Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 5175–5178, August 1984
Genetics

Isolation of *HLA* locus-specific DNA probes from the 3′-untranslated region

(*HLA-A* and *-B*/*HLA* loss mutants/high-stringency hybridization)

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Communicated by D. Bernard Amos, April 23, 1984

ABSTRACT **When human class I cDNA clones containing coding sequences are used to probe genomic DNA, 15–20 fragments, each containing a complete class I gene or pseudogene, are seen. Identification of which genomic DNA segments encode the HLA-A and -B antigens has to date required transfection of mouse L cells with cloned class I genes or analysis of *HLA* loss mutants. In this report we show that under high-stringency conditions, probes constructed from the 3′-untranslated region can be used to specifically identify the segments of DNA that encode the HLA-A and -B antigens in the human lymphoblastoid cell line 721. Examination of DNA from unrelated individuals indicates that these probes are locus specific and will permit identification of *HLA-A* and *-B* genes in the population.**

The definition of the HLA system arose from the discovery of leukocyte agglutinins in the sera of polytransfused individuals (1) and multiparous women (2, 3). Two antigenic groups were initially described; the group Four, or HLA-B, and LA, or HLA-A. As more sera were described, it was found that the antigens fell into two series, HLA-A and -B, each of which behaved as if controlled by a set of alleles at two closely linked loci (4). Today, >20 *HLA-A* and >40 *HLA-B* alleles have been described (5). As well as providing a major barrier to transplantation, the HLA antigens are responsible for restriction of T-cell-mediated cytotoxicity to virus-infected target cells (reviewed in ref. 6).

Both HLA-A and -B antigens are integral membrane glycoproteins of about 44,000 daltons expressed on the cell surface of most tissues in noncovalent association with β_2-microglobulin (reviewed in ref. 7). Protein sequence analysis of products of the *HLA-A* and *-B* loci shows overall homology of 86% (8). However, few of the differences appear to be locus specific. Comparison of the protein sequences of *HLA-A2* and *-B7* shows that most of the differences between these two proteins are found clustered in three areas of their NH_2-terminal regions. Between amino acid residues 65 and 80, homology drops to 30% and from residues 105–116 and 177–194, 50% homology is seen. However, these areas are also poorly conserved between the *HLA-A2* and *HLA-A28* heavy chains (9). Moreover, residues that distinguish *HLA-B7* from *HLA-A2* are found in another *-A* allele, *HLA-A28*. Thus, since positions that differ from *HLA-A* and *-B* also vary between alleles of a single locus, the limited sequences available have not been useful in identifying residues unique to *HLA-A* or *-B* alleles (9, 10). Monoclonal antibodies reacting with the protein products of only one allele of each locus have been isolated (e.g., ref. 11). Monoclonal antibodies that react with the protein products of a number of *HLA* alleles, but not necessarily of the same locus, have also been described (12). Thus, although monoclonal antibodies to HLA class I antigens have been described, none uniquely defines the alleles according to locus.

Two mechanisms have been invoked to explain the apparent lack of locus-specific sequences. The first suggests that divergence in the population is limited by functional constraints placed on the antigens. Thus, at any given position only a limited spectrum of amino acids will result in a functional molecule. The second suggests that much of the polymorphism seen in class I genes is the result of gene conversion. Gene conversion could result in a movement of sequences from one class I locus to another, resulting in both polymorphism and loss of locus-specific sequences that may have evolved since gene duplication.

The high level of sequence conservation in HLA class I proteins extends to the DNA level. Over 15 different fragments generated by restriction endonucleases hybridize with cDNA probes for class I coding sequences (13–15). Hybridization of these fragments with probes from both the 5′ and 3′ regions of the class I cDNA clones indicates that they contain an entire gene. The large number of class I genes that hybridize these cDNA probes has made identification of the *HLA-A* and *-B* genes difficult. Two approaches have been used. Class I genomic clones have been transfected into mouse L cells and assayed for their ability to direct synthesis of HLA-A and -B surface antigens (16, 17). An alternate approach has been to analyze a series of *HLA* loss mutants derived from lymphoblastoid cell line (LCL) 721 (18). Correlation of loss of restriction enzyme-generated fragments of genomic DNA hybridizing with class I cDNA probes, with loss of reactivity with specific HLA sera, has resulted in the identification of the *HLA-A1*, *-A2*, and *-B8* genes in this cell line (19).

