THE IDENTIFICATION OF BIOLOGICAL MATERIALS FOR FORENSIC ANALYSIS: APPLICATION OF NEW DNA TECHNOLOGY TO AN OLD PROBLEM

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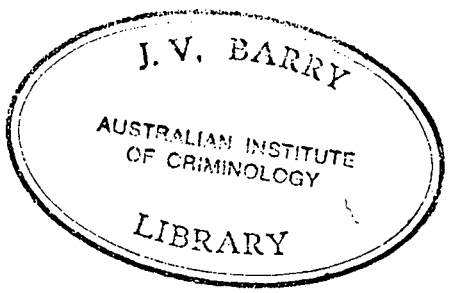
Simon Easteal, PhD
The John Curtin School of Medical Research
The Australian National University



Address for Correspondence:

Dr Simon Easteal
Human Genetics Group
Division of Clinical Sciences
John Curtin School of Medical Research
The Australian National University
Canberra, A.C.T. 2601

Telephone: (06) 249 4719
Fax: (06) 249 4712

Email Simon.Easteal@anu.edu.au

INITIAL PROJECT OBJECTIVES

1. To develop methods of detecting genetic variation at HLA Class II genes for the purpose of individual identification in the context of forensic investigations, with particular emphasis on the HLA DPB1 gene.

2. To determine the frequencies of these genetic variants in an Australian Caucasian sample.

3. Identify and characterise any new genetic variants that might be detected in the above sample or in the course of other work and develop routine methods for detecting these.

RESEARCH RESULTS

The results of the research funded by the grant have been reported in the publications that are listed at the end of this report and which are attached. One final publication has not been completed. What follows is an overview of the entire project.

The initial objectives of the project have all been achieved. When the project began there are 19 HLA DPB1 alleles officially recognised by the World Health Organisation. A simple and accurate method of detecting these alleles was developed (Dekker and Easteal, 1990a, b). The method is based on amplification of the variable portion of the DPB1 gene by the polymerase chain reaction (PCR) followed by restriction enzyme digestion of the PCR product at sequence-specific restriction sites. The method, now commonly referred to as PCR-restriction fragment polymorphism (RFLP) analysis, has a number of advantages over alternative typing methods in terms of accuracy , reliability, efficiency and sensitivity that are particularly relevant in the context of forensic investigations. The reliability of the method has been tested by applying it to the typing of cell lines prepared for the Tenth International Histocompatibility Workshop, that had previously been typed by other methods.

There are now 44 recognised HLA-DPB1 alleles and the typing method has been modified and updated to enable all the new alleles to be detected. The principles on which the typing method is based are explained in Easteal (1990a, b). The combination of DNA fragments produced using the four restriction enzymes on which the method is primarily based are shown in Figure 1a-d. The way in which these fragments can be used to distinguish the different alleles is shown in Figure 2.

The HLA-DPB1 typing method has been applied to samples of unrelated individuals from ten populations from the Asia-Pacific region and the frequencies of the different alleles have been determined (Table 2). In the process, a number of new alleles were discovered, one of which is described in Dekker et al. (1992).

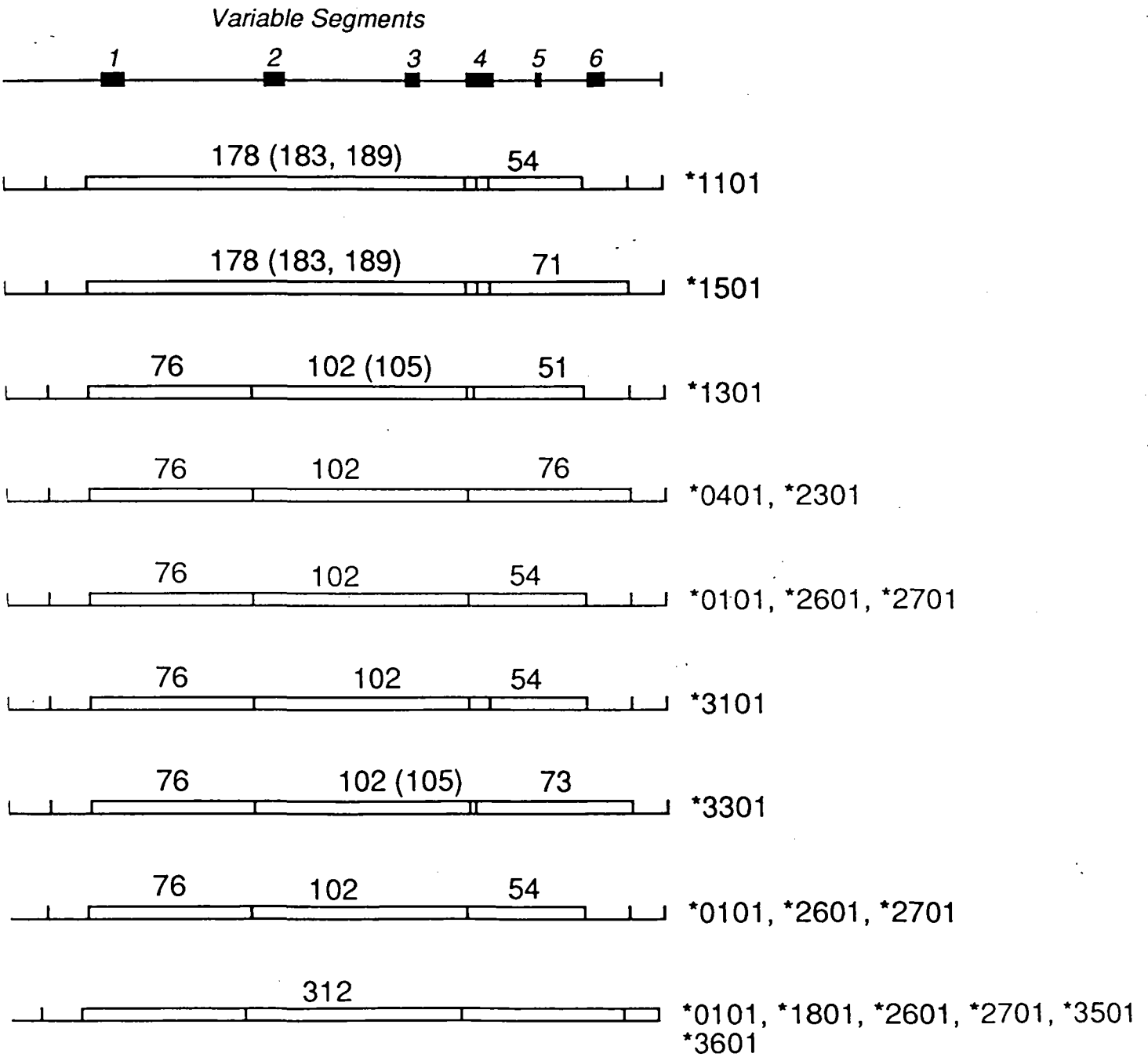
IMPLICATIONS AND PRACTICAL OUTCOME FROM THE STUDY

HLA-DPB1 is one of the most polymorphic genes in the human genome and therefore of great potential use in forensic DNA typing. With 44 known alleles it is, for example, much more polymorphic than HLA-DQA1 at which only six alleles are routinely detected in forensic typing. A reliable and efficient method of detecting variation at this locus has now been developed. The method is based on simple routine procedures and requires very little in the way of specialist equipment or reagents. It is already proving useful in clinical applications and should be equally useful in a forensic context.

Figure 1. Map of HLA-DPB1 exon 2 showing variable segments, the position of the recognition sequences of the four restriction enzymes used for typing the HLA-DPB1 alleles, and the restriction fragments generated by digesting the PCR-amplified exon 2 of the different alleles with these enzymes.

