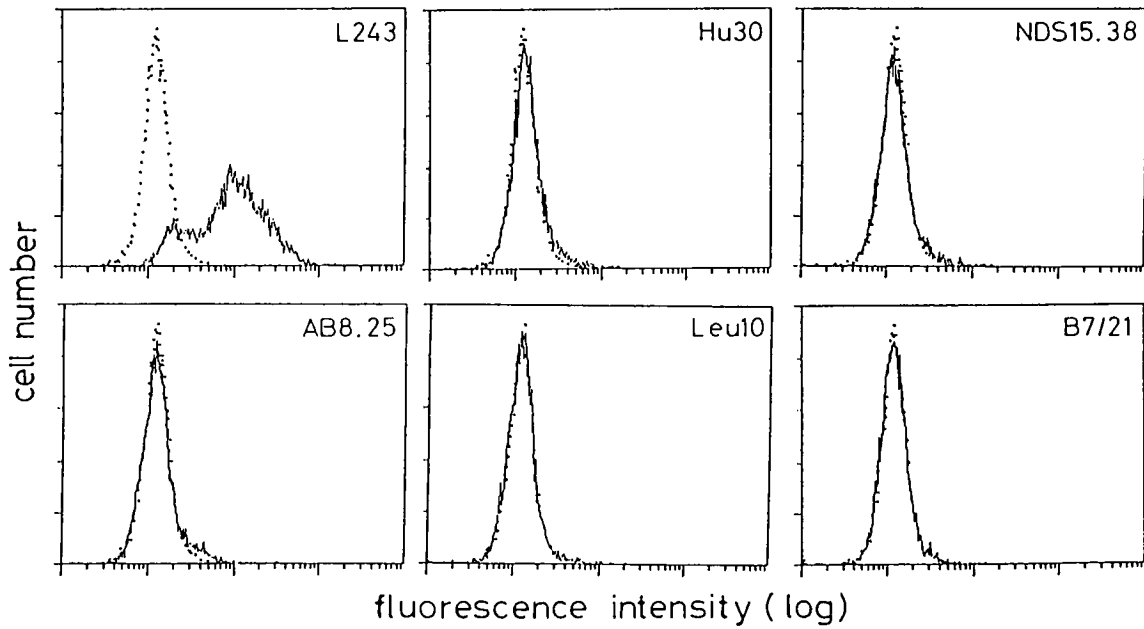


Fig.13

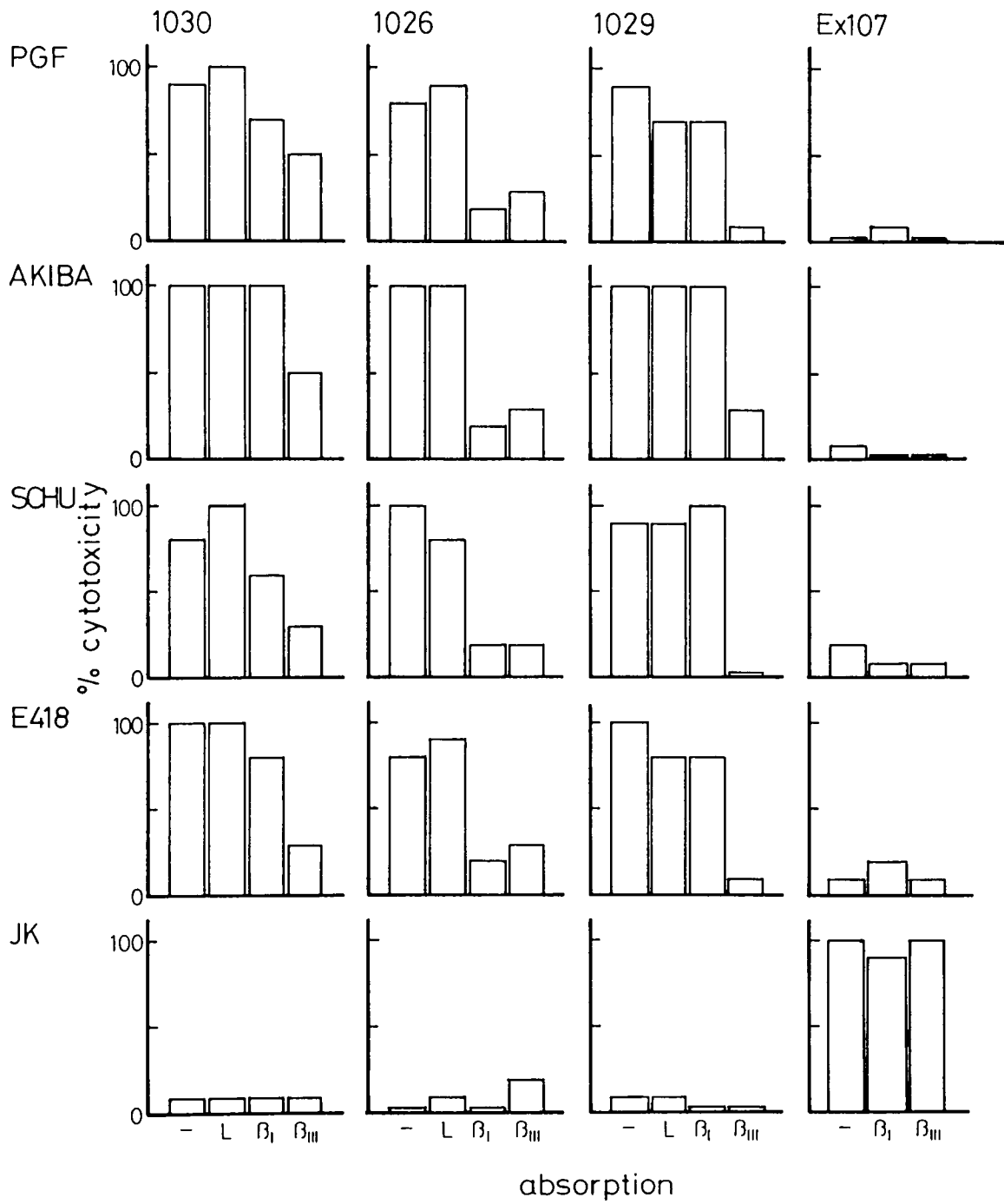


Fig. 14

Analyses of gene structures and antigen determinants
of human class II major histocompatibility antigens.

II. Subtype frequencies in type I diabetic patients
with DR2 haplotype

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ABSTRACT

The serologically defined HLA-DR2 specificity is divided into several Dw subtypes determined by the mixed leukocyte reaction, Dw2, Dw12, Dw21 and Dw blank. HLA-DR2 allele is negatively associated with insulin-dependent diabetes (IDDM) in Japanese, suggesting that resistance related factors are involved in DR2 haplotype. It is still unknown, however, which Dw specificities in DR2 correlate with that resistance. I investigated the genomic clones and restriction fragment length polymorphism (RFLP) from DR2 specificities to show an ability of Dw typing in DR2 by genomic Southern blotting with the DQ β cDNA probe. Furthermore, I compared Dw subtype frequencies in IDDM patients carrying DR2 haplotype to that of healthy individuals in Japanese, but failed to observe statistical significance.

INTRODUCTION

The major histocompatibility complex (MHC) codes for at least three classes of products involved in the immune response (class I, class II, and class III) (Klein et al., 1983). Class II products are membrane glycoproteins composed of two chains, α and β . Three class II products, DR, DQ, and DP antigens, which respectively are encoded by their genes, HLA-DR, HLA-DQ, and HLA-DP genes, are expressed on antigen presenting cells, such as, B-cells, macrophages, and dendritic cells.

IDDM is resulted from the selective destruction of insulin-producing islet cells of the pancreas. It has been argued that disease development involves an autoimmune response to an islet antigen(s) (Eisenbarth, 1986). The IDDM is clearly polygenic in inheritance (Thomson et al., 1984) and it has been estimated that the HLA-D region contributes to much of the heritability (Rotter and Landlaw, 1984). About 95% of IDDM patients possess either HLA-DR3 or -DR4, compared to 45-54% of the normal population in Caucasian (Svejgaard et al., 1983; Thomson et al., 1984). At least one IDDM susceptibility gene was mapped in the MHC, especially in the HLA-D region, by population and family studies (Winter et al., 1987; Bertrams et al., 1984; Winearls et al., 1984; Rubinstein et al., 1981; Bertrams et al., 1984). The serological (Kim et al., 1983; Schreuder et al., 1985) and RFLP (Owerback et al., 1983; Arnheim et al., 1985; Bohme et al., 1986; Nepom et al., 1986)

RESULTS and DISCUSSION

Detection of polymorphic fragments specific for HLA-Dw specificities

Serological DR haplotypes are divided into several Dw subtypes determined by the mixed lymphocyte reaction (Back, 1985), DR2 into Dw2, Dw12, Dw21 and Dw blank. Genomic cosmid clone analysis of DR2 specificities showed RFLPs between Dw2 (PGF) and Dw12 (AKIBA) (Kawai, 1990). I analyzed the possibility of Dw typing by Southern blotting using HLA class II gene cDNAs as probes in representative DR2 homozygous typing cell lines including Dw2, Dw12 and Dw21. In an attempt to establish standard Southern blot patterns for different Dw specificities with HLA class II antigen probes, genomic DNAs carrying DR2 specificity were digested with various restriction endonucleases (EcoRI, PstI, BamHI, TaqI and MspI) and probed with the HLA-DR β , DQ α , and DQ β cDNA probes. Fig. 1-3 showed the polymorphic restriction fragments of some DNA samples from three Dw subtypes of DR2 (Dw2, Dw12 and Dw21) hybridizing the DQ α , DQ β and DR β cDNA probes, respectively, after restriction endonucleases digestion. Multiple hybridizing fragments were encountered in DNA from all homozygous typing cell lines with the DR β , and DQ α and DQ β cDNA probes. The Southern blot patterns were illustrated in Fig. 4 schematically. Each individual homozygous typing cell lines corresponded to a characteristic pattern of hybridizing fragments

