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Genetics

Polymorphic restriction endonuclease fragment segregates and correlates with the gene for HLA-B8

(polymorphism)

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ABSTRACT **Cellular DNA from HLA-typed individuals was digested with the restriction endonuclease *Eco*RV. After electrophoresis and transfer to a hybridization membrane, the restriction endonuclease fragments were probed with cDNA carrying the nucleotide sequence encoding a class 1 HLA gene. Polymorphism for presence or absence of various *Eco*RV fragments was noted in a panel of unrelated HLA-typed individuals. A polymorphic 8.6-kilobase pair *Eco*RV fragment was found which correlated in the panel with the serologically defined gene HLA-B8. A family study revealed that this fragment segregated with the haplotype carrying the HLA-B8 gene. This fragment may carry the gene for HLA-B8 or it may represent another class 1 gene (or pseudogene) in linkage disequilibrium with HLA-B8.**

The HLA chromosomal region, the human major histocompatibility complex (MHC), is a cluster of genes localized to the short arm of chromosome 6 (1, 2). Genes in this cluster code for at least three different classes of polymorphic proteins, of which two are membrane glycoproteins involved in cell–cell interaction of the immune system. Class 1 proteins are found on all nucleated cells. Class 2 proteins are found mainly on cells involved in the immune response. Class 3 proteins consist of complement components. By phenotypic (serologic) analysis, three loci have been demonstrated to code for class 1 cell surface proteins, HLA-A, -B, and -C (1); these loci show extensive polymorphism. The origin, maintenance, and function of this rich polymorphism have posed questions of major theoretical interest in modern human genetics.

Direct analysis of the DNA of the HLA region should provide additional understanding of the gene cluster and its polymorphism. Analysis of human DNA with restriction endonucleases provides a set of useful markers for mapping the human genome (3). Polymorphism of restriction endonuclease sites has been found (4), and restriction fragment-length polymorphisms (RFLPs) are being used in our laboratory to analyze the HLA region.

Our initial experiments indicate that RFLPs of class 1 genes are readily detectable and that such polymorphic DNA fragments can be correlated with the polymorphism of class 1 gene products.

MATERIAL AND METHODS

Human Cellular DNA. DNA was prepared by standard methods (4) from peripheral blood leukocytes of individuals who have been typed extensively and repeatedly for HLA antigens in this laboratory. Briefly, leukocytes were lysed in a solution containing proteinase K (Boehringer Mannheim) at 0.2 mg/ml and 0.2% $NaDodSO_4$. The lysates were extracted three times with a mixture of 3 vol of phenol and 1 vol of chloroform/isoamyl alcohol, 24:1 (vol/vol), once with chloroform/isoamyl alcohol, and once with ether. The aqueous phase of the ether extractions was dialyzed against 10 mM Tris·HCl, pH 7.6/1 mM EDTA. The samples were then: (*i*) adjusted to a final concentration of 10 mM NaCl, incubated (37°C) with RNase (Boehringer Mannheim) and then with proteinase K and $NaDodSO_4$, and extracted once with phenol and chloroform/isoamyl alcohol and once with ether; or (*ii*) centrifuged in a CsCl/ethidium bromide gradient at 47,000 rpm for 16–41 hr in a Ti 50.2 angle-head rotor and extracted (four times) with butanol. All the samples were then dialyzed exhaustively. After dialysis, NaCl was added to each sample (final concentration, 20 mM) and DNA was precipitated with 2–3 vol of cold 100% ethanol. The DNA precipitate was then dissolved in 10 mM Tris·HCl/1 mM EDTA at concentrations varying from 0.5 to 1 $\mu g/\mu l$. The mean total yield of DNA from a 20-ml blood sample was approximately 200 μg.

Restriction Endonuclease Digestion of Cellular DNA. The restriction endonuclease *Eco*RV was used as directed by the manufacturer (Boehringer Mannheim). The nucleotide sequence of the recognition site for this enzyme is G-A-T-A-T ↓ C (5). Usually, 16 μg of human cellular DNA was incubated at 37°C for 18 hr with *Eco*RV at enzyme concentrations of 6–10 units/μg of DNA.

