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Isolation and identification of a cDNA clone corresponding to an HLA-DR antigen β chain

(hybrid selection/histocompatibility antigens/membrane protein)

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ABSTRACT **The *HLA-D* locus in the major histocompatibility complex controls the expression of the genetically polymorphic HLA-DR antigens. mRNA coding for the β chains of these antigens was partially purified from the human lymphoblastoid cell line Raji. The mRNA was copied into double-stranded cDNA and cloned in *Escherichia coli*. One clone, pDR-β-1, obtained by hybrid selection, carries a 1070-base-pair insert comprising all of the coding region except the signal sequence and a substantial portion of the untranslated region. To identify pDR-β-1, highly purified HLA-DR antigen β chains derived from Raji cells were subjected to NH_2-terminal amino acid sequence determination. This sequence displayed extensive homology with that deduced from the nucleotide sequence at the 5′ end of the pDR-β-1 coding region. Taken together, the amino acid and nucleotide sequences strongly argue in favor of Raji cells containing at least two β-chain loci.**

HLA-DR histocompatibility antigens are cell surface proteins present on different subclasses of lymphocytes and macrophages (1). However, recently it was demonstrated that expression of these antigens is not strictly limited to cells of the immune system: they also occur on other types of cells such as epithelial cells in various organs (2). The molecular functions of the HLA-DR antigens, and of their murine counterparts the Ia antigens, are largely unknown. However, their role in several immunobiological phenomena is well documented. Expression of Ia antigens on the antigen-presenting cell seems to be a prerequisite for the proper activation of T-helper cells (3). Likewise, the cooperation between T-helper cells and B lymphocytes is controlled by the HLA-DR (Ia) antigens (4).

HLA-DR antigens are composed of two noncovalently linked, glycosylated, polypeptide chains with apparent molecular weights of 35,000 and 29,000, respectively (5). The smaller one, the β chain, displays most of the extensive genetic polymorphism (6, 7). Such polymorphism is a puzzling, common feature of molecules controlled by the major histocompatibility complex. The other chain, the α chain, is much less polymorphic than the β chain (6, 7).

The murine Ia antigens are controlled by two distinct subloci called *I-A* and *I-E/C* (8). Limited NH_2-terminal amino acid sequence analyses have revealed that $I\text{-}A_\alpha$, $I\text{-}A_\beta$, $I\text{-}E/C_\alpha$, and $I\text{-}E/C_\beta$ chains display unique primary structures (8, 9). Corresponding structural analyses of highly purified HLA-DR antigens have revealed only the existence of E/C-like sequences (10). However, recent data based on serological tests and two-dimensional electrophoretic analyses suggest that HLA-DR antigens may be controlled from more than one locus (11, 12).

Structural analyses of HLA-DR antigens lag behind those of HLA-A, -B, and -C antigens (13). In this communication we describe the isolation and partial characterization of an HLA-DR antigen β-chain cDNA clone. The insert in the clone corresponds to most if not all of the translated portion of a β chain.

MATERIALS AND METHODS

Materials. S1 nuclease and terminal deoxynucleotidyl transferase were obtained from Bethesda Research Laboratories; DNA polymerase I was from Boehringer Mannheim. Polynucleotide kinase and restriction enzymes were purchased from New England BioLabs. $(dT)_{12-18}$ and oligo (dT)-cellulose were from Collaborative Research (Waltham, MA). Rabbit reticulocyte lysate, [^{35}S]methionine, and EN^{3}HANCE were the products of New England Nuclear. [α-^{32}P]Deoxynucleotides (>400 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and adenosine [γ-^{32}P]triphosphate (>2000 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, England). Nitrocellulose filters (BA 85) were purchased from Schleicher & Schuell.

Antiserum. An antiserum against HLA-DR antigen β chains was raised in a rabbit by immunization with highly purified β chains. HLA-DR antigens were purified to homogeneity and α and β chains were separated from each other by preparative $NaDodSO_4$/polyacrylamide gel electrophoresis. The antiserum obtained did not crossreact with HLA-DR antigen α chains.

