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Research Article

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Molecular Cloning of a Polymorphic DNA Endonuclease Fragment Associates Insulin-dependent Diabetes Mellitus with HLA-DQ

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Abstract

A BamHI 3.7-kilobase (kb) fragment detected by an HLA-DQ β-chain complementary DNA (cDNA) probe and negatively associated with insulin-dependent diabetes mellitus (IDDM) was cloned and sequenced to localize the polymorphism to BamHI sites in intervening sequences of an HLA-DQ β-chain gene. A probe of the first intervening sequence (IVS 1) showed the BamHI 3.7-kb fragment in 6 of 17 HLA-DR3/4 controls but in 0 of 13 DR-identical IDDM patients. All IDDM patients (13 of 13) had BamHI fragments of 12 and 4 kb, detected in 9 of 17 controls (P < 0.02). The simple restriction fragment length polymorphism pattern of the IVS 1 probe was exploited by comparing 113 IDDM patients with 177 healthy controls to show increased prevalences in IDDM of the 12-kb (P < 0.0001) and 4-kb (P < 0.0001) fragments. In IDDM patients younger than 20 yr at onset, 98% were 12- and/or 4-kb positive, compared with 63% of controls (P < 0.0001), giving a relative risk of 91.8 for individuals with both fragments. The 12-kb fragment was linked to HLA-DR4, and the 4-kb fragment to HLA-DR3. Both serologic markers were split and a non-DR3/non-DR4 IDDM patient was 4-kb positive. HLA-DQ seems therefore closer, than HLA-DR, to an IDDM susceptibility gene.

Introduction

Type 1 (insulin-dependent) diabetes mellitus (IDDM)[1] affects primarily children and young adults and is the most common chronic metabolic disorder in childhood (see references 1–3 for reviews). The etiology is still unknown although virus or other environmental factors (1–4) have been implicated. The pathogenesis involves autoimmune phenomena, including the presence of insulitis (5, 6), islet cell autoantibodies (reviewed in references 2 and 3), or other organ-specific autoimmune diseases (2, 7). In contrast to the common view of an acute and dramatic onset, IDDM seems to evolve after immune abnormalities are present but long before the clinical onset (8, 9). The specific evanescence of the pancreatic β cells has yet to be clarified, but it is possible that certain autoantigens, such as an M_r 64,000 protein detected by IDDM sera (10), direct the immune system to the β cells. The disease seems to run in families, but the mode of inheritance has not been clarified. A familial hyperautoreactivity may be a prerequisite for IDDM to develop (11), and the adverse autoimmune reaction against the pancreatic β cells may be controlled by HLA genes in the major histocompatibility complex, inasmuch as > 90% of IDDM patients are HLA-DR3 and/or 4 positive (12, 13).

Although these HLA-DR specificities account for nearly all IDDM patients, their frequencies in the background population amount to nearly 60% (12, 13). The HLA-DR3/4 genotype is associated with the highest risk of developing IDDM. The concordance rate for IDDM among monozygotic twins remains, however, below 50% (14) and the risk of an HLA-DR–identical sibling developing IDDM is only 12%–24% (2, 13). HLA typing is therefore insufficient in predicting a development of IDDM also because recent epidemiologic data indicate that only 13% of new IDDM patients have an affected parent or sibling (15). The question, therefore, remains whether there exist other genes linked to HLA-DR, which would confer a greater risk for IDDM to develop. The present study describes an approach by which to define disease susceptibility genes at the genomic level by the isolation of locus-specific genomic DNA probes derived from a polymorphic DNA fragment.

Recently, we (16, 17) and others (18, 19) described HLA-DQ–region β-chain DNA endonuclease fragments that differ among HLA-DR–identical healthy and IDDM individuals. These observations are important, because they suggest that DNA polymorphism may define better the susceptibility to develop IDDM or other disorders found to be associated with HLA. The restriction fragment length polymorphisms (RFLP) detected by an HLA-DQ β-chain complementary DNA (cDNA) probe are extensive and reveal complex endonuclease fragment patterns (16–24). Because we found that HLA-DR–identical control and IDDM individuals differ with respect to a BamHI 3.7-kb fragment detected with an HLA-DQ β-chain cDNA probe (16, 17), we decided to clone this fragment from an HLA-DR4+ chromosome of a healthy individual (a) to establish its origin, (b) to determine the molecular basis of the RFLP, and (b) to test whether a fragment of the cloned BamHI 3.7-kb fragment could be used to obtain a less complex fragment pattern to increase the precision of RFLP analyses used for the detection of individuals that risk developing IDDM.

Methods

Subjects. Frozen lymphocytes from HLA-DR3/4+ individuals were obtained from 13 patients with IDDM and 17 healthy individuals. The samples were kindly made available for study by Drs. A. Svejgaard and P. Platz, the Tissue Typing Laboratory, Rigshospitalet, Copenhagen and by F. Kissmeyer-Nielsen, Tissue Typing Laboratory, Kommune Hospitalet, Aarhus, Denmark. Blood collected in EDTA was also obtained from 177 healthy blood donors at the Blood Bank, Gentofte County Hospital, Gentofte, Denmark. There were 42 (24%) females and 131 (76%) males. At routine clinical visits at the Steno Memorial Hospital,

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1. *Abbreviations used in this paper:* IDDM, insulin-dependent diabetes mellitus; NIDDM, non–insulin-dependent diabetes mellitus; RFLP, restriction fragment length polymorphism.

Gentofte, Denmark, 10–20 ml of blood, collected in the presence of heparin, was obtained at random from 113 patients, 38 (34%) females, 75 (66%) males, with ages varying between 2 and 66 yr at onset of IDDM (Fig. 1), and also from 46 patients with non–insulin-dependent diabetes mellitus (NIDDM). The age distribution at the time of admittance (Fig. 1) was compatible with that previously reported for Denmark (25). All the blood samples were obtained during routine visits, and were coded and analyzed in a random fashion. The fragments detected in the RFLP analysis were scored and recorded without the observer knowing the origin of the sample.

