Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 5966–5970, October 1982
Genetics

Isolation of a cDNA clone for the human HLA-DR antigen α chain by using a synthetic oligonucleotide as a hybridization probe

(B-cell histocompatibility antigen/restriction fragment polymorphism)

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Communicated by Hugh O. McDevitt, June 17, 1982

ABSTRACT **We have used a synthetic 20-nucleotide hybridization probe to isolate a cDNA clone encoding the α chain of the HLA-DR antigen from a cDNA library constructed from membrane-bound poly(A)$^+$mRNA. A set of synthetic 11-nucleotide fragments, potentially complementary to the codons for amino acids 11–14 of the HLA-DR α chain, were used to prime a cDNA synthesis reaction on various poly(A)$^+$mRNA templates. Extension of the primers in the presence of a single dideoxynucleotide triphosphate resulted in an 18-nucleotide cDNA product whose sequence corresponded to the NH_2-terminal amino acids of the HLA-DR α chain. An oligonucleotide was synthesized based on this sequence information and its specificity for HLA-DR α mRNA was confirmed by primer extension and blot analysis. The cDNA library made from mRNA from the lymphoblastoid cell line CA-SC was probed with ^{32}P-labeled cDNA synthesized on poly(A)$^+$mRNA from a B-cell line (CA-SC) or from a T-cell line (Molt-4) to enrich for B-cell-specific clones. A set of cDNA clones that hybridized preferentially with the B-cell probe was screened with the ^{32}P-labeled 20-nucleotide probe. The cDNA clone isolated by this procedure is 1,100 nucleotides long; the nucleotide sequence of the 5′ end of the cDNA insert corresponds to the amino acid sequence of the HLA-DR α chain. Hybridization of this cDNA clone to genomic blots suggests that the HLA-DR α chain is encoded by a single-copy gene. One of the restriction endonucleases used in genomic DNA digests reveals a restriction fragment polymorphism.**

The major histocompatibility complex (MHC) of mammals is a highly polymorphic genetic region composed of a set of tightly linked genes encoding several cell surface glycoproteins. Genes that map within the MHC control a variety of basic immunological functions; in particular, immune responsiveness to defined antigens has been mapped to the *I* region of the mouse MHC, the *H-2* complex (1). The human MHC, the *HLA* complex, is located on chromosome 6 and consists of the related loci *HLA-A*, *HLA-B*, and *HLA-C*, which code for glycoproteins of ≈45,000 daltons present on the surface of all nucleated cells, and the *HLA-D* locus, which codes for noncovalently associated glycoproteins of ≈34,000 (α) and ≈29,000 (β) daltons (the HLA-DR antigens) present primarily on B cells. The *HLA-D* locus is homologous, by a variety of criteria, to the mouse *I* region (2–4) and, like the *HLA-A*, *-B*, and *-C* loci, is highly polymorphic. The HLA-DR antigens are the presumptive human analogues to two of the four biochemically identified mouse *I*-region-associated (Ia) antigens (Eα and Eβ; 3, 4). The serologic polymorphism for the HLA-DR and the mouse Ia antigens appears to reside primarily in the β chain (2–5). Many recent studies have revealed striking associations of specific serologically defined alleles at *HLA* loci and a large number of human diseases (6). However, the allosera that detect polymorphic HLA-D-associated antigens (DR specificities) are serologically complex and limited in availability, making analysis of HLA-D polymorphism difficult. The molecular analysis of *HLA* genetic organization, afforded by cloned DNA probes, will allow the definition of alleles at the DNA level; it may also elucidate the mechanism of the disease associations and provide insights into the biological function of the *HLA* gene products. The isolation of a cDNA clone for the α chain of the HLA-DR antigen, reported here, provides a specific probe for analyzing the genetic organization, polymorphism, and regulation for one of the *HLA-D* locus-encoded antigens.