In this report, we describe probes isolated from the 3′-untranslated region of *HLA-A* and *-B* genes that, under high-stringency conditions, hybridize only to one locus in LCL 721, demonstrating that locus-specific sequences can be identified for members of the class I multigene family. Hybridization of these probes with genomic DNA from individuals with different *HLA-A* and *-B* types suggests that the locus specificity of the 3′-untranslated region is not restricted to the *HLA-A* and *-B* alleles found in LCL 721. Thus, these probes can be used to identify all *HLA-A* and *-B* genes.

MATERIALS AND METHODS

Cell Lines. LCL 721 (*HLA-A1*, *-B8*, *-A2*, *-B5*) is an Epstein–Barr virus-transformed human B LCL. *HLA* loss mutants were derived from LCL 721 after γ-irradiation (300 rad; 1 rad = 0.01 gray) and selection with complement and alloantisera (18, 20) or monoclonal antibodies against specific HLA antigens (15, 19).

Southern Blot Analysis. Genomic DNA was isolated from LCL 721 and its mutants as described (19). Restriction en-

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Abbreviations: LCL, lymphoblastoid cell line; bp, base pair(s); kb, kilobase(s).

zymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim, and New England BioLabs and used according to supplier specifications. Gel electrophoresis was performed as described (17) and blotting was according to Southern (21). Filter prehybridization and hybridization were as described (19). Filters were washed initially at room temperature in 0.15 M sodium chloride/0.015 M sodium citrate, pH 7/0.5% $NaDodSO_4$, followed by washes in either 15 mM sodium chloride/1.5 mM sodium citrate, pH 7, at 42°C (low stringency) or 15 mM sodium chloride/1.5 mM sodium citrate, pH 7, at 65°C (high stringency).

Probes. An *HLA-B* locus-specific probe, pHLA-1.1 (Fig. 1*b*), was prepared by digesting cDNA clone pHLA-1 (22) with restriction endonucleases *Pvu* II and *Pst* I. The DNA digest was subjected to electrophoresis in a 5% acrylamide gel. A 358-base-pair (bp) fragment was isolated from the acrylamide gel according to Maxam and Gilbert (23). This fragment contains only 3′-untranslated sequences and does not include the poly(A) tail or poly(A) addition site. An *HLA-A* locus-specific probe, pHLA-2a.1, was prepared from the *HLA-A2* genomic clone pHLA-2a (Fig. 1*a*). A 490-bp *Pvu* II–*Msp* I fragment of pHLA-2a was subcloned into the *Acc* I and *Sma* I sites of pUC9 (24). By sequence analysis, this subclone contains most of the *HLA-A2* 3′-untranslated region and 72 bp of 3′-flanking DNA. The 490-bp insert was isolated as described above (23). Probes were radiolabeled by nick-translation (25) of 0.5 μg of insert DNA.

RESULTS AND DISCUSSION

***HLA-A* Locus Probe.** The *HLA-A* locus probe pHLA-2a.1 was prepared from the genomic clone pHLA-2a, which by analysis of *HLA* mutants (19), sequence analysis, and transfection studies (unpublished data), has been shown to encode the HLA-A2 antigen. Sequence analysis and comparison to pHLA-1 indicated that the 490-bp subclone of pHLA-2a (Fig. 1*a*) contains all of the *HLA-A2* 3′-untranslated region and 72 bp of 3′-flanking DNA. When this subclone was used to probe *Hin*dIII-digested genomic DNA from LCL 721 at low stringency (15 mM sodium chloride/1.5 mM sodium citrate, pH 7, at 42°C), a pattern very similar to that seen with an *HLA-B7* cDNA clone (15) was obtained (Fig. 2, lane a). Only two of the bands visualized with the *HLA-B7* cDNA probe failed to hybridize with this *HLA-A2* subclone. When the stringency was increased (15 mM sodium chloride/1.5 mM sodium citrate, pH 7, at 65°C), a markedly different result was obtained. Although the *HLA-B7* cDNA probe continued to hybridize to all but a few of the *Hin*dIII-generated fragments at high stringency (unpublished data), only two bands continued to bind to the pHLA-2a.1 subclone (Fig. 2, lane e). One of these, the 5.1-kilobase (kb)