Cloning of restriction fragment. In our previous family study it was demonstrated that the RFLP observed after hybridization with an HLA-DQ β-chain probe was linked to HLA-DR (17) and that the BamHI 3.7-kb fragment appeared on three of six HLA-DR4–containing chromosomes in healthy individuals, but in 0 of 13 of the diabetic DR4–containing chromosomes (17). We selected one informative Swedish family without history of either IDDM or NIDDM to clone a BamHI 3.7-kb fragment from an HLA-DR4–containing chromosome (26). The individual donating blood for cloning was a healthy HLA-DR2/4, 3.7-kb-fragment-positive mother with two daughters. Her husband is HLA-DR3/7 and their two daughters are HLA-DR2/3 and 4/7, respectively. The HLA-DR4/7 daughter also has the BamHI 3.7-kb fragment, which is therefore present on the HLA-DR4–containing chromosome of the mother. Mononuclear cells were obtained from 30 ml of blood by Ficoll-Hypaque gradient centrifugation and the DNA was extracted as described below. ~ 50 μg of DNA was digested with BamHI and electrophoresed in a 1% agarose gel along with appropriate molecular weight markers. The 3.4–3.8-kb region was sliced from the gel and the DNA fragments recovered by electroelution. This fraction of fragments was ligated into the BamHI site of pUC8 and used to transform *Escherichia coli* JM10528. Recombinant plasmids were recognized as those giving rise to white colonies on L-broth plates with 20 μg/ml each of isopropyl thiogalactoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and were screened for HLA-DQ–related sequences by in situ hybridization on nitrocellulose filters with the nick-translated DQβ cDNA probe (26).

DNA preparation. Mononuclear cells, obtained from 10 ml of blood by Ficoll-Hypaque gradient centrifugation, were digested overnight at 37°C in 0.02% proteinase K and 1% (wt/vol) SDS in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA. After phenol and chloroform extractions, the DNA was precipitated with ethanol and resuspended in Tris-EDTA buffer.

Genomic blots. Lymphocyte DNA (10–20 μg) was digested with restriction enzymes according to the suppliers (Boehringer, Mannheim, Federal Republic of Germany) specifications, electrophoresed overnight at 40 V in 1% flatbed agarose gels, and then transferred to HybondN nylon membranes (Amersham International, Buckinghamshire, United Kingdom) using the methods described by the supplier.
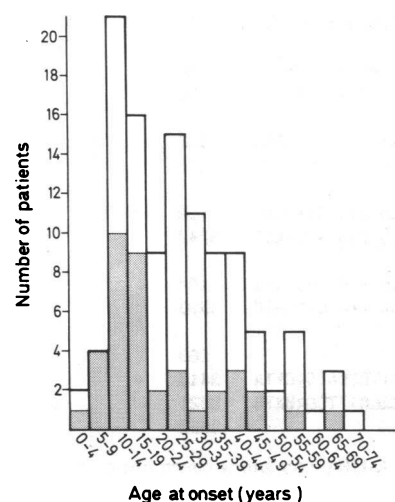

Figure 1. Age variation among the 113 IDDM patients studied for RFLP polymorphism with the HLA-DQ β-chain IVS 1 gene probe. The dotted bars represent the 39 (35%) patients who were positive for the 12- and 4-kb BamHI restriction fragments detected with the IVS 1 gene probe.

Age at onset (years)
Number of patients

Hybridizations. Filters were prehybridized for 2–16 h in 50% formamide, 5 × standard saline citrate (SSC), 5 × Denharts solution, 50 mM Na₂PO₄ (pH 6.5), and 0.5 mg/ml denatured salmon sperm DNA. Hybridization was performed overnight in 50% formamide, 1 × Denharts solution, 20 mM Na₂PO₄ (pH 6.5), 10% dextran sulphate, 0.2 mg/ml denatured salmon sperm DNA, and 10⁶ dpm/ml denatured probe. Washing stringency was 0.1 × SSC at 55°C.

Probes. The DQβ cDNA probe was derived from the plasmid pII-β1 (27) by digestion with PstI and EcoR1, after which the 800-base pair (bp) fragment was eluted from agarose melting at low temperature (BioRad Laboratories, Richmond, CA).

A subclone of the BamHI 3.7-kb fragment cloned in pBR322 was used to construct the first intervening sequence (IVS 1) probe. The recombinant plasmid was digested with the endonuclease RSA1 to yield a fragment, which contains 154 bp of intervening sequence and 12 bp of the second exon which codes for the first domain of the HLA-DQ β-chain, as well as the portion of pBR322 that ranges from the BamHI site in position 375 to the RSA1 site in position 2281. This 2.072-bp fragment was purified after agarose gel electrophoresis in a low-temperature melting-point agarose. Labeling of probes was by nick-translation, using α-[³²P]deoxy CTP (Amersham International) at 30 TBq/mmol (DQβ probe) or 110 TBq/mmol (IVS 1 probe).