Figure 1a

MnlI



*EcoN*I

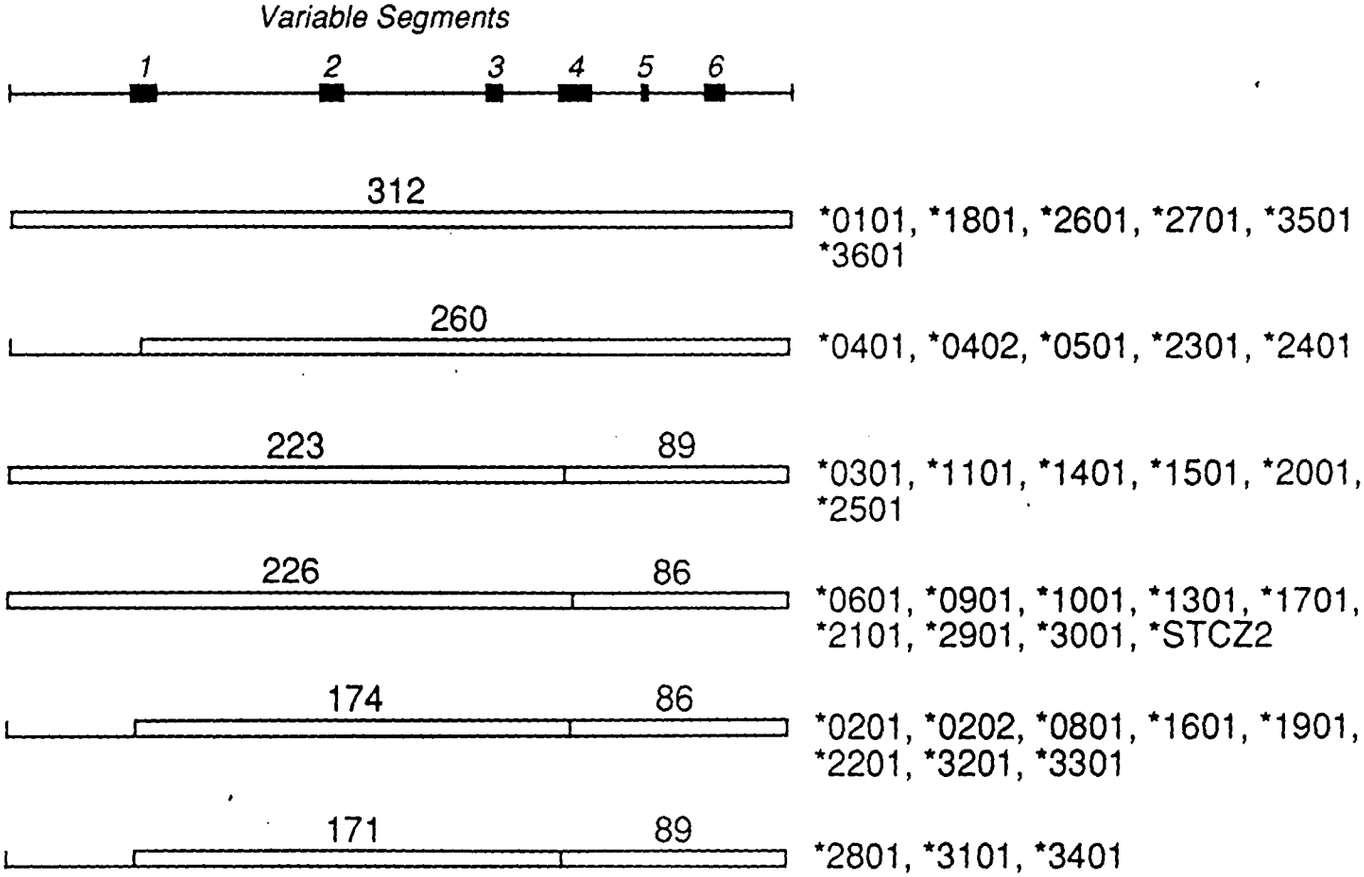


Figure 1b

Rsa I

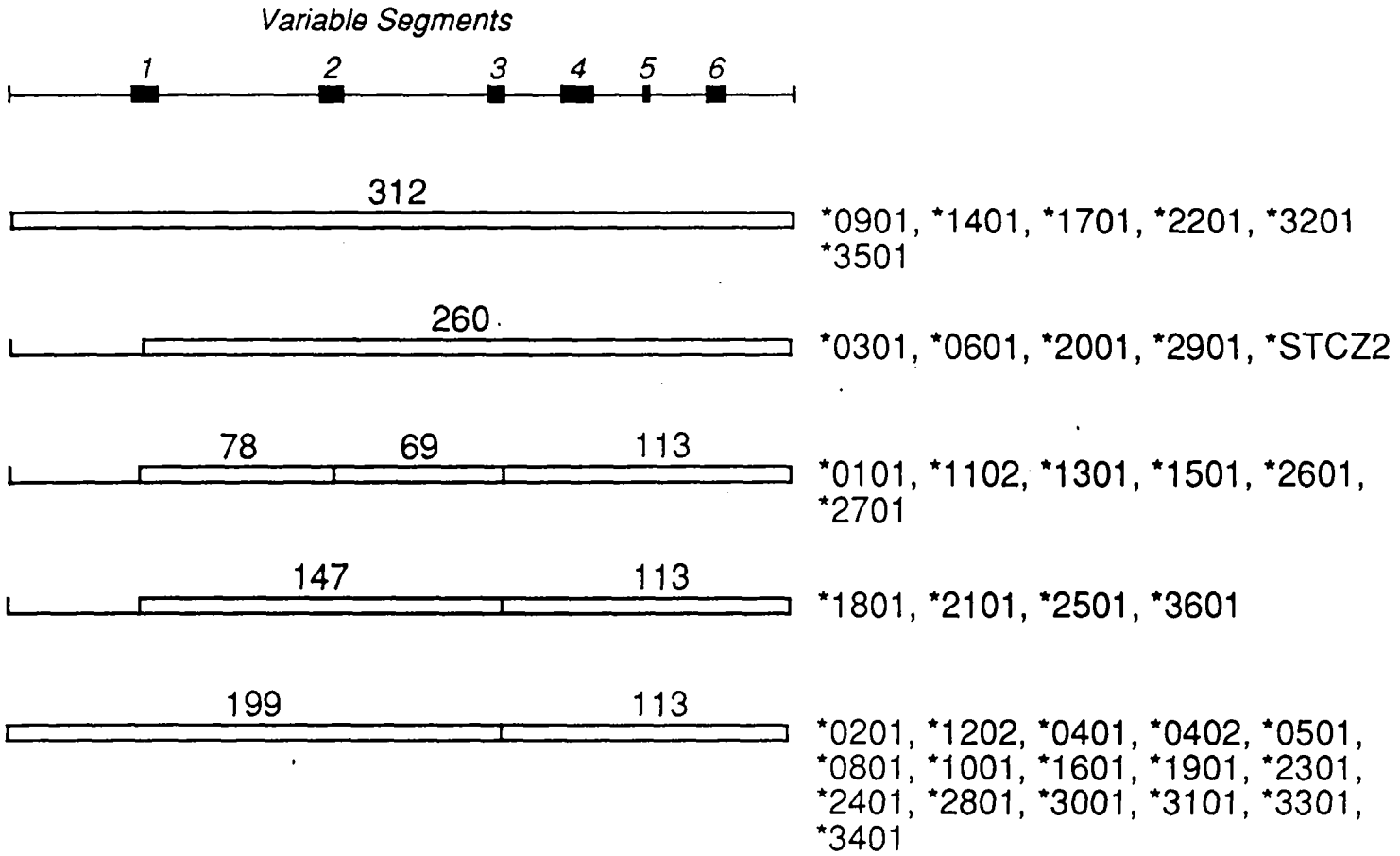
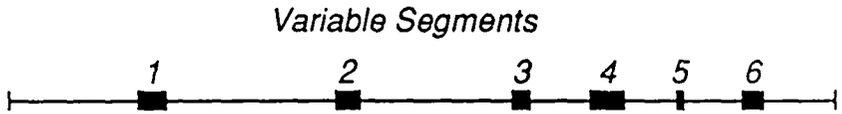
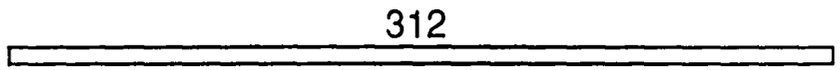


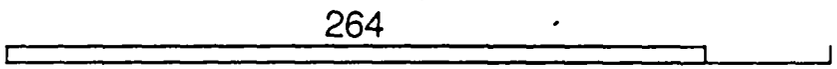
Figure 1c

*Fok*I

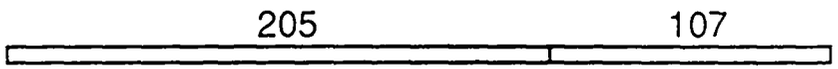




*0301, *1401, *2501, *2901, *STCZ2



*0601, *1101, *1501, *2001, *2801
*3101, *3401



*0101, *0801, *0901, *1001, *1301,
*1901, *2601, *3501

*0201, *0202, *0401, *0402, *0501,
*1601, *1701, *1801, *2101, *2201,
*2301, *2401, *2701, *3001, *3201,
*3301, *3601