Cultivation of Raji Cells. The human lymphoblastoid cell line Raji (HLA-Dw 3 and 6) was maintained in roller-flask cultures containing RPMI-1640 medium (GIBCO) and 10% neonatal calf serum. RNA was isolated from cells in logarithmic growth phase.

Isolation of mRNA. Microsomal mRNA was isolated from Raji cells according to a protocol described elsewhere (14). Enrichment of mRNA coding for HLA-DR antigen subunits was accomplished by centrifugation of the mRNA in a 10–30% aqueous sucrose gradient for 11 hr at 39,000 rpm in a Beckman SW 40 rotor at 15°C. Distribution of HLA-DR antigen mRNA in the gradient was determined by cell-free translation (15).

Preparation of cDNA Clones. Sucrose density gradient fractions enriched for HLA-DR antigen mRNA were transcribed into cDNA with use of avian myeloblastosis virus reverse transcriptase (a generous gift of J. W. Beard, National Institutes of Health). The second strand was synthesized with DNA polymerase I. The conditions for first- and second-strand synthesis described by Wickens *et al.* were used (16). After treatment of the cDNA with S1 nuclease as outlined by Hoeijmakers *et al.*

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(17), the material was size fractionated on a 5–23% sucrose gradient (17). Molecules longer than 600 base pairs were tailed with deoxycytidine (18), and the tailed cDNA was hybridized to *Pst* I-digested pBR322 (19) elongated with deoxyguanosine (18). The resulting hybrid molecules were used to transform *Escherichia coli* strain 294 (20, 21). Preparation of competent cells was performed according to Dagert and Erlich (22).

Purification of Plasmid DNA. Tetracycline-resistant colonies were grown in L broth and amplified by the addition of chloramphenicol (200 μg/ml) at an optical density of 0.6 at 600 nm. Cleared lysates were prepared as described (23). Plasmids were purified by centrifugation in a CsCl/ethidium bromide density gradient. For restriction mapping and sequence determination, plasmid DNA was further purified by centrifugation in a 5–40% sucrose gradient (24) or by chromatography on a column of Sepharose 4B.

mRNA Selection by Hybridization on Nitrocellulose Filters. Plasmid DNA was sonicated into pieces ranging from 0.3 to 1 kilobase. The DNA was denatured in 0.1 M NaOH for 10 min on ice. After neutralization with NaH_2PO_4, the DNA (60 μg) was immobilized onto 25-mm-diameter nitrocellulose filters. Four micrograms of mRNA was hybridized to each filter. The conditions for the hybridization have been described (25). For rapid screening, plasmid DNA from eight different recombinant clones was immobilized on the same filter. When a positive signal was obtained, the clones were rescreened individually.

Cell-Free Translation. The eluted mRNA was translated in a rabbit reticulocyte lysate system in the presence of dog pancreas microsomes (15). An aliquot (2 μl) of the translated products was characterized directly by $NaDodSO_4$/polyacrylamide gel electrophoresis (15). The remainder (23 μl) was subjected to immunoprecipitation using an antiserum specific for HLA-DR antigen β chains. In some cases, proteins translated in the presence of microsomes were subjected to proteinase K treatment to digest the cytoplasm tail of membrane-integrated proteins (15). Precipitated proteins were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography. The detailed procedures are described in ref. 15.

Restriction Mapping and DNA Sequence Analysis. The restriction map was constructed as described (26). For DNA sequence determination, the DNA was cleaved with a suitable restriction endonuclease and labeled with [γ-^{32}P]ATP at the 5′ ends (27). DNA fragments were then cleaved a second time to produce fragments with only one labeled end. Purification of DNA fragments for sequence determination was accomplished by polyacrylamide gel electrophoresis and electroelution into dialysis bags. Sequence determination was performed according to Maxam and Gilbert (27) and Maat and Smith (28).

Amino Acid Sequence Determinations of HLA-DR Antigen β Chains. Raji cells were separately labeled with [^{3}H]leucine, [^{3}H]phenylalanine, [^{3}H]tyrosine, and [^{35}S]methionine (Radiochemical Centre, Amersham, England) according to a published protocol (29). Radiolabeled HLA-DR antigen β chains were isolated and subjected to amino acid sequence determinations. In each case the β chains contained only a single type of radioactive amino acid. The details of the methods are outlined elsewhere (29).