Construction of a series of progressive deletions for sequencing. The BamHI 3.7-kb genomic insert was subcloned in the BamHI site of pUC19 (28). This construct was then linearized with the restriction enzymes Sph1 and HindII. Sph1 digestion between the BamHI site in pUC19 and the attachment site for the reverse-sequencing primer (Amersham International) was carried out to leave a four-base 3′ overhang, which is inaccessible to Exonuclease III (29) (Pharmacia PL Biochemicals, Uppsala, Sweden). HindII digestion within the polylinker sequence of pUC19, between the Sph1 site and the BamHI cloning site, was used to generate a blunt end, susceptible to Exonuclease III. There were no sites for Sph1 and HindII in the insert. Unidirectional deletions, ranging from the primer attachment site in pUC19 to various points within the insert, were isolated by digestion of the plasmid DNA with Sph1 and HindII, followed by phenol/chloroform extraction and ethanol precipitation. The linearized plasmid DNA was resuspended to 0.1 g/liter in 6.6 mM Tris-HCl (pH 7.4) containing 6.6 mM MgCl₂ and ncubated at 37°C for 5 min before Exonuclease III was added to a final concentration of 10 U/μl. In 15-s intervals, 35 10-μl aliquots were transferred to 30 μl of 0.2 M NaCl containing 5 mM EDTA and heated to 70°C for 10 min to inactivate the enzyme. The DNA was precipitated by the addition of 120 μl of ethanol and centrifuged and each pellet was resuspended in 80 μl of 0.05 M NaOAc (pH 4.5) containing 0.1 M NaCl, 30 mM ZnSO₄, and 75 Vogt U/ml S1 nuclease (Boehringer) to remove by incubation for 30 min at room temperature the single-stranded 3′- and 5′-end protutions generated by Sph1 and Exonuclease III, respectively. The reaction was terminated by phenol/chloroform extraction followed by ethanol precipitation. The pellets were resuspended in 10 μl 25 mM Tris-HCl (pH 7.4) containing 5 mM MgCl₂, 5 mM dithiothreitol (DTT), 0.25 mM spermidine, 1 mM ATP, 10 μg/ml BSA, and 700 U/ml T4 DNA ligase (Amersham), and ligated at room temperature overnight. *E. coli* KM109 was transformed with 5 μl of each of the ligated fractions according to Hanahan (30) and the bacterial cells spread on L-broth plates containing 50 μg/ml ampicillin. About 10–20 transformants from each aliquot were selected for characterization of deletion size by agarose gel electrophoresis. Each clone was grown overnight in a 5-ml L-broth culture in the presence of 50 μg/ml ampicillin. Small-scale plasmid preparations were made by the alkaline lysis method (31), with the following modifications. After precipitation with potassium acetate, supernatants were centrifuged for 2 min, transferred to fresh tubes containing 0.6 vol of isopropanol, and centrifuged for 5 min at 4°C. The pellets were washed with 80% ethanol and resuspended in Tris-EDTA buffer. An equal volume of 4 M LiCl was added. After 5 min at 0°C, the precipitates were collected by centrifugation for 2 min. The supernatants were incubated for 30 min at 37°C with 25 μg/ml RNase A, followed by phenol/chloroform extraction. The plasmid DNA was then precipitated by incubating the samples for 10 min at 0°C in 2.5 vol of ethanol. After centrifugation, pellets were

washed once each in 80% and 99% ethanol, air dried, and resuspended in Tris-EDTA buffer. The deletion breakpoints were estimated by agarose gel electrophoresis of appropriate restriction digest of the plasmid DNA. This analysis made it possible to decide which clones should be further characterized by sequencing.

Sequencing. Selected clones from the preparations described above were sequenced by the chain termination method, using double-stranded, supercoiled plasmid DNA (32). Most clones from each aliquot had deletion breakpoints positioned within a range of 50–100 bp. The distance between fractions was roughly 100–200 bp.

Statistical evaluation. The difference in frequency between control and test samples was estimated by the Fisher exact test or the χ^2 test with Yate's correction. The level of significance was accepted to be P

< 0.05 after the P value was corrected for the number of variable fragments observed between individuals. The relative risk (RR) was calculated from the formula: RR = (positive patients $\times$ negative controls)/(negative patients $\times$ positive controls), and the absolute relative risk (ARR) from ARR = (positive patients $\times$ total controls)/(positive controls $\times$ total patients) $\times$ prevalence of IDDM. The prevalence of IDDM in Denmark was 0.38% (25).

Results

Sequence of the polymorphic BamHI 3.7-kb fragment. The entire nucleotide sequence of the cloned 3.7-kb fragment from an HLA-

GGATCCCAGGTCTGCAGCGCGAGGCACGGGCCGGCGGGAACTGGAGGTCGCGCGGGCGGTTCCACAGCTCCGGGCCGGGTCAGGGCGGCGGCTGCGGGGT   100

								Asp Phe Val Tyr Gln Phe Lys Ala Met Cys Tyr		16

GGCCGGGCTGGGGCCGGGCCGGGGCCGGACTGACCGGCCGGTGATTCCCCGCAG AG GAT TTC GTG TAC CAG TTT AAG GCC ATG TGC TAC   189

Phe Thr Asn Gly Thr Glu Arg Val Arg Tyr Val Thr Arg Tyr Ile Tyr Asn Arg Glu Glu Tyr Ala Arg Phe Asp Ser   42
TTC ACC AAC GGG ACG GAG CGC GTG CGT TAT GTG ACC AGA TAC ATC TAT AAC CGA GAG GAG TAC GCA CGC TTC GAC AGC   267

Asp Val Glu Val Tyr Arg Ala Val Thr Pro Leu Gly Pro Pro Asp Ala Glu Tyr Trp Asn Ser Gln Lys Glu Val Leu   68
GAC GTG GAG GTG TAC CGG GCG GTG ACG CCG CTG GGG CCG CCT GAC GCC GAG TAC TGG AAC AGC CAG AAG GAA GTC CTG   345

