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Immunology

Structural organization of the DR subregion of the human major histocompatibility complex

(class II antigen/cosmid cloning/DNA and protein sequence)

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ABSTRACT **Two clusters of overlapping cosmid and λ phage clones comprising 205 kilobases (kb) have been isolated from the DR subregion of the human major histocompatibility complex from a *DR4* haplotype. A single *DRα* and three *DRβ* genes were identified. In one cluster (135 kb), the *DRα* gene is 90 kb distant from the *DRβ* gene encoding a molecule that carries the MT3 serological specificity. In the second cluster (70 kb), the *DRβ* gene determining the *DR4* specificity is located 22 kb apart from a *DRβ* pseudogene (*DRβψ*). A 3- to 4-kb sequence located at the 5′ end of the *DRβ(MT3)* gene is common to all three *DRβ*-chain genes. In addition, three more copies of this sequence are spaced between the *DRα* and the *DRβ* (*MT3*) genes in the first cluster and one of these, at least, is associated with a DRβ1 exon, suggesting that additional genes could be encoded in this region and that multiple duplication events have led to its evolution.**

The class II antigens of the major histocompatibility complex are heterodimeric transmembrane glycoproteins expressed on B cells, activated T cells, and macrophages. They are composed of an α (heavy) and a β (light) chain, both comprising two extracellular domains (α1, α2 and β1, β2), a membrane spanning segment, and a cytoplasmic tail. Characteristically, these molecules display extensive sequence variation, mainly within the β1 domain of the light-chain subunit. This polymorphism defines specific determinants involved in central events of immunorecognition (reviewed in refs. 1–3).

Three subsets of class II antigens, DR, DQ(DC), and DP(SB), have been identified by serological and biochemical studies and assigned to the HLA-D region on chromosome 6. They are encoded in distinct subregions, which include most of the six α- and seven β-chain genes so far detected. Several cDNA and genomic clones containing DR-, DQ-, and DP-related α- and β-chain genes have been isolated and characterized. In an attempt to investigate the genetic structure of the entire HLA-D region, cosmid cloning has provided recent insights into the complexity of the *DQ* and *DP* gene clusters (reviewed in ref. 1). The organization of the genes in the DR subregion encoding the predominant and most polymorphic class II antigens is, however, still unknown.

This paper reports the isolation of two series of overlapping cosmid clones containing a single *DRα* and three *DRβ* genes from a DR4 homozygous cell line. Two *DRβ* genes have been identified by comparison of DNA and protein sequence data. They encode the β chains carrying the DR4 and MT3 serological specificities, respectively (4, 5). The third *DRβ* gene represents a recently described pseudogene (6). In addition, a conserved element containing *DRβ* 5′ flanking sequences has been found to occur in a total of six repeats within the cosmids. The results provide a detailed picture of the molecular structure of the HLA-DR subregion and implications regarding its evolutionary development.

MATERIALS AND METHODS

Isolation and Characterization of Genomic Clones. The construction of cosmid and λ phage libraries from the *DR4* homozygous B-lymphoblastoid cell line Priess has been described in detail (7). Libraries were screened with cDNA and genomic DNA fragments labeled with [^{32}P]XTPs by nick-translation (8). Colony hybridization was carried out in 6× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M Na citrate)/5× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/10% dextran sulfate/0.1% $NaDodSO_4$/sonicated salmon sperm DNA (100 μg/ml) for 16 hr at 65°C. Filters were washed in 0.1× NaCl/Cit/0.05% $NaDodSO_4$ at 65°C, and were exposed to Kodak XAR-5 film. Isolated clones were characterized by restriction mapping and Southern blot hybridization using exon-specific cDNA probes and defined genomic DNA fragments.

cDNA Probes. The DRα probe used was the cDNA clone α-15 isolated from a DR4,w6 cell line (9). The 1.1-kilobase (kb) insert was obtained by digestion with *Pst* I. The DRβ probe was the cDNA clone DRβI derived from the same DR4,w6 cell line (10). The 800-base-pair (bp) fragment obtained by *Hin*dIII/*Sst* I cleavage comprises the β1, β2, transmembrane, and cytoplasmic domain sequences. Exon-specific subfragments were generated by *Hin*dIII/*Pst* I/*Sst* I digestion. The signal sequence and the β1 and β2 exons are included within a 140-bp, a 260-bp, and a 450-bp fragment, respectively. The DQ1β cDNA clone DK30 isolated from the cell line LB (D. Kappes, personal communication) contains a 900-bp *Eco*RI insert encoding the entire β2, transmembrane, cytoplasmic, and 3′ untranslated sequences.