Figure 1d

	Restriction Fragment Length (bp)																													
	Mnl I												EcoN I						Rsa I						Fok I					
Allele	178	113	105	102	76	76	73	71	69	66	54	51	312	260	226	174	89	86	312	260	199	147	113	78	312	264	205	107	59
0101				♦	♦						♦		♦										♦	♦			♦	♦	
0201					♦		♦		♦							♦		♦			♦		♦				♦		♦
0202					♦		♦			♦						♦		♦			♦		♦				♦		♦
0301					♦				♦		♦				♦		♦			♦					♦				
0401				♦	♦	♦								♦							♦		♦				♦		♦
0402					♦	♦			♦					♦							♦		♦				♦		♦
0501					♦					♦	♦			♦							♦		♦				♦		♦
0601					♦				♦			♦			♦			♦		♦						♦			
0801					♦				♦			♦				♦		♦			♦		♦				♦	♦	
0901					♦				♦			♦			♦			♦	♦								♦	♦	
1001					♦				♦			♦			♦			♦			♦		♦				♦	♦	
1101	♦										♦				♦		♦						♦	♦		♦			
1301			♦	♦								♦			♦			♦					♦	♦			♦	♦	
1401					♦				♦		♦				♦		♦		♦						♦				
1501	♦							♦							♦		♦						♦	♦		♦			
1601					♦				♦			♦				♦		♦			♦		♦				♦		♦
1701					♦				♦			♦			♦			♦	♦								♦		♦
1801					♦	♦			♦				♦										♦	♦			♦		♦
1901					♦					♦		♦				♦		♦				♦		♦			♦	♦	
2001					♦				♦		♦				♦		♦			♦						♦			
2101					♦					♦		♦			♦			♦				♦	♦				♦		♦
2202					♦					♦		♦				♦		♦	♦								♦		♦
2301				♦	♦	♦								♦							♦		♦				♦		♦
2401					♦	♦				♦				♦							♦		♦				♦		♦
2501					♦				♦		♦				♦		♦					♦	♦		♦				
2601				♦	♦						♦		♦										♦	♦			♦	♦	
2701				♦	♦						♦		♦										♦	♦			♦		♦
2801					♦			♦	♦							♦	♦				♦		♦			♦			
2901					♦				♦			♦			♦			♦		♦					♦				
3001					♦					♦		♦			♦			♦			♦		♦				♦		♦
3101				♦	♦						♦					♦	♦				♦		♦			♦			
3201					♦		♦		♦							♦		♦	♦								♦		♦
3301			♦	♦	♦		♦									♦		♦			♦		♦				♦		♦
3401		♦		♦	♦			♦								♦	♦				♦		♦			♦			
3501					♦				♦		♦		♦						♦								♦	♦	
3601					♦					♦	♦		♦									♦	♦				♦		♦

Figure 2. Typing scheme used to detect DPB1 alleles by digestion with four restriction enzymes.

Table 1

HLA-DPB1 ALLELE FREQUENCIES (%) IN ASIA-PACIFIC POPULATIONS

	EUR	IND	CHIN	JAVA	PNGC	POLY	MICN	FIJI	SCZ	PNGH
Allele	(178)	(128)	(132)	(144)	(84)	(136)	(100)	(72)	(52)	(90)
0101	5	6	1	6						1
0201	11	26	11	7	25	24	18	35	27	19
0202	2		10	7	1					1
0301	9	3	9	13	2	9	1			11
0401	47	37	6	20	63	18	35	40	42	42
0402	15	7	4	10			1			
0501	3		45	13	7	49	44	24	10	24
0601	2				1					
0801		1								
0901		3		1						
1001	2	2								
1101	2									
1301	2	11	7	18	1		1			
1401	1	2	3					1		
1501	1	1								
1601	1			1						
1701	1	1								1
1801		1								
1901	1									
2201										
3101				3						
2801				1						
4401									21	
2101	1		5	1						

Publications Resulting from the Work

Dekker, J. & **S. Easteal**. 1990. HLA-DP typing with AFLPs. *A.S.E.A.T.T.A Newsletter* 1990(1): 5-13.

Dekker, J. & **S. Easteal.** 1990. HLA-DP typing by amplified fragment length polymorphisms (AFLPs). *Immunogenetics* **32**:56-59.

Dekker, J.W., L. Croft & **S. Easteal**. 1992. The nucleotide sequence of a novel HLA-DPB1 allele. *Immunogenetics* **36**:341-343.

Easteal, S., J. W. Dekker & L.Croft. HLA-DPB1 allelic variation in the Asia-Pacific region. (Manuscript in Preparation, to be submitted to *Human Immunology).*

Conference Papers Describing Aspects of the Work

Dekker, J & **S. Easteal.** HLA-DP, -DQ, and -DR typing using amplified fragment length polymorphisms (AFLPs). *Australasian & South East Asian Tissue Typing Association* Annual Meeting, Aukland, New Zealand, 1990 (invited paper).

Easteal, S. HLA-DPB1 amplified fragment length polymorphism in human populations in the Asia-Pacific region. *Genetics Society of Australia* Annual Meeting, Melbourne, 1991.

Easteal, S. HLA-Class II typing by amplified fragment length polymorphisms (AFLPs). *Australasian & South East Asian Tissue Typing Association* Annual Meeting, Bangkok, Thailand, 1991 (invited paper).

Easteal, S. The molecular and evolutionary basis of HLA-DPB1 allelic variation. *14th Annual Conference on the Organization and Expression of the Genome*, Lorne, 1992.

Easteal, S. The molecular evolutionary genetics of the *HLA-DPB1* locus. *Boden Conference on the Evolution of Gene Families*, Threadbo, 1993.

Invited Seminars Describing Aspects of the Work Presented at:

Division of Biochemistry and Molecular Biology, The Faculties, ANU

Department of Clinical Immunology, Royal Perth Hospital

Research School of Biological Science, ANU

John Curtin School of Medical Research, ANU

CRC 19/89

Immunogenetics 32: 56-59, 1990

Immuno-genetics

HLA-DP typing by amplified fragment length polymorphisms (AFLPs)

James W. Dekker and Simon Easteal

Human Genetics Group, John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601, Australia

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The *HLA-DP* genes form one of the three sets of gene subfamilies that code for the class II antigens of the human major histocompatibility, or *HLA* complex. HLA class II antigens are cell surface glycoproteins involved in the presentation of antigenic peptides to T lymphocytes. The HLA-DP antigens are heterodimers consisting of α and β chains coded by the *HLA-DPA1* and *HLA-DPB1* genes, respectively. Most *HLA-DP* allelic variation occurs in the second exon of *HLA-DPB1* which codes for the cell distal, extracellular domain of the β chain. This domain is involved in antigenic peptide binding and T-cell recognition. DNA sequencing (Bugawan et al. 1988, 1989) has shown that there is far more *HLA-DP* allelic variation than is indicated by the six HLA-DP specificities identified by primed lymphocyte typing (PLT).

Although the *HLA-DP* genes are expressed at lower levels than the *HLA-DR* and *-DQ* genes, host T cells are generated against allogeneic DP molecules (Bonneville et al. 1988), and the risk of graft-vs-host disease in bone marrow transplants can be significantly increased by DP mismatches (Odum et al. 1987). HLA-DP specificities have also been shown to be associated with several autoimmune diseases (Stephens et al. 1989; Bugawan et al. 1989).

HLA-DP typing has traditionally been by PLT and more recently by restriction fragment length polymorphism (RFLP) analysis, both of which are difficult to interpret and time-consuming. Angelini and co-workers (1989) have recently described a method of HLA-DP typing based on polymerase chain reaction (PCR) amplification of the DPB1 second exon and hybridization with sequence-specific oligonucleotides (SSOs). The method is more accurate, sensitive, and efficient than PLT or RFLP typing. Here we present an alternative PCR-based method of HLA-DP typing that involves analysis of electrophoretically separated restriction fragments, or amplified fragment length polymorphisms (AFLPs), produced by digestion of PCR-amplified DPB exon 2 sequences at allele-specific restriction sites.