MATERIALS AND METHODS

Construction of cDNA Library. Poly(A)$^+$mRNA from membrane-bound polyribosomes was prepared by modifications of published procedures (7, 8) from CA-SC cells obtained from Hugh O. McDevitt. CA-SC has been HLA-DR typed as DR1/DR2 by Carl Grumet. Double-stranded cDNA was synthesized from 10 μg of poly(A)$^+$mRNA from membrane-bound polyribosomes of CA-SC cells and inserted into the *Pst* I site of pBR322 with G·C tailing by using modifications of published procedures (9). This material was used to transform *Escherichia coli* CS412 (9).

cDNA Synthesis Primed by Synthetic Oligonucleotides. Oligonucleotides were chemically synthesized by the triester method (10) and phosphorylated with [γ-^{32}P]ATP (New England Nuclear) and T4 polynucleotide kinase (Biogenics Research, Dublin, CA). The sequences of the oligonucleotides were confirmed by the two-dimensional electrophoresis/homochromatography technique (11) or by a modification of the Maxam–Gilbert technique (12). Total poly(A)$^+$mRNA was prepared from the cell line LG2 for use as template in a cDNA synthesis reaction primed by synthetic oligonucleotides; 160 μg of poly(A)$^+$mRNA was mixed with 1,100 pmol of ^{32}P-labeled primer and the dideoxynucleoside triphosphate-terminated cDNA reaction was carried out as described (13). The individual cDNA products were characterized by two-dimensional electrophoresis/homochromatography (11) or by the Maxam–Gilbert technique (12).

cDNA Screen of cDNA Library. [^{32}P]cDNA was synthesized as described (14) from poly(A)$^+$mRNA from the T-cell line Molt-4 and from sucrose-gradient size-fractionated poly(A)$^+$mRNA from the B-cell line CA-SC. Fractions corresponding to the peak of translational activity for proteins with the expected two-dimensional gel (15) mobility of HLA-DR antigens were the gift

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Abbreviations: MHC, major histocompatibility complex; NaCl/Cit, 0.15 M NaCl/0.015 M Na citrate; kb, kilobase(s); bp, base pair(s).

of Marie-Francoise Aellen-Schulz and were used as template for the cDNA synthesis reaction. Colonies from the cDNA library constructed from membrane-bound B-cell mRNA were replica plated from microtiter dishes onto duplicate nitrocellulose filters as described (16) and hybridized with the ^{32}P-labeled cDNA probes from B-cell and T-cell mRNA as described (17).

Oligonucleotide Screen of cDNA Library. Plasmid DNA was prepared by CsCl/ethidium bromide equilibrium density centrifugation from members of the B-cell specific cDNA subset, denatured with NaOH and heat, neutralized, and spotted onto nitrocellulose. The filter was hybridized in 0.75 M NaCl/0.075 M Na citrate (6× NaCl/Cit)/10× Denhardt's solution (18)/0.2% NaDodSO$_4$ containing the ^{32}P-labeled 20-nucleotide probe at 3.3×10^5 cpm/ml for 60 hr at 37°C, washed at 20°C with 4× NaCl/Cit/0.2% NaDodSO$_4$, and autoradiographed.

mRNA and Genomic DNA Blot Analysis. Poly(A)$^+$mRNA prepared by modifications of published procedures (7, 8) was electrophoresed on a 1.5% agarose slab gel in the presence of 6% formaldehyde as described (19) and transferred to a nitrocellulose filter (20). The filter was hybridized with the ^{32}P-labeled 20-nucleotide probe for 36 hr at 37°C in 4× NaCl/Cit/5× Denhardt's solution/0.2 mM EDTA/0.1% NaDodSO$_4$ and washed in 2× NaCl/Cit/0.1% NaDodSO$_4$ at room temperature. The filter was boiled in 5 mM EDTA for 5 min to remove the bound ^{32}P-labeled 20-nucleotide probe, hybridized with ^{32}P-labeled nick-translated (21) pDRα-1 plasmid DNA as described (17), and washed as described (20).

High molecular weight DNA was prepared by using modifications of published procedures (22), digested with restriction endonucleases, electrophoresed on a 0.6% agarose gel, transferred to nitrocellulose by published procedures (23, 24), and hybridized (17) and washed (20) as described.

Nucleotide Sequence Analysis of the cDNA Clone. *Pst* I digestion of pDRα-1 yields a 520-base-pair (bp) and a 580-bp insert fragment. The 520-bp insert fragment was purified from a 5% acrylamide gel. The 3'-recessed ends of a *Sau*3A/*Pst* I subfragment and a *Hin*fI/*Hin*fI subfragment of the 520-bp *Pst* I fragment were labeled with DNA polymerase I (Klenow fragment). Strand separation of the *Hin*fI/*Hin*fI subfragment was carried out by standard techniques (12). The sequence of the labeled fragments was determined by the technique of Maxam and Gilbert (12).