regardless of whether the DR β , the DQ β or the DQ α cDNA probe was used. Hybridization with the DR β and DQ α cDNA probe generally gave identical patterns with DNA from different Dw specificities carrying an identical DR type (DR2). In contrast, the DQ β cDNA could easily distinguish different Dw specificities. Hybridization of EcoRI-digested DNAs with the DR β probe gave six bands, 20, 9.0, 6.0, 5.0, 4.0 and 3.4kb long (Fig. 1 and 4). All the hybridizing bands correspond to each of the class II β chain genes reported in the accompanying paper (Kawai, 1990) and other reports. Thus, the EcoRI 20kb band corresponded to the DR β III gene, 9.0kb to DP β 1 and DP β 2 genes (Ando et al., 1986), 6.0kb to DR β I and isolated β 1 exon next to the DR α gene, 5.0kb to DR β I, β II and β III genes, 4.0kb to DR β I and isolated leader sequence downstream from DR β III gene, and 3.4kb to DR β III gene (see Kawai, 1990). Clone analysis suggested that probes of DR β and DQ α cDNAs would show RFLPs, but polymorphic bands were hidden by other nonpolymorphic bands. For example, 3.4kb EcoRI of PGF (Dw2) and 4.2kb EcoRI fragment of AKIBA (Dw12) expected to show polymorphism by use of the DR β 5'UT specific probe were hidden by nonpolymorphic bands derived from DR β II and DR β I genes, respectively. Similarly the DQ α cDNA probe also gave less polymorphisms and every hybridizing bands observed could correspond to that of the DQ α and the DX α genes. In contrast, the DQ β cDNA probe generated highly polymorphic

blot patterns using each restriction enzyme, EcoRI, PstI, MspI and TaqI (Fig. 3), suggesting the ability of the Dw typing. The restriction maps of cosmid clones encoding HLA class II genes suggested that all of the polymorphic bands except of EcoRI 10kb band of AKIBA which seemed to encode the DP β 1 gene (Ando et al., 1986), were derived from the DQ β gene. Hybridizing patterns that 9.6kb and 2.6kb bands were seen with EcoRI digestion in Dw2, as well as 18kb band in Dw12, was coincident with the results of the DQ cosmid clones (Kawai, 1990) Eleven polymorphic bands selected for Dw typing in DR2 as follows; EcoRI 18kb, EcoRI 2.6kb, PstI 6.5kb, PstI 4.6kb, PstI 1.4kb, MspI 5.0kb, MspI 0.4kb, TaqI 7.0kb, TaqI 5.4kb, and TaqI 0.7kb.

Dw typing of IDDM patients and healthy controls

Serological studies showed that the frequencies of the specificities DR3 and DR4 are increased in IDDM patients as compared to normal controls, while the frequency of DR2 is decreased, suggested relevant susceptibility- or resistance-related factors are involved in DR2 haplotypes (Svejgaard et al., 1984; Bertrams and Baur 1984). The results of statistical analysis of DR frequencies in IDDM patients were shown in Table II, clearly indicating the remarkable decrease of Table II DR2-positive IDDM patients ($\chi^2=162$) I tried to investigate the genetical bases of the resistance of DR2 against IDDM.

Genomic DNAs from fifteen IDDM patients' and fourteen healthy controls' peripheral blood lymphocytes with DR2 specificity which was determined at the Department of Transplantation, School of Medicine, Tokai university, were digested with four different restriction enzymes (EcoRI, PstI, TaqI and MspI) and probed with the DQ β cDNA. Autoradiograms using EcoRI and MspI as restriction enzyme were shown in Fig. 5. Fig. 5

Neither specific band nor unexpected band only for the IDDM patient in comparison with the result of homozygous typing cell lines was found, suggesting that IDDM patients might have no specific fragment observed by Southern blot. Dw typing panel were shown in Fig. 6. Twenty six out of twenty nine Dw subtypes Fig. 6 derived from DR2 of the IDDM patients and healthy controls could be determined clearly. For example, IDDM patient, #23, has six informative bands as follows, P1.4, P4.6, T5.4, E2.6, P6.5 and M5.0. First three bands (P1.4, P4.6, T5.4) were Dw21 specific and last four bands (T5.4, E2.6, P6.5, M5.0) were specific for Dw2, and no additional band specific for Dw12 was observed, clearly suggesting that he had Dw2/Dw21 specificities. Haplotypes of three persons, #412, #172 and KAT, were undetermined, because that serological DR typing might be mistaken or new Dw haplotype presently unknown. Table III showed Dw type frequencies in DR2 positive IDDM patients and healthy controls. Although frequency of Dw12 and Dw21 in IDDM patients was apparently increased in comparison of that of