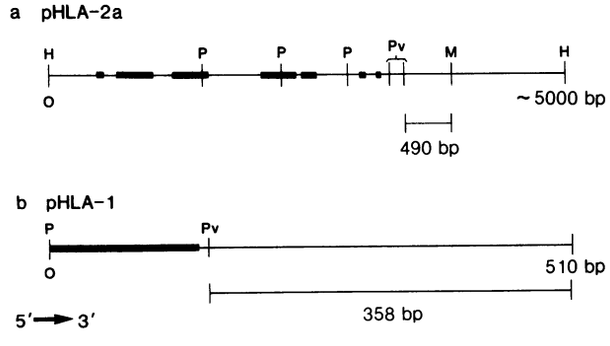

FIG. 1. Localization of subclones used as locus-specific probes from the genomic clone pHLA-2a (*a*), which encodes *HLA-A2* from LCL 721 (unpublished data), and from the HLA class I cDNA clone pHLA-1 (*b*) (19). Restriction endonuclease-cut sites indicated are H, *Hin*dIII; M, *Msp* I; P, *Pst* I; Pv, *Pvu* II. Coding regions of each clone are indicated by solid boxes. Both clones are depicted 5′ → 3′.

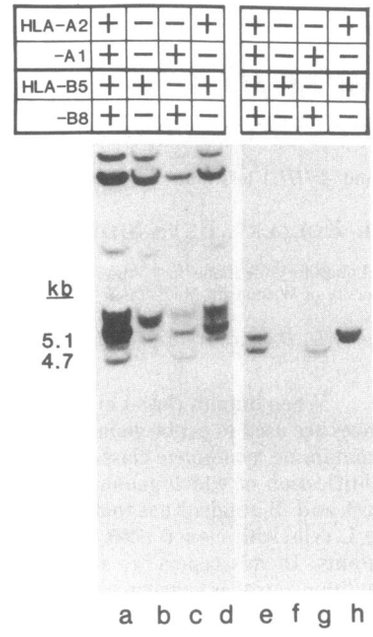

FIG. 2. Genomic DNAs from LCL 721 (lanes a and e), mutant 721.144 (lanes b and f), mutant 721.45.1 (lanes d and h), and mutant 721.52 (lanes c and g) were digested with *Hin*dIII and electrophoresed on a 0.7% agarose gel. The Southern blot was probed with pHLA-2a.1. This fragment consists of the 3′-untranslated region of the *HLA-A2* gene and 72 bp of 3′-flanking DNA. A stringency wash of 15 mM sodium chloride/1.5 mM sodium citrate, pH 7, at 42°C was used for lanes a–d and 15 mM sodium chloride/1.5 mM sodium citrate, pH 7, at 65°C was used for lanes e–h. The HLA-A and -B phenotypes of LCL 721 and the mutants used are indicated above the appropriate lanes.

fragment, correlated with the expression of the HLA-A2 antigen (19). The second, a 4.7-kb band, corresponded by both an analysis of mutants and population studies to *HLA-A1* (19). To further verify the specificity of the *HLA-A* probe, DNA was prepared from *HLA* loss mutants derived from LCL 721. If pHLA-2a.1 is locus specific, hybridization of this probe to these mutant lines should correlate with their expression of *HLA-A* genes. When *Hin*dIII-digested DNA from 721.52, an *HLA-A2*, *-B5*, *-DR1* loss mutant (26), was examined, only the 4.7-kb band seen in the parental line 721 was detected (Fig. 2, lane g). Conversely, when we examined *Hin*dIII-digested DNA from the mutant 721.45.1, from which the *HLA-A1*, *-B8*, *-DR3* haplotype had been deleted (18), the band at 4.7 kb was no longer seen while the 5.1-kb band remained (Fig. 2, lane h). To ensure that hybridization was *HLA-A* locus specific, the *HLA-A* null cell line 721.144 was examined. This line was obtained after γ-irradiation of the *HLA-A1*, *-B8*, *-DR3* loss mutant 721.45.1, followed by immunoselection for cells no longer expressing HLA-A2 (ref. 15; unpublished data). Both the 4.7-, and 5.1-kb fragments were absent from *Hin*dIII-digested DNA from line 721.144 (Fig. 2, lane f). Thus, the hybridization data obtained from LCL 721 and the *HLA* loss mutants show that at high stringency the 3′-untranslated region of *HLA-A2* can be used as an *HLA-A* locus-specific probe for LCL 721.