Glu Arg Thr Arg Ala Glu Leu Asp Thr Val Cys Arg His Asn Tyr Gln Leu Glu Leu Arg Thr Thr Leu Gln Arg Arg   94
GAG AGG ACC CGG GCG GAG TTG GAC ACG GTG TGC AGA CAC AAC TAC CAG TTG GAG CTC CGC ACG ACC TTG CAG AGG CGA   423
V
G GTGAGCGTCGTCGCCCCGCTGCGAGGCCCACTCTTGGCCGGGGCCCGAGTCTCTGCGCTAGGAGGGACGAGGGGGGCGAAGCCTCTGGAACCTGAGCCGCG   525
TTCGTTCCACCCCAGGGGGACAGGAGTTGGCGGCGTGGGTGGTGGGGGCAGGGACGGGGGGCAGGCAGAGCAGGGGTXACAGGTAGAGTTG   628
GTCAAACTGCCTAGTTTTGCCCCAGCCTTCCCGTCCGTCAGCCTCGCCCTCTGCTGTGCACGTTCTCGCCTCGTGCCTTATGCGTTTGCCTCCTCGTGCCTTG   731
CCTTTGCTAAGCAGTCCTCTCTGCCCCCAATTTCTGCCCTCTTCCCCTGCCCGCCCGCCCCGCTAGCACTGCCCCACCCAGCAAGGTCCACGTGCACTCGTCG   834
CGCCGCAGGAAGCTTCAGCTTGGCCTGGTGGAGTTAGGGCTGCTCCACAACTGTGCGCAGGGCATCCAGCAATTACAGTTGTGAAATAAGATATTTTGACTTT   937
TGGCTTCAAATTATTATTCATCGTAATTCCGTTTTCTTAAATGGCTCTCATTCATGGCGGAGCTCTTTGAGGTGAGAGTTTTTTAATCATTTTATGGCCGGTA   1040
CCTGACACATTGACTGGCATGTGGTATAAGCTCAATAATCTTCTGTTAAATTAATGAATAAATGTGCTCAGCTGCCAATCCACTTAGGCTCAAGAAAAANCAG   1143
AGGTAAACAGAACCTTAAAAATGGACTTTTATTAATTATTTTCTATCATTTTGCTTAATTCTTTAAAGTAAACTCTTATTGACTTGGATCTAAATAGTTTGTG   1246
AATACAAAGTCTGAGGAAAAAAATGTTTGCTAAAAATAAAAACAACACTTGAATGATGTTTGTAAGGCAGTTTTAATTTCTTAGAAAAAGCTGAACAAATGGCA   1349
CAATGCAAAGAGCAGAAGTTTTGGAATAAAATAGATTGAAGCCATTAAATTATTGGATAAAAATAGTTTCAGGTTGCTTTTGGCCTAGGTTCTCCCCTCCCCCC   1452
ATCACTATCCACTTCAGGAATAAACATTCTGAAAGTTAATTTTACCCATATAGTGAGCACTTATTTCTAAGTTGCCTTATCAAATACCATCTATGTTATGTCA   1555
TTTAATCTCGCAGTTGCCTGTGCATTAGAGATTAGCATCACCACTTTATATATCCTAATATTAGTACATGATAAACACTTTAAGTAATCAGCCCACAAGTACT   1658
CACCCAAGACCTTAAGCCTCCCAAAGTACACAACATTCTTACGTTCCTCACTACACATCTGTAGAGNNNAAGGGACATAAAGCCTTGTTAAAGCCAGTTTTGAC   1761
CAGAAGCAGCAATGGTCTCTTCCTGTTTGATCTCCATGTTAATGGGACAAAATGATACTTTCAAGGCATTGAAAATTTATGATTAATCAATCAAATTTATGAT   1864
TAATCAATCCCTAGTCTGACTCCAGTGAGATTCACAAAACTTTCAGTTTACTTTATACTCCCTTGCCTTCTTTTGACTCACATCATAGTGCCGGCAAGTACTT   1967
ATATTTTTGCTTATTTCAGTTCTATTTCCATAAAATTTATTTTATCATCTTTTCTCATAAATTTGTGCCCTCTATTTTTACTCCCAATCTGTGTAAGATGAAC   2070
AAATCTTATAAGACCACATAGCTGACTGTGATTTCAGGTGGACTCCAGGAAGGAGAACCAAAGAAAACTTTCAGTCTTTTTAAGATGAACAAATCTTGTAAGT   2173
CCCCACATAGCTGACTGTGATTTCAGGTCGACTCCAGGAAGGAGAACCAAAGAAAAGTTCAAGTCCAAGCAGAAACCGTGATTCTTCCGGATGATGGCTCATG   2276
AGTGCCATTTAATTGGGGTGCCACCTGCTGTCCTCAGCAAATCCCAGCTATATGTATATGTTCGCATTACAGGCTCATTAACCTAGGCTGACCTCTGCAAGGA   2379
TCTCAGAATATTTTCTACAGAGAACATACATGATAATATCTGATTTTAGGACAAAAAAGTAATTCTCAATAGCAAGGGAATGGAGTAGGGTAGACAGCTAGTA   2482
ATTAAACTCACTTGTGTGTTAAAAATAAATTAAGGAAAAAAGAAAATGAGAGAACATATTACTAAATAAAGAAAGCATACATTAAATATTTACTATAGTTTTA   2585
CACTAAGAGAATAAAGGAAATGCAATAAAGTGGCCTGAAAGGTAAAGGATGAGATGTGTAAAAGAGGCGGGGAAAGATGTGTCATTTTTTTTTTACTATGAGC   2688
AGCAATCTGAGAAGATAAAGGAATCGAGTTATGGGCAGACATGATGTTTGATCAGTGTTATTTGTTTTCAAGGCCTGCCTACTTTTTTTTTCAAATATTACAAA   2791
CTTTTGAAGTCACATTCTTTTTGTTTTTTGCTGTCTGTTACTAGATCGCACATTCTGTAAAGGCAGGGACCATGGTATGTTGTTTATCTTTGGATTCTCAGTG   2894
ATTGTCATATTTATGTTTGTTGAATGAATCTTAATCCAAGACTTGGGCTCCAGGTATCTTTCCATTCTGGTTCCAAGGAGGGNCCTTCCTACAGCAGGCCTGC   2997

					al Glu Pro Thr Val Thr Ile Ser Pro Ser Arg Thr		106

TGTGTGGTCTCACATCTCACTCCTATATCTTTCCCTGTCTGTTACTGCCCTCAG TG GAG CCC ACA GTG ACC ATC TCC CCA TCC AGG ACA   3086

Glu Ala Leu Asn His His Asn Leu Leu Val Cys Ser Val Thr Asp Phe Tyr Pro Ala Gln Ile Lys Val Arg Trp Phe   132
GAG GCC CTC AAC CAC CAC AAC CTG CTG GTC TGC TCA GTG ACA GAT TTC TAT CCA GCC CAG ATC AAA GTC CGG TGG TTT   3164