We evaluated this method by applying it to 30 cell lines from the panel designed for the Tenth International Histocompatibility Workshop (Yang et al. 1989). For each cell line, exon 2 of *DPB1* was amplified by PCR from 0.5 µg genomic DNA using the primers DPB13 (5′-GATGAAGCCCCTCCCCGCAGAGAATTAC-3′) and DPB14 (5′-GATGAAGCTCACTCACCTCGGCGCTG-CAG-3′) in a 100 µl reaction volume containing 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% (w/v) gelatin, 200 µ*M* of each dNTP, 0.2 µ*M* each primer, and 2.5 units *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Connecticut), overlaid with 40 µl mineral oil. The reaction mixtures were incubated for 40 cycles, consisting of 45 s at 95 °C, 10 s at 60 °C, and 45 s at 72 °C in a Programmable Cyclic Reactor (Ericomp, San Diego, California). The cycles were preceded by an extended denaturation step (5 min) at 95 °C and followed by an extended primer extension step at 72 °C (7 min). Between 5 and 10 µl of the PCR product was digested for at least 4 h with the following restriction endonucleases: *Bst* UI (4 units), *Dde* I (2 units), *Eco* NI (4 units), *Fok* I (2 units), *Mnl* I (6 units), *Rsa* I (2 units), *Sau* 96I (6 units), according to the manufacturer's instructions. The restriction fragments were separated on 10% polyacrylamide gels and visualized by staining with ethidium bromide. The restriction fragments were interpreted by reference to the fragment sizes predicted from the known *DPB1* allele second exon sequences (Bugawan et al. 1988, 1989; Begovich et al. 1989).

As shown in Figure 1, all 19 reported *DPB1* alleles can be distinguished from each other. Alleles coding for the six recognized PLT-defined DP specificities can be distinguished using a single restriction enzyme, *Mnl* I, with the exception of *DPw3* and *w6* which require a further digest using *Fok* I. The other 13 known alleles of *DPB1*, including those coding for subtypes of *DPw2* and

Address correspondence and offprint requests to: J. Dekker.

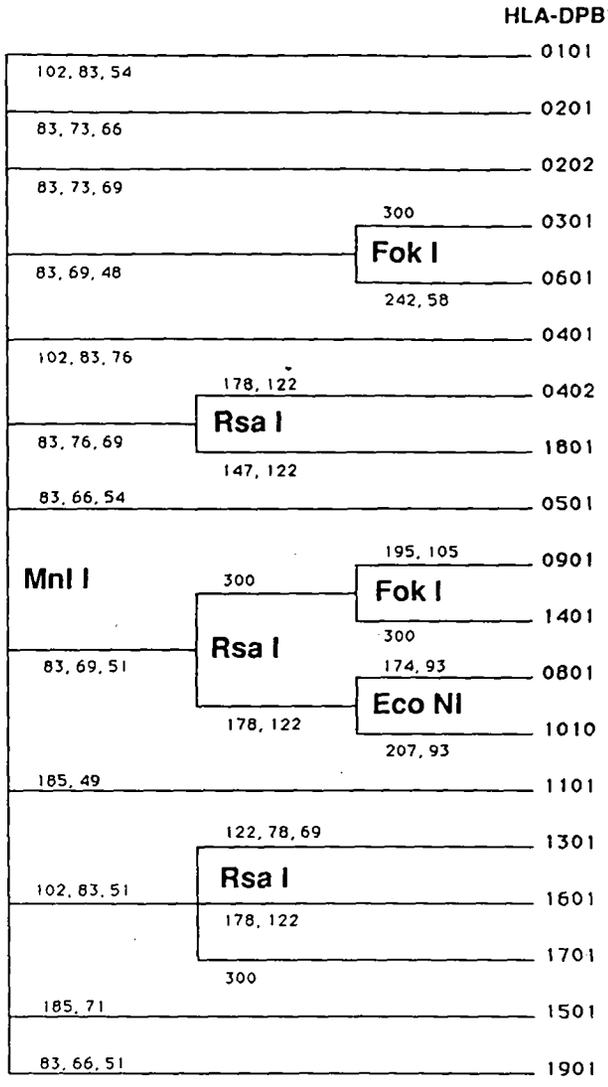

Fig. 1. Diagram showing typing scheme used to define 19 *DPB1* alleles by progressive restriction enzyme digests. *Numbers* refer to expected fragment sizes in bp.

w4 (*DPB1*0201*, **0202*, **0401*, and **0402*) can all be distinguished by digestion with three more enzymes.

Rsa I separates all the remaining alleles except *DPB1*0801* and **1001* which may be split on the basis of an *Eco* NI digest, and *DPB1*0901* and **1401* which are split on the basis of a *Fok* I digest. The three other enzymes, *Sau* 96I, *Bst* UI, and *Dde* I, were used to confirm the results obtained using the scheme in Figure 1 and to resolve ambiguities associated with the typing of heterozygotes. *Sau* 96I digestion of *DPB1*0301*, **0601*, **1101*, and **1401* produces 196 and 104 base pair (bp) fragments; digestion of *DPB1*0201*, **0202*, **0401*, **0402*, and **1801* produces 260 and 39 bp fragments; digestion of *DPB1*1501* produces 196, 64, and 39 bp fragments and the remaining alleles are not cut by this enzyme. *Bst* UI digestion of *DBP1*0101*, **0401*, **1301*, and **1501* produces 186, 55, and 57 bp fragments while the remaining alleles produce 245 and 55 bp fragments. *Dde* I only cuts *DPB1*0202*, **0501*, and **1901*, producing 169 and 131 bp fragments, and does not cut any other alleles. Examples of diagnostic AFLPs are shown in Figure 2.

For AFLP typing, the identities of the cell lines were obscured to avoid any potential bias due to prior knowledge of the typing results by other methods. The AFLP typing results and their comparison with the results of PLT, RFLP, and SSO typing are summarized in Table 1. All the cell lines had previously been PLT-typed, all but three had been RFLP-typed, and 14 had been typed by SSO. The cell lines included all but one (DPw6) of the six PLT-defined HLA-DP specificities at the Tenth International Histocompatibility Workshop, as well as a number of "blank" types. AFLP typing agreed with PLT and RFLP typing in all but a few cases. There was complete agreement between AFLP and SSO typing results.

Cell line 9063 was PLT-typed as DPw2, and identified as a novel type by RFLPs. It is typed as DPB1*0801 by both SSOs and AFLPs. Cell line 9077 was identified as a *DPw5* homozygote by PLT typing and a *DPw3/6,w5* heterozygote by RFLP typing. AFLPs indicated that it is

Table 1. DPB1 assignments determined by AFLP typing compared with SSO assignments and RFLP and PLT-determined DP types for a subset of Tenth International Histocompatibility Workshop (IHW) cell lines.

Tenth IHW cell line	PLT* DPw	RFLP† DPw	AFLP DPB
9023*, 9058*	1	1	0101
9038*, 9050*	2	2	0201
9017*	2, 4	2, 4	0201, 0401
9045	2, 4	2, 4	0201, 0402
9074	2, 5	2, 5	0201, 0501
9020*	2	2	0202
9019*	0	2	0202
9018	3	3/6	0301
9079	3, 4		0301
9087	3, 4	3/6, 4	0301, 0401
9071	3, 4	3/6, 4	0301, 0402
9004, 9014, 9075*	4	4	0401
9031*, 9051*, 9035	4	4	0401
9027	4	4	0401, 0402
9001	4		0402
9013	0	4	0402
9010, 9064	0	new	0402
9024	5		0501
9055*	5	new	0501
9077	5	3/6, 5	0501, new
9063*	2	new	0801
9048*	0	new	0901
9034*	0	new	1001

* Yang and co-workers 1989.
† Consensus from Easteal and co-workers 1989, Hyldig-Nielsen and Svejgaard 1989, and Mitsuishi and co-workers 1989.
* SSO typed by Angelini and co-workers 1989.

