Major Histocompatibility Complex–linked Diabetogenic Gene of the Nonobese Diabetic Mouse

Analysis of Genomic DNA Amplified by the Polymerase Chain Reaction

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Abstract

Inheritance of insulin-dependent diabetes mellitus (IDDM) is polygenic, and at least one of the genes conferring susceptibility to diabetes is tightly linked to the MHC. Recent studies have suggested that DQB1 of humans and I-A_β of mice are closely associated with susceptibility and resistance to IDDM. For further characterization and localization of the MHC-linked diabetogenic gene, we studied the genomic sequence of the A_β gene of the nonobese diabetic (NOD) mouse, an animal model of IDDM, in comparison with those of its sister strains, nonobese nondiabetic and cataract Shionogi (CTS) mice, and the original strain, outbred Imperial Cancer Research (ICR) mice. Genomic DNAs from these strains were amplified in vitro by the polymerase chain reaction with thermostable Taq polymerase. The amplified sequences were analyzed by restriction endonuclease digestion, hybridization with allele-specific oligonucleotide probes, and direct sequencing. The unique I-A_β sequence of NOD mice was observed in the sister strain, CTS mice, and in one mouse of the original strain, outbred ICR mice. These data together with the results of MAb typing of MHC molecules and restriction mapping of the I-A region suggest that the unique class II MHC of NOD mice is not the result of a recent mutation, but is derived from the original strain. Since class I MHC of CTS mice is different from the MHC of NOD mice at both the K and D loci, CTS mice are a naturally occurring recombinant strain with NOD type class II MHC and non–NOD type class I MHC. Thus, breeding studies in crosses of NOD with CTS mice should provide biological information on whether the unique class II MHC of NOD mice is diabetogenic. (*J. Clin. Invest.* 1990. 85:18–24.) diabetogenic gene • insulin-dependent diabetes mellitus • major histocompatibility complex • nonobese diabetic mouse • polymerase chain reaction

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Introduction

Insulin-dependent diabetes mellitus (IDDM)[1] appears to result from autoimmune β-cell destruction in the setting of a genetic predisposition (see references 1–3 for reviews). In both man (4) and animal models (5, 6) inheritance of IDDM is polygenic, and at least one of the genes conferring susceptibility to diabetes has been mapped in the MHC (4–6). Recent studies in Caucasian patients with IDDM suggest that the HLA-DQB1 locus (7–11), in particular, amino acid residue 57 of the DQB1 chain, is closely associated with both susceptibility and resistance to the disease: DQB1 alleles with aspartic acid at position 57 provide resistance to IDDM, whereas the absence of aspartic acid at position 57 results in susceptibility to IDDM (12). No biological information is as yet available, however, on whether the DQB1 gene itself causes diabetes in combination with other susceptibility genes.

A suitable animal model with a similar MHC sequence to that in human IDDM patients is essential for studies on whether the DQB1 sequence is a MHC-linked diabetogenic gene. Nonobese diabetic (NOD) mice develop IDDM secondarily to autoimmune beta cell destruction (13, 14). Recent breeding studies (6, 15–18) suggested that the development of diabetes in NOD mice is regulated by at least two, and probably three or more, recessive diabetogenic genes, and that at least one of these is linked to the MHC of the NOD mouse. NOD mice have unique class II MHC molecules with no expression of surface I-E molecules and I-A molecules different from any known I-A characterized so far (6, 16). Recent sequence studies (19) indicated that unique sequences for NOD mice are located in the first external domain of I-A_β chain, while other parts of the I-A_β chain and the I-A_α chain are identical to those in BALB/c (H-2^d) mice. In particular, NOD mice have a unique serine residue at position 57 of the I-A_β chain instead of the aspartic acid residue conserved in other strains. This characteristic of the I-A_β sequence is the same as that in human IDDM in which amino acid residue 57 of DQB1, the human homologue of mouse I-A_β, is closely associated with susceptibility or resistance to diabetes.

The aim of the present study was further characterization and localization of the MHC-linked diabetogenic gene of NOD mice. For this purpose we analyzed the genomic sequences of the MHC of NOD mice in comparison with those of nondiabetic sister strains and the original strain. In particular, we tried to find the unique class II MHC of the NOD mouse in nondiabetic NOD-related strains.