RESULTS

Enrichment of mRNA Used as Template for cDNA Library. RNA from membrane-bound polyribosomes and free polyribosomes was prepared from the human lymphoblastoid B-cell line CA-SC; the amount of RNA extracted from the free polyribosomes was typically ≈10-fold greater than the amount of membrane-bound RNA. The partition of specific mRNAs into membrane-bound and free fractions was monitored by two-dimensional gel analysis (15) of the ^{35}S-labeled products of *in vitro* translation (25) and by immunoprecipitation of oocyte translation products. The pattern of the translation products of membrane-bound mRNA was reduced in complexity and enriched for a number of specific spots having the electrophoretic mobility of MHC-associated antigens. The monoclonal antibody, 2.06 (26), precipitated HLA-DR antigens from oocyte translation products of membrane-bound mRNA but not of free mRNA (unpublished results). We estimate that the membrane-bound mRNA fraction is enriched ≈10-fold for *HLA-D*-related mRNA species. Double-stranded cDNA was prepared from the membrane-bound mRNA and inserted into pBR322.

Strategy for Synthesis of the 20-Nucleotide Probe. The sequence of a set of four 11-nucleotide fragments was based on limited published data available for the NH_2-terminal amino acid sequence of HLA-DR α chain derived from the cell line RPMI-4265 (4). The oligonucleotides, synthesized by the triester method (10), were all potentially complementary to the codons for the tetrapeptide Glu-Phe-Tyr-Leu at position 11–14 (Fig. 1). These amino acids show minimal codon degeneracy, with ambiguities at positions 2, 3, 6, and 9 (Fig. 1). Four out of a possible 16 different sequences were synthesized, incorporating adenosine or guanosine at position 6 and thymidine or cytidine at position 9. Guanosine was chosen for positions 2 and 3 to minimize the destabilizing effect of potential mismatched bases.

Since the synthetic oligonucleotides were complementary to codons for amino acids 11–14, oligonucleotide-primed cDNA synthesis from the *HLA-DR* α-chain mRNA would be expected to generate a product of 150–200 nucleotides. This estimate is based on a presumptive leader sequence of ≈75 nucleotides (27) and assumes a 5'-untranslated region of 75–125 nucleotides. The specificity of the four 11-mers was compared by using them individually as primers in cDNA synthesis reactions on membrane-bound, free, and total poly(A)$^+$mRNA from B cells and total poly(A)$^+$mRNA from T cells. Only the oligonucleotide A-G-G-T-A-G-A-A-C-T-C primed a cDNA product of the expected size (≈175 nucleotides), which was observed only in reactions with B-cell membrane-bound mRNA template (unpublished results). The specificity of this 11-nucleotide fragment was confirmed by extending the primer in a cDNA synthesis reaction in the presence of a dideoxynucleotide triphosphate, an approach that had proved successful in the isolation of the *HLA-B7* cDNA clone (13). In the presence of dideoxy ATP, a minor cDNA band corresponding to a predicted 18-nucleotide primer extension product was observed. The additional seven nucleotides were determined by two-dimensional electrophoresis/homochromatography (11) to be G-G-C-C-T-G-A, corresponding to the HLA-DR α-chain amino acids at positions 10, 9, and 8 (Fig. 1). The subsequent two nucleotides at the 3' end, A-T, could be inferred from the codon for isoleucine.

A 20-nucleotide fragment corresponding to this sequence was then synthesized by the triester method (10). The specificity

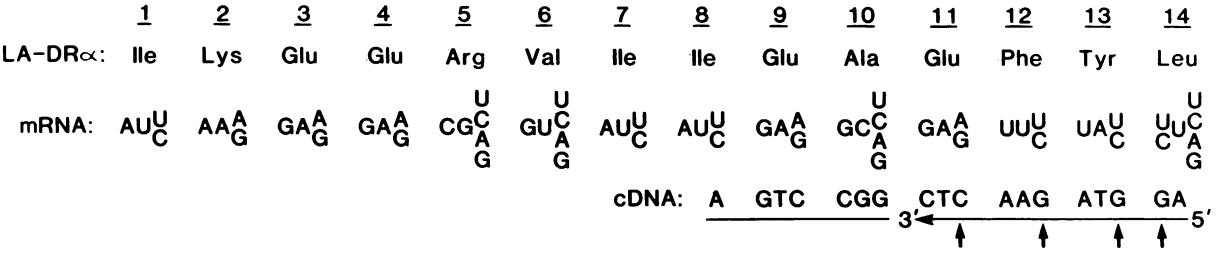

FIG. 1. Nucleotide sequence of a dideoxy ATP-terminated cDNA product primed by an 11-nucleotide fragment. Arrows indicate degenerate positions.