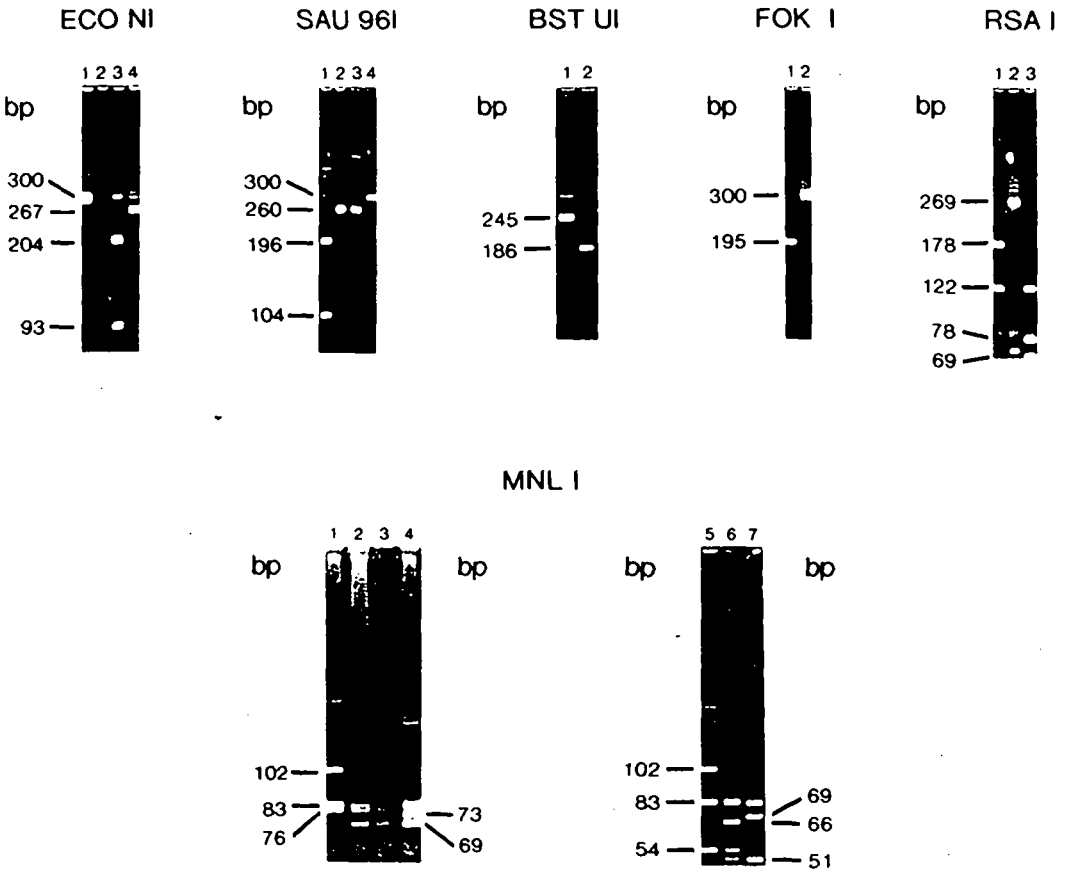

Fig. 2. Diagnostic restriction fragments used in AFLP typing for six restriction endonucleases separated on 10% polyacrylamide gels. For example, the *Eco* NI results show the 300 bp uncut fragment descriptive of *DPB1*0101* and **1801* (lane 1). The 204 and 93 bp bands (lane 3) are descriptive of *DPB1*0301*, **0601*, **1401*, and **1501* (*DPB1*0901*, **1001*, **1101*, **1301*, and **1701* present a 207 bp band that cannot be distinguished from the 204 bp band at this resolution). The 267 bp band (lane 4) defines *DPB1*0401*, **0402*, and **0501*. Not shown are the 174 and 93 bp bands specific to *DPB1*0201*, **0202*, **1701*, and **1901*. The *Fok* I 195 bp fragment (lane 1) is associated with all alleles except *DPB1*0301* (uncut 300 bp band, lane 2) and *DPB1*0601*, **1101*, and **1501* (242 and 58 bp bands, not shown). The *Rsa* I 188 and 122 bp fragments (lane 1) are diagnostic of all alleles except *DPB1*0301* and **0601* (269 bp band, lane 2), *DPB1*1101*, **1301*, and **1501* (122, 78, and 69 bp bands, lane 3), *DPB1*1801* (147 and 122 bp bands, not shown), and *DPB1*0901*, **1401*, and **1701* (300 bp uncut fragment, not shown).

a *DPB1*0501*, "new allele" heterozygote. The new allele has the same AFLP pattern as *DPB1*0201* except *Rsa* I (new band approximately 130 bp) and *Mnl* I (102 and 69 bp bands). The novel *Rsa* I band also occurs in cell line 9074, which otherwise types by AFLP, SSO, RFLP, and PLT as DPB1*0201,0501. For of this reason, and because the allelic association of the novel *Rsa* I fragment cannot be determined, the cell line was provisionally typed as DPB1*0201,0501. The novel RFLP pattern observed for the cell line 9055 does not appear to correlate with any *DPB1* allelic variant. Cell line 9055 was typed by SSO and AFLPs as DPB1*0501 and by PLT as DPw5. Cell lines 9034, 9048, 9010, 9064 were all typed as DPw blank by PLT. Cell lines 9010 and 9064 are typed by AFLPs as DPB1*0402. Cell lines 9034 and 9048 are typed as DPB1*1001 and *0901, respectively, by both SSO and AFLPs; both were identified as having novel RFLP patterns.

There are a number of factors that may make AFLPs the preferred alternative as a sensitive and efficient method of HLA typing. First, AFLPs are technically simple, involving digestion with a small number of restriction endonucleases and no hybridization. Second, since they involve the identification of differences in the outcome of an endonuclease digestion, rather than the occurrence or nonoccurrence of hybridization, as in the case of SSOs, experimental failure (i. e., lack of digestion) can be readily detected. Where the separation of two alleles involves a lack of digestion of one of them, as in the case of *Fok* I digestion of *DPB1*0301* and **0601*, and where homozygotes are being typed, an allelic sequence known to contain a restriction site for the enzyme can be included in the digestion as an internal control. Third, the interpretation of AFLPs is relatively unaffected by PCR contamination. Since ethidium bromide staining is far less sensitive for DNA detection than hybridization, con-

tamination is less likely to be detected. Furthermore, since a PCR product and its contaminant are subjected to exactly the same experimental regime of PCR amplification and restriction enzyme digestion, a contaminant, where it is observed, can be identified by its relatively lower concentration. AFLPs should be particularly useful for HLA-DP typing given the limitations of PLT and RFLPs but should, however, be generally useful as a method of HLA typing.

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Note added in proof:
A scheme similar to the one described here has recently been published for the typing of 10 *HLA-DPB1* alleles using a different combination of restriction enzymes (Maeda, M., Uryu, N., Murayama, N., Ishii, H., Ota, M., Tsuji, K., and Inoko, H.: A simple and rapid method for HLA-DP genotyping by digestion of PCR amplified DNA with allele-specific restriction endonucleases. *Hum Immunol 27:* 111–121, 1990).

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Immuno-genetics

Nucleotide sequence of a novel *HLA-DPB1* allele

James W. Dekker, Lynn Croft, and Simon Easteal

Human Genetics Group, John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T., Australia

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The 1991 report of the WHO Nomenclature Committee (1991) included four *HLA-DPA1* and 20 *HLA-DPB1* alleles. Of the latter, 19 are distinguished from other alleles by nonsilent nucleotide sequence differences in the second exon of the *DPB1* gene. This exon codes for the antigen binding site domain of the DPβ polypeptide. The sequence of another allele has recently been described which is identical to *DPB1*0301* except at codon 76 (Marsh and Bodmer 1991) where the G and A in the first and third positions are replaced by A and G respectively (De Koster et al. 1991). The existence of five other alleles has been implicated in *DPB1* typing with sequence-specific oligonucleotides (SSOs) (Fernandez-Vina et al. 1991; Gao et al. 1991) but the DNA sequences for these are not yet available. With a few minor exceptions, *DPB1* allelic variation within exon 2 is confined to six distinct variable segments (VSs), and allelic differences consist of different combinations of a small number of sequence motifs at each of the VSs (Bugawan et al. 1988, 1990).

During the development of procedures for *HLA-DPB1* typing by amplified fragment length polymorphisms (AFLPs; Dekker and Easteal 1990), a novel AFLP pattern was observed in 10th International Histocompatibility Workshop typing cell 9077 (T7527), which was of Hong Kong Chinese origin (Yang et al. 1989). Restriction mapping of exon 2 with *Eco* NI, *Fok* I, *Mnl* I, and *Rsa* I, following amplification by polymerase chain reaction (PCR), indicated the presence of a new allele, in heterozygous combination with *DPB1*0501*. The new allele appeared to contain a combination of the VS sequences similar to as *DPB1*0501* except at HVR1 where it appeared to share sequences with *DPB1*0301*, **0601*, **1101*, and **1301* and at VS4 where it resembles **0201*, **0202*, **0801*, **0901*, **1001*, **1701*, and **1901*.

The nucleotide sequence data reported in this paper have ben submitted to the GenBank nucleotide sequence database and have been assigned the accession number M80300.

Address correspondence and offprint requests to: S. Easteal, Human Genetics Group, John Curtin School of Medical Research, Australian National University, G.P.O. Box 334, Canberra, A.C.T., Australia.