Southern Transfer and Blot Hybridization. Total genomic DNAs (10 μg) were digested with different restriction enzymes. Fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose filters (8). Hybridization and washing conditions were as described above.

Subcloning and DNA Sequencing. Fragments containing the β1 exons from the *DRβ* genes encoded in the cosmid T8B and λ133 were obtained by *Bam*HI/*Hin*dIII digestion and ligated with pUC12. For sequence determination, these subclones were cleaved with either *Bam*HI, *Ava* I, or *Bss*HII (only λ133), and 3′-end-labeled using the Klenow fragment of DNA polymerase I. After digestion with a second enzyme, the fragments were purified and sequenced by the chemical degradation procedure (11). To gather overlapping sequences for the β1 exon derived from the cosmid T8B, further subclones were constructed by treatment of the 1.2-kb *Bam*HI/*Hin*dIII insert with the BAL-31 exonuclease. Sequence data were obtained from both strands.

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Abbreviations: kb, kilobase(s); bp, base pair(s); mAb, monoclonal antibody.

Amino Acid Sequence Determination. The *DR4* homozygous cell line WT51 was intrinsically labeled in the presence of 5 mCi (1 Ci = 37 GBq) of different radioactive amino acids ([^{3}H]arginine, [^{3}H]glutamine, [^{3}H]leucine, [^{3}H]phenylalanine, [^{3}H]proline, and [^{35}S]cysteine) as described (12). Cells were lysed by using 1% Triton X-114, and the membranes were isolated following the protocol of Bordier (13). Immunoprecipitations were performed with the monoclonal antibodies L243 (14) and 109d6 (15). The α and β chains were separated and sequenced by automated Edman degradation as described (12).

RESULTS

Isolation and Characterization of Clones Containing *DRα* and *DRβ* Genes. Several cosmid libraries from the *DR4* homozygous cell line Priess, comprising ≈3 million clones, were screened by colony hybridization using nick-translated DRα and DRβ cDNA fragments. Positive clones were analyzed by blot hybridization under stringent conditions. The nonpolymorphic *DRα*-chain gene was identified in two overlapping cosmids, B2G and T9C (Fig. 1*a*). The restriction mapping and blotting data were in accordance with the structure formerly reported for the DRα clone λDR6A (16).

Two sets of cosmids encoding *DRβ* light-chain genes were found. Their structural organization was determined by using defined DRβ cDNA fragments individually as exon-specific probes. The cosmids B16A and L11A include a single complete *DRβ* gene, the six exons of which are distributed over 15 kb (Fig. 1*a*). The β1 and β2 exons are contained in *Eco*RI fragments of 3.6 and 4.1 kb, respectively, and they were localized by mapping these fragments. The signal sequence and the β1 exon are separated by an unusually large intron of ≈9 kb. The second intron (β1, β2) is ≈3.5 kb. The intron–exon boundaries of the transmembrane, cytoplasmic, and 3′ untranslated region segments could not be precisely distinguished.

Additional DRβ coding sequences related to two different genes were identified in the cosmids T7D and T8B (Fig. 1*b*). The exact locations of the β1 and the β2 exons were derived from a 1.2- and a 1.9-kb *Bam*HI/*Hin*dIII subclone. A *DRβ* gene common to both cosmids is truncated in the immediate vicinity of the β1 exon and thus lacks most of the first intron and the signal sequence. The size of the second intron is 3.5 kb. In addition, a signal sequence was detected ≈22 kb downstream of the 3′ untranslated region of this preceding gene in the cosmid T7D (Fig. 1*b*).