1. *Abbreviations used in this paper:* ASO, allele-specific oligonucleotide; CTS, cataract Shionogi; ICR, Imperial Cancer Research; IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; NON, nonobese nondiabetic; PCR, polymerase chain reaction.

Methods

Mice. NOD mice and sister strains, nonobese nondiabetic (NON) and cataract Shionogi (CTS) mice, were originally derived from outbred Jcl-Imperial Cancer Research (ICR) mice (Fig. 1; 13, 16). The nuclei of the NOD/Shi/Jos, NON/Shi/Jos, and CTS/Shi/Jos mice were originally obtained from the colony of Dr. Makino at Aburahi Laboratories, Shionogi Co., Ltd., Shiga, Japan, bred by brother-sister mating at the Animal Resources Center and the Radioisotope Center of Kyoto University (Kyoto, Japan), and raised in our animal facility at the Joslin Diabetes Center. The NOD, NON, and CTS mice in our colonies at the time of study were in the 40th, 44th, and 84th generation, respectively. Jcl-ICR mice were obtained from Clea Japan, Inc. (Tokyo, Japan) and were from the same colony as that from which the NOD, NON, and CTS strains were derived. Female mice 4–16 mo of age were used.

MHC typing. The expression of MHC molecules was determined by indirect immunofluorescence assay as reported previously (16). Briefly, splenocytes were isolated from NOD, NON, CTS, and ICR mice with Ficoll-400 (Lympholyte-M; Cederlene, Ontario, Canada) and incubated with MAbs to class I or class II MHC molecules for 45 min at 4°C. The cells were then washed three times with RPMI 1640 medium and stained with FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (gamma and light chain specific; Tago Inc., Burlingame, CA) for class I or with FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (Fc fragment gamma chain specific; Cappel Laboratories, Cochranville, PA) for class II MHC molecules. Stained cells were analyzed with an EPICS flow cytometer (Coulter Electronics Inc., Hialeah, FL). Nonspecific MAb (P3 × 63) was used as a negative control. MAbs to class I MHC molecules were purchased from Litton Bionetics (Charleston, SC). MAb 10-2-16 was kindly provided by Dr. C. A. Janeway, Jr. (Yale University School of Medicine, New Haven, CT) and MKD6, 14-4-4 was a gift from Dr. L. H. Glimcher (Harvard School of Public Health, Boston, MA).

Restriction mapping. High molecular weight genomic DNA was extracted from the liver as described (18). The DNA was digested with a restriction endonuclease, Bam HI, Hind III, or Eco RI, and the digests were subjected to electrophoresis on 0.9% agarose gel, transferred to nitrocellulose paper, and hybridized with ^{32}P-labeled probes as described elsewhere (20). The A_β probes used for restriction mapping were a 2.1-kb genomic fragment of H-2^k encoding the β_2, transmembrane, and intracytoplasmic domains, and a 2.5-kb genomic fragment of H-2^d encoding part of the intracytoplasmic domain and the 3′ untranslated region. The probes were kindly provided by Dr. J. G. Seidman (Harvard Medical School, Boston, MA).

Polymerase chain reaction (PCR). Genomic DNA was amplified by the PCR with a thermostable DNA polymerase, Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) (21). Two oligonucleotide primers (PES166-21 and PEA367-20) were synthesized (Fig. 2) in 381A DNA synthesizer (Applied Biosystems Inc., Foster City, CA). These primers were designed to flank a 202-bp segment of the A_β gene containing the NOD-specific sequence at amino acid residues 56 and 57. Amplification was performed in 100 μl of reaction mixture containing 1 μg of genomic DNA in 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μM concentrations of each deoxyribonucleotide triphosphate (dNTP; dATP, dCTP, dGTP, dTTP), and a 1-μM concentration of each primer. After initial denaturation at 94°C for 2 min, samples were centrifuged at 12,000 *g* for 10 s and 2.5 U of Taq polymerase was added. The samples were then overlaid with 100 μl of mineral oil and subjected to 25 cycles of amplification as follows. The samples were incubated at 60°C for 2.5 min (to anneal primers), at 72°C for 3.5 min (to extend the annealed primers), and at 94°C for 1.5 min (to denature the DNA). At the end of the 25th cycle the heat denaturation step was omitted and all samples were incubated for an additional 7 min to ensure that the final extension step was complete. The amplified DNA was then precipitated with ethanol and resuspended in 50 μl of TE (10 mM Tris, pH 7.4, 0.1 mM EDTA) buffer. Then one-tenth of each sample was subjected to electrophoresis on a composite mini-gel of 3% NuSieve and 0.75% SeaKem agarose (FMC BioProducts, Rockland, ME) in Tris-acetate buffer and stained with ethidium bromide.