healthy controls, no statistical significance was observed. Frequency of Dw subtype Dw21 in DR2 is reported to be significantly increased in Caucasian (Bohme et al., 1986; Segall, 1986, Sorretino et al., 1985). The subtype Dw21 accounts for only about 6% of normal DR2 haplotypes, 77% of IDDM DR2 chromosomes, however, are Dw21. In the present study. I analyzed samples of 17 DR2 IDDM patients and 14 DR2 healthy individuals in Japanese. All of Dw haplotypes except three persons were clearly determined by Southern blot analysis using the DQ β cDNA probe and no statistical significance of Dw frequencies was observed (Table III) It is, however, interesting that only two IDDM patients, but no healthy individual, have Dw21 specificities. Some reports (Todd et. al. 1987, 1988, Morel et. al. 1988) suggested that IDDM susceptibility is largely dependent on the identity of amino-acid residue 57 of the DQ β chain. Thus, amino acid, aspartic acid (Asp) at position 57 in the DQ β chain is negatively associated with this disease. DQ β chain of Dw2 and Dw12 have Asp at position 57, on the other hand, Dw21 has a residue, serine. Recently, however, single amino-acid, aspartic acid at position 57, is not sufficient for development of insulinitis, but that the amino acid at position 57 of the β chain is involved in the interaction between MHC class II molecules and the T-cell receptor of the autoreactive T cells in the transgenic NOD mice (Miyazaki et al., 1990; Slattery et al.,

1990; Lund et al., 1990) This implies that conformation of the DQ molecule is involved in the IDDM development. No significant increase of the frequency of Dw21 in Japanese patients might be resulted from more participation of three-dimensional conformation of the DQ gene antigen in IDDM than single amino acid at position 57. The DQ transfectant reported accompanying paper (Kawai, 1990) remains valuable for precise analysis of the mode of the DQ antigen in the disease process.