Primers JDPU2 (5′-GAGAGTGGCGCCTCCGCT-CAT-3′) and JDPB3 (5′-TCACTCACCTCGGCGCTGC-AG-3′), tagged at their 5′ ends with the M13 forward and reverse universal sequencing primers respectively were used to PCR amplify *DPB1* exon 2 from 1 μg of T7527 genomic DNA in a 100 μl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 10% DMSO, 20 mM each dNTP, 0.2 mM each primer and 2.5 units *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT). Thermocycling consisted of 30 cycles of 1 min each at 95 °C, 60 °C, and 72 °C. The resultant PCR products were subjected to asymetric PCR amplification, using a fifty-fold lower than normal concentration of one or other of the primers, to produce single-stranded PCR products, which were purified by retention in a centricon 100 filter (Amicon, Danvers, MA). Four independent single-stranded PCR products were sequenced by the dideoxy chain-termination method using the Applied Biosystems cycle-sequencing kit (Applied Biosystems, Foster City, CA) with dye-labeled M13 universal sequencing primers. Thermocycling consisted of 15 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. Sequencing products were resolved in 6% denaturing polyacrylamide gels using an Applied Biosystems 370A automated DNA sequencer.

It was not possible to determine the complete, individual sequences of the two alleles from the resultant composite electropherograms. It was however possible to confirm that the sequences predicted to occur at VS1 by the restriction mapping were those associated with *DPB1*0501* (CTTTTCCAGGG) and with *DPB1*0301*, **0601*, **1101*, and **1301* (GTGTACCAGTT). Unambiguous results were also obtained for the eight nucleotides 5′ to VS1.

A PCR primer (JDPU3; 5′-CGCAGAGAATTACG-TGTACCAGTT-3′), with specificity to the *DPB1*0301*, **0601*, **1101*, **1501* VS1 sequence and tagged with the M13 universal sequencing primer was used, in combination with JDPB3 to PCR amplify T7527. Digestion of the resultant PCR product with *Rsa* I produced only the 147

```
                    10                                  20
AG AAT TAC GTG TAC CAG TTA CGG CAG GAA TGC TAC GCG TTT AAT GGG ACA CAG
   Asn Tyr Val Tyr Gln Leu Arg Gln Glu Cys Tyr Ala Phe Asn Gly Thr Gln

                        30                                  40
CGC TTC CTG GAG AGA TAC ATC TAC AAC CGG GAG GAG CTC GTG CGC TTC GAC AGC
Arg Phe Leu Glu Arg Tyr Ile Tyr Asn Arg Glu Glu Leu Val Arg Phe Asp Ser

                        50
GAC GTG GGG GAG TTC CGG GCG GTG ACG GAG CTG GGG CGG CCT GAG GCG GAG TAC
Asp Val Gly Glu Phe Arg Ala Val Thr Glu Leu Gly Arg Pro Glu Ala Glu Tyr

      60                                70
TGG AAC AGC CAG AAG GAC ATC CTG GAG GAG GAG CGG GCA GTG CCG GAC AGG ATG
Trp Asn Ser Gln Lys Asp Ile Leu Glu Glu Glu Arg Ala Val Pro Asp Arg Met

                80                                  90
TGC AGA CAC AAC TAC GAG CTG GAC GAG GCC GTG ACC CTG CAG CGC CGA G gt
Cys Arg His Asn Tyr Glu Leu Asp Glu Ala Val Thr Leu Gln Arg Arg
```

Fig. 1. Nucleotide sequence (and inferred amino acid sequence) of *HLA-DPB1* exon 2 of the novel allele found in XIHW cell line T7527. Codon numbering is as in Marsh and Bodmer (1991). Variable segments are *underlined*.

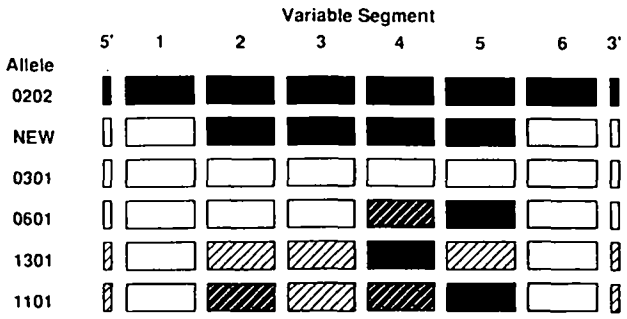

Fig. 2. Comparison of exon 2 variable segment sequences and flanking region sequences inferred from restriction fragment length polymorphism analysis between the novel allele and *DPB1*0202*, **0301*, **0601*, **1101*, and **1301*.

and 113 base pair (bp) fragments predicted for the new allele. The absence of the 113 and 183 bp fragments, predicted for *DPB1*0501*, confirmed that allele-specific amplification had been achieved. The products of three independent PCR reactions were subjected to further asymetric PCR amplification, to produce a single-stranded DNA template for direct sequencing as described above.

In addition, a fourth primer (DPB10; 5'-AGCCGG-CCCAAAGCCCTC-3'), tagged with the M13 reverse universal sequencing primer, and with complementarity to the sequence commencing three bases from the 5' end of intron 2 was used in combination with JDPU3 to PCR amplify T7527. The products of three of these PCR reactions were used, independently, as templates for DPB10 primed direct sequencing. This was done in order to obtain the sequence of the 13 nucleotides at the 3' end of exon 2 which are included in JDPU3.

The results of the sequencing reactions showed that in the region between, but not including, VS1 and VS6, the allele is identical in sequence to *DPB1*0201*, while at VS6, as at VS1, the allele is the same as *DPB1*0301*, **0601*, **1101*, and **1301* (Fig. 1.). The sequence of the new allele is different from any of the sequences predicted from novel SSO patterns (Fernandez-Vina et al. 1990; Gao et al. 1991).

The pattern of VS sequences in the new allele suggests that either it or *DPB1*0202* arose by nonreciprocal or double recombination involving the central portion of *DPB1* exon 2, which the two alleles share, and the terminal portions of exon 2 of either *DPB1*0301*, **0601*, **1101*, or **1301* (Fig. 2). Restriction fragment length polymorphism (RFLP) analysis indicates that the recombination was with either *DPB1*0301* or **0601*. RFLP typing with *HLA-DPA1* and *-DPB1* probes, which detect restriction site variation 5' and 3' to *DPB1* exon 2 respectively, showed that T7527 has a pattern of restriction site variation seen in *DPB1*0301* (or **0601*), **0501* heterozygotes (Easteal et al. 1989), indicating that the sequence identity between the new allele and *DPB*0301* and **0601* extends from the terminal VSs of exon 2 into the flanking regions. Although *DPB1*1101* and **1301* have the same VS1 and VS6 sequences as *DPB1*0301*, **0601*, and the new allele, this identity does not extend into the regions flanking *DPB1* exon 2. Two Caucasian samples, typed by AFLPs as *DPB1*0401*, **1301*, and another sample, typed as *DPB1*0401*, **1101*, all had the same RFLP patterns consistent with the presence of both *DPB1*0401* and an allele not corresponding to any of the then defined primed lymphocyte typing (PLT) specificities. This pattern is quite distinct from that associated with *OPB1*0301* and **0601*. Involvement of *DPB1*1101* and **1301* in the recombination event(s) that gave rise to the new allele is thus ruled out. Additional data on population distributions and frequencies of the new allele and of *DPB1*0202* are needed before any further inference can be made as to which is the ancestral and which the derived allele.

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DNA Fingerprinting by PCR Amplification of HLA Genes

Simon Easteal
Human Genetics Group
John Curtin School of Medical Research
Australian National University, Canberra

The term 'DNA fingerprinting' could be applied to any approach to detecting individual variation through analysis of DNA. It has, however, come to be associated with a particular method based around the technique of 'Southern' blotting. The application of this procedure has been widely acclaimed as a major breakthrough in forensic science, although its use has not been without criticism in some instances (Lander 1989). The power of the method to discriminate between individuals derives from the use of DNA probes that identify highly variable regions of the genome, usually short tandemly repeated sequences or minisatellites (Jeffreys et al. 1985; Nakamura et al. 1987; Wong et al. 1987). These regions are sufficiently variable that the probability of false identity is negligible, thus allowing positive identification to be made with confidence (Gill et al. 1985; Jeffreys et al. 1985).

Because this approach is potentially so powerful, one might be tempted to think that we have gone as far as we need in our efforts to develop methods of identifying individual genetic variation for forensic purposes. Existing methods do, however, have a number of limitations and new techniques are now available that can overcome these. These techniques are already widely used in many areas of human genetics and will undoubtedly become the principle method of analysis in forensic studies in the near future. The techniques are based around a procedure known as the polymerase chain reaction (PCR) (Saiki et al. 1985; Saiki et al. 1988). This procedure allows the multi-million-fold replication, in vitro, of specific DNA regions. Starting with as little as the unpurified DNA contents of a single cell, PCR produces, within hours, microgram amounts of a specific segment of DNA, enough to allow genetic variation to be analysed in a number of ways.