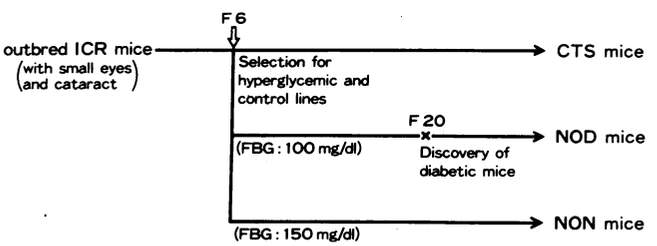

Figure 1. Genealogy of NOD, NON, and CTS mice. The three strains originated from an outbred colony of Jcl-ICR mice and have been bred by brother-sister mating for > 40 generations.

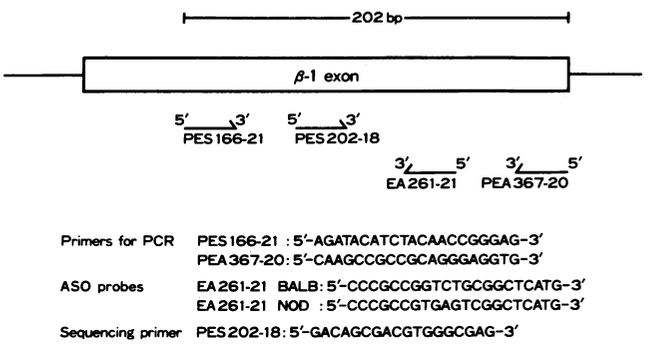

Figure 2. Primers for the PCR and allele-specific oligonucleotide probes (ASO probes). The PCR primers are flanking the 202-bp region of the A_β gene, including the NOD-specific sequence between nucleotides 248 and 252. PES166-21 is 21 bases long and corresponds to nucleotides 166–186 of the A_β gene. PES367-20 is 20 bases long and is complementary to nucleotides 348–367 of the A_β gene. The ASO probes are complementary to allele-specific sequences of NOD (EA261-21NOD) and BALB/c (EA261-21BALB) mice between nucleotides 241 and 261, corresponding to amino acid residues 54–60 of the I-A_β chain.

Analysis of amplified samples. Portions of one-tenth of the amplified samples were digested with Dde I or Hae III, precipitated with ethanol, and resuspended in 10 μl of TE buffer. The samples were then subjected to electrophoresis on 3% Nusieve/0.75% SeaKem agarose mini-gel and stained with ethidium bromide. Southern blot transfer was performed as described (20) onto GeneScreen Plus nylon membranes (DuPont, Boston, MA). An 18-base oligonucleotide probe (PES202-18) corresponding to the conserved sequence (nucleotides 202-219) (22–25) of the A_β gene was labeled at the 5′ end with gamma-[^{32}P]ATP and T4 polynucleotide kinase and hybridized to the filters.

Analysis with allele-specific oligonucleotide (ASO) probes was performed as follows (12, 26, 27). Portions of one-tenth of amplified samples were denatured with 0.3 M sodium hydroxide in a final volume of 400 μl in 10 mM Tris-Cl (pH 7.0) and 1 mM EDTA at 70°C for 30 min. The mixtures were neutralized with an equal volume of 2 M ammonium acetate (pH 7.0), loaded into the wells of a Manifold II slot-blotter (Schleicher & Schuell, Inc., Keene, NH) and blotted onto nitrocellulose filters according to the manufacturer's instructions. Two ASO probes (Fig. 2) complementary to nucleotides 241–261 of the A_β gene were synthesized. EA261-21BALB is complementary to the conserved sequence for H-$2^{b,d,k,q,u,s}$, while EA261-21NOD is complementary to the NOD-specific sequence with a five nucleotide substitution corresponding to amino acid residues 56 and 57. Filters were prehybridized in 6× standard saline citrate (SSC), 5× Denhardt's solution,

and 0.5% SDS for 1 h at 37°C, and hybridized overnight at 37°C by the addition of ^{32}P end-labeled ASO probes to the prehybridization mixture. After hybridization the filters were washed first with 6× SSC/0.1% SDS at 30°C for 10 min, and then with 6× SSC/0.5% SDS at 74°C for EA261-21BALB or at 72°C for EA261-21NOD (12, 28), and exposed for 24 h at −70°C.

