*Proc. Natl. Acad. Sci. USA*
Vol. 82, pp. 8614–8618, December 1985
Immunology

# *HLA-B* locus polymorphism: Studies with a specific hybridization probe

(*HLA* class I genes/Southern blotting/expression in murine L cells)

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*Contributed by Hugh O. McDevitt, April 19, 1985*

ABSTRACT **The large number of class I histocompatibility genes (*HLA*) and their extensive homology has made it difficult to assign bands on genomic Southern blots to known genes. Therefore, we have tried to obtain nucleic acid probes for class I genes that are locus specific or have restricted locus specificity. Computer sequence-homology analysis was used to compare the nucleic acid sequences of two genomic clones, one coding for the HLA-B7 antigen (JY150) and one containing a class I pseudogene (pHLA12.4). A sequence in the 3′ untranslated region with very low homology was identified. This sequence from the *HLA-B7* gene was subcloned into M13 phage. This fragment, JY150/C5, hybridized with two genomic bands in DNA from human *HLA* homozygotes—presumably the *HLA-B* locus gene and a closely related gene. The probe was used to assess restriction fragment polymorphism at the *HLA-B* locus in homozygous consanguineous cell lines. This analysis permitted the association of certain polymorphic restriction enzyme fragments with some alleles of this locus. However, many *HLA-B* alleles have identical restriction fragments produced by a number of restriction endonucleases.**

The major histocompatibility complex (MHC), *HLA* in the human, *H-2* in the mouse, plays an important role in regulation of the immune response. In humans, at least three classes of products are encoded by the MHC: class III products (involved in the complement cascade); class II products (which mediate cellular interaction in the cooperation between macrophages, B cells, and T cells in the immune response); and class I products required for T-cell cytolysis (1–4). The class I products are glycoproteins of 44,000 Da associated with a smaller polypeptide, $\beta_2$-microglobulin (12,000 Da) (5, 6). Comparison of amino acid sequence between many different HLA and H-2 molecules has shown a high degree of homology in their primary structures (7).

In the human, three class I loci, *HLA-A*, *-B*, and *-C* have been identified by serological analysis. At each locus, there is a large number of different alleles. Analysis of genomic DNA with a cDNA probe reveals the presence of many closely related class I genes (*25–40*). The class I multigene family has two remarkable characteristics: all members of this multigene family have considerable overall sequence homology (70–80%) (8), yet several individual loci are very highly polymorphic (1). In particular, the *HLA-A*, *-B*, and *-C* loci have 10–40 alleles. These two characteristics could be explained by gene conversion between both allelic and nonallelic genes (9–11). Although this hypothesis has never been proven in the human system, gene conversion may also explain why "A-ness" and "B-ness" have been difficult to find at the protein or DNA level.

In this report we show that the 3′ untranslated region from a genomic clone coding for HLA-B7 is apparently specific for the *HLA-B* locus. A probe constructed from this region specifically hybridizes to *B* locus genes, allowing correlation of the pattern of restriction enzyme fragment length polymorphism with particular alleles of this locus on genomic blots. In addition, another class I gene or pseudogene (a potential *B* locus duplication) can be detected with this probe. A probe with this specificity will be useful for further study of MHC polymorphism at the DNA level and the association between the MHC and various diseases.

## MATERIALS AND METHODS

**Cell Lines.** The cell lines used in this study have the following *HLA* alleles: (*i*) consanguineous mating-derived homozygous cell lines are PGF, *A3, B7*; WT49, *A2, B17*; THR, *A25, B18*; MVL, *Aw32, B27*; LBF, *Aw30, B13*; AVL, *A1, B8*; OOS, *A26, Bw22*; DKB, *Aw24, B40*; IBW4, *A3, Bw35*; WVD, *A2, Bw16*; WT51, *A9, B14*; LG2, *A2, B27*; and (*ii*) heterozygous cell lines are JY, *A2, B7*; MWF, *A2, B8, w35*; VC, *A3, w24, B7, 27*; KCA, *A11, w24, B27, w44*.

The cell lines PGF, WT51, IBW4, WT49, VC, KCA, and JY were grown in RPMI 1640 medium with 10% fetal calf serum. Several samples of homozygous consanguineous peripheral blood cells were transformed with Epstein–Barr virus (AVL, WVD, THR, OOS, MVL, and DKB). The homozygous consanguineous cell lines had a fully documented genealogy and were known to be derived from the offspring of first-cousin marriages or more closely related matings.