Arg Asn Asp Gln Glu Glu Thr Thr Gly Val Val Ser Thr Pro Leu Ile Arg Asn Gly Asp Trp Thr Phe Gln Ile Leu   158
CGG AAT GAC CAG GAG GAG ACA ACC GGC GTT GTG TCC ACC CCC CTT ATT AGG AAC GGT GAC TGG ACC TTC CAG ATC CTG   3242

Val Met Leu Glu Met Ser Pro Gln His Gly Asp Val Tyr Thr Cys His Val Glu His Pro Ser Leu Gln Asn Pro Ile   184
GTG ATG CTG GAA ATG TCT CCC CAG CAT GGA GAC GTC TAC ACC TGC CAC GTG GAG CAC CCC AGC CTC CAG AAC CCC ATC   3320

Thr Val Glu Trp   188
ACC GTG GAG TGG C GTAAGGGGATATTGAGTTTCTGTTACTATGGGCCCCACAAGACAAAGGGCAGAGCTCATTCTGACCCATCCCTTCCCATCTCTTA   3418
TCCCTGATGTCACTACTGAGCTGGGTTTCACAGGAGACTAGAGCACTTCTTCCTCCATGGCAAGTGCATCAGAAGAATCCTGATCTCATCACCTTTCCAGATG   3521
CTAGGGAAATTACTCTACATACTGTTTCTCTGGATCC   3558

Figure 2. The nucleotide sequence of the cloned BamHI 3.7-kb DNA endonuclease fragment from an HLA-DR4–containing chromosome of a healthy individual.

Table I. Sequence Comparison between DQ β3.7 and Published HLA-DQ β-Chain Sequences

Sequence of DQ β 3.7	Comparison	Nucleotides	Amino acids
IVS 1	cos II-102	94	—
	DC3β	91	—
β₁ exon	cos II-102	98	96
	DC3β	91	80
	pII-β-1	90	80
	pII-β-2	88	78
	DC1β	—	85
	KT3c21	91	85
β₂ exon	cos II-102	98	97
	DC3β	98	96
	pII-β-1	96	96
	pII-β-2	97	94
IVS 3	cos II-102	96	—
	DC3β	96	—

Comparison of nucleotide and amino acid sequences of the Bam 3.7-kb fragment (DR4 haplotype) with the IVS 1, coding regions for the first domain (β₁ exon), second domain (β₂ exon), and the IVS 3 of cosII-102 (DW4/DR4) (33), DC-3β (DR3, 3) (34), pII-β1 (27), pII-β-2 (35) (DR3, 6), DC1β (DR2, 2) (36), and KT3c21 (DR 4, 4) (37).

DR4-containing chromosome showed the fragment, to which we refer as DQβ 3.7, to be 3,558 bp in length (Fig. 2). Comparing this sequence (Table I) to previously published HLA-DR and DQ β-chain genes (27, 33–37) allowed us to identify two coding regions that were nearly identical to the cosmid clone cos II-102 HLA-DQ β-chain gene (33). The coding regions of the first and second domains showed 88%–98% homology to other HLA-DQ β-chain genes. The 154 bp of the first intervening sequence (IVS 1) and the 225 bp from the third (IVS 3) also compare favorably (91%–96%) to the cos II-102 (33) and DC3β (34) HLA-DQ β-chain genes (Table I). All four splice junctions conformed with the GT/AG rule described in Breatnach and Chambon (38).

The predicted amino acid sequence (Figs. 2 and 3) in the second exon coding for part of the NH₂-terminal end and the β1 domain of the protein showed only four amino acids in positions 13, 26, 45, and 57 (Fig. 3) to differ from the cos II-102 (33), whereas there were 11 amino acid substitutions compared with the other HLA-DR–associated DQ β-chain sequence deduced from the KT3c21 cDNA clone (37). It is noted that the amino acid substitutions in different HLA-DQ β-chains primarily occur in positions 25–60 and 65–80 (Fig. 3). In contrast, only two amino acid substitutions between DQβ 3.7 and cos II-102 were found in the second domain (Fig. 3). It was concluded, therefore, that the BamHI 3.7-kb fragment represents an HLA-DQ β-chain gene, and that the two BamHI sites defining the fragment were located in the first and third intervening sequences, respectively (Fig. 4).

The IVS 1 probe detects differences between HLA-DR3/4 healthy and IDDM individuals. A probe was constructed from the cloned DQ_β 3.7 fragment (Fig. 4). This short (166-bp) IVS 1 probe was expected to hybridize only to closely related DQ/DX-related β-chain genes. Furthermore, because the IVS 1 probe primarily represents noncoding sequences, which may be less conserved than their coding counterparts, we assumed little cross-hybridization to alleles of different genes and loci. We first tested 30 HLA-DR3/4⁺ Danish individuals (Table II), 13 of them were IDDM patients and 17 were healthy controls, to find a simple hybridization pattern (Fig. 5) of only five fragments being 12, 10, 4, and 3.7 and 3.2 kb (Table II). The 10-kb fragment was present among all individuals and all (13 of 13) IDDM patients had the 12-kb fragment compared with 10 of 17 (59%) of controls ($P < 0.02$). In fact, all IDDM patients were 12- and 4-kb positive although four different restriction enzyme patterns were found among the controls (Table II).