Direct sequencing of the amplified sequences was performed by the triple primer method of Wrischnik et al. (29) with minor modification. Amplified samples were purified on an Elutip-d affinity column (Schleicher & Schuell, Inc.) and precipitated with ethanol. Sequencing primer (PES202-18; 18 bases long, nucleotides 202–219) was 5′ end-labeled with gamma-[^{32}P]ATP. Approximately 0.2–0.25 pmol of the amplified sequence was then mixed with 2–2.5 pmol of ^{32}P-labeled primer (PES202-18) in 10 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, and 30 mM NaCl. The template-primer mixture was denatured in a boiling water bath for 10 min, centrifuged at 12,000 *g* for 10 s, and placed in a water bath at 50°C for at least 15 min. Aliquots of 2 μl of appropriate dddNTP mixtures in 10 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, and 30 mM NaCl were placed in a set of four 1.5-ml Eppendorf microcentrifuge tubes. These mixtures were as follows. dddATP mixture: 0.02 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 3.6 mM dideoxy(dd)ATP; dddCTP mixture: 0.2 mM dATP, 0.02 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTC, 1.6 mM ddCTP; dddGTP mixture: 0.2 mM dATP, 0.2 mM dCTP, 0.02 mM dGTP, 0.2 mM dTTP, 2 mM ddGTP; and dddTTP mixture: 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.02 mM dTTP, 1.6 mM ddTTP. 5 U of DNA polymerase I Klenow fragment and then primer-template mixture were added to each dddNTP tube, and the reactions were allowed to proceed for 50 min at 50°C. Then 1 μl of 1 mM solution of the respective dNTP was added to each tube as cold chase solution and the reaction was allowed to proceed for an additional 15 min. The reaction mixtures were then dried and the residues were suspended in 3 μl of water, mixed with 3 μl of formamide-dye mixture (0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM EDTA, and 95% deionized formamide), and boiled for 3 min. They were then subjected to electrophoresis on 8 M urea/8% acrylamide sequencing gel (30). The gels were dried and exposed to x-ray film at −70°C for 24 h.

Results

MAb typing of MHC molecules. In previous studies using a panel of MAbs, we found that CTS mice appear to have the same I-A molecules as NOD mice and, like NOD mice, lack surface I-E molecules (16). We therefore studied the MHC molecules of the original strain, ICR mice, to see whether these class II MHC molecules were derived from the original strain. Table I shows the result of MHC typing of 10 ICR mice. Of 10 ICR mice examined, one mouse (No. 9) showed the same class II reactivity as NOD and CTS mice. The I-A molecules of the ICR mouse No. 9 reacted with anti–I-A MAb 10-2-16, but not with MKD6, suggesting that I-A of this mouse is the same as that of NOD mice. I-E molecules of this animal did not react with MAb 14-4-4. Since MAb 14-4-4 should react with all MHC haplotypes expressing surface I-E molecules (6, 16, 31), these data suggest that ICR No. 9 lacked surface I-E molecules as NOD mice do.

Restriction mapping. Studies using three restriction endonucleases (Bam HI, Eco RI, and Hind III) and two genomic probes for the A_β gene showed that the restriction maps of the A_β gene of the CTS mice and ICR mouse No. 9 were identical to that of NOD mice, while that of NON mice was different (Fig. 3). These data suggest that the genomic organizations of the A_β gene in CTS mice and ICR mouse No. 9 are the same as that in NOD mice.