Milligram amounts of highly purified HLA-DR antigens from Raji cells (5) were subjected to preparative $NaDodSO_4$/polyacrylamide gel electrophoresis to separate α and β chains. The HLA-DR antigen subunits were extracted from the gel, dialyzed against distilled water, and lyophilized. The β-chain fraction was subjected to automatic amino acid sequence determination in a Beckman 890 C sequencer (30). Phenylthiohydantoin derivatives of the amino acids were identified by high-pressure liquid chromatography (31).

Biosafety. All work involving recombinant plasmids was done under conditions conforming to the standards outlined in the National Institutes of Health guidelines for recombinant DNA research.

RESULTS

Construction of Plasmid pDR-β-1. RNA coding for HLA-DR antigen chains was isolated from a crude microsome fraction of Raji cells. After selection for poly(A)-containing mRNA, an aliquot was subjected to cell-free translation. Approximately 0.01% of the [^{35}S]methionine-labeled product was precipitated by the antiserum against HLA-DR antigen β chains. After size separation of the mRNA by sucrose gradient centrifugation, the purity of the β-chain mRNA increased 10-fold as evidenced by cell-free translation, immunoprecipitation, and $NaDodSO_4$/polyacrylamide gel electrophoresis. Fractions containing β-chain mRNA were combined and concentrated.

From the fractions containing HLA-DR antigen β-chain mRNA, 10 μg was copied into double-stranded cDNA and cloned into *Escherichia coli*. Double-stranded cDNA molecules recombined with *Pst* I-digested pBR322 yielded 1500 tetracycline-resistant, ampicillin-sensitive colonies after transformation. Plasmid DNA that was purified from individual colonies and combined into groups of eight was immobilized onto nitro-

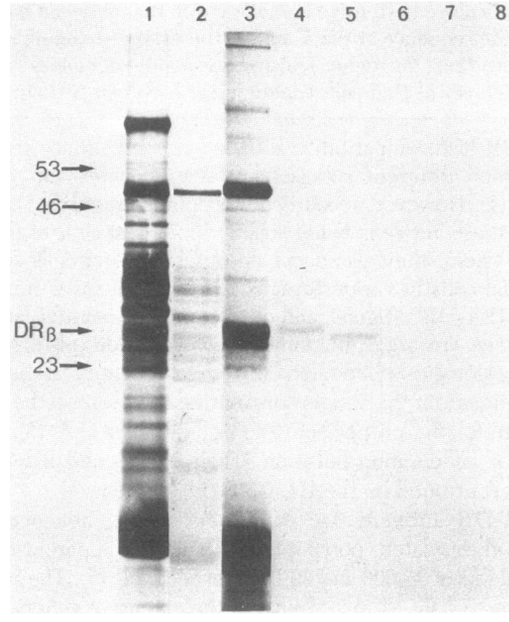

FIG. 1. Identification of pDR-β-1 by hybridization-selection and cell-free translation. Plasmid DNAs were immobilized onto nitrocellulose filters and hybridized with Raji mRNA. After elution, the mRNA was translated in a cell-free system. Translation products were characterized by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. Lanes: 1, total translation products, in the absence of microsomes, of Raji mRNA used for hybridization to pDR-β-1 DNA; 2, as lane 1 but in the presence of microsomes; 3, total translation products in the presence of microsomes of Raji mRNA hybridized to pDR-β-1 DNA; 4, as lane 3 but after precipitation with an anti-HLA-DR antigen β-chain antiserum; 5, after translation of Raji mRNA hybridized to pDR-β-1, the microsomes were treated with proteinase K (15) prior to immunoprecipitation of the β-chain; 6, 7, and 8, translation products of Raji mRNA hybridized to two separate recombinant plasmids containing unidentified inserts and to pBR322, respectively. All translations were carried out in the presence of microsomes except in lane 1. Translation products were precipitated with an HLA-DR antigen β-chain antiserum except in lanes 1–3. *Arrows*, positions of markers (kilodaltons).