Association between IDDM and the IVS 1 probe restriction fragments in a population study. The simplified restriction pattern and the likelihood that we were probing a limited number of DQ/DX-related β-chain genes made it possible to test whether the IVS 1 probe would resolve differences between randomly selected IDDM patients and healthy individuals. Such a study would allow an analysis of the strength of a possible association between IDDM and the HLA-DQ locus. It was also important to establish whether the IVS 1 probe would reveal a simple hybridization pattern in the background population to permit studies on the relative risk of developing diabetes without previous knowledge of HLA types. Therefore, we compared 177 healthy, adult blood donors (controls) with 113 IDDM patients.

Figure 3. Comparison between the deduced amino acid sequence of the DQ 3.7 fragment with published HLA-DQ β-chain sequences. See Table I for reference to previously published sequences.
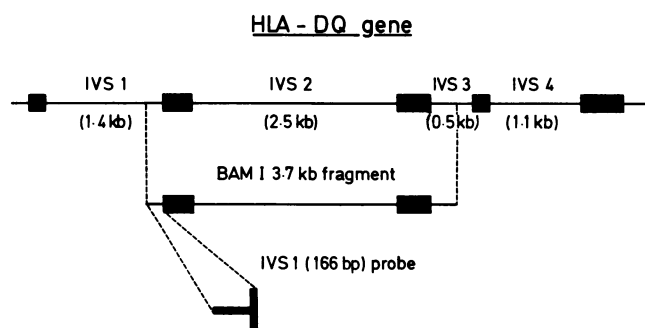
HLA - DQ gene

Figure 4. An HLA-DQ β-chain gene and the localization of the BamHI 3.7-kb fragment detected by DNA–DNA hybridization using an HLA-DQ β-chain cDNA probe. The genomic probe, IVS 1 probe, representing 154 bp of the first intervening sequence and 12 bp of the β$_1$-domain coding sequence, is indicated.

Table II. RFLP after BamHI Digestion

	Prevalence among		
Restriction fragment size	IDDM ($n = 13$)	Controls ($n = 17$)	P value
kb	*n (%)*	*n (%)*	
12–4	13 (100)	9 (53)	0.02
12–4–3.2	0	1 (6)	NS
4–3.7	0	6 (35)	NS
4	0	1 (6)	NS

Detection of RFLP was done with the HLA-DQ β-chain gene IVS 1 probe in HLA-DR3/4–identical IDDM and control individuals. P values corrected for number of variable fragment patterns.

The presence of distinct hybridization signals were seen in all individuals (Fig. 5) to yield a single nonpolymorphic fragment of 10 kb and only six variable fragments. Among the 113 IDDM patients and including the 10-kb fragment, 25 (21%), 76 (65%) ($P < 0.001$), and 16 (14%) ($P < 0.001$) had two, three, or four restriction fragments, respectively, compared with 25 (14%), 79 (45%), and 71 (40%) among the controls. Only two controls had as many as five and six fragments, respectively. Analysis of the frequency of individual fragments among IDDM patients and controls revealed additional differences (Table III). Correcting the P values for the number of variable fragments observed, it was demonstrated that the 12-kb ($P < 10^{-4}$) and 4-kb ($P < 10^{-4}$) fragments were increased among the IDDM patients whereas the 7.5-kb ($P < 10^{-4}$) and 3.7 kb ($P < 10^{-3}$) fragments were decreased. As many as 108 of 113 (92%) of the IDDM patients had the 12- and/or the 4-kb fragment compared with 112 of 177 (63%) of the controls ($P < 10^{-4}$) (Table IV). The simultaneous

presence of the 12- and 4-kb fragments was found among 40 of 113 (34%) IDDM compared with 17 of 177 (10%) control individuals ($P < 10^{-4}$). There were no differences between sex in the frequency distribution of the different fragments.

We analyzed the frequency of the different fragments in relation to age at onset (Fig. 1, Table IV). Among the 43 IDDM patients with age at onset below 20 yr, 42 (98%) were 12- and/or 4-kb positive compared with 112 of 177 (63%) of the controls ($P < 10^{-4}$). The 12–4-kb-positive individuals amounted to 24 (56%), found only among 17 of 177 (10%) controls ($P < 10^{-4}$). The relative risk for a young 12–4-kb-positive individual to develop IDDM amounts, therefore, to 91.8 compared with the control individuals who are negative for both of these fragments. The absolute relative risk was 2.2%.

The presence or absence of the 12-kb and/or 4-kb fragments were also analyzed in a group of 46 patients with NIDDM (Table IV). Neither individual fragments nor the fragment combinations
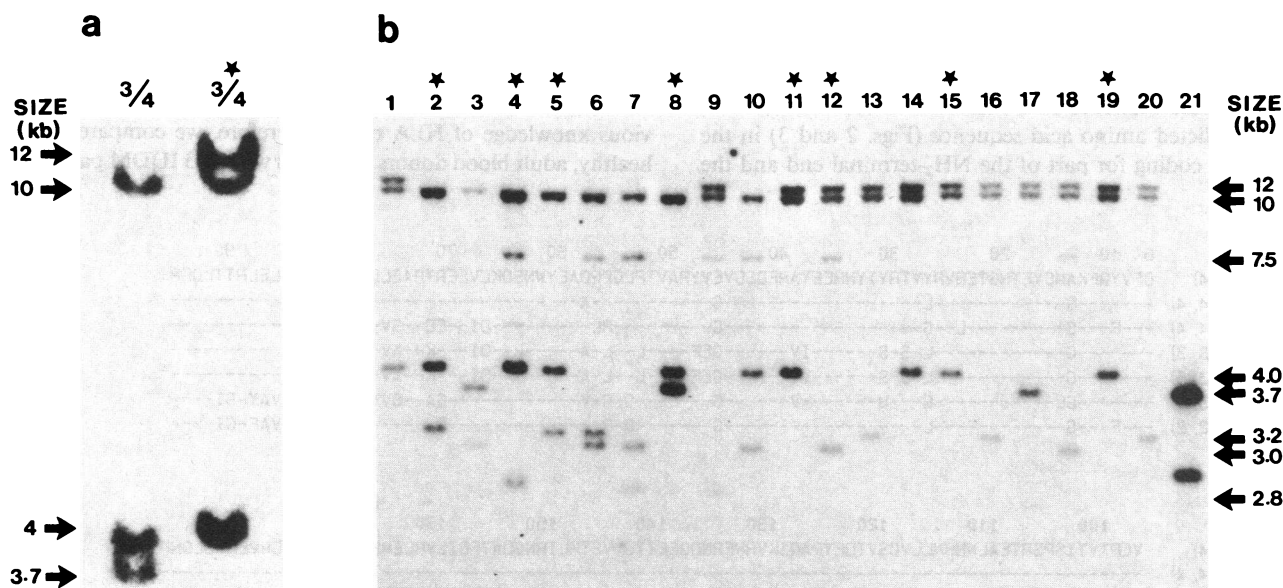