PCR. To determine the optimal conditions for the PCR with these particular primers, we examined the effects of different annealing temperatures on the specificity of amplification (Fig. 4). Samples of 1 μg of genomic DNA from NOD mice were subjected to 25 cycles of the PCR at annealing temperatures at 37, 55, and 60°C. After annealing at 37 (lane *1*) and 55°C (lane *2*), nonspecific bands were observed in addition to the target sequence at 202 bp, whereas after annealing at 60°C fewer nonspecific bands were visible. Therefore, an annealing temperature of 60°C was used in subsequent experiments. This temperature is close to the dissociation temperatures of the PCR primers (PES166-21, 62°C and PEA367-20, 70°C) calculated by the equation T_d(°C) = (number of GC) × 4 + (number of AT) × 2 (12, 28).

When genomic DNA from BALB/c mice (the I-A_β sequence of which shows the highest homology with that of NOD mice) was amplified for 25 cycles, the amplified sequence had a higher molecular weight (208 bp; Fig. 5, lane *2*) than that of NOD mice (202 bp, lane *1*). This was due to a

Table I. Reactivity of Splenocytes from ICR Mice with Anti–Class I and –Class II MAbs

						ICR mice									
	MAb	MHC	NOD	NON	CTS	1	2	3	4	5	6	7	8	9	10
Class I	28-13-3S*	K	−	+	−	−	−	−	−	−	−	−	−	−	−
	31-3-4S	K	+	−	+	−	−	−	−	+	−	+	−	+	−
	16-1-11N	K	−	+	+	+	+	+	+	+	+	+	+	+	+
Class II	10-2-16	I-A	+	+	+	−	−	−	+	−	+	−	−	+	+
	MKD 6	I-A	−	+	−	+	+	+	+	+	+	+	−	−	+
	14-4-4	I-E	−	+	−	−	−	−	+	−	−	−	+	−	+
Class I	28-14-8S	D	+	+	−	−	+	+	+						
	34-4-21S	D	−	−	−	−	−	−	−			NT			
	12-2-2S	D, K	−	−	−	−	−	+	−						

* The specificities of the MAbs were as follows: 28-13-3S, K^b, crossreaction with H-2^f; 31-3-4S, K^d; 16-1-11N, K^k, crossreactions with H-$2K^q$, H-$2^{p.r}$; 10-2-16, I-$A^{k.f.r.s}$; MKD6, I-A^d; 14-4-4, I-$E^{k.d.p.r}$ but not strains lacking surface I-E such as H-$2^{b.f.q.s}$; 28-14-8S, D^b, crossreactions with H-$2L^d$, D^q, and/or H-$2L^q$; 34-4-21S, D^d; 12-2-2S, D^k, K^k, crossreactions with H-$2K^q$, H-$2^{p.r}$.
NT, not tested.

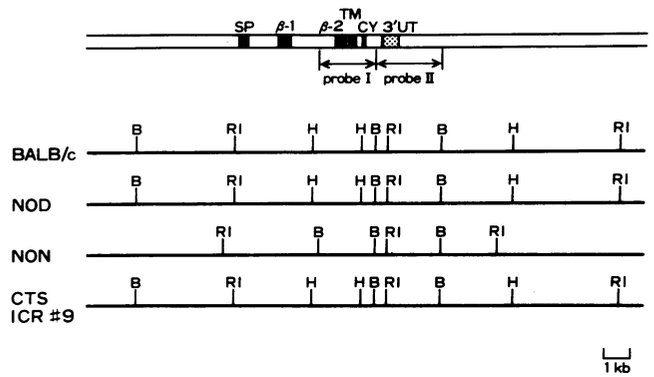

Figure 3. Restriction mapping of the A_β region of the MHC of NOD, BALB/c, NON, and CTS mice and ICR mouse No. 9. The restriction map of CTS mice and the ICR mouse No. 9 are the same as that of NOD mice, which is identical with that of BALB/c mice. *B,* Bam HI; *H,* Hind III; *RI,* Eco RI.