**DNA Isolation.** Genomic DNA was obtained from $10^8$ fresh or previously frozen cells (12) and was precipitated sequentially with ethanol, spermine (final concentration, 1.5 mM), and again with ethanol. The precipitate was dried and resuspended in TE buffer (10 mM Tris/1 mM EDTA) and stored at 4°C or at −70°C in 80% ethanol.

**DNA Digestion.** DNA (10–15 μg) was digested for 5 hr with a 3-fold excess of restriction enzyme. The digested fragments were precipitated with 2 vol of ethanol and electrophoresed in 0.8% agarose gels in TAE buffer (40 mM Tris acetate/2 mM EDTA) at 40 V for 17 hr. Restriction enzymes were purchased from International Biotechnologies (New Haven, CT) and New England Biolabs.

**Southern Blotting and End Labeling of DNA.** Southern blotting was performed as described (13) with Genetran paper (Plasco, Woburn, MA). The insert JY150/C5 was excised from an M13 phage clone by digestion with *Hin*dIII and *Eco*RI and was purified by electrophoresis in a 1.2% agarose gel followed by electroelution. DNA was labeled by 3′ end extension of the restriction enzyme fragment by *Escherichia coli* DNA polymerase large fragment. The labeling was

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Abbreviations: MHC, major histocompatibility complex; kb, kilobase(s).

performed with 50 μCi (1 Ci = 37 GBq) of [$^{32}$P]ATP and [$^{32}$P]CTP (specific activity, 3000 Ci/mmol) (New England Nuclear) and 4 units of Klenow polymerase (Bethesda Research Laboratories). The resulting $^{32}$P-labeled DNA had a specific activity of $5 \times 10^8$ cpm/μg.

**Hybridization.** Prehybridization was performed overnight at 42°C in 3× NaCl/Cit (0.45 M NaCl/0.045 M sodium citrate) containing 5× Denhardt's solution (0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin), 50% formamide, 2% $NaDodSO_4$, 25 mM $Na_2HPO_4/NaH_2PO_4$, pH 7.4, salmon sperm DNA at 100 μg/ml. The hybridization buffer was similar except that 5% dextran sulfate was added. Hybridization was performed in 10 ml with $^{32}$P-labeled DNA probe at $10^6$ cpm/ml. Washing was performed in two steps: the first wash was at room temperature (1 hr in 0.1× NaCl/Cit/0.1% $NaDodSO_4$), and the second wash was at 65°C for 1 hr in the same solvent. Filters then were dried and exposed for 5 to 10 days to Kodak XAR-5 films at −70°C.

**Construction of Genomic DNA Library.** A genomic library was made in the phage λ vector Charon 4. Vector DNA (30 μg) was digested with *Eco*RI, treated overnight with 100 units of T4 ligase, and the cloning arms were purified over a 5–20% sucrose gradient. The eukaryotic DNA 20-kilobase (kb) fragments were made from DNA isolated from a heterozygous *HLA-B7, -B27* cell line (VC) and were purified and ligated into the Charon 4 cloning arms (14). The packaging and amplification of the libraries were performed as described (15).

**DNA Transfections into Eukaryotic Cells.** Transfections were performed by the calcium phosphate precipitation technique as described by Herman *et al.* (16).

## RESULTS

**Localization of a Sequence with Potential for *HLA-B* Locus Specificity.** A computer "dot-plot" analysis was used to compare the nucleic acid sequences of genomic clones containing different class I genes. Comparison of a genomic clone, JY150, coding for HLA-B7 and of a class I pseudogene, pHLA12.4 (17, 18), showed extensive homology between these clones but also revealed a region of little homology contained within the 3′ untranslated region of these genes (Fig. 1). A *Pvu* II–*Pvu* II 544-base-pair fragment (JY150/C5) was obtained from this region of the JY150 clone and used to study the specificity of this region further.