Figure 5. (a) RFLP in HLA-DR3/4 healthy and IDDM (*star*) individuals detected with the HLA-DQ β-chain IVS 1 probe. (b) RFLP detected with the HLA-DQ β-chain IVS-1 gene probe after BamHI digestion of blood mononuclear cell DNA from 19 randomly selected blood donors and IDDM patients (*star*). Lane *1* is DNA from a lymphoblastoid cell line of an HLA-DR3/4 IDDM patient and lane *21* is the plasmid DNA containing the cloned BamHI 3.7-kb fragment.

Table III. Frequency of Restriction Fragments after BamHI Digestion and Hybridization with the HLA-DQ IVS 1 Probe in IDDM, NIDDM, and Control Individuals

Fragment size	Controls ($n = 177$)	IDDM ($n = 113$)	P value	RR	ARR	NIDDM ($n = 46$)	P value vs. controls	P value vs. IDDM
kb	n (%)	n (%)			%	n (%)		
12	65 (37)	73 (65)	10^{-4}	3.1	0.7	19 (42)	NS	0.03
10	117 (100)	112 (99)	NS		0.4	46 (100)	NS	NS
7.5	89 (50)	21 (19)	10^{-4}	0.2	0.1	23 (50)	NS	10^{-3}
4	64 (36)	71 (63)	10^{-4}	3.0	0.7	17 (37)	NS	0.01
3.6	52 (29)	13 (12)	10^{-3}	0.3	0.2	7 (15)	NS	NS
3.2	52 (29)	20 (18)	NS	0.5	0.2	17 (37)	NS	0.04
3.0	65 (37)	7 (7)	10^{-3}		0.1	13 (28)	NS	0.002
2.8	19 (11)	11 (10)	NS	0.9	0.4	13 (28)	NS	0.02

All P values are corrected for the number of variable fragments. ARR, absolute relative risk; RR, relative risk.

of 12 and 4 kb differed in frequency between the NIDDM and the control individuals. We noted the absence of both the 12- and 4-kb fragments was 7 of 70 (10%) among the IDDM patients with age at onset older than 20 yr compared with 17 of 46 (37%) of NIDDM patients ($P < 10^{-4}$).

In our current family study (in preparation), we found a family with an IDDM proband who was HLA-DR3/4 and 12 kb positive. HLA-DR typing alone was not informative because the mother was DR3/4 and IVS-1 12-, 7.5-, and 3.0-kb positive and the father, DR3, IVS 1 4-kb positive. However, the analysis of their five children demonstrated that the father's DR3 was associated with a 4-kb fragment on both chromosomes while the mother's DR3-containing chromosome had the IVS 1 fragments 7.5–3.0 kb. The analysis in Table III shows that these two fragments were decreased in frequency among the IDDM patients. The IVS 1 probe therefore appears to resolve the HLA-DR3 haplotype into subtypes of greater or lower propensity to develop IDDM. Further, in our family analysis, we have also identified one IDDM patient who is HLA-DR -/7 but was found

to have the BamHI IVS 1 probe 4-kb fragment associated with his HLA-DR blank allele (data not shown).

Discussion

The present study, which to our knowledge is the first report of a successful cloning of a disease-associated RFLP fragment detected by an HLA-D–region cDNA probe, allows two major conclusions: (a) by sequencing the cloned polymorphic BamHI 3.7-kb fragment (16, 17), we establish that this polymorphism is due to a structural difference in an HLA-DQ β-chain gene; (b) by using the IVS 1 probe, we establish that BamHI 3.7-kb fragment-negative IDDM patients are BamHI 12-kb fragment positive. The less complex restriction pattern of the HLA-DQ β-chain IVS 1 probe (Fig. 5) then allowed us, in our family study (in preparation), to show that not only HLA-DR4–but also HLA-DR3–containing chromosomes may differ between controls and IDDM patients. Finally, in one HLA-DR -/7 IDDM individual, the IVS 1 probe detected a 4-kb fragment. Therefore, these data

Table IV. Frequency of the 12 and 4-kb BamHI Restriction Fragments Detected after Hybridization with the HLA-DQ IVS 1 Probe in Patients with IDDM Dependent on Age at Onset, NIDDM, and in Control Individuals

	n	Fragment size (kb)				
		12	4	12–4	12 and/or 4	Neither 12 nor 4
Controls	177	65 (37%)	64 (36%)	17 (10%)	112 (63%)	65 (37%)
IDDM, onset below 20 yr of age	43	32 (74%)	34 (79%)	24 (56%)	42 (98%)	1 (2%)
P value		10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
RR		5.0	6.7	11.9	24.4	0.04
RR*		—	—	91.8	—	—
ARR		0.8%	0.8%	2.2%	0.6%	0.02%
IDDM, onset above 20 yr of age	70	41 (59%)	37 (53%)	15 (21%)	63 (90%)	7 (10%)
P value		0.002	0.02	0.01	10^{-4}	10^{-4}
RR		2.4	1.4	2.6	5.2	0.19
RR*		—	—	8.2	—	—
ARR		0.6%	0.6%	0.8%	0.5%	0.3%
NIDDM	46	19 (42%)	17 (37%)	6 (13%)	29 (63%)	17 (37%)
P value		NS	NS	NS	NS	NS