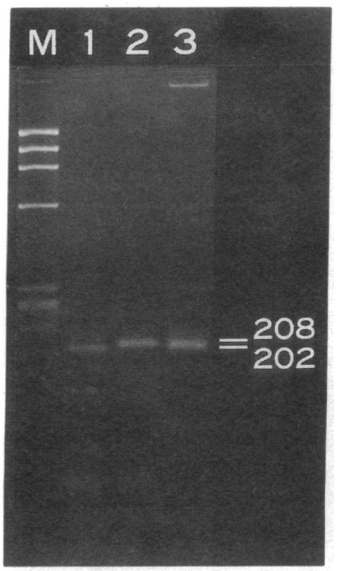

Figure 5. Electrophoretic analysis of amplified products of NOD, BALB/c, and F1(NOD × BALB/c) DNA. The amplified band of the NOD mouse was at 202 bp (lane *1*), while that of the BALB/c mouse was at 208 bp (lane *2*). F1(NOD × BALB/c) gave a broad band corresponding to 208 and 202 bp (lane *3*). See Fig. 4 for molecular size marker (lane *M*).

6-nucleotide insertion at nucleotide residues 274 and 280 of the H-$2^{b,d,q}$ haplotypes (Fig. 6) (22–25). When F1(NOD × BALB/c) DNA was amplified, a broad band of 208–202 bp was observed, suggesting that the I-A_β sequences on both chromosomes were amplified. These data indicate that a difference of as little as 6 bp can be identified without use of radioactivity by a combination of the PCR and NuSieve agarose gel electrophoresis.

Restriction site analysis of amplified sequence. Genomic DNAs from NOD, NON, and CTS mice and ICR mouse No. 9 were subjected to 25 cycles of the PCR in comparison with those of control strains, C3H/He (H-2^k), C57BL/6 (H-2^b), and BALB/c (H-2^d). As expected, the DNAs of C57BL/6 and BALB/c mice gave an amplified band at 208 bp, while those of C3H/He and NOD mice showed a band at 202 bp (Fig. 7 *A*). The DNAs of NON and CTS mice and ICR mouse No. 9 gave an amplified band at 202 bp, suggesting that these mice have the same sequence at nucleotides 274–276 as that of NOD mice and k, q, u, r, s haplotypes.

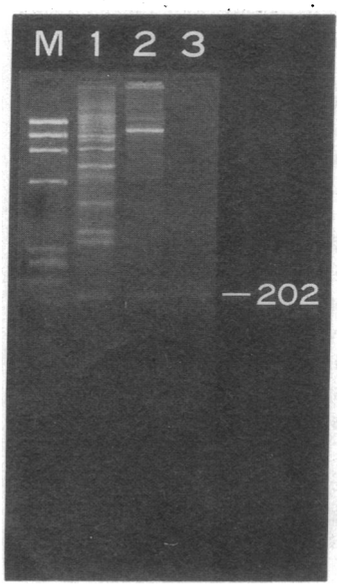

Figure 4. Effect of annealing temperature on the specificity of amplification. Genomic DNA from a NOD mouse was amplified by 25 cycles of the PCR with primer annealing temperature at 37 (lane *1*), 55 (lane *2*), or 60°C (lane *3*). The molecular size markers (lane *M*) are Hae III–digest of ϕX174 RF DNA. Relevant sizes are as follows: 1,353, 1,078, 872, 603, 310, 281/271, 234, 194, 118, and 72 bp.

Since the five nucleotide substitutions at nucleotides 248–252 in the DNA of NOD mice creates a new restriction site for Dde I at nucleotide 249 (Fig. 8), amplified sequences were digested with restriction endonuclease Dde I. As expected, the NOD sequence gave two bands at 118 and 84 bp (Fig. 7 *B,* lane *4*), while the C3H/He, C57BL/6, and BALB/c sequences remained at the original position (Fig. 7 *B,* lanes *1–3*). When NON, CTS, and ICR No. 9 DNAs were digested with Dde I, the CTS and ICR No. 9 sequences gave the same two bands as DNA of NOD mice (Fig. 7 *B,* lanes *6* and *7*), while the NON sequence did not (lane *5*). These data suggest that CTS mice and ICR mouse No. 9 have the same sequence at nucleotides 248–252 as that of NOD mice, whereas that of NON mice is different.