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Table I

DR/Dw haplotypes of HTC's

HTC	HLA	
	DR	Dw
PGF	DR2	Dw2
Ms-7	DR2	Dw2
Ci	DR2	Dw2
AKIBA	DR2	Dw12
TOK	DR2	Dw12
FJO	DR2	Dw21

Table II

HLA-DR haplotypes in Japanese IDDM patients and healthy controls*

	DR2	not DR2	
IDDM	41	394	
healthy	162	310	$\chi^2=162$

* HLA-DR haplotypes were serologically determined at the department of plantation, school of medicine, Tokai Univ.

Table III

Dw type frequency in DR2 IDDM patients and healthy controls.

		Dw2	Dw12	Dw21	others
IDDM	(N=15)*	6	11	2	2
control	(N=14)	7	6	0	1

* Number of investigated individuals.

FIGURE LEGENDS

Figure 1

Autoradiograms of blot hybridization analysis of HLA homozygous DR2-positive B cell lines with the DR β cDNA, pDR β 101, as probe after digestion of EcoRI (A), PstI (B) and BamHI (C)

Figure 2

Autoradiograms of blot hybridization analysis of HLA homozygous DR2-positive B cell lines with the DQ α cDNA, pDC α 07. as probe after digestion of EcoRI (A), PstI (B), TaqI (C) and MspI (D)

Figure 3

Autoradiograms of blot hybridization analysis of HLA homozygous DR2-positive B cell lines with the DQ β cDNA, pDC β 101, as probe after digestion of EcoRI (A), PstI (B) MspI (C) and TaqI (D).

Figure 4

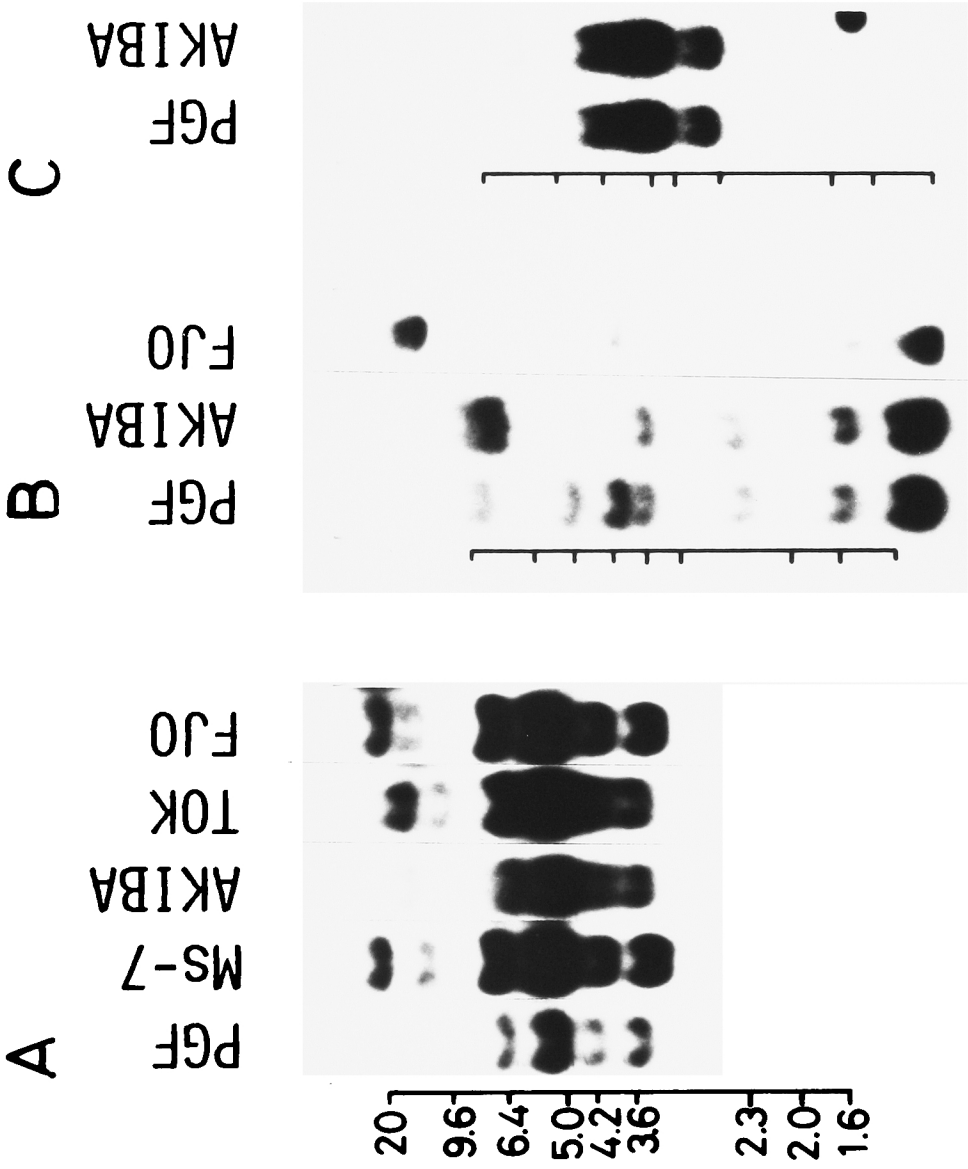
Hybridization patterns of Dw subtypes of DR2 using the DR β , DQ α and DQ β probes are represented schematically. Endonucleases used are shown at the top, and the Dw specificities of DR2 are shown at the bottom. Dotted lines represents faint bands. Polymorphic bands selected for Dw typing are indicated by the asterisks.