ARR, absolute relative risk; RR, relative risk. * RR is relative risk compared with controls with neither the 12- nor the 4-kb fragments.

strongly suggest that an IDDM susceptibility gene would seem closer to HLA-DQ than HLA-DR. Comparing the RFLP patterns obtained with cDNA probes for HLA-DR and HLA-DQ β-chains, Böhme et al. (24) have recently reached the same conclusion. Because the HLA-DQ RFLP patterns do not conform with currently available serologic reagents (21, 23, 24), our approach of using the IVS 1 probe to analyze IDDM and control individuals, without previous knowledge of their HLA types, is an acceptable approach. It also means that the relative risk estimates will not be comparable to those obtained by serologic typing, in that all individuals will show a RFLP fragment pattern whereas ∼ 20% of the background population are assigned as blanks, owing to the lack of suitable HLA-DR typing sera. Several recent investigations, using methods for serology, primed lymphocyte typing, biochemical analysis, and restriction fragment length polymorphism, have described several subtypes of HLA-DR4 (21, 23, 24, 39–41). Combining all four techniques to analyze DR4⁺ homozygous cells, the presence of at least nine different haplotypes of HLA-DR4 (42, 43) was revealed. The difference in HLA-DR4 haplotypes may explain the controversy (12, 13) over whether IDDM is associated with the DR rather than with the DW locus.

The IVS 1 probe seems to be specific for the HLA-DQ/DX locus because we found only one to three variable fragments per individual. The nonpolymorphic 10-kb fragment presumably represents the HLA-DX β-chain gene. We note that the 7.5-, 3.0-, and the 2.8-kb fragments never occur alone, and that the 3.0-kb always appears together with the 7.5-kb fragment. Some of these fragments may therefore occur in the same haplotype.

The high relative risk value of 91.8 for the simultaneous presence of the 12- and 4-kb fragments (Table IV) may be due to the ability of the IVS 1 probe to differentiate between subtypes of both HLA-DR4 and DR3. Because the 166-bp IVS sequence was most closely related to previously described DQ β-chain gene sequences (27, 33–37), we argue that, the DQ β-chain gene either represents a diabetes-susceptibility gene or is closer to a putative susceptibility gene than the HLA-DR locus. This would be particularly true for individuals developing IDDM below the age of 20 yr (Table IV). This conclusion is supported by the recent observation that the HLA-DQ-specific monoclonal antibody, TA 10 (42), differentiates between HLA-DR4 identical IDDM and control individuals (Tait, personal communication).

We compared the intervening sequences of our BamHI 3.7-kb fragment with the two published DQ intervening sequences which are associated with HLA-DR4 (cos II-102) (33) and HLA-DR3 (DC3β) (34), respectively. Because the DC3β was cloned from a DR3/3 homozygous cell line, we would predict the presence of a BamHI fragment of ∼ 4 kb rather than 3.6 kb. However, both BamHI restriction sites in the IVS 1 and IVS 3 (Fig. 2) were preserved in the DC3β sequence (34). Therefore, when analyzing the IVS 2 sequence of cos II-102 with that of DC3β we found that the latter was 300 bp larger, because of the insertion of an Alu sequence which was not reported by the authors (34). It is important to note that the DQ 3.7-kb fragment had no sequences corresponding to the Alu sequence of the DC3β. Furthermore, plasmid DNA, containing the DQ 3.7 fragment, did not hybridize with the Alu-sequence probe BLUR 2 (44) (data not shown). Therefore, RFLP associated to the development of IDDM, or other diseases associated to HLA, may be linked not only to the absence or presence of certain restriction enzyme sites but to insertion of sequences as well.

The two BamHI restriction sites in the IVS 1 and IVS 3 of the DQ 3.7-kb fragment (Fig. 1) are missing in the cos II-102 (33). The restriction enzyme map of this cosmid clone suggests that the IVS 1 probe sequence is contained within a BamHI fragment that is larger than 6 kb. Because cos II-102 is from an HLA-DR4⁺ individual, its DQ β-chain gene may contain a BamHI 12-kb fragment. The BamHI 3.7-kb polymorphism would therefore be caused by either point mutations or the presence of unknown inserted sequences within noncoding regions. However, even though these events have occurred in noncoding sequences, it is notable that all three alleles of the HLA-DQ β-chain gene also differ in their coding regions (Fig. 3).

Our further studies involve cloning and sequencing of the 12- and 4-kb fragments from IDDM patients. This is important, because these two approaches will not only give further structural information on the HLA-DQ β-chain genes but also allow us to derive yet other probes from IDDM patients and permit further dissection of disease susceptibility, both in population and family studies. Sequence information should also resolve whether the 12-kb fragment on HLA-DR4–containing chromosomes is related to the HLA-DR2–associated BamHI 12-kb fragment found to be positively associated with multiple sclerosis (18).

Our approach in cloning RFLP fragments should eventually lead to the identification of an HLA-D locus gene that gives maximal susceptibility to develop IDDM. It has been previously shown that HLA-DR3 and 4 have different regulatory functions on the proliferative T lymphocyte response to mumps and coxsackie B4 (45), two viruses implicated in the etiology of IDDM (4). Class II antigens confer restriction in antigen presentation perhaps by direct binding of processed antigen (46). Isolation and structural analysis of these HLA-D class II molecules will therefore be of central importance in testing the hypothesis (2, 47, 48) that the pathogenesis of IDDM is conferred by the ability of these molecules to present antigens that induce an abnormal immune response against the pancreatic β cells.

Acknowledgments

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