Despite extensive attempts, additional cosmids required for the completion of these genes were not obtainable, possibly because of the apparent preferential cleavage of the genomic DNA in the course of the preparation of the libraries. The subsequent screening of a λ-phage library yielded a number of recombinants representing subclones of the cosmid B16A (λ133, λ150, λ152; Fig. 1*a*). However, previously undetected DRβ2 and 3′ sequences were found within the 17-kb insert of the isolated λ136 (Fig. 1*b*) and were presumed to be located downstream of the signal sequence encoded in the cosmid T7D. This assumption was substantiated by comparison to the physical map of a DRβ cosmid (cosII-801) from a *DR4* haplotype, described by Larhammar *et al.* (6). The very similar arrangement of six *Bam*HI, two *Kpn* I, and a particularly rare *Xho* I restriction site conclusively defines regions of overlap shared by cosII-801 with the cosmid T7D and λ136. The clone λ136 contains two additional *Bam*HI and four additional *Kpn* I restriction sites not found in cosII-801; these differences are presumably attributable to subtypic variability. As has been shown by nucleotide sequencing, the β-chain gene included in cosII-801 displays several deleterious mutations rendering it a nonfunctional pseudogene (*DRβψ*) (6).

The existence of three *DRβ* gene loci in the *DR4* as well as in most other haplotypes is consistent with previous Southern blot analyses (9, 12). None of the isolated clones appear to be allelic; multiple overlapping clones have always proven to be identical.

Linkage of the *DRα* with a *DRβ* Gene. To connect the three nonoverlapping groups of cosmid and λ-phage clones so far described (B16A, L11A, λ133, λ150, and λ152; B2G and T9C; T7D, T8B, and λ136), single or low copy number probes from terminal locations were sought. However, suitable "walking fragments" were not obtainable because of a very high frequency of repetitive elements throughout the cosmids