***HLA-B* Locus Probe.** pHLA-1 (22) is a cDNA clone obtained from the cell line LKT (*HLA-A1*, *-B8* homozygous). Sequence homology between pHLA-1 and an *HLA-B7* cDNA clone suggests that pHLA-1 originates from an *HLA-B* locus mRNA (27). When pHLA-1 was digested with the restriction enzyme *Pvu* II, two fragments were obtained. The 150-bp fragment contains the sequence encoding the cytoplasmic region and a small portion of the transmembrane region. The larger 358-bp fragment consists entirely of the 3′-

untranslated region. When the latter fragment, pHLA-1.1, was used to probe at high stringency DNA from LCL 721 digested with *Bgl* II, only two bands were seen (Fig. 3, lane a), one at 7.0 kb and another at 5.8 kb. Examination of haplotype loss mutants correlated the 7.0-kb band with the *HLA-A1*, *-B8* haplotype and the 5.8-kb band with the *HLA-A2*, *-B5* haplotype—that is, *HLA-A1*, *-B8* loss mutants no longer contained a 7.0-kb band while retaining the 5.8-kb band (Fig. 3, lanes d and e). In *HLA-A2*, *-B5* loss mutants, the 5.8-kb band was no longer detected, while the 7.0-kb band remained (Fig. 3, lanes h and i). To determine which locus on the *HLA-A1*, *-B8* haplotype hybridized the probe, *Bgl* II-digested DNAs from five *HLA-B8* loss mutants—721.5, 721.16, 721.10, 721.18, and 721.25—were examined (Fig. 3, lanes b, c, and j–l) and four were shown to have lost the 7.0-kb band while retaining the 5.8-kb fragment. Mutant 721.16 (Fig. 3, lane c) retained both *Bgl* II-generated fragments. Earlier hybridization studies using an *HLA-B7* cDNA clone as a probe indicated that *HLA-B8* single-loss mutants can be divided into two groups (19), those whose loss of expression of the *HLA-B8* gene can be correlated with loss of DNA hybridizing with the *HLA-B7* cDNA probe and those in which no loss of class I DNA is apparent. The *HLA-B8* loss mutant 721.16 falls into this later category (19).

As indicated above, the 5.8-kb *Bgl* II-generated DNA band correlated with the *HLA-A2*, *-B5* haplotype in LCL 721 and therefore was present in the *HLA-A1*, *-B8*, *-DR3* loss mutant 721.45.1 (Fig. 3, lane e). When mutant 721.45.1 was mutagenized further with γ-rays to create the *HLA-A* null line 721.144, the 5.8-kb *Bgl* II-generated band was retained (Fig. 3, lane f). As line 721.144 has lost the *HLA-A2* structural gene (17), the 5.8-kb fragment that hybridizes with pHLA-1.1 cannot originate from the *HLA-A2* gene. Therefore, the 5.8-kb *Bgl* II-generated DNA fragment contains the *HLA-B5* gene or a gene closely associated with it. The strong evidence that pHLA-1 is a clone of the *HLA-B8* gene makes it likely that the 5.8-kb band does contain the *HLA-B5* gene. Therefore, at high stringency a probe obtained from the 3′-untranslated region of an *HLA-B* cDNA clone, pHLA-1, hybridizes to class I genes of LCL 721 in a locus-specific manner.