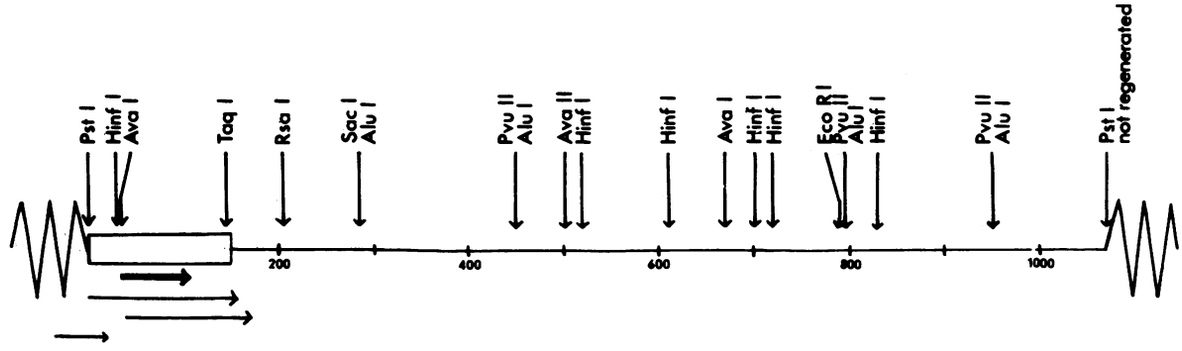

FIG. 2. Restriction map of the pDR-β-1 cDNA insert. The 1070-base-pair insert is drawn as a straight line with flanking pBR322 sequences, as it is oriented in the plasmid (with the *Eco*RI site close to the right-hand side of the insert). The 5′ end of the coding strand is to the left. The part of the insert whose sequence was determined is shown as a box, and the strategy is indicated by the arrows. The bold arrow indicates sequence obtained by the method of Maat and Smith (28). Thin arrows indicate sequence obtained by the Maxam and Gilbert method (27). Fragments were isolated from pDR-β-1 as follows: (*i*) cut with *Pst* I, labeled, recut with *Eco*RI, 790-base-pair fragment isolated; (*ii*) cut with *Ava* I, labeled, recut with *Sac* I, 250-base-pair fragment isolated; (*iii*) cut with *Ava* II, labeled, recut with *Ava* I, 140-base-pair fragment isolated. Fragment *i* was analyzed by both methods.

cellulose filters for the selection by hybridization of mRNA coding for HLA-DR antigen β chains. The hybridized mRNA was eluted and then translated in a cell-free system containing dog pancreas microsomes. After translation, the microsomes were isolated and analyzed for content of newly synthesized HLA-DR antigen β chains by immunoprecipitation and $NaDodSO_4$/polyacrylamide gel electrophoresis.

By using this selection method, 1 of 40 filters tested was positive (i.e., hybridized to β-chain mRNA). Plasmid DNA from each one of the eight bacterial colonies comprising the mixture present on the positive filter was immobilized separately onto nitrocellulose filters. The selection procedure was subsequently repeated and one positive clone was obtained. The mRNA eluted from this plasmid DNA directed the synthesis of a major polypeptide chain with an apparent molecular weight of about 28,000 (Fig. 1, lane 3). This polypeptide was precipitated by the antiserum against HLA-DR antigen β chains (lane 4) and comigrated with core-glycosylated β chains of Raji cells (not shown). Moreover, proteinase K digestion of the microsomes after the translation removed approximately 1000 daltons from the putative β chain (lane 5).

This result is in agreement with the observation that the β chain is a transmembrane protein. Other plasmids and the vector (Fig. 1, lanes 6–8) did not give rise to any immunoprecipitable polypeptide chain. The identified plasmid, named pDR-β-1, was used to generate restriction enzyme fragments of the insert. Colony hybridization was carried out with such labeled fragments (32). From the original 1500 clones, 2 more were identified as positive by this procedure. Detailed analyses of these clones will be reported elsewhere.

Partial Characterization of the pDR-β-1 Insert. A restriction map of the pDR-β-1 cDNA insert is shown in Fig. 2. The insert contains 1070 base pairs. Only the left *Pst* I site was reconstituted. Three cleavage sites for *Pvu* II, two for *Ava* I, and one for *Sac* I, *Taq* I, *Ava* II, and *Eco*RI were found in the insert.