The I-A_β sequences of H-$2^{b,d,k,q,u,r,s}$ haplotypes have a Hae III recognition site at nucleotide 246 (Fig. 8). The five nucleotide substitutions in NOD mice at nucleotides 248–252 destroy this Hae III recognition site at nucleotide 246 (Fig. 8). Since the amplified sequences of the NOD and control strains have a conserved recognition site for Hae III at nucleotide 292 (298 for H-$2^{b,d,q}$ due to a 6-nucleotide insertion; 19, 22–25), Hae III digestion of amplified DNA of the NOD mouse generated bands at 127 and 75 bp (Fig. 7 *C,* lane *4;* the 75-bp band is not clearly seen in this figure because of the high background in the low molecular weight region). The amplified sequences of H-$2^{b,d,k}$ haplotypes have an additional restriction site for Hae III within this 127-bp band at nucleotide 246 (22–25), so Hae III digestions of the C3H/He (H-2^k), C57BL/6 (H-2^b), and BALB/c (H-2^d) sequences generate smaller bands (75 bp for C3H/He, 81 bp for C57BL/6 and BALB/c) than those of the NOD mouse sequence, and the 127-bp band is not seen (Fig. 7.

Amino acid =	62	63	64	65	66	67	68	69
Nucleotide =	265							282
NOD	AAT	AAG	CAG	***	TAC	***	CTG	GAG
k, u, s, f	---	---	---	***	---	***	---	---
d, b, q	--C	-GC	---	CCG	G-G	ATC	---	---

Figure 6. Comparison of nucleotide sequences of the A_β gene between nucleotides 265 and 282 of the NOD mouse (19) and those of b,d,k(22),f,q,u, and s(25) haplotypes. *Dashed lines,* identity with the NOD sequence; *asterisks,* gaps inserted into sequences to achieve maximal alignment.