FIG. 2. cDNA product primed by the 20-nucleotide fragment. The upper arrow indicates the *HLA-DR* α-chain primer extension product (≈175 nucleotides); the lower arrow indicates the 20-nucleotide primer.

of this fragment as a primer was examined in a cDNA synthesis reaction on poly(A)$^+$mRNA from the B-cell line LG2. A major cDNA band, ≈175 nucleotides long, was synthesized (Fig. 2); the nucleotide sequence of the eluted band, determined by the Maxam–Gilbert technique, corresponded to the expected sequence for the HLA-DR α chain (see Fig. 4). An arginine to histidine substitution at position 5 and a glutamic acid to glutamine change at position 9 represent the only discrepancies with the published NH_2-terminal sequence; these differences may reflect errors in the original amino acid assignments rather than genetic polymorphism. The sequence agrees, however, with amino acid sequence data for the HLA-DR α chain from cell line LG2 (28).

The specificity of the 20-nucleotide fragment as a hybridization probe was analyzed on a RNA blot of poly(A)$^+$mRNA (Fig. 3*A*). A unique band of ≈1,400 nucleotides was observed after hybridization of mRNA from the B-cell line CA-SC with the ^{32}P-labeled 20-nucleotide probe. This ≈1,400 nucleotide band was absent in mRNA from the T-cell line Molt-4 but was present in mRNA from another B-cell line, Namalwa. Membrane-bound mRNA from Namalwa was enriched for mRNA that hybridized to the 20-nucleotide probe.

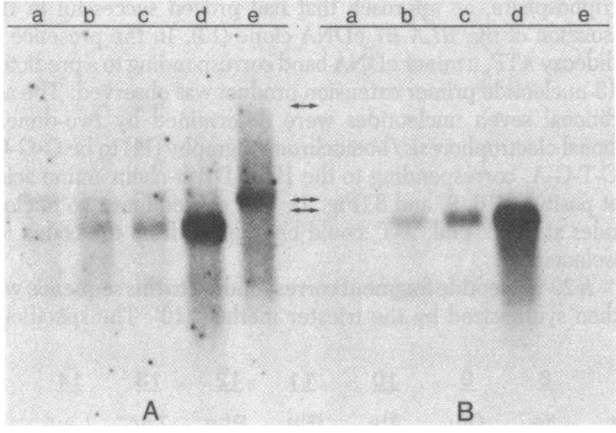

FIG. 3. Hybridization of the RNA blot with the 20-nucleotide probe and the pDRα-1 probe. RNA was electrophoresed, blotted to nitrocellulose, and hybridized with end-labeled 20-nucleotide probe (*A*) or with nick-translated pDRα-1 (*B*). Lanes: a, 5 μg of free poly(A)$^+$mRNA from the B-cell line Namalwa; b, 5 μg of membrane-associated Namalwa poly(A)$^+$mRNA; c, 5 μg of Namalwa total poly(A)$^+$mRNA; d, 10 μg of CA-SC total poly(A)$^+$mRNA; e, 15 μg of total poly(A)$^+$mRNA from the T-cell line Molt-4. Size markers (arrows): 28S rRNA (≈5 kb), 18S rRNA (≈2 kb), *HLA-B7* mRNA (≈1.7 kb). The band in lane e reflects nonspecific binding of the probe to 18S rRNA.

Screening the cDNA Library Constructed from Membrane-Bound mRNA. The extent of enrichment for mRNAs encoding rare membrane proteins in the membrane-bound mRNA preparation was assessed by hybridization of the cDNA library with several specific probes for such sequences. A cDNA clone encoding the HLA-B7 antigen, previously described (13), hybridized to about 4% of the cDNA clones in the library and a mouse β_2-microglobulin cDNA clone (29) hybridized to about 1% of the clones. Since both of these cDNA clones encode rare (<0.1%) membrane proteins, these results show the substantial enrichment afforded by using membrane-bound mRNA as the template for construction of a cDNA library. To further enrich for *HLA-D*-associated mRNA sequences, the library was hybridized with cDNA probes expected either to contain or to lack these sequences.