**Characterization of a Putative *HLA-B* Locus-Specific Probe.** The specificity of the JY150/C5 fragment was tested by hybridization of the fragment to three genomic clones containing *HLA* class I sequences. Two of these clones coded for the HLA-A2 and HLA-B7 antigens (B3.2 and JY150), and the other is of unknown specificity (LN11A) (19). Under stringent conditions (65°C in 0.1× NaCl/Cit), the JY150/C5 probe specifically hybridized to the *HLA-B7* (JY150) genomic clone but not to the two other clones (data not shown).

Southern blots with genomic DNA from one heterozygous cell line (MWF) and one homozygous cell line (LBF) allowed a comparison of the hybridization pattern of the JY150/C5 probe with that of a full-length class 1 cDNA clone, B7-cDNA (this cDNA clone was obtained from a cDNA library made with mRNA from a *HLA-B7* homozygous cell line, and its predicted amino acid sequence corresponds to the amino acid sequence of the HLA-B7 antigen). The cDNA-B7 clone used as a probe hybridizes to a large number of class I genes (20). After hybridization, the B7-cDNA probe detected 8–10 bands in each cell line, whereas only one band (*Bam*HI digest) or two bands (*Hin*dIII digest) were detected with the JY150/C5 probe (Fig. 2).

These results show that the JY150/C5 probe recognizes only a fraction of the *HLA* class I genes that hybridize to the B7-cDNA probe. The combination of these results with those described above with genomic clones indicates that, under stringent conditions, the JY150/C5 probe recognizes a small subset of two class I genes including the *HLA-B* locus gene and a second, presumably closely related gene.

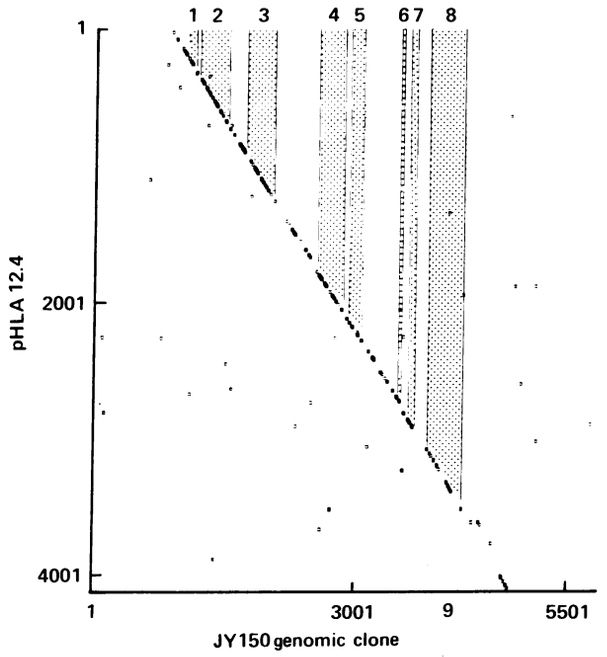


FIG. 1. "Dot-plot" analysis of JY150 and pHLA12.4 sequences. The relative position of exons and introns are indicated by clear (intron) and stippled (exon) areas. Exons: 1, peptide signal; 2, first domain; 3, second domain; 4, third domain; 5, 6, and 7, intracytoplasmic and transmembrane region; 8, 3′ untranslated region. The JY150/C5 probe is localized to the area 9.

**The JY150/C5 Probe Detects Restriction Enzyme Polymorphism in Homozygous Consanguineous Cell Lines.** To further characterize the JY150/C5 probe specificity, its pattern of hybridization with restriction enzyme digests of DNA from human B-cell lines was examined by genomic Southern blotting. The 11 cell lines used were derived from individuals who are *HLA* homozygous offspring of consanguineous marriages. DNA preparations from 8 to 11 of these cell lines