Nucleotide sequence determinations were carried out from both ends of the insert. No stretch of poly(A) residues was observed. However, stop codons were found in all reading frames in the sequence close to the right-hand side of the insert. This is a strong indication that this portion of the cDNA corresponds to the noncoding region of the mRNA. Consequently, the left-hand side of the insert corresponds to the coding portion. The sequence of 150 nucleotides at the left-hand side of the insert is depicted in Fig. 3. Following 21 or 22 guanosines belonging to the poly(G)tail, the sequence of the insert most likely begins. Only one open reading frame exists. That reading frame provides unambiguous information for the amino acid sequence shown in the figure apart from the NH_2-terminal glycine (see below).

NH_2-Terminal Amino Acid Sequence Determination of Raji HLA-DR Antigen β Chains. Detergent-solubilized HLA-DR antigens were isolated from Raji cells. During the isolation procedure, which involved several fractionation steps (5), the occurrence of HLA-DR antigens was monitored both by a radioimmunoassay method and by $NaDodSO_4$/polyacrylamide gel electrophoresis. When no contaminating proteins could be detected in the HLA-DR antigen preparation, α and β chains were separated by preparative $NaDodSO_4$/polyacrylamide gel electrophoresis. The β-chain fraction was subjected to automatic NH_2-terminal amino acid sequence analysis. In 33 of the 35 degradation cycles performed, phenylthiohydantoin amino acid derivatives could be detected (Fig. 4). For seven positions, more than a single amino acid residue was found, supporting the fact that Raji cells are heterozygous at the *HLA-D* locus.

Because of the known genetic polymorphism of the HLA-DR antigen β chains (6, 7) we wished to confirm that the protein sequence was representative for the Raji cells. Accordingly, HLA-DR antigen β chains derived from Raji cells separately

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                                25                          50                          75
pDR-β-1      (G)22AG GGC AGA GAC TCT CCC GAG GAT TTC GTG TAC CAG TTT AAG GGC ATG TGC TAC TTC ACC AAC GGG
                     Gly-Arg-Asp-Ser-Pro-Glu-Asp-Phe-Val-Tyr-Gln-Phe-Lys-Gly-Met-Cys-Tyr-Phe-Thr-Asn-Gly-

                                100                         125                         150
             ACA GAG CGC GTG CGT CTT GTG AGC AGA AGC ATC TAT AAC CGA GAA GAG GTC GTG CGC TTC GAC
             Thr-Glu-Arg-Val-Arg-Leu-Val-Ser-Arg-Ser-Ile-Tyr-Asn-Arg-Glu-Glu-Val-Val-Arg-Phe-Asp
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FIG. 3. Nucleotide sequence at the 5′ end of the pDR-β-1 insert and the predicted amino acid sequence.