Figure 5

Autoradiograms of blot hybridization analysis of DR2 positive IDDM patients and healthy controls using the DQ β cDNA as probe. DNA from peripheral blood lymphocytes were digested with EcoRI(A) and MspI(B). Individual DR haplotypes were listed at the top.

Figure 6

Diagrammatic patterns of DQ β DNA fragment length of IDDM patients and healthy controls. Dw types and their polymorphic fragments are listed at the top. E, P, T and M represent the restriction endonucleases, EcoRI, PstI, TaqI and MspI, respectively and the numbers indicate length of hybridizing bands in kb. Symbols as follows; +, positive; /, unable to read or not done. Bands seemed to be derived from other haplotype than DR2 (Maeda et al., 1989) were shown by (+)



B

C

A

21
Fig. 2

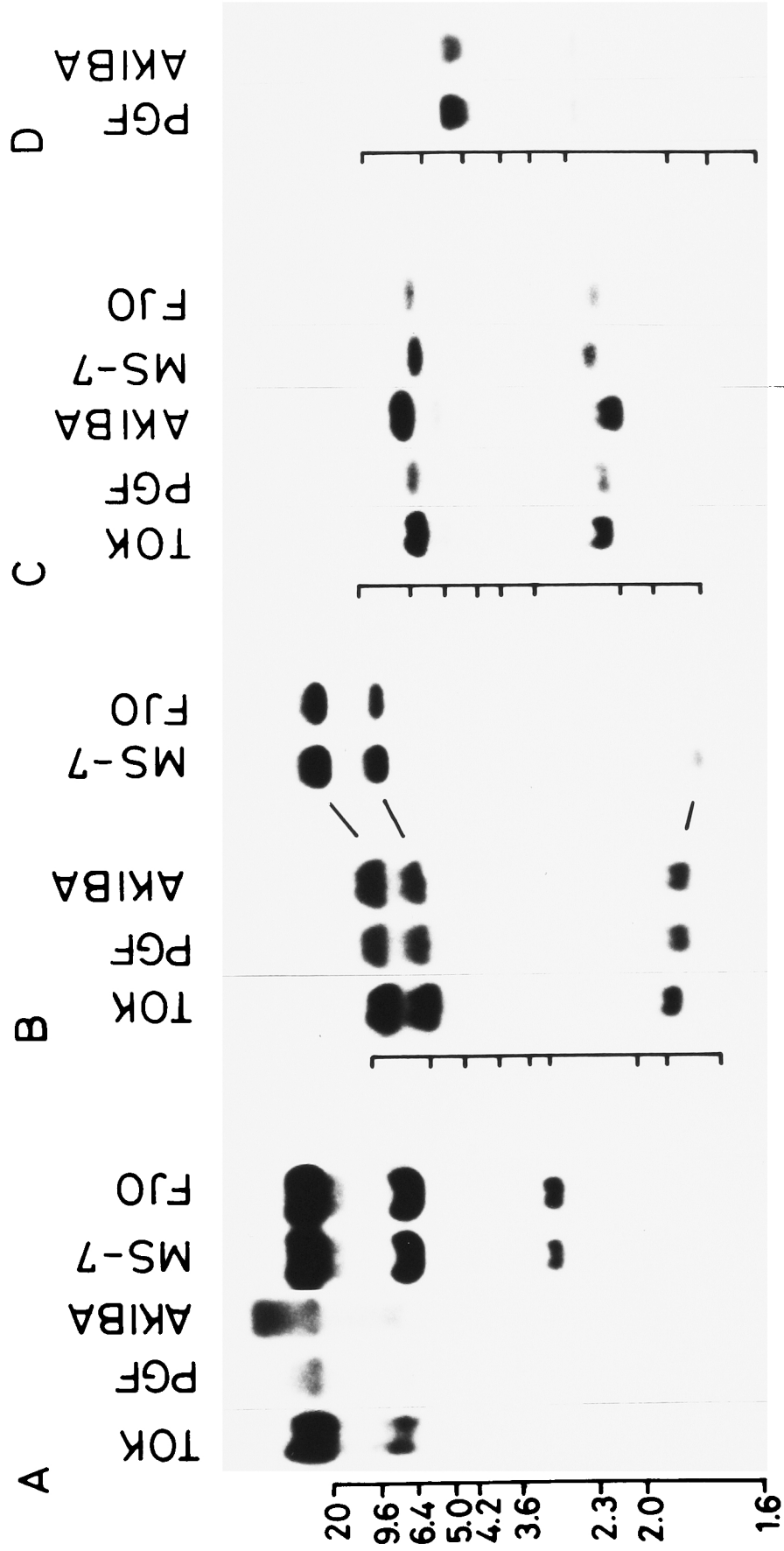
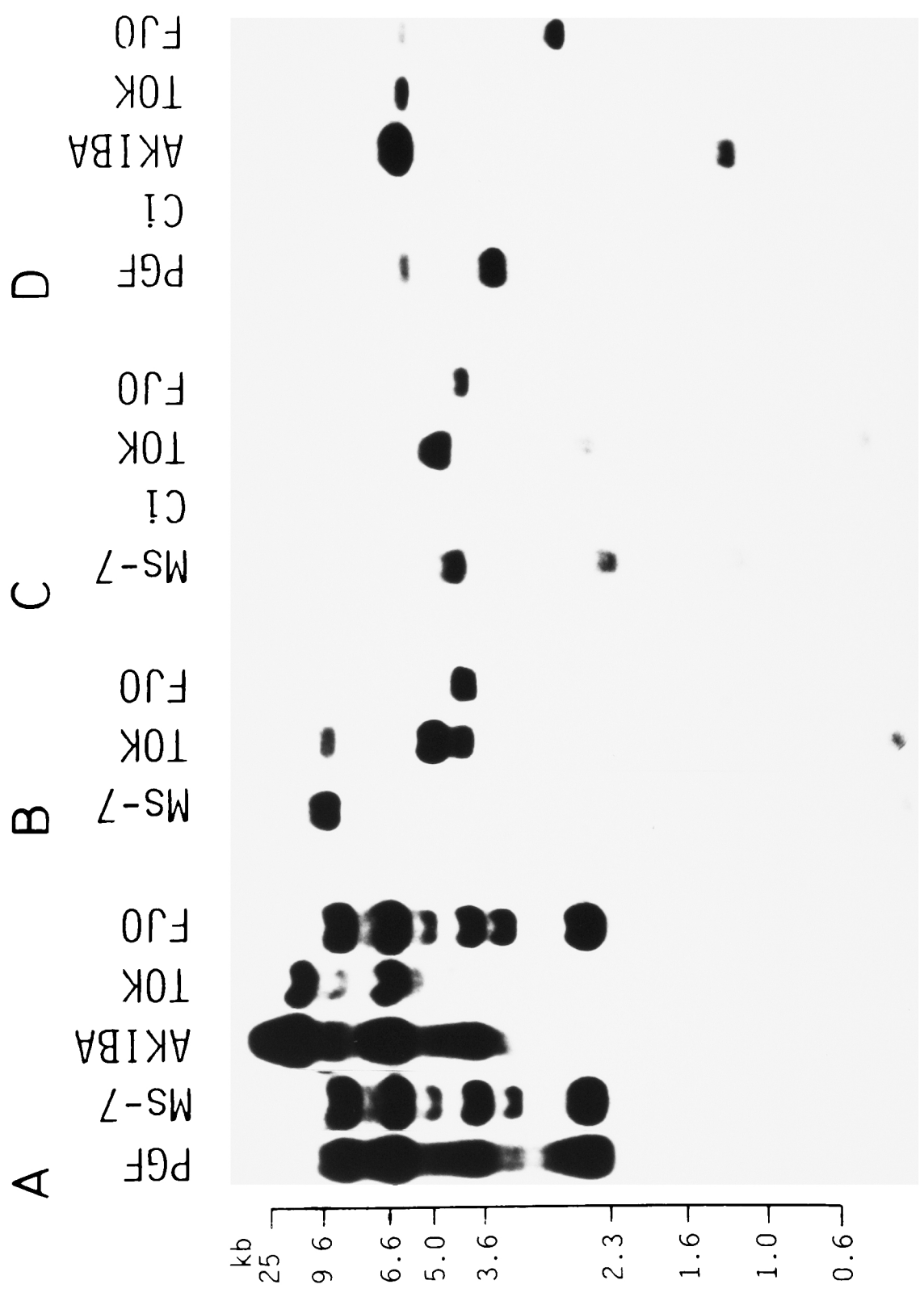
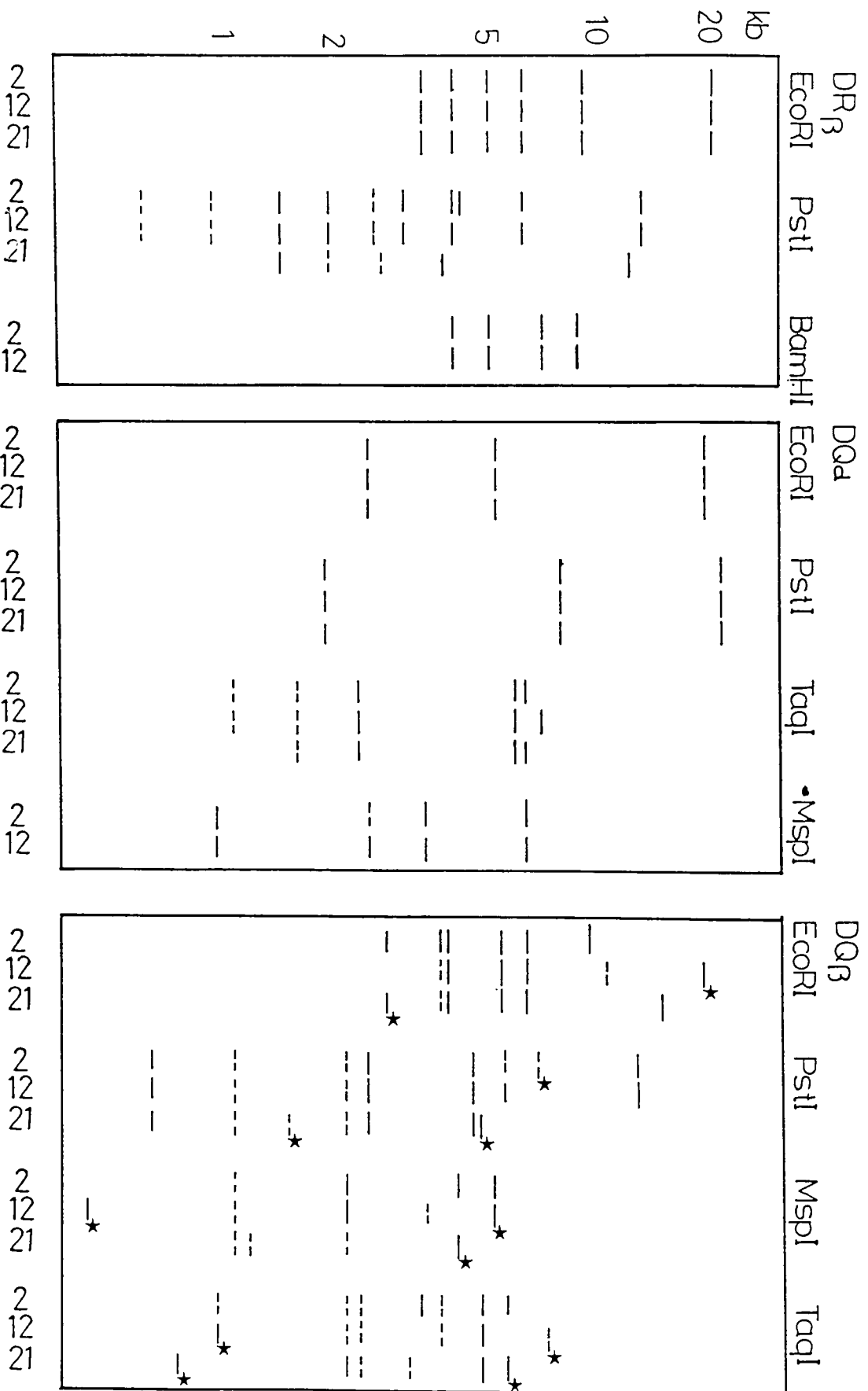