Duplicate sets (≈4,000 clones per set) of colonies were transferred to nitrocellulose filters and hybridized with [^{32}P]cDNA probes prepared from size-fractionated B-cell mRNA and T-cell mRNA template. A subset of ≈350 clones containing sequences of B-cell-specific mRNAs of the selected size range and derived from membrane-bound polyribosomes was defined by this screening.

Plasmid DNA prepared from members of this subset was spotted onto nitrocellulose and hybridized with the ^{32}P-labeled 20-nucleotide probe. One clone, pDRα-1, was found to hybridize strongly to the probe (data not shown).

Characterization of the cDNA Clone. In a RNA blot experiment, the pDRα-1 probe hybridized to a B-cell mRNA of the same size (≈1,400 nucleotides) as the band complementary to the 20-nucleotide probe. This mRNA was enriched in membrane-bound mRNA from B cells and was absent in T-cell mRNA (Fig. 3*B*). In addition, mRNA selected by hybridization to pDRα-1 DNA was shown to encode an acidic protein of ≈32,000 daltons in an *in vitro* translation experiment (unpublished result), as expected for the HLA-DR α chain.

Confirmation of identity was provided by DNA sequence analysis. Digestion of the pDRα-1 plasmid with *Pst* I gave fragments of 580 and 520 nucleotides, indicating a cDNA insert of 1,100 nucleotides with an internal *Pst* I site. The presumptive 5′ end of the cDNA insert, which should correspond to the published NH_2-terminal amino acid sequence, was defined by hybridization to the 20-nucleotide probe and its sequence was determined by the Maxam–Gilbert technique (12). The pDRα-1 DNA sequence predicts an amino acid sequence that matches the NH_2-terminal data for the HLA-DR α chain and is identical to the sequence determined for the 175-nucleotide primer extension product derived from LG2 mRNA (Fig. 4) at all but two nucleotides. The 5′ end of the cDNA insert is about 95 nucleotides from the 5′ end of the mRNA and falls within the twelfth amino acid of the presumptive leader sequence. The complete sequence of pDRα-1, which appears to be truncated at the 3′ end since no poly(A)$^+$ sequences were found, will be reported elsewhere.

Analysis of Genomic *HLA-DR* α Chain Genomic Sequences. Genomic DNA from two human cell lines, CA-SC and T5-1, was digested with a variety of restriction endonucleases, resolved by electrophoresis on agarose gels, transferred to nitrocellulose by the method of Southern (23), and hybridized to ^{32}P-labeled pDRα-1. In most enzyme digests, a single genomic restriction fragment hybridized to the pDRα-1 probe (Fig. 5), in contrast to the complex multilocus pattern observed with a cDNA probe