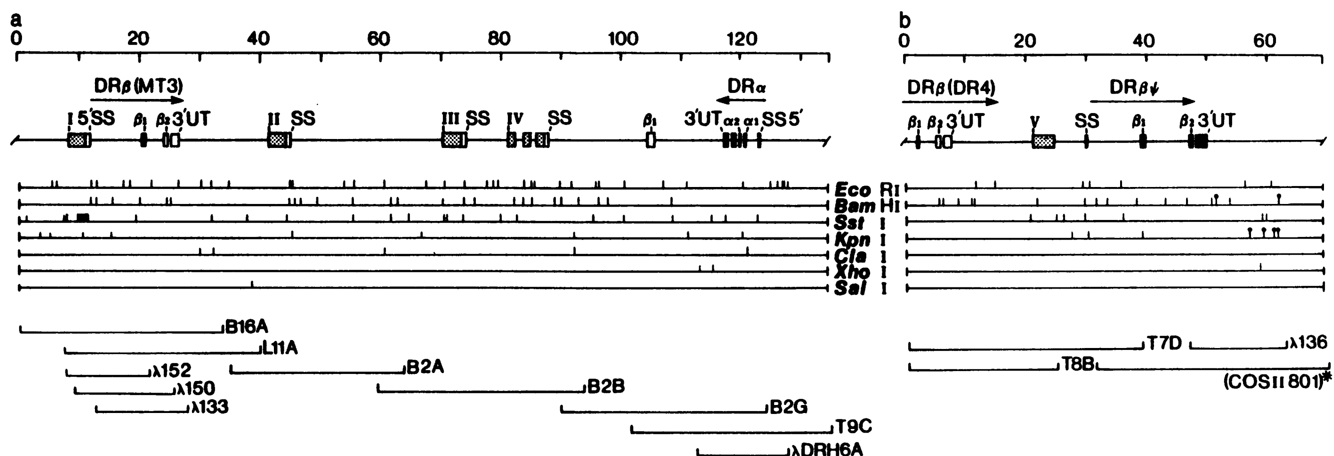

FIG. 1. Molecular organization of the HLA-DR subregion from a DR4 haplotype. (*a*) Linkage of the *DRα* with the *DRβ* gene determining the MT3 specificity. (*b*) Linkage of the *DRβ* gene, which encodes the protein carrying the *DR4* allospecificity, with a *DRβ* pseudogene (*DRβψ*). The top line gives the scale (in kb). Stippled boxes indicate five copies of a repeated element (I–V) hybridizing with a 2-kb *Sst* I fragment (closed box on the line showing the *Sst* I restriction sites) from the cosmid B16A. This probe contains the *DRβ* signal sequences (5′ SS) and its 5′ flanking region. The homologous repeats (I–V) also hybridize to the adjacent 5′ 1.7-kb *Sst* I fragment from B16A. Closed boxes refer to exons whose DNA sequences have been determined. Arrows show direction of transcription of the four genes. The 135- and the 70-kb stretches of DNA are defined by a number of overlapping cosmid and λ-phage clones shown below the cleavage sites of the restriction enzymes *Eco*RI, *Bam*HI, *Sst* I, *Kpn* I, *Cla* I, *Xho* I, and *Sal* I. λDRH6A was isolated from a library of an untyped individual (16). cosII-801* is a cosmid described by Larhammar *et al.* (6). It contains *DRβψ* and links T7D and λ136. Restriction sites present in λ136 that are lacking in cosII-801 are labeled by dots. Further explanations are given in the text. UT, untranslated.

except within the coding regions. Finally, a 2-kb *Sst* I fragment located upstream of the DRβ signal sequence in the cosmid B16A (Fig. 1*a*) was shown to hybridize with at least six bands on a Southern transfer of total Priess cell DNA under stringent conditions (Fig. 2*a*). By using this probe, the cosmids B2A and B2B were isolated and characterized (Fig. 1*a*). Both cosmids contain sequences homologous with the 2-kb *Sst* I fragment, although in different locations. Surprisingly, the cosmid B2A overlapped the DRβ cosmid L11A by 5.5 kb, as indicated by the restriction enzyme maps (Fig. 1*a*). The cosmid B2B was shown to share 4.2 kb with the DRα cosmid B2G (Fig. 1*a*). In fact, it includes two copies of the probe. Moreover, the cosmids B2A and B2B also overlap each other by 4.3 kb (Fig. 1*a*). These results were confirmed by terminal-fragment blot hybridizations. Thus, a contiguous cosmid cluster of 135 kb was formed, linking the *DRα* and a *DRβ* gene. The distance between these two loci is 90 kb. Relative to each other, they are oriented 3′ to 3′ with regard to the direction of transcription.

The reiterated 2-kb sequence appears to occur exclusively within the DR subregion. It comprises the DRβ signal sequence and the promoter. Cross-hybridization to cosmids encoding the *DQ* and *DP* genes (7, 17) was not detected. The 135-kb cosmid cluster includes four copies of this repeat, all of them in the same direction (Fig. 1*a*). They hybridized independently with the 5′ end of the DRβ cDNA and with two different subfragments from the 2-kb *Sst* I probe generated by digestion with *Hin*cII—namely, a 0.75-kb fragment including the signal sequence, and a 1.1-kb fragment located further upstream (Fig. 2 *b* and *c*). In all cases, homology was shown to extend 1–2 kb further upstream from the 2-kb *Sst* I fragment, using the adjacent 1.7-kb *Sst* I fragment from the cosmid B16A as a probe (Fig. 1*a*). In the cosmid B2B, one of the two repeats was found to be discontinuous over 7.5 kb (Fig. 1*a*). This entire 3- to 4-kb region is also present in the cosmid T7D, although here it is not directly associated with the DRβ signal sequence. The latter and its 5′ flanking probes were 4 kb apart (Fig. 1*b*). It has been reported that *DRβψ* contains a *Kpn* family repeat inserted 5′ to the promoter, suggesting an implication in the genesis of this pseudogene by means of transcriptional inactivation (6). This finding is