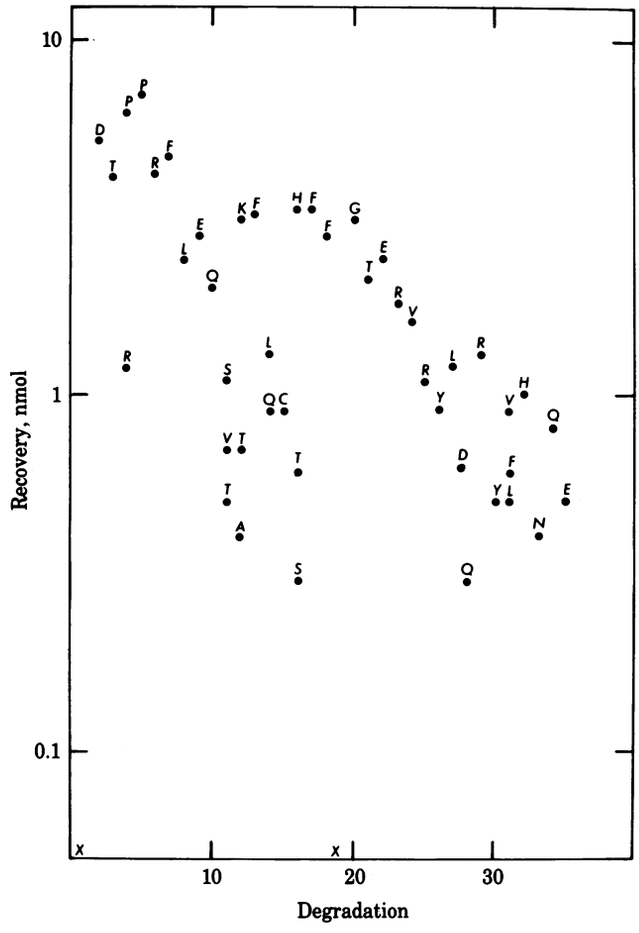

FIG. 4. Yields of phenylthiohydantoin derivatives in the amino acid sequence determination on 14 nmol of HLA-DR antigen β chain. The one-letter amino acid code is used. X, unidentified residue.

labeled with [^{35}S]methionine, [^{3}H]phenylalanine, [^{3}H]tyrosine, and [^{3}H]leucine were subjected to automatic amino acid sequence analyses. By this procedure, 11 of 30 positions in the NH_2-terminal portion of the Raji β chains were assigned one or more amino acid residues (Fig. 5). Seven of the 11 positions displayed the same residues as those obtained by analyzing the unlabeled β chains.

The varying yields of the radioactive phenylthiohydantoin derivatives clearly demonstrated that the Raji β chains were heterogeneous in amino acid sequence. For instance, the tyrosine in position 7 gave a lower yield than that in position 10. Likewise, the phenylalanine in position 11 was recovered in smaller amounts than that in position 13. These data together with the fact that 4 of the 11 positions analyzed by the radiochemical method displayed amino acid residues not found in the sequence of the unlabeled β chains strongly indicate that the β-chain family of Raji cells is greater than revealed by the sequence analysis of the unlabeled material.

Identification of pDR-β-1 As an HLA-DR Antigen β-Chain-Like Clone. Fig. 5 shows the NH_2-terminal amino acid sequences of HLA-DR antigen β chains as determined on the unlabeled (middle row) and on the biosynthetically labeled subunit (bottom row). The amino acid sequence deduced from the nucleotide sequence of the pDR-β-1 insert is also shown (top row). In the region available for comparison (i.e., 33 positions), pDR-β-1 displays an overall homology to the combined protein sequences of 64%. To maximize the homology, the penultimate arginine of pDR-β-1 has to be aligned with the NH_2 terminus of the protein sequences. This suggests that the NH_2-terminal glycine of pDR-β-1 represents the last residue of the signal sequence. However, the glycine residue should be regarded as tentative because a single adenosine separates the poly(G)tail from three additional guanosines, two of which are part of the glycine codon. Thus, the adenosine may be an artifact which arose during the tailing reaction.

The homology between the protein sequence of the unlabeled material and pDR-β-1 is 48% whereas the homology between the radiochemical sequence and pDR-β-1 is 64%. Those positions in the unlabeled β-chain sequence corresponding to the known ones in the radiochemical sequence show only 18% homology with pDR-β-1.

DISCUSSION

To clone HLA-DR antigen β-chain cDNA, we used methods that proved successful in cloning H-2 antigen cDNA (14). There seems to be three key features related to using this protocol. First, the mRNA has to be enriched because Raji cells contain relatively small amounts of β-chain mRNA. Although size separation by sucrose gradient centrifugation is an efficient means for enrichment of the mRNA, the use of microsomal mRNA rather than total mRNA as the starting material was probably just as important. Second, cell-free translations were always carried out in the presence of dog pancreas microsomes (15). In our experience the microsomes promote the translation, protect the protein against proteolytic degradation, and, by removing the signal sequence and by adding the core sugars, allow the expression of most if not all antigenic determinants present on the cell surface form of the protein. Third, the availability of a specific antiserum strongly reactive against the microsomal form of the β chain was important. It seems that the use of $NaDodSO_4$/polyacrylamide gel electrophoresis, which denatures the protein to some extent, to isolate β chains for immunization provided us with an antiserum that reacted better with the isolated β chains than with the intact HLA-DR antigens.