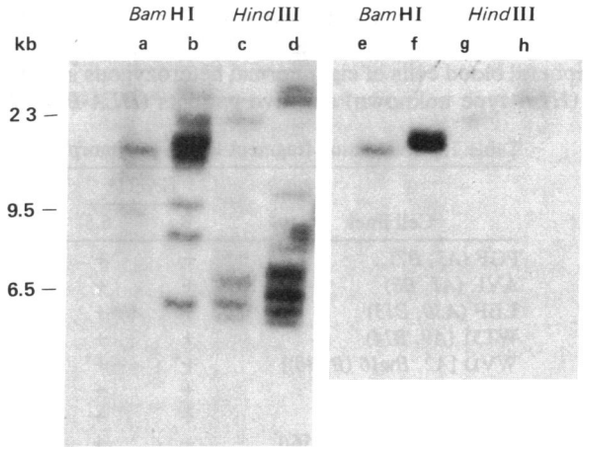


FIG. 2. Specificity of the JY150/C5 probe. Two cell lines, LBF (*HLA-B13*) (lanes a, c, e, and g) and MWF (*HLA-B8, -w35*) (lanes b, d, f, and h) were digested with *Bam*HI or *Hin*dIII. The digested DNA was split in two parts, electrophoresed, and hybridized either with the HLA-B7 full-length cDNA probe (lanes a–d) or with the JY150/C5 probe (lanes e–h).

were digested with four restriction endonucleases: *Eco*RI, *Bgl* II, *Hin*dIII, *Bst*EII. The results are summarized Table 1.

After *Eco*RI digestion, two fragments of 8 and 6.5 kb that hybridize with JY150/C5 were observed for all of the cell lines tested. No polymorphism was observed for 11 cell lines, except the cell line WVD (*Bw16*) which shows two fragments with a slightly different size. In contrast, polymorphism was detected in several cell lines after digestion with the other endonucleases.

After *Hin*dIII digestion, two bands of 21 and 27 kb were observed for the *HLA-B7*, *-B8*, -B14, -B17, -Bw22, -B27, and *-Bw35* alleles. Different patterns were seen for cell lines expressing *HLA-B40* (27 and 10 kb bands) and *-Bw16* (28- and 24-kb bands). A light band of 1.7 kb was also observed in the pattern for all these cell lines. An example of these different patterns is shown in Fig. 3 *Top* (*Eco*RI digestion) and *Middle* (*Hin*dIII digestion).

Thus, the JY150/C5 probe hybridizes with two fragments after *Eco*RI digestion and three fragments after *Hin*dIII digestion. These results confirm that the JY150/C5 probe recognizes a restricted subset of the *HLA* class I genes. Two cell lines, WVD (*HLA-A2, -Bw16*) and WT49 (*HLA-A2, -B17*) express identical *HLA-A* alleles and different *HLA-B* alleles. However, their patterns of hybridization with the JY150/C5 probe are different. Thus, the polymorphism detected after *Hin*dIII digestion is more likely to be linked to the *HLA-B* locus than to the *HLA-A* locus. A linkage between a 10-kb band and *HLA-B40* has been shown in a previous study (21).

After *Bgl* II digestion two patterns of hybridization were observed. One showed a single band of 7 kb and was obtained from cell lines expressing the *HLA-B8, -B14, -B17, -Bw35*, and *-B40* alleles. The other pattern consisted of two bands of 7 and 5.3 kb and was found with the cell lines expressing *HLA-B7, -B13, -Bw16, -B18, -Bw22*, and *-B27* (an example of these patterns is shown in Fig. 3 *Bottom*). No correlation with the two public serologic antigen groups HLA-Bw4 and -Bw6, defined on the basis of cross reactions in lymphocytotoxicity or complement fixation between different alleles of the *HLA-B* locus, could be made with these two subgroups (22).

After *Bst*EII digestion, a single band of 5.5 kb was observed for all of the cell lines tested except those expressing the *HLA-B7* alleles, and one out of two cell lines expressing the *-B27* allele which gave an additional band of 1.3 kb (an example of these patterns is shown in Fig. 4 *Left*). To study further the two bands observed for HLA-B7 and -B27, a similar analysis with *Bst*EII was made with DNA from three cell lines that express *HLA-B7* and/or *-B27* [KCA (*B27, Bw44*), VC (*B7, B27*), and JY (*B7*)], with DNA from peripheral blood cells of eight normal heterozygous individuals (*HLA* type unknown) and five patients (*HLA-B27*) suffering from ankylosing spondylitis. In every case, a band that hybridizes with JY150/C5 at 5.5 kb was observed. In addition to this band, the DNA from one normal individual, from three patients suffering from ankylosing spondylitis, and from JY showed a second band at 1.3 kb; the DNA from one normal individual and from KCA showed a second band at 2 kb, and the DNA from VC showed two bands of 2 and 1.3 kb in addition to the 5.5-kb band (an example of these patterns is shown in Fig. 4 *Right*). Thus, within this group of cells, four different patterns of hybridization were seen.