Effective use of this approach in forensic context requires firstly, the appropriate choice of highly variable regions of DNA for amplification by PCR and secondly, the development of efficient and sensitive methods for detecting variation in the PCR amplified product. This paper will describe the PCR approach to detecting genetic variation and discuss how the above requirements can be met to allow the approach to be effectively used in forensic investigations. Before doing that, some aspects of the currently used 'Southern' blotting method will be considered to highlight its limitations and allow it to be compared with the PCR approach.

Limitations of the Present Method

There is a requirement in 'Southern' blotting analysis for 0.05 - 1 μg of purified DNA, depending on the nature of the DNA probes being used to detect variation (Jeffreys et al. 1988). This amount of DNA can be obtained from approximately 10,000 - 200,000 nucleated cells, or from one or two hair roots, 2 - 40 μl blood, or 25 - 500 μl saliva. More than this may be required as some DNA is usually lost during purification. There will also be a requirement for more DNA when the analysis needs to be duplicated (for example, by prosecution and defence) or repeated (because of experimental failure or ambiguous results) or when the DNA is cut with more than one enzyme in the course of the analysis.

This lower limit to the amount of DNA required for 'Southern' blotting does not pose a major problem in areas of civil law such as paternity testing or the resolution of immigration disputes. If a sample can be obtained in these situations it can usually be obtained in sufficient quantity. The limit does however have considerable implications in criminal investigations, where only small amounts of residue may be available for analysis.

It is not unusual for the results of 'Southern' blot analysis to be other than completely clear and sometimes experiments need to be repeated. There are many possible causes of experimental failure. One, tissue degradation, is of particular importance in the forensic context because it is likely to be encountered and it may not be possible to overcome.

When DNA is degraded it is cut into fragments. The sizes of these fragments decrease as degradation progresses. Eventually a point is reached where there are no longer any fragments remaining that are long enough to span the distance between the sites in the DNA that are cut by restriction enzymes. The result is that the restriction fragments that normally occur in a region of DNA being studied are no longer detectable when probed with DNA from that region. 'Southern' blot analysis is thus limited not only by the amount of required DNA, but also by a need for relatively undegraded DNA.

A Better Alternative

The PCR approach is substantially less limited in both respects. PCR amplification can be achieved from a single cell (Jeffreys et al. 1988; Li et al. 1988), that is from 10,000 - 200,000 times less DNA than is required for 'Southern' blots. PCR amplification can also be achieved from DNA that is extensively degraded. The extent of permissible degradation depends on the length of the DNA fragment being amplified, however fragments of only a few hundred bases are large enough to permit extensive genetic variation to be analysed following amplification. As long as there are some fragments of at least this length spanning the target region in a sample of DNA, successful amplification is possible. This compares with the 'Southern' blot requirement for substantial numbers of fragments at least tens of thousands of bases long. The extent to which PCR can be successfully carried out on degraded material is illustrated by the amplification of DNA from the 13,000-year-old remains of the extinct giant sloth (Pääbo 1989) and the brain of a 7000-year-old mummy (Pääbo et al. 1988) as well as the remains of many other long-dead specimens (Pääbo 1989; Pääbo & Wilson 1988; Kocher et al. 1988).

PCR has a number of additional advantages over 'Southern' blotting. The small amount of DNA required for PCR will make it easier for multiple independent tests to be conducted. It will also greatly facilitate the acquisition of DNA from suspects and other individuals, as this can be done non-invasively by sampling hair or mouthwash (Higuchi et al. 1988; Lench et al. 1988). This

should make consent much easier, obviate the need for medical supervision and reduce the risk of infection (by, for example, hepatitis B or HIV).

The PCR and associated techniques do not involve recombinant DNA nor do they need to involve radioactive material (Bugawan et al. 1988; Kaiser et al. 1989). There is thus no need for recombinant DNA or radioactive waste disposal and containment facilities. The techniques are much faster, taking a total of less than two days, as compared with at least the best part of two weeks for 'Southern' blotting. They are less labour intensive and considerably cheaper.

The Choice of Genes

There are two reasons why the minisatellites that are used as hybridisation probes in the 'Southern' blot method are not well suited as targets for PCR (Jeffreys et al. 1988). First, they are generally too long. There is a limit of a few kilobases to the length of DNA that can be amplified by PCR and many minisatellites are longer than this. Second, because minisatellites consist of repeated sequences, out of register alignment may occur during PCR amplification making results difficult to interpret.

A better source of variation are the genes of the HLA or Major Histocompatibility gene complex. These genes have three important attributes:

1. The genes are extremely variable. The products of the HLA genes are the histocompatibility antigens responsible for the phenomenon of rejection in organ transplants. The reason that suitable donors are so hard to find for transplants is that we are almost all different from each other with respect to HLA genotypes. This variation which presents such a problem to transplantation biologists and surgeons does, of course, have enormous potential usefulness in forensic genetics. There are at least six highly variable genes in the HLA gene complex which have from 11 to 57 known variants (Bodmer et al. 1987). When all of these variants are considered, bearing in mind that each individual has two sets of the genes, there are at least 240,000,000,000 possible HLA genotype combinations. In fact variants of the different genes tend to occur in characteristic combinations, and thus not all the potential genotype combinations are found.

2. Much is known about the genes and their variants. In addition to their role in transplant rejections, HLA gene variants are associated with many autoimmune diseases such as insulin-dependent diabetes mellitus, multiple sclerosis and rheumatoid arthritis. Largely because they are clinically important in these two ways, the genes have been extensively investigated. Specifically, from the forensic perspective we know two important things: firstly, the sequences of the variable regions of a large proportion of the variants (Bell et al. 1987; Todd et al. 1987; Bugwan et al. 1988; Parham et al. 1988, Pohla et al. 1988); that is, we know the precise molecular nature of the variation. This has important implications in terms of the way in which the variation can be assayed; and secondly, we know the frequencies of many of the variants in different human populations (Albert & Baur 1984) including most of the major ethnic groups occurring in Australia. This is important in terms of determining the probabilities of random misidentifications in forensic comparisons.

3. The genes are widely studied. There is a large body of scientists and clinicians whose work in some way involves the HLA genes. International Histocompatibility Workshops are held every four years in addition to regional workshops and society meetings at which information is shared and, more importantly, nomenclature and procedures for identifying and assaying variations are assessed and standardised. Data on HLA variation obtained in a forensic context can thus be understood and evaluated within an already existing, well-organised scientific infrastructure and against an extensive background of scientific knowledge. Nothing comparable exists for any other region of the human genome.

This has two important implications:

- The scientific infrastructure makes it possible for data obtained using different assay procedures at different times and in different laboratories to be compared and communicated by means of a well-established and standardised, internationally recognised system of nomenclature (Bodmer 1987). It also provides a good source of expert advice and opinion.

- The extensive underpinning of scientific knowledge about the HLA system should make interpretation of data on HLA variation obtained for forensic purposes less subject to ambiguities and more open to critical evaluation.

Assaying the Variation

Knowledge of the sequences of HLA variants allows three options for assaying the variation and makes possible comparisons of data obtained using the different assay procedures.

The three procedures are:

- Dot-blot hybridisations with sequence specific oligonucleotides (Bugwan et al. 1988).

- Digestion at sequence-specific restriction sites (Saiki et al. 1985).

- Direct sequencing (Gyllensten & Erlich 1988).

If the sequence of a particular variant is known, then a short segment of DNA, or oligonucleotide, complementary to the region of the variant that distinguishes it from other variants, can be synthesised and used as a means of testing for the presence of the variant through hybridisation. If the variant is present the oligonucleotide hybridises, otherwise it does not. If the oligonucleotide is labelled either radioactively or non-radioactively and the amplified PCR product is bound to a membrane, the occurrence of hybridisation, and thus of the variant in question, can be identified. This procedure is known as dot blotting. By using a series of oligonucleotides specific to each of the known variants, sample genotypes can be determined.

Another approach is to identify restriction sites that are specific to particular variants and digest the PCR product with restriction enzymes that produce variant specific restriction fragments which can be separated by electrophoresis and visualised by fluorescent staining.

Both these methods are indirect and the results obtained require extrapolation about the underlying sequence. A third, more direct approach is to determine the sequences of the PCR amplified variants. In addition to being more direct this approach has the advantage that it allows previously undescribed sequence variation to be immediately identified.

The three approaches to detecting variations are based on different principles. Results obtained using one method can thus be used to check those obtained using another.