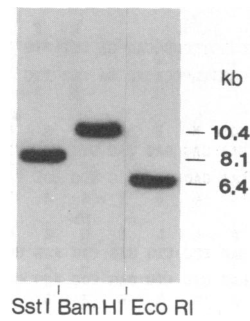

FIG. 3. Demonstration of a single DRβ1 exon 10–16 kb downstream of the *DRα* gene. B2G was digested with *Sst* I, *Bam*HI, and *Eco*RI, respectively, and hybridized with a DRβ1-specific cDNA fragment under stringent conditions. The final wash of the blot was in 0.4× NaCl/Cit at 65°C.

well-documented by the present results, providing further support to the linkage of the clones T7D and λ136 by cosII-801. Another cosmid, V11B, was isolated, including a sixth copy of the repeated sequence that might be related to the 5′ region of the truncated *DRβ* gene encoded in the cosmids T7D and T8B (Fig. 2 *b* and *c*). An overlap between the cosmids T7D and V11B, however, has not been detected. In a search for additional β-chain genes, the entire 135-kb cosmid cluster was probed with DRβ and DQβ cDNA fragments under relaxed conditions. A single domain was found that hybridized with a DRβ1 exon probe, even under increased stringency (Fig. 3). It is located within a 6.4-kb *Eco*RI fragment 10–16 kb downstream from the *DRα* transcriptional unit (Fig. 1*a*).

Analysis of the *DRβ* Sequence Polymorphism. The availability of multiple *DRβ*-chain genes from a single haplotype provided the unique opportunity to examine the extent of pseudoallelic sequence divergence within the polymorphic β1 domains. Nucleotide sequences were determined for the β1 exons from the cosmid T8B and λ133. The intron–exon boundaries were localized after alignment of the sequences with published data derived from cDNA clones (10, 18). The