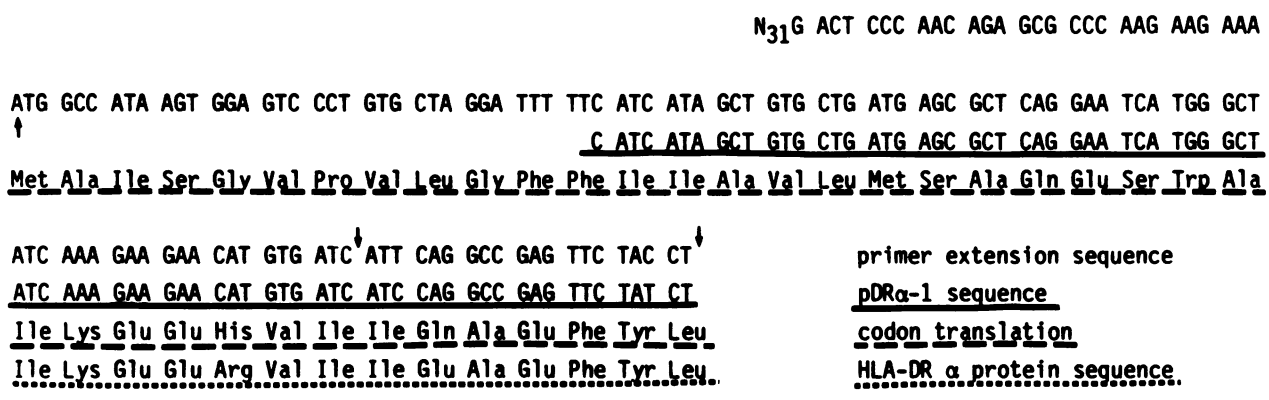

FIG. 4. Comparison of the amino acid sequence of the HLA-DR α-chain with the nucleotide sequence of the pDRα-1 cDNA clone and the DNA complement of the ≈175-nucleotide primer extension product. Downward arrows delimit the sequence of the 20-nucleotide primer. Upward arrow indicates the initiating methionine codon of the leader sequence.

encoding the HLA-B7 antigen (13). The simplest interpretation of these data is that the HLA-DR α chain is encoded by a unique-copy sequence. The two bands observed with *Pst* digestion are consistent with this interpretation since there is a *Pst* I site in the middle of the cDNA clone. The pDRα-1 probe hybridized to a unique 3.5-kilobase (kb) *Eco*RI fragment (Fig. 5). However, similar analysis of a genomic clone (to be described elsewhere) with the 175-nucleotide cDNA primer extension product has revealed an additional 4.5-kb band due presumably to an *Eco*RI site in an intervening sequence near the 5′ end of the gene (unpublished results). The data obtained from genomic blots and from this genomic clone suggest that the *HLA-DR* α-chain gene, like the *HLA* genes (30, 31), is contained within a 3- to 4-kb fragment of DNA.

One of the enzymes tested, *Bgl* II, reveals a restriction fragment polymorphism between DNA from CA-SC and from T5-1, a cell line apparently heterozygous for the polymorphic *Bgl* II site. One of the two bands present in the *Bgl* II digest of T5-1 DNA is absent in the deletion variant, 6.3.6 (32), derived from T5-1 (unpublished results).

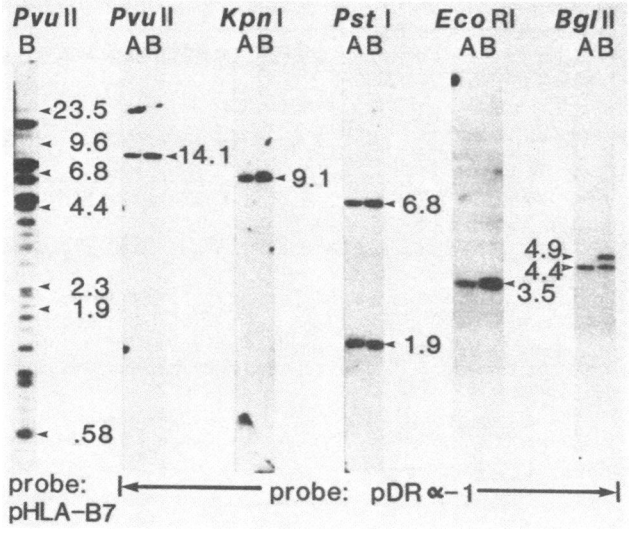

FIG. 5. Hybridization of genomic blots with the pDRα-1 probe. Seven-microgram samples of CA-SC (lanes A) or T5-1 (lanes B) were digested with various restriction endonucleases, electrophoresed, blotted to nitrocellulose, and hybridized with ^{32}P-labeled pDRα-1 or pHLA-B7. Arrows indicate restriction fragment sizes in kilobase pairs.

DISCUSSION

The cell surface antigens encoded by MHC loci represent less than 0.1% of the cellular protein and are presumably encoded by rare mRNA species. The molecular cloning of rare mRNA sequences such as those specifying the HLA and H-2 antigens and β_2-microglobulin has recently been achieved by using a specific antibody to screen the translation products of mRNAs hybridizing to cloned cDNA sequences (29, 33, 34). After the work reported here was completed, Lee *et al.* (35) reported the identification of an *HLA-DR* α-chain cDNA clone by using this technique. This approach, however, requires the availability of sera or monoclonal antibodies capable of reacting specifically with the *in vitro* translation products of a unique mRNA. An alternative approach using a mixture of synthetic oligonucleotides has recently been used to identify mouse *H-2* and human β_2-microglobulin clones (36, 37). For the identification of an *HLA-DR* α-chain cDNA clone, we adopted a strategy using an individual oligonucleotide as a hybridization probe, an approach successfully used in the isolation of an *HLA-B* cDNA clone (13). The oligonucleotide sequence was based on the limited NH_2-terminal amino acid sequence data available for the HLA-DR α chain. Consequently, this approach ensures that any *HLA-DR* α-chain cDNA clone isolated with the probe will contain the 5′-cDNA sequences necessary for a definitive identification using available NH_2-terminal protein sequence information.