Mutant 721.53 is phenotypically *HLA-B* null (28). Since previous studies failed to establish a correlation between the *HLA-B5* allele and a restriction fragment hybridizing to the *HLA-B7* cDNA probe, it was not possible to determine

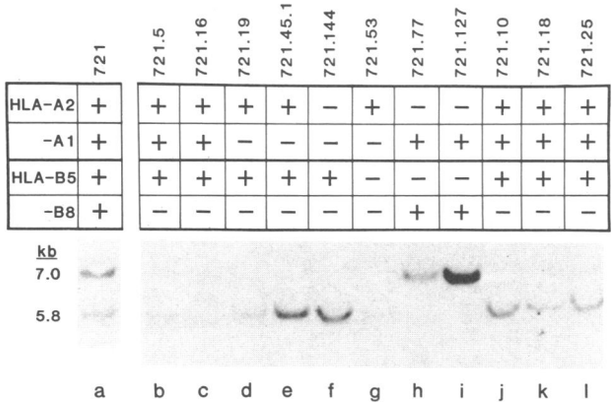

FIG. 3. *Bgl* II digests of genomic DNA from LCL 721 and several of the mutants derived from it were electrophoresed on a 0.7% agarose gel. The Southern blot was probed with pHLA-1.1 at high stringency with a final wash at 65°C in 15 mM sodium chloride/1.5 mM sodium citrate, pH 7. The probe contains DNA from only the 3′-untranslated region of pHLA-1. The HLA-A and -B phenotypes of LCL 721 and the mutants used are indicated above the appropriate lanes.

FIG. 4. *Hin*dIII digests of genomic DNA from LCL 721 (lanes a, n, and o) and from 23 unrelated individuals (lanes b–m and p–z) were electrophoresed on a 0.7% agarose gel. The Southern blot was probed with pHLA-2a.1 and washed at high stringency. The *HLA-A* types of the individuals are indicated above the appropriate lanes.

whether loss of *HLA-B5* expression in mutant 721.53 was due to a deletion of the *HLA-B5* gene (20). Although our interpretation is limited because the fragment to which the probe from the 3′-untranslated region of pHLA-1 hybridized may not contain the complete gene, the presence of the 5.8-kb band in mutant 721.53 (Fig. 3, lane g) suggests that the loss of *HLA-B5* expression in mutant 721.53 may not be due to a γ-ray-induced deletion of the *HLA-B5* gene.

As studies thus far have been restricted to the LCL 721, it is possible that pHLA-1.1 and pHLA-2a.1 hybridize only to the genes of a limited array of *HLA-A* and *-B* alleles. To address this question, pHLA-2a.1 was used to probe genomic DNA from 23 unrelated individuals (Fig. 4, lanes b–m and p–z). Two hybridization patterns were observed. In some individuals, two bands were seen. In others, only one band was visualized; however, this band has an intensity double that of a single copy gene. These data, and similar studies with pHLA-1.1 (unpublished data), support the contention that probes from the 3′-untranslated region can be used to identify *HLA-A* and *-B* genes.

The *HLA-A* and *-B* genes are part of a multigene family with >15 members (13–15). The high level of sequence conservation within the class I family has made it difficult to use class I cDNA probes containing coding information to identify and study the products of any given class I locus independent of other family members, at either the RNA or DNA level. This paper describes the construction and characterization of probes from the 3′-untranslated region that can circumvent these problems. These probes provide a means for rapid assignment of cloned class I genes to either the *HLA-A* or *-B* locus. Restriction fragment-linked polymorphisms can be studied for either the *HLA-A* or *-B* locus. This may be especially useful for examining the basis of disease associations that have been identified for several class I alleles (reviewed in ref. 29). As the two probes described were constructed from the 3′-untranslated region, they will also be useful for studying the expression of either *HLA-A* or *-B* genes at the mRNA level.

We acknowledge the technical assistance of M. Longtine, G. Hahn, and G. Johnson. This is paper no. 2713 from the Laboratory of Genetics, University of Wisconsin, Madison, WI 53706. This research was supported by NIH Grants AI-18124, AI-15486, and P30 CA 14520 and by grants from the Searles' Scholar Program and the Leukemia Society of America to H.T.O.

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