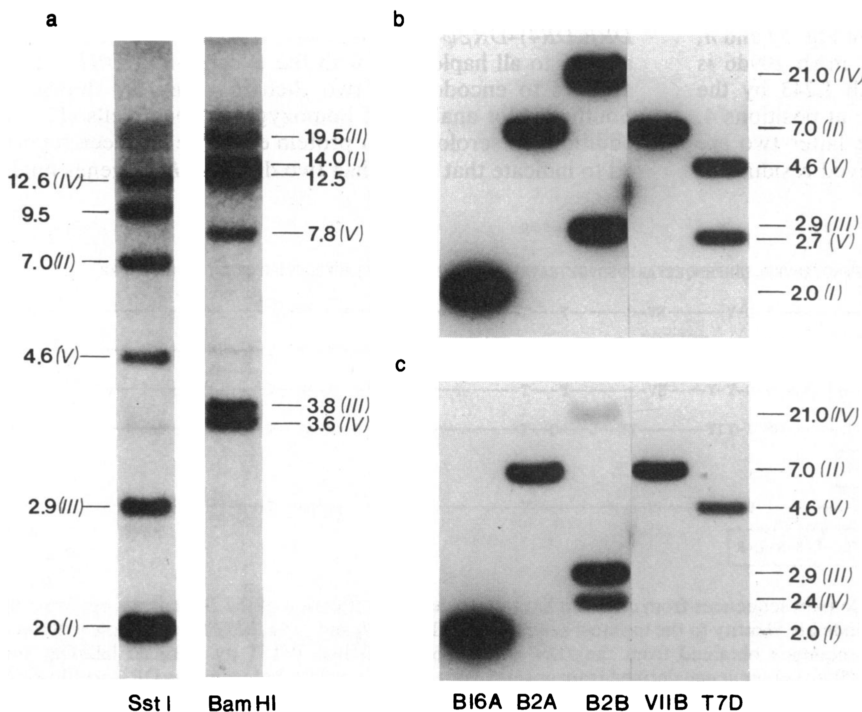

FIG. 2. Demonstration of a repeated element within the DR subregion containing the *DRβ* signal sequence and 5′ flanking probes. (*a*) Blot hybridization of total Priess cell DNA (10 μg) digested with *Sst* I and *Bam*HI, respectively, and probed with the 2-kb *Sst* I fragment from B16A (Fig. 1*a*). Five of the minimum of six bands (I–V) correspond with the locations of the repeated sequence within the cosmids as depicted in Fig. 1. (*b*) Blot hybridization of the *Sst* I-digested cosmids B16A, B2A, B2B, V11B, and T7D with a 1.1-kb *Hin*cII subfragment located at the 5′ end of the 2-kb *Sst* I probe from B16A. B2B includes two copies of the probe. The repeats I–III and V match the fragments on the genomic blot. The 21.0-kb band representing repeat IV in B2B corresponds with the 12.6-kb genomic *Sst* I fragment. The difference in size is due to the terminal truncation of B2B within the 12.6-kb *Sst* I fragment. V11B contains a sixth copy of the repeated element. It appears to share a homologous 7.0-kb *Sst* I fragment with B2A. However, these two cosmids are very different by restriction enzyme mapping and do not overlap each other (data not shown), suggesting the possibility that the 7.0-kb *Sst* I band in *a* contains two comigrating bands. (*c*) Same as in *b*, but using a 0.75-kb *Hin*cII subfragment located at the 3′ end of the 2-kb *Sst* I fragment as a probe that includes the DRβ signal sequence.

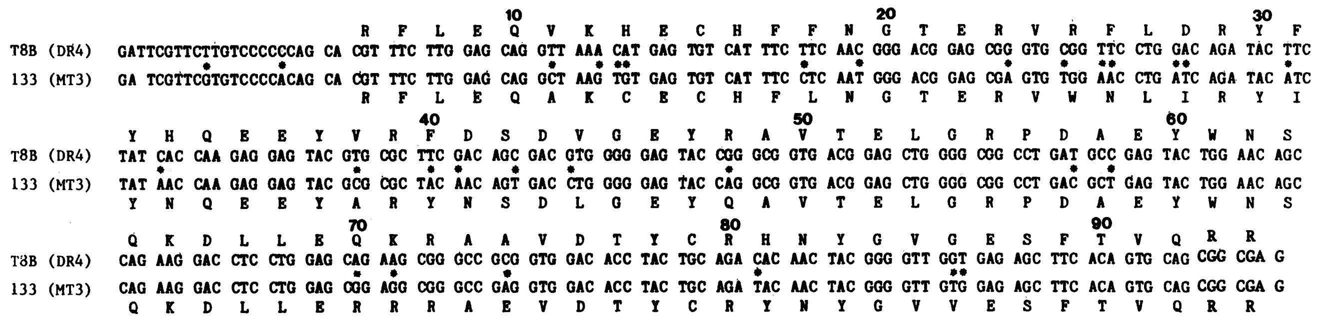

FIG. 4. Nucleotide and predicted amino acid sequences (designated by single-letter code) of the β1 exons and adjacent partial intron sequences from *DRβ(DR4)* and *DRβ(MT3)*. These genes are contained in the clones T8B and λ133, respectively. Nucleotide substitutions are marked by asterisks.

first five amino acids of the β1 domains are lacking because they are encoded in the preceding exon, including the 5′ untranslated region and the signal sequence. These β1 exons share 90% nucleotide sequence homology. They differ at 28 positions, 22 of which constitute 18 amino acid substitutions (Fig. 4). This relative variation correlates quantitatively with the polymorphism observed among different *DRβ* alleles, although a distinction between alleles and pseudoalleles is not always possible (Fig. 5). Most of the amino acid replacements are accumulated within three sections—at positions 9–13, 25–33, and 70–77 (Fig. 5). Conserved regions are marked by the two cysteine residues involved in disulfide loop formation at positions 15 and 79 (19), and by the glycosylation site N-G-T at position 19 (20). Moreover, a strictly conserved region ranges from position 52 to 66. A peculiarity of the sequence from λ133 is the presence of an extra cysteine at position 13 (Fig. 4), which is an uncommon feature in human class II antigens but not in the mouse Eβ chains (21, 22).