The cDNA clone pDR-β-1 contains an insert of 1070 base

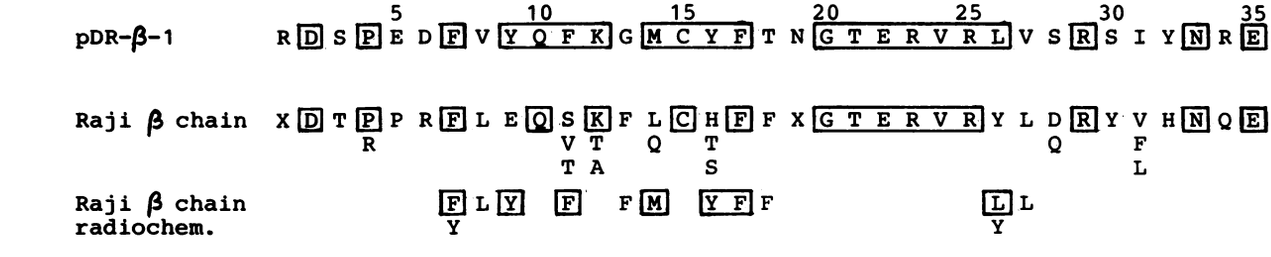

FIG. 5. Predicted amino acid sequences of pDR-β-1, Raji HLA-DR antigen β-chain sequence determined on unlabeled material, and radiochemical amino acid sequence of the HLA-DR antigen β chains biosynthetically labeled with leucine, phenylalanine, tyrosine, and methionine. Amino acid sequence homologies are denoted by boxes.

pairs. Because pDR-β-1 comprises the nucleotide sequence corresponding to the NH_2 terminus of a β chain and because β chains should be composed of about 230 amino acids, as calculated from the apparent molecular weight of nonglycosylated β-chains, pDR-β-1 should provide the entire nucleotide sequence of the translated portion of a β chain. In addition, approximately 300 base pairs of untranslated sequence should be obtained. This is obviously less than the complete untranslated region because our preliminary nucleotide sequence analysis at the right-hand side of the pDR-β-1 insert fails to reveal a poly(A) site. Apart from providing the protein sequence for a β chain, the pDR-β-1 clone should prove useful in analyses of the structure and organization of the *HLA-D* locus and its genes.

Data on HLA-DR antigen β-chain amino acid sequences are scarce. In fact, 15 positions in the NH_2-terminal region appear to have been identified (10, 33, 34). This situation together with the fact that β chains display an extensive genetic polymorphism prompted us to elucidate the NH_2-terminal sequence of HLA-DR antigen β chains derived from Raji cells. The amino acid sequence found for unlabeled β chains demonstrated the heterogeneity of the material because multiple residues were obtained in some positions. We expected to find two amino acid residues in some positions because the Raji cells express two alleles, *Dw3* and *Dw6*, at the *HLA-D* locus. However, three amino acid residues were obtained for four positions. This suggests that the HLA-DR antigens may comprise more than two types of β chains. In fact, recent data strongly indicate that the *HLA-D* region may contain two loci (11, 12). The present observations are compatible with such an idea because in three of the four positions where the amino acid sequence determination revealed three amino acid residues, pDR-β-1 contained yet a fourth amino acid residue. Consequently, Raji cells may be heterozygous also at a second *HLA-D* locus.

It is well established that the murine counterpart of the *HLA-D* locus, the *I* region, contains two subloci. A similar situation has been noted in the rat (35) and in the chicken (36). Unfortunately, not enough amino acid sequence information is available relative to murine I-A and I-E/C β chains to allow any conclusion as to whether pDR-β-1 may be the equivalent of an I-A or an I-E/C β chain.

The NH_2-terminal amino acid sequence determinations were carried out by two entirely different techniques. This had to be done because the isolation procedures used may have provided highly purified HLA-DR antigens that were not quantitatively representative of the Raji cell HLA-DR antigens. Indeed, the two protein sequences only displayed 64% homology at the positions available for comparison. It is interesting to note that the four positions in the radiochemical sequence that differed from the corresponding amino acid sequence of the unlabeled β chains were identical to the pDR-β-1. Therefore, the two protein sequences firmly establish that the isolated cDNA clone corresponds to the mRNA of an HLA-DR antigen β chain.

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