## DISCUSSION

Several points of interest can be noted from the results obtained in this study. No differences were found in the restriction enzyme fragment pattern of several homozygous consanguineous and heterozygous cell lines. Indeed, some cell lines were indistinguishable in their patterns of hybridization after digestion with four different restriction enzymes. This indicates that the flanking region around the *HLA-B* locus has been highly conserved at least for these sites. This situation is different from that found in the class II MHC genes, where extensive polymorphism can be detected between different *HLA-DR* alleles (23, 24).

The JY150/C5 probe hybridizes with two separate large fragments in DNA obtained from several homozygous consanguineous cell lines. A polymorphism caused by a difference between the two parental chromosomes is unlikely because the genealogy of these cell lines is well documented. Two hypotheses could then explain these results. First, the enzymes used could cut within the sequence to which the probe hybridizes and, thus, two bands would be generated after hybridization. This explanation is not likely to be correct because all of the enzymes used are infrequent cutters and the restriction map of the probe, which is derived from the *HLA-B7* sequence, has been determined and it does not contain any restriction sites for *Eco*RI, *Hin*dIII, *Bgl* II or *Bst*EII. Furthermore, after digestion with *Eco*RI, *Bgl* II, or *Hin*dIII, the hybridization pattern of JY DNA is not different from most of the homozygous consanguineous cell lines tested. Therefore, it is likely that JY150/C5 also hybridizes with a second *HLA-B*-related class I gene.

Four different patterns of hybridization were observed after *Bst*EII digestion, but this restriction endonuclease polymorphism cannot be correlated with any known polymorphism of the *HLA* loci including *HLA-B7* and *-B27*. For example, it has been possible with two monoclonal antibodies, M1 and M2, to divide HLA-B27 into two distinct serological subtypes (25). VC expresses one subtype ($M1^+$, $M2^-$) and KCA, MVL and LG2 express the other subtype

Table 1. Restriction fragment length polymorphism between different *HLA-B* alleles

| | *Eco*RI* | | *Bgl* II* | | *Hin*dIII* | | *Bst*EII* | |
|---|---|---|---|---|---|---|---|---|
| Cell lines | 8 | 6.5 | 7 | 5.3 | 21 | 27 | 5.5 | 1.6 |
| PGF (*A3, B7*) | + | + | + | + | + | + | + | + |
| AVL (*A1, B8*) | + | + | + | − | + | + | + | − |
| LBF (*A30, B13*) | + | + | + | + | NT | | NT | |
| WT51 (*A9, B14*) | + | + | + | − | + | + | + | − |
| WVD [*A2, Bw16 (Bw38)*] | +† | +† | + | + | 24 | 28 | + | − |
| WT49 (*A2, B17*) | + | + | + | − | + | + | + | − |
| THR (*A25, B18*) | + | + | + | + | NT | | + | − |
| OOS [*A26, Bw22 (Bw56)*] | + | + | + | + | + | + | + | − |
| MVL (*Aw32, B27*) | + | + | + | + | + | + | + | + |
| IBW4 (*A3, Bw35*) | + | + | + | − | + | + | + | − |
| DKB [*Aw24, Bw40 (Bw60)*] | + | + | + | − | 10 | + | + | − |

NT, cells not tested.
*Fragment sizes are in kb.
†The band observed after *Eco*RI digestion of the DNA from WVD were slightly larger than with the other alleles.