Fig. 3





23
 Fig. 4

A

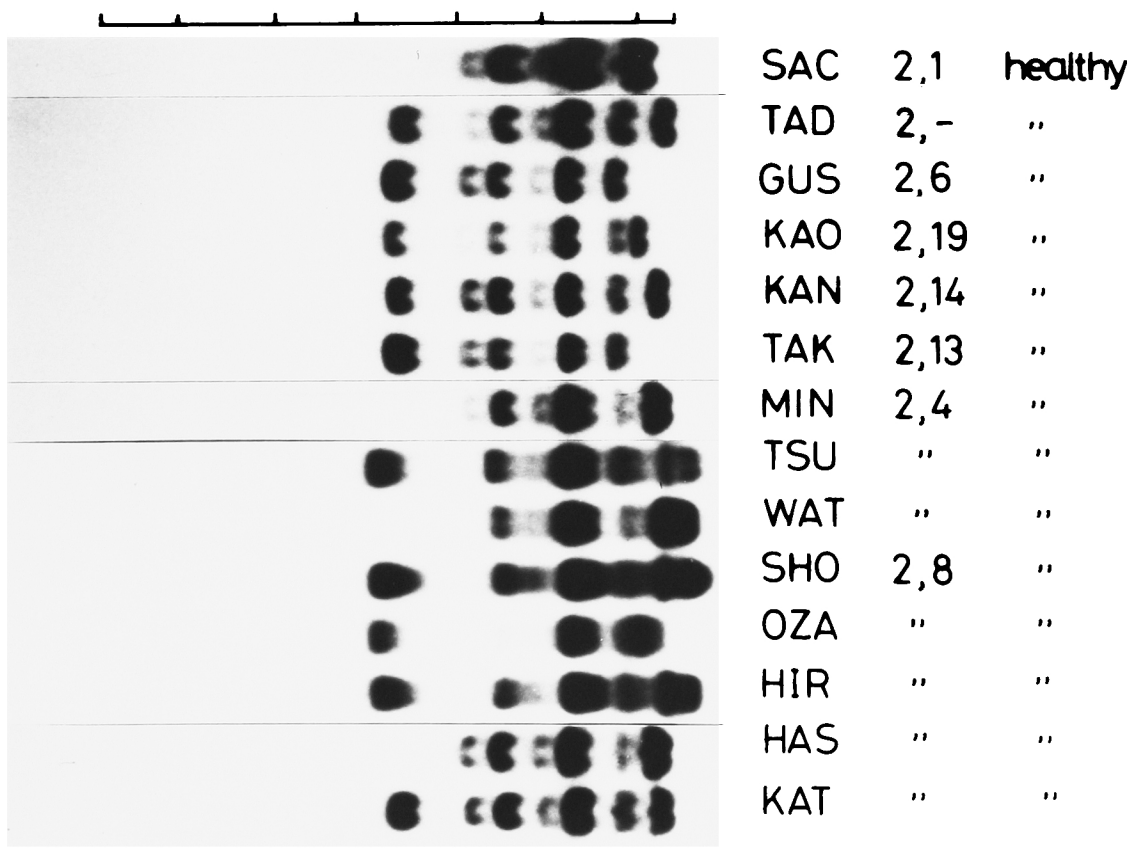
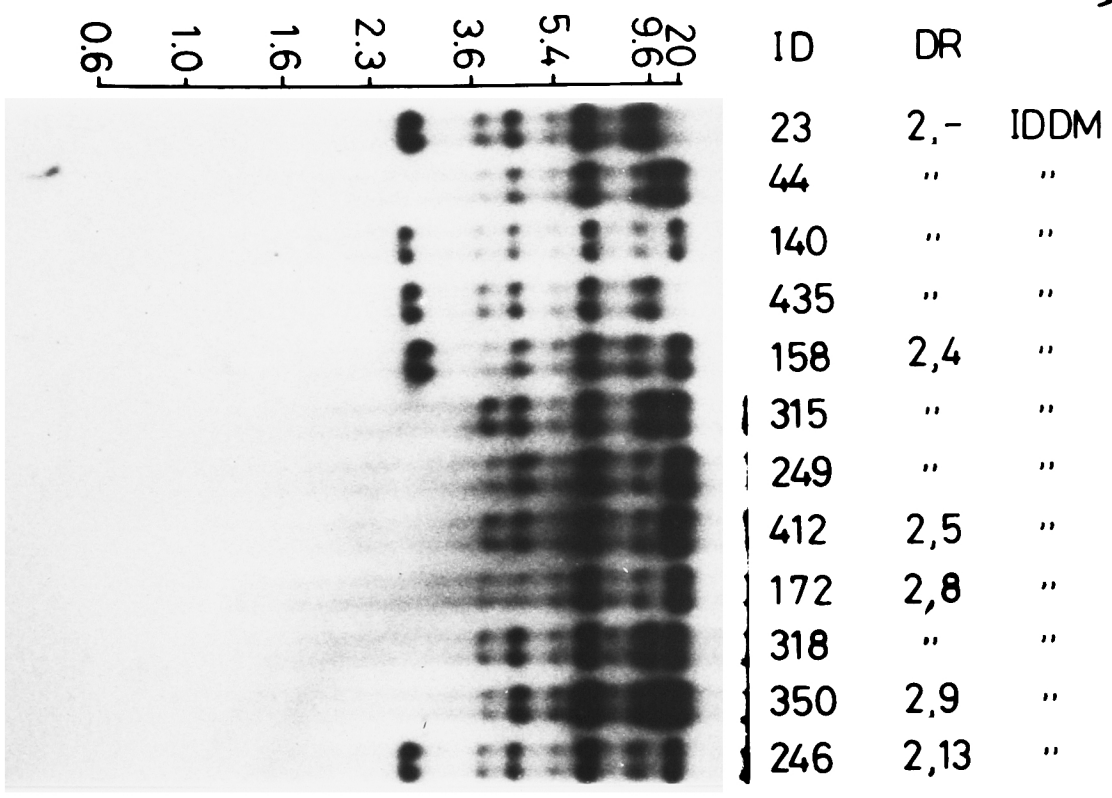


Fig. 5
24

Fig. 5