Electrophoresis and Transfer of DNA Fragments. Restriction endonuclease DNA fragments were subjected to electrophoresis in 0.7% agarose gels. Each electrophoresis procedure included a sample of *Hin*dIII-digested, ^{32}P-labeled λ phage DNA for molecular weight markers. After electrophoresis, the DNA fragments were depurinated, denatured and neutralized, and then transferred to a hybridization transfer membrane (GeneScreen, New England Nuclear) by the method of Southern (6) as modified by the manufacturer.

Probe for Class 1 DNA Sequence. The detection of class 1 MHC genes among restriction endonuclease DNA fragments was made possible by the use of the cDNA clone described by Sood *et al.* (7). This cDNA clone, about 1,400 base pairs in size, contains most or all of the coding sequence for an HLA-B gene, most likely HLA-B7. For use as a probe, the cDNA insert was excised from the pBR322 vector with *Pst* I, polymerized with T4 ligase, and labeled with [^{32}P]dGPT and [^{32}P]dCTP by nick-translation (8, 9). The specific activity of this probe was at least 1.1×10^8 cpm/μg of DNA.

Prehybridization Treatment and Hybridization. Prehybridization treatment of the membranes carrying restriction en-

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Abbreviations: kb, kilobase pair(s); RFLP, restriction fragment-length polymorphism; MHC, major histocompatibility complex.

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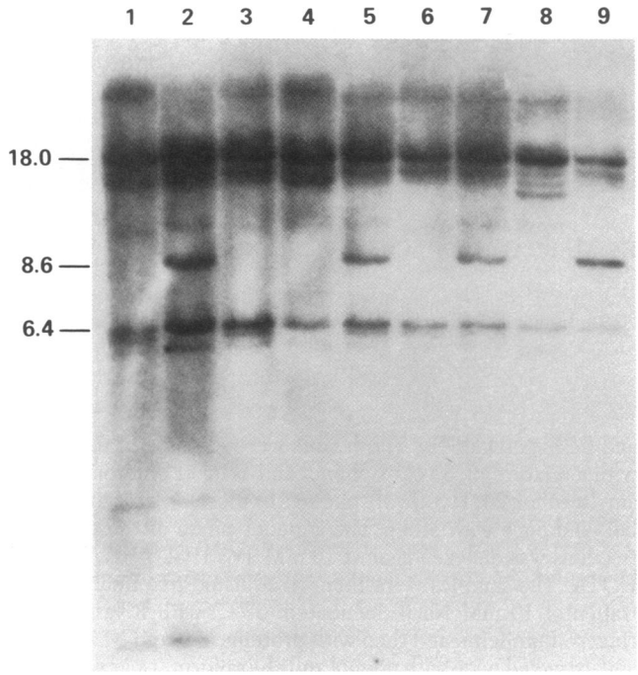

FIG. 1. Autoradiogram of *Eco*RV fragments of cellular DNA from nine individuals after agarose gel electrophoresis, transfer to a hybridization membrane, and hybridization with a nick-translated cDNA clone containing the nucleotide sequence of a class 1 HLA gene. Lanes: 1, individual who does not carry the gene for HLA-B8; 2, individual who carries the gene for HLA-B8; 3, 4, and 6, three offspring of individuals of lanes 1 and 2 (none of these three offspring carry the gene for HLA-B8); 5 and 7, two offspring of individuals of lanes 1 and 2 (each carries the gene for HLA-B8; see Table 2 for analysis of haplotype segregation in this family); 8, individual who does not carry the gene for HLA-B8; 9, individual who carries the gene for HLA-B8. The individuals of lanes 8 and 9 are not genetically related nor are they related to individuals of lanes 1–7. Numbers to the left indicate size of DNA fragments in kb.

donuclease DNA fragments was carried out in a mixture containing 50% (vol/vol) formamide and 1 M NaCl for 12–16 hr at 42°C. Hybridization was performed in 50% formamide/0.6 M NaCl/5% (wt/vol) dextran sulfate (10). Sufficient radiolabeled probe was added to the hybridization mixture to provide about 1×10^6 cpm/ml, which represents 10 ng or less of cDNA per ml. Hybridization was performed at 42°C for at least 48 hr. After hybridization, the membranes were washed at 60°C with 0.3 M NaCl (four times, 30 min each), with 0.03 M NaCl (once, 30 min), and finally with 0.015 M NaCl. This was followed by a short rinse in 3 mM Tris at room temperature.