Identification of the Loci Encoding the DR4 and MT3 Allospecificities. Two different DR heterodimers are expressed on the surface of DR4 homozygous cell lines. These products have been serologically correlated with the DR4 and MT3 allospecificities (4, 5, 12). NH_2-terminal radiochemical amino acid sequencing of the *DRβ* light chains from the *DR4* homozygous cell line WT51 was already in progress when the β1 exon sequences from the T8B and λ133 clones became available. Immunoprecipitations were carried out with the MT3 specific monoclonal antibody (mAb) 109d6 and the DR reactive mAb L243, respectively. As shown in Fig. 5 *f* and *h*, the sequence of the protein recognized by mAb 109d6 is distinguished from that recognized by mAb L243 by the presence of glutamine, cysteine, and leucine at positions 4, 13, and 18, respectively, of which only the latter two are encoded in the β1 exon. All the protein-derived residues of the mAb 109d6-related sequence match the sequence deduced from the clone λ133, including the cysteine at position 13 (Fig. 5 *e* and *f*). Moreover, all the positions available for the DR4β-chain protein are identical to the sequence deduced from the cosmid T8B (Fig. 5 *g* and *h*).

DISCUSSION

A single *DRα* and three *DRβ* genes have been isolated from a *DR4* homozygous cell line and mapped to two clusters of overlapping cosmids comprising 135 and 70 kb, respectively. One of the *DRβ* genes represents a nonfunctional pseudogene (*DRβψ*) and has previously been analyzed in detail (6). In the cells examined, it displays four defective splice junctions, two premature termination codons within the β1 exon, and a translational frame shift in the β2 exon. The other two *DRβ* genes express molecules carrying the serologically defined DR4 and MT3 allospecificities, respectively. The gene encoding the *DRβ* chain, which carries the MT3 specificity, is linked to the *DRα* gene. These two loci are 90 kb distant from each other and are inverted with respect to the directions of transcription (3′ to 3′) (Fig. 1*a*). The β-chain gene determining the *DR4* allospecificity is located 22 kb apart from *DRβψ*, both showing the same transcriptional orientation (Fig. 1*b*). A direct succession of the three β-chain genes in opposite polarity to the α-chain gene would be homologous to the arrangement of the genes in the murine I–E region (23). Thus, a presumptive order of the four DR subregion genes is *DRβ(DR4)*–*DRβψ*–*DRβ(MT3)*–DRα. This number of loci applies to all haplotypes with the exception of *DR1*, which appears to encode only two β-chain genes, as shown by Southern blot analysis of homozygous typing cells (12). In addition, no serological or protein evidence has been reported to indicate that more than two different *DRβ* genes might