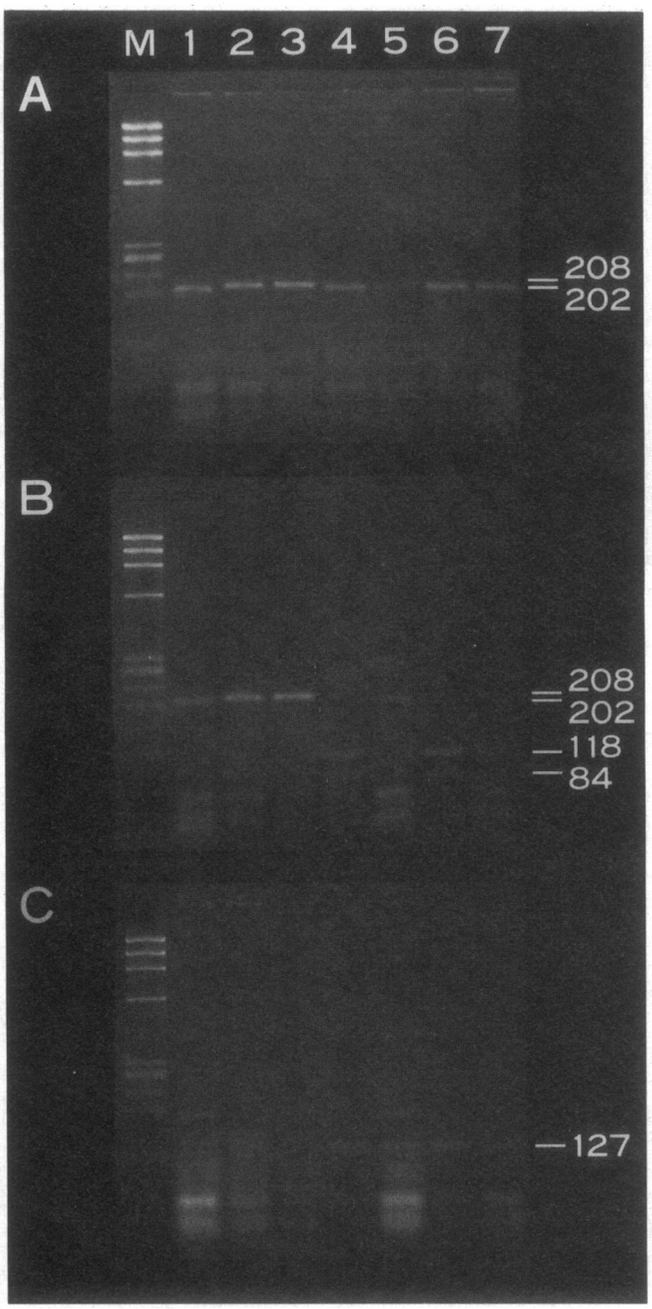

Figure 7. Restriction site analysis of amplified A_β sequences of a NOD mouse and related strains in comparison with results on control strains. *A,* Amplified products from C3H/He (lane *1*), C57BL/6 (lane *2*), BALB/c (lane *3*), NOD (lane *4*), NON (lane *5*), CTS (lane *6*), and ICR No. 9 (lane *7*). C57BL/6 and BALB/c gave bands at 208 bp, whereas C3H/He, NOD, NON, CTS, and ICR No. 9 (lane *7*) gave bands at 202 bp. *B,* Dde I digestion of amplified sequences. On Dde I digestion, the NOD sequence gave two bands at 118 and 84 bp (lane *4*), while the sequences of C3H/He (lane *1*), C57BL/6 (lane *2*), and BALB/c (lane *3*) remained at the original position. The sequences of CTS (lane *6*) and ICR No. 9 (lane *7*) generated the same bands as those of the NOD mouse, while that of the NON mouse (lane *5*) remained at the original position. *C,* Hae III digestion of amplified sequences. Hae III digestion of the NOD sequence generated a band at 127 bp (lane *4*), while digestions of those of C3H/He (lane *1*), C57BL/6 (lane *2*), and BALB/c mice (lane *3*) generated smaller bands at 75 bp (C3H/He) and 81 bp (C57BL/6 and BALB/c), which are not clearly seen in this figure. The sequences of NON (lane *5*), CTS (lane *6*), and ICR No. 9 (lane *7*) gave the same 127-bp band as that of the NOD mouse (lane *4*). See Fig. 4 for molecular size marker (lane *M*).

Amino acid #	54	55	56	57	58	59
Nucleotide #	241					258
NOD	GGG	CGG	CAC	TCA	GCC	GAG
			↑ Dde I			
d, b, k, q, u, s	GGG	CGG	CCA	GAC	GCC	GAG
			↑ Hae III			
f	GGG	CGG	TCA	GAC	GCC	GAG

Figure 8. Comparison of the nucleotide (241–258) and amino acid (54–59) sequences of the A_β gene of the NOD mouse with those of control strains. The A_β sequence of the NOD mouse has a Dde I site at nucleotide 249, whereas the A_β sequences of H-$2^{b,d,k,q,u,s}$ haplotypes do not. In contrast, the A_β sequences of H-$2^{d,b,k,q,u,s}$ haplotypes have a Hae III site at nucleotide 246, while that of the NOD mouse does not. The A_β sequence of the H-2^f haplotype has no Dde I or Hae III site.

C, lanes *1–3*). Hae III digestion of the amplified sequences of NON and CTS mice and ICR mouse No. 9 generated a 127-bp band identical to that of NOD mice (Fig. 7 *C,* lanes *5–7*), suggesting that, like NOD mice, these strains lack the Hae III recognition site at nucleotide 246. The amplified bands and bands of digestion products in Fig. 7 were confirmed to be target sequences by Southern blot hybridization with the ^{32}P-labeled probe PES202-18.

These restriction analyses with Dde I and Hae III indicated that CTS mice and ICR mouse No. 9 have the same recognition sequence for these endonucleases as in NOD mice, and suggested that these mice have the same unique amino acid substitution as NOD mice at amino acid residues 56 and 57. In contrast, NON mice do not have restriction sites for either Dde I or Hae III, indicating that the NON sequence in this region is different from that in NOD mice and control strains of H-$2^{b,d,k,q,u,s}$ haplotypes. The only possible sequence fitting the restriction site characteristics of NON mice is the H-2^f haplotype, which has no restriction site for Dde I or Hae III (Fig. 8; 25).

ASO slot blot analysis. Fig. 9 shows a slot blot of amplified DNA hybridized with ^{32}P-labeled ASO probes for the NOD-specific sequence (lane *A*) and BALB/c-specific sequence (lane *B*). As expected, the NOD sequence hybridized with the NOD probe but not with the BALB/c probe (sample *1*), whereas the BALB/c sequence hybridized with the BALB/c probe but not with the NOD probe (sample *2*). The F1(NOD × BALB/c) sequence hybridized with both probes (sample *3*). The NON sequence did not hybridize with either probe (sample *4*), suggesting that this sequence differed