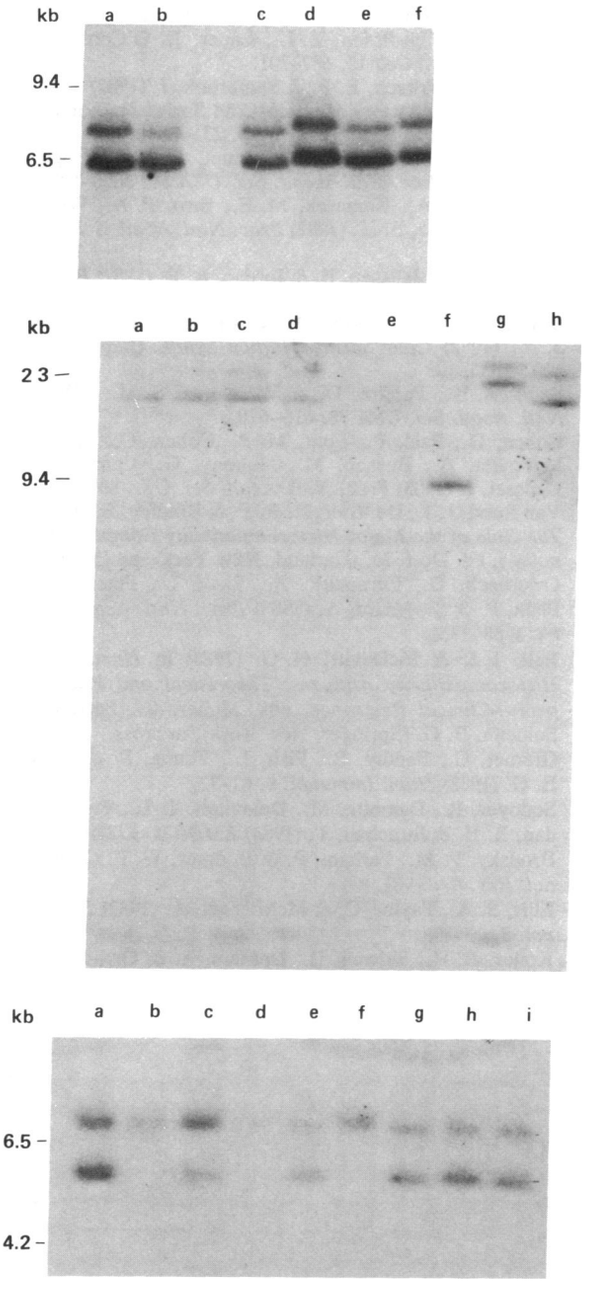


FIG. 3. Restriction fragment patterns of several homozygous typed consanguineous cell lines digested with different endonucleases. (*Top*) Digestion with *Eco*RI. Lanes: a, PGF (*A3, B7*); b, DKB (*Aw24,B40*); c, OOS (*A26, Bw22*); d, WVD (*A2, wB16*); e, LBF (*A30, B13*); f, IBW4 (*A3, Bw35*). (*Middle*) Digestion with *Hin*dIII. Lanes: a, AVL (*A1, B8*); b, MVL (*Aw32, B27*); c, OOS (*A26, Bw22*); d, IBW4 (*A3, Bw35*); e, WT51 (*A9, B14*); f, DKB (*Aw24, B40*); g, WVD (*A2, Bw16*); h, PGF (*A3, B7*). (*Bottom*) Digestion with *Bgl* II. Lanes: a, LBF (*A30, B13*); b, WT51 (*A9, B14*); c, MVL (*Aw32, B27*); d, WT49 (*A2, B17*); e, OOS (*A26, Bw22*); f, IBW4 (*A3, Bw35*); g, THR (*A25, B18*); h, PGF (*A3, B7*); i, WVD (*A2, Bw16*).

(M1$^+$, M2$^+$). VC and KCA share bands of 5.5 and 2 kb in their hybridization patterns after *Bst*EII digestion; VC and MVL also share two bands of 5.5 and 1.3 kb after digestion with the same enzyme. Thus, none of these bands can be correlated with one subtype of HLA-B27.

To establish the existence of a second *HLA-B*-related class I gene, a genomic library was constructed from an *HLA-B7, -B27* heterozygous cell line (VC). This library was amplified and then screened with JY150/C5 and a HLA-B7 full length