The results obtained from all three methods can be interpreted in the context of the existing system of HLA nomenclature and stored on computer in that form. Thus data obtained by any of the methods can be compared to those obtained by any other.

Concluding Remarks

At present these methods can be applied to the HLA class II genes. In principle they could also be applied to class I genes although there is still much work to be done before that will be possible. The importance of developing methods of detection of class I variation is that they will greatly increase obtainable exclusion probabilities, and thus provide better quality evidence in criminal investigations.

There is a need for research and development in this area as well as in the implementation of a system for detecting class II variation. The potential advantages of the PCR approach are so great that this research should be encouraged. Furthermore, the establishment of laboratories and other facilities, and the consideration of the issues of standardisation and evaluation of data should be made with the expectation that the PCR technology will be firmly established as the main means of detecting genetic variation in forensic investigations within the next few years.

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Tissue Typing

HLA class II typing using AFLPs

Simon Easteal

The advent of the polymerase chain reaction (PCR) has had a major impact on many areas of biological and medical science. One of the first applications of the PCR was the amplification of the genes coding for the HLA class II antigens. The PCR has subsequently been widely used in the characterization and detection of HLA variation. The first, and now well established, PCR based method for detecting HLA variation was dot-blot hybridization with sequence specific oligonucleotides (SSOs). An alternative approach is now being developed that has a number of advantages over the SSO method. It involves analysis of electrophoretically separated restriction fragments, or amplified fragment length polymorphisms (AFLPs), produced by digestion of PCR products at allele specific restriction sites.

HLA class II antigens are cell surface glycoproteins that function in the regulation of the immune response through the presentation of foreign antigenic peptides to T lymphocytes. The HLA class II genes are enormously variable and there is considerable clinical interest in this variation because of its involvement in the rejection of organ transplants and the association of specific HLA class II antigens with a number of autoimmune diseases. HLA variation is also of great interest to population and forensic biologists.

Four class II genes, HLA-DPB1, -DQA1, -DQB1, and -DRB1 contain most of the allelic variation and within these genes the variation is largely confined to the second exons which code for the antigenic peptide-binding and T-cell recognition sites of the class II antigens [1]. The allelic variation appears to have functional significance and there is now substantial evidence that it has been maintained in the population by balancing natural selection.

The ability to specifically amplify the second exons of the variable class II genes has allowed a total of 21 DPB1, 8 DQA1, 13 DQB1, and 43 DR alleles to be sequenced either directly following PCR or after cloning of PCR products. The knowledge of these sequences has made possible the development of the SSO and AFLP methods for HLA class II typing, both of which involve the detection of specific nucleotide differences between alleles.

AFLP methods have now been described for typing DPB1 [2, 3] and DQA1 [4] alleles. A DQB1 typing procedure has also been developed (unpublished data) and DR protocols are currently being tested.

Both the SSO and AFLP methods are more accurate, sensitive, and efficient than conventional serological, cellular and RFLP typing. Of the two, there are a number of factors that make AFLPs the preferred alternative. First, AFLPs are technically simple, involving digestion with a small number of restriction enzymes (five for DPB1 typing and four for DQA1 and DQB1 typing) and no hybridization. Second, SSO typing involves the detection of the presence or absence of hybridization, however the absence of hybridization may also result from experimental failures and this is difficult to control for. With AFLPs, experimental failure can readily be detected because, with a few exceptions, allelic identification involves detection of specific products of endonuclease digestion. Third,

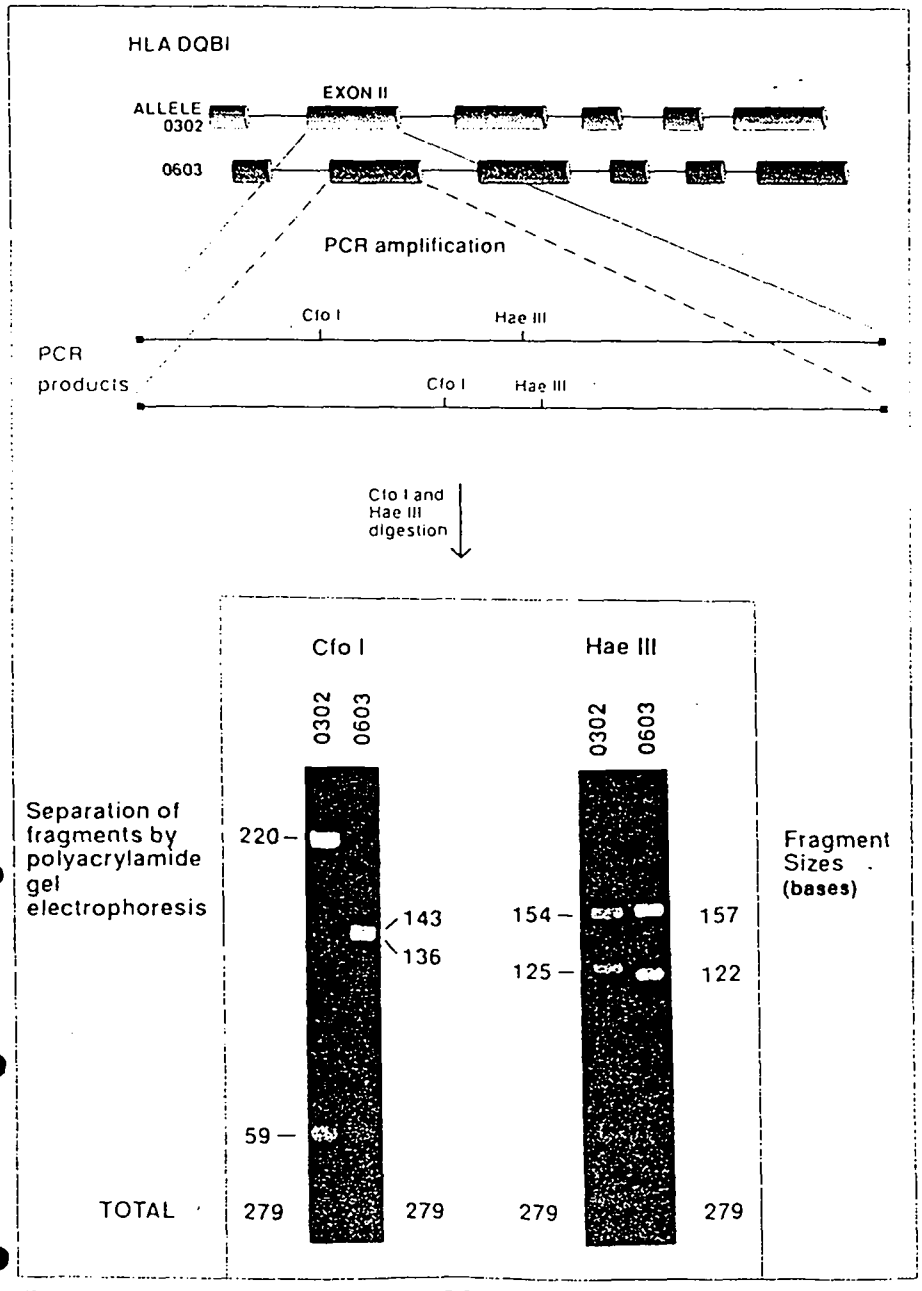

Figure 1. AFLP identification of HLA-DQB1 alleles from two samples homozygous for DQB1*0302 and *0603. Alleles are inferred by reference to the AFLPs predicted from the known allelic sequences.

AFLP detection is by ethidium bromide staining which is much less sensitive than hybridization. This means that AFLP results are less affected by PCR contamination. Furthermore, when contaminating fragments are present they can readily be identified because the expected total sizes of the true PCR product is known.

HLA typing by AFLPs can easily be completed within two days and with further refinement this time could certainly be reduced. This should make it a particularly attractive method of typing when speed is important, for example when transplant organs are obtained from cadaver donors. The technical precision of the AFLP method combined with the ability of the PCR to make DNA available for analysis from minute amounts of degraded material, should also greatly facilitate the exploitation of the extensive genetic variation in the HLA region for forensic identification. This should prove particularly useful in the area of criminal law where large fresh samples are often hard to come by. There have been considerable problems in the implementation of DNA fingerprinting using Southern blotting techniques. These could almost entirely be overcome by the use of PCR and AFLPs to assay HLA variation [5].

Conventional methods of HLA class II typing will continue to be practised, but they can now be complemented, and in some applications replaced, by this simple, quick, and accurate procedure made possible by the development of the PCR. The method is already attracting interest from tissue typing and forensic laboratories.

Simon Easteal is with the Human Genetics Group, John Curtin School of Medical Research, The Australian National University, Canberra.

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