The restriction endonuclease fragments were visualized by autoradiography with Kodak XAR-5 film in a cassette with Dupont Lightning Plus intensifying screens.

RESULTS

***Eco*RV Restriction Fragment Polymorphism Among Class 1 MHC Genes.** The autoradiogram of the pattern of *Eco*RV DNA fragments after digestion of cellular DNA from nine individuals, electrophoresis, transfer to a hybridization membrane, and probing with the cDNA clone carrying a class 1 sequence is shown in Fig. 1. The usual pattern of fragments consisted of 12 or 13 bands ranging in size from approximately 28 kilobase pairs (kb) to 2 kb. Polymorphism (presence or absence) in the population sample studied (35 unrelated individuals) was noted for restriction endonuclease fragments of sizes 15, 13.5, 13.4, 8.6, 6.3, and 4.6 kb. Polymorphism for the 13.4-kb, 8.6-kb, and 6.3-kb fragments is apparent in Fig. 1 which shows the patterns of restriction endonuclease fragments for four unrelated individuals and the five offspring of two of them.

Correlation Between a Polymorphic *Eco*RV 8.6-kb Fragment and HLA-B8. Cellular DNA from each of 35 unrelated individuals was screened for RFLP with *Eco*RV. The HLA phenotype and genotype (i.e., haplotypes) were known for each of these 35 individuals, and this information was used to analyze the RFLPs. A relationship was noted between an 8.6-kb fragment and the antigen HLA-B8. *Eco*RV generated the 8.6-kb fragment in the cellular DNA from nine individuals, each of whom carried an HLA-B8 antigen. This fragment was not present in the autoradiographic pattern of restriction fragments from the 26 other individuals studied; none of these 26 individuals was HLA-B8. An autoradiogram of restriction fragments from cellular DNA of 28 of the 35 unrelated individuals studied is shown in Fig. 2. The 8.6-kb fragment is seen in lanes that show restriction fragments from individuals with an HLA-B8 antigen. The 8.6-kb fragment is not present in lanes that show restriction fragments from individuals who do not carry HLA-B8. The 2×2 table of association (Table 1) for the 8.6-kb fragment and HLA-B8 is highly significant ($P < 10^{-6}$). Associations between the 8.6-kb fragment and other class 1 HLA genes (serologically defined) including those in linkage disequilibrium with the gene for HLA-B8—e.g., HLA-A1 and HLA-Cw7—were less strong, indicating a poorer correlation than that of this fragment and HLA-B8. The nine individuals with the 8.6-kb fragment carried no HLA class 1 gene in common other than HLA-B8. Each of the other class 1 genes carried by these individuals was also found among the 26 people without the fragment.

None of the other *Eco*RV polymorphic fragments correlated, positively or negatively, with the 8.6-kb band. Cellular DNA from 28 of the 35 individuals studied—9 with the 8.6-kb frag-

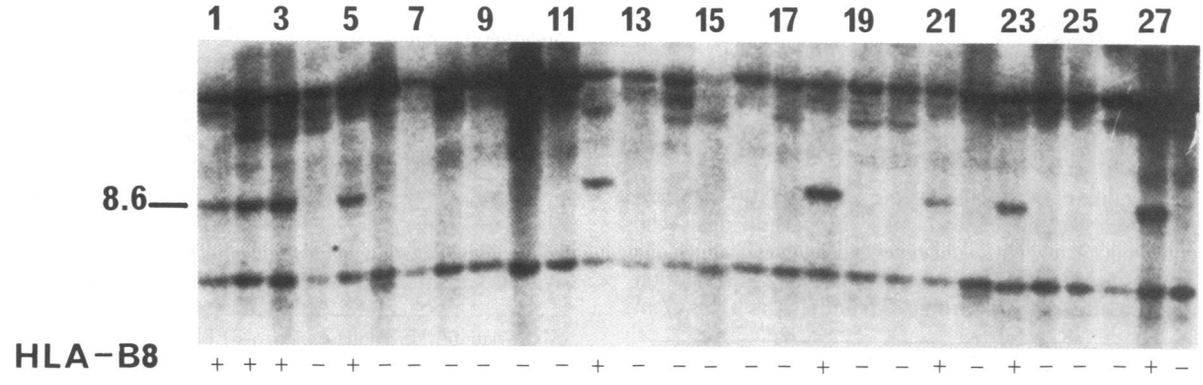

FIG. 2. Autoradiogram of *Eco*RV fragments from 28 unrelated individuals, showing correlation between the fragment and antigen HLA-B8.