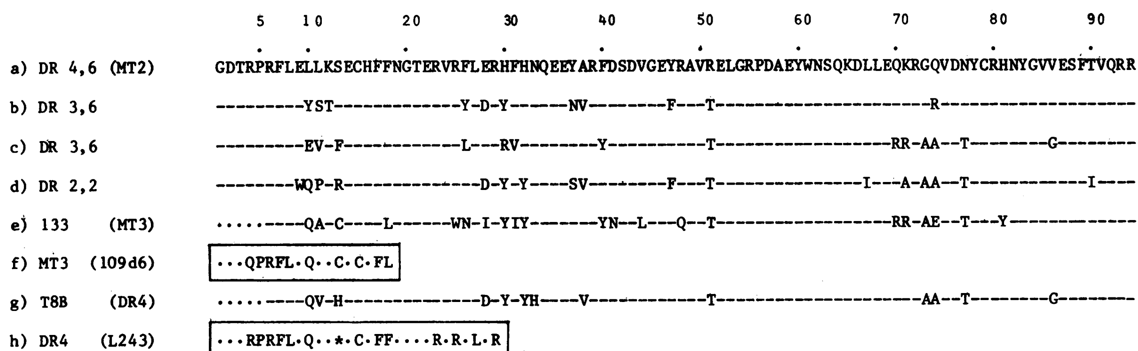

FIG. 5. Comparison of the polymorphic *DRβ1* domain sequences from different haplotypes and identification of the *DRβ* genes determining the DR4 and MT3 serological specificities. Dashes indicate identity to the topmost sequence. (*a*) 10, 29; (*b* and *c*) 18; (*d*) 30; (*e*) sequence derived from λ133; (*f*) partial NH_2-terminal amino acid sequence obtained from the *DR4* homozygous cell line WT51 by intrinsic labeling and immunoprecipitation using the MT3-specific mAb 109d6; (*g*) sequence derived from cosmid T8B; (*h*) same as in *f*, but using the DR-specific mAb L243 for protein isolation. Asterisk indicates the absence of cysteine at position 13. Amino acids are designated by single-letter code.

be expressed in a homozygous cell line. These facts suggest a similar organization of the DR subregion in most other haplotypes, including the presence of $DR\beta\psi$.

The existence of multiple $DR\beta$ genes implies that they were derived from a single ancestral gene by duplications and that they subsequently diverged. The results obtained in this study demonstrate that such events resulted in a large expansion of the DR subregion. Most human and murine class II α- and β-chain genes have been localized in close proximity to each other, not exceeding a distance of 15 kb (7, 17, 23). A conserved 3- to 4-kb element, including the signal sequence, the promoter, and presumably also other control sequences required for transcription, has been found at the 5′ end of each of the $DR\beta$ genes. Moreover, three additional copies of this sequence in the same orientation occur within the 90-kb region (Fig. 1*a*). Their presence may suggest the duplication of formerly intact genes, followed by the degeneration of their coding regions. This loss of genes is reflected by the existence of a single remaining DRβ1 exon 10–16 kb distant from the $DR\alpha$ gene (Fig. 1*a*). The murine $E\beta 1$, $E\beta 2$, and $E\alpha$ genes are encoded within ≈42 kb (22, 23). It is conceivable that two sequential duplications of a unit comprising equivalents of $E\beta 1$ and $E\beta 2$ took place in the evolution of the DR subregion. Three of the six resulting genes have either degenerated because of loss of function or they have extensively diverged.

The intriguing conservation of the three repeated elements between the $DR\alpha$ and the $DR\beta(MT3)$ genes needs to be accounted for by function. It is possible that they exert a role in the coordinate expression of the $DR\alpha$ and $DR\beta$ genes. Interesting in this respect is the presence of a tissue-specific enhancer within 2.5 kb upstream of the $E\beta 1$ gene (24). Homologous promoter regions have already been implicated in the induction and the control of transcription of *DR* and *I–E* genes (reviewed in ref. 1).

The presence of two functional $DR\beta$ genes raises the question of whether they differ in the extent of their polymorphism. The DR determinant encoded at one distal flank of the DR subregion is haplotype specific and therefore highly polymorphic. To date, 14 different alleles have been described (25). In addition, DR4 molecules even show structural heterogeneity among different DR4 cell lines (26, 27). By contrast, the MT3 determinant, encoded in closer association with the nearly invariant $DR\alpha$ gene, constitutes a less polymorphic "supertypic" specificity in that it is shared among the *DR4*, *-7*, and *-w9* haplotypes (4). No difference has been observed in the MT3 molecule on DR4 and DR7 cell lines (12).

The correlation of defined $DR\beta$ genes with well characterized serological specificities contributes to the understanding of the polymorphism that defines the determinants involved in the restriction of T-lymphocyte recognition. A T-cell clone specific for antigen and $I–E^b$ as well as $I–E^k$ has also been found to respond to the antigen kLH (keyhole limpet hemocyanin) in the context of MT3 (28). A common feature of all these molecules is a free cysteine at position 12 in $I–E^k$ and $I–E^b$ (21, 22) and at position 13 in DRβ(MT3) (Fig. 4). Gene-transfer experiments combined with *in vitro* mutagenesis using the $DR\beta(MT3)$ gene will provide a means to identify the actual sites functioning in T-lymphocyte recognition.

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