Given the ambiguities in the codons for the chosen tetrapeptide, Glu-Phe-Tyr-Leu, it was necessary to synthesize a set of potentially complementary 11-nucleotide fragments. The synthesis of a cDNA primer extension product of a predicted length, either a full-length product of 150–200 nucleotides or a dideoxy ATP-terminated product of 18 nucleotides, was monitored as a test of primer specificity. Sequence analysis of the primer extension product provided definitive evidence of specificity. By comparing the sequence of the cDNA clone and the four oligonucleotides, we could, in retrospect, evaluate directly their degree of complementarity. The 11-nucleotide primer that appeared, on the basis of the primer extension studies, to be the best matched of the four primers tested proved to have the correct nucleotides at the variable positions 6 and 9 and the invariant position 2. The choice of guanosine at the invariant position 3 was incorrect.

The 20-nucleotide fragment, whose synthesis was based on the sequence of the 18-nucleotide primer extension, was used to probe a highly enriched cDNA bank. The enrichment for HLA-D-associated antigens in the membrane-bound mRNA used for cDNA synthesis was estimated to be ≈10-fold. Six other clones in this cDNA bank hybridized strongly to the

pDR-α probe under stringent conditions; these clones presumably represent cDNA copies of *HLA-DR* α-chain mRNA sequences. The observed frequency of presumptive *HLA-DR* α-chain clones (7/4,128 or 0.17%), assuming the enrichment estimate of $\approx$10-fold, suggests that the level of *HLA-DR* α-chain mRNA in the CA-SC cell is $\approx$0.02%. Within the subset of clones that hybridized preferentially to the cDNA probe made from size-fractionated B-cell mRNA, the frequency of presumptive *HLA-DR* α-chain clones was $\approx$1.7% (6/350). Thus, an additional enrichment factor of $\approx$10-fold was afforded by the size-selected "B vs. T" screening. Under conditions of low stringency, 11 other cDNA clones hybridized weakly to the pDRα-1 probe; these are candidates for clones encoding additional HLA-D-associated α-chain antigens, expected by analogy with the mouse Ia antigens (3).

Genomic blots of total human DNA hybridized under stringent conditions with the pDRα-1 probe revealed a single band with most of the restriction endonucleases tested. This genomic blot pattern is much simpler than the multilocus pattern obtained with probes encoding the HLA antigens (Fig. 5; refs. 30, 38). The observation of a single genomic restriction fragment complementary to pDRα-1 indicates that the α chain of the HLA-DR antigen is probably encoded by a single genetic locus but cannot exclude a related set of loci containing conserved restriction sites.

One of the enzymes tested, however, does reveal a restriction site polymorphism near or within the *HLA-DR* α-chain gene. Since the HLA-DR α chain, unlike the polymorphic HLA-DR β chain, exhibits little haplotype-specific variation, MHC linkage has been difficult to demonstrate. By using the mutant cell line, 6.3.6 (32), which contains a small deletion on chromosome 6 covering the MHC, we have utilized restriction fragment polymorphism to map the *HLA-DR* α-chain gene to a region on the short arm of chromosome 6 (unpublished results). In addition, by using recombinant congeneic mice, we have mapped DNA sequences complementary to the pDRα-1 probe to the *I-E* region of the mouse MHC (unpublished results).

MHC cDNA clones such as the one described here should provide useful probes for analyzing the genetic organization, polymorphism, and regulation of loci in the MHC.

We are grateful to Hugh McDevitt for his encouragement and advice and to Marie-Francoise Aellen-Schulz, Tom St. John, and Chander P. Bahl for helpful discussions. We thank Dan Denney and Kathy Lee for valuable technical assistance and Elizabeth Jarvis for careful preparation of the manuscript. H.D. was supported by a fellowship grant from the Cancer Research Institute.

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