Table 1. 2 × 2 tables of association for the *Eco*RV 8.6-kb fragment and class 1 HLA serologically defined genes

Class 1 gene for	Finding*			
	+ +	− +	+ −	− −
HLA-B8	9	0	0	26
HLA-A1	5	3	4	23
HLA-CW7	8	4	1	22
HLA-A2	4	11	5	15
HLA-A3	2	5	7	21
HLA-AW23	2	1	7	25
HLA-A25	1	4	8	22
HLA-A26	2	1	7	25
HLA-AW32	1	1	8	25
HLA-CW6	1	3	8	23
HLA-BW51	2	2	7	24
HLA-B7	1	4	8	22
HLA-BW44	1	9	8	17
HLA-B14	1	1	8	25
HLA-B18	1	9	8	17

* + +, 8.6-kb fragment present, HLA gene present; − +, 8.6-kb fragment absent, HLA gene present; + −, 8.6-kb fragment present, HLA gene absent; − −, 8.6-kb fragment absent, HLA gene absent.

ment and 19 without—was digested with *Eco*RV in at least two separate experiments. Concordance for presence or absence of the fragment was noted for each individual tested more than once.

Segregation of the *Eco*RV 8.6-kb Fragment with HLA-B8. A family (parents and five offspring) was studied for segregation of the *Eco*RV 8.6-kb fragment and the gene HLA-B8 (Fig. 1). The mother and two offspring, one male and one female, carry HLA-B8; *Eco*RV digestion of cellular DNA from each of these individuals generated the 8.6-kb fragment (lanes 2, 5, and 7). The father and three other offspring do not carry HLA-B8, and *Eco*RV digestion did not generate the 8.6-kb fragment from their cellular DNAs (lanes 1, 3, 4, and 6). Table 2 presents the genotype of this family in relation to presence (+) or absence (−) of the fragment. This genetic analysis permits us to conclude that offspring 3 and 5 have inherited from their mother the gene for HLA-B8 and the sequence(s) generating the *Eco*RV 8.6-kb fragment. Offspring 1, 2, and 4 have inherited neither this *Eco*RV sequence nor the HLA-B8 gene from their mother. In this family the 8.6-kb fragment segregates with the maternal haplotype carrying the gene for HLA-B8. The other polymorphic *Eco*RV fragment (6.3 kb) noted for this family did not segregate with either maternal haplotype but did segregate with the paternal haplotype designated *a*.

Table 2. Segregation of HLA haplotypes (class 1 genes) and the *Eco*RV fragment

Family member*	Haplotype	Genotype†	*Eco*RV, 8.6-kb fragment
Father	*a*	*HLA A2, CW7, BW21*	−
(lane 1)	*b*	*HLA AW24, CW2, B40*	
Mother	*c*	*HLA A25, C-, B18*	+
(lane 2)	*d*	*HLA A1, CW7, B8*	
Offspring 1	*a*	*HLA A2, CW7, BW21*	−
(lane 3)	*c*	*HLA A25, C-, B18*	
Offspring 2	*b*	*HLA AW24, CW2, B40*	−
(lane 4)	*c*	*HLA A25, C-, B18*	
Offspring 3	*a*	*HLA A2, CW7, BW21*	+
(lane 5)	*d*	*HLA A1, CW7, B8*	
Offspring 4	*a*	*HLA A2, CW7, BW21*	−
(lane 6)	*c*	*HLA A25, C-, B18*	
Offspring 5	*a*	*HLA A2, CW7, BW21*	+
(lane 7)	*d*	*HLA A1, CW7, B8*	

* Identified by lane number in Fig. 1.
† *C-* is an undefined allele ("blank") at the *HLA-C* locus.