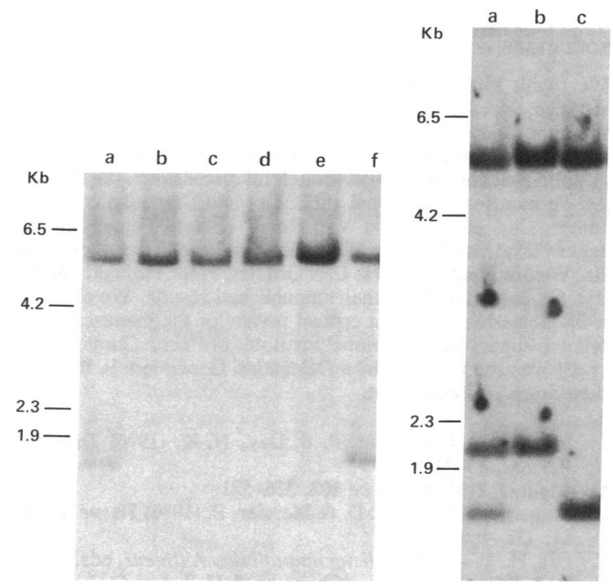


FIG. 4. (*Left*) Digestion with *Bst*EII. Lanes: a, MVL (*Aw32, B27*); b, LG2 (*A2, B27*); c, THR (*A25, B18*); d, OOS (*A26, Bw22*); e, WVD (*A2, Bw16*); f, PGF (*A3, B7*). (*Right*) Polymorphism with *HLA-B7* and *-B27* alleles. Lanes: a, VC (*B7, B27*); b, KCA (*B27, Bw44*); c, JY (*B7*).

cDNA probe. From the VC library a single clone, C4/VC5.1 was isolated. This clone contained a 15-kb insert and, when digested with *Eco*RI, yielded an 8-kb fragment that contains an entire class I gene, on the basis of the restriction map (data not shown). Surprisingly, the restriction map of the C4/VC5.1 clone showed more homology with the restriction map of the HLA-Cw3-encoding gene (26) than with the restriction map of the HLA-B7-encoding gene. To determine if the C4/VC5.1 gene coded for a class I product, it was transfected into murine L cells by the calcium phosphate precipitation technique. A genomic clone coding for HLA-B7 (JY150) was used as a positive control in this experiment. After transfection, the positive control was strongly positive with a monomorphic anti-HLA-A, -B, -C monoclonal antibody (MB40.5) and with a polymorphic anti-HLA-B7, -B27, -B22 monoclonal antibody (ME1) but was negative with an irrelevant monoclonal antibody (X63) (27, 28). The cells transfected with C4/VC5.1 were also positive with MB40.5 but negative with ME1 and X63, suggesting that this clone was expressed at the cell surface, although its expression level was lower than the expression level of JY150. These results suggest that the JY150/C5 probe is also able to hybridize with *HLA-C* locus genes.

Koller *et al.* recently reported isolation of a 385-bp *Pvu* II–*Pst* I fragment from the 3′ untranslated region of an HLA-B8 cDNA clone. This probe gave a single band when tested on hemizygous *HLA-B* region deletion mutants (29). Since this probe and JY150/C5 come from the same part of the *HLA-B* 3′ untranslated sequence, it is not clear why the probe of Koller *et al.* did not pick up a second genomic band. The difference may be explained by choice of restriction enzyme. Thus, JY150/C5 gives a single band with *Bgl* II digests of *HLA-B8* homozygous typing cells (Table 1).

Our results indicate that probes of restricted specificity may be derived from the 3′ untranslated region of class I genes, where homology between members of this multigene family is low. Single- or low-copy-number probes will prove useful in several ways. First, they will assist in cloning *HLA-B* class I genes. Although confirmation of identity by expression is still an essential step, the number of clones required to be tested in this way is greatly reduced. This type

of probe will also make possible more precise studies of *HLA* associations with susceptibility to disease.

We thank Mrs. Linda Fish and Mrs. Dolly Ness for technical assistance, Mr. David Austin for his help with the computer, Dr. Christophe Benoit for his help in the preparation of the library, Dr. John Monaco for fluorescence-activated cell sorter analysis, and Drs. John Bell and Judy Ways for helpful discussions. We thank Dr. Peter Parham for monoclonal antibodies and Drs. Julia and Walter Bodmer, Carl Grumet, Jon Van Rood, Hilliard Festenstein, and Angelo Carbonara for gifts of cell lines and typed peripheral blood cells. We are grateful to Drs. Carl Grumet, Peter Parham, and Judy Ways for communicating their unpublished results. We also thank Dr. Nicholas Holmes for a critical review of the manuscript. This work was supported by National Institutes of Health Grant AI 11313. H.L.C. was the recipient of a Delegation Generale a la Recherche Scientifique Training Grant.

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