DISCUSSION

At least 13 *Eco*RV-generated DNA fragments hybridize with cDNA from a class 1 MHC gene. This observation, similar to observations noted for murine and human class 1 genes with other restriction endonucleases (11, 12), is evidence for a multiplicity of class 1 gene loci, which exceeds the number known from phenotypic (serologic) analysis. The present study indicates that polymorphism for *Eco*RV nucleotide recognition sequences occurs relatively frequently and is readily detectable in DNA homologous with a class 1 probe. We would expect that most of this DNA comprises part of the HLA chromosomal region. Results from this laboratory suggest that RFLP from class 1 genes is readily detectable with other restriction endonucleases.

In this study, we observed correlation of an 8.6-kb *Eco*RV polymorphic fragment from class 1 DNA sequences with a serologically defined class 1 gene, HLA-B8. In a family study, we found that the 8.6-kb fragment segregated with this gene. The fragment segregated with the haplotype (maternal) carrying HLA-B8, and the correlation data indicated that the fragment is related to the specific gene. We were unable to find an allelic *Eco*RV fragment—i.e., one that segregates with the HLA-B18, the maternal allele at the HLA-B locus. It is possible that one or more serologically defined class 1 genes not present in the population sample analyzed could be associated with an *Eco*RV 8.6-kb fragment.

Two simple hypotheses are consistent with the observation of segregation and correlation of HLA-B8 with the *Eco*RV 8.6-kb fragment which carries a sequence homologous with class 1 genes. This fragment could carry the gene or part of the gene that codes for HLA-B8. An 8.6-kb fragment is sufficiently large to carry a 4- to 5-kb class 1 MHC gene (13). Alternatively, the *Eco*RV fragment could represent part or all of a class 1 gene (or pseudogene) in linkage disequilibrium with HLA-B8. The size of the HLA region is approximately 2–3 × 10^3 kb (2–3 centimorgans, genetic map distance), and the cluster of class 1 genes may be about one-third to one-half this size. Linkage disequilibrium is favored in clusters of closely linked genes and has been detected in the HLA region by phenotypic analysis (14).

These two hypotheses may be distinguished in two ways: additional family studies and transfection experiments.

Family studies that demonstrate an occasional offspring who carries HLA-B8 but not the *Eco*RV site that generates the 8.6-kb fragment or one with the fragment but not HLA-B8 would favor the second hypothesis; such rare individuals presumably would be carrying recombinant haplotypes. Admittedly, the small distance likely to exist between two class 1 genes in the MHC does not favor finding such recombinant individuals readily, and this type of study is not a statistically efficient discriminant between the two hypotheses.

DNA-mediated gene transfer of class 1 MHC genes has now been demonstrated in the mouse (15) and in humans (16). The expression of HLA-B8 on cells transformed with an isolated gene associated with the *Eco*RV 8.6-kb fragment will demonstrate the first hypothesis. Failure of cells to express the antigen after transfection might be construed to favor the second hypothesis, although technical failure will confound proper interpretation. To be convincing, transformation experiments aimed at demonstrating the allotype would require appropriate cell lines as DNA recipients, specific techniques for selecting transformants (17, 18), appropriate HLA-B8 reagents, and multiple demonstrations by different techniques of expression of the antigen.

Moreover, cloning, sequence determination, and transfor-

mation experiments could raise a third possibility for the findings reported here: *Eco*RV 8.6-kb fragments could be associated in the same individual with several functional and nonfunctional genes in linkage disequilibrium.

The findings reported here raise new practical possibilities and have fundamental implications. DNA polymorphic restriction fragments represent a new tool which will permit the definition of groups within the population that may or may not be similar to the already-defined serological groups. These newly defined groups (unpublished data) in particular may detect polymorphism of the noncoding part of the genome (introns or flanking sequences) or even of pseudogenes. RFLPs that will correspond to serologically defined MHC polymorphisms might be used for HLA typing instead of batteries of human alloantisera which are difficult to collect and characterize.

From a fundamental point of view, RFLPs will contribute to a better understanding of the genetic organization of the human MHC, one of the most thoroughly studied regions of the mammalian genome. Hypotheses concerning generation and maintenance of polymorphism can be evaluated. We can expect additional understanding of the evolution of this region which, because of its polymorphism, is responsible for the individual differences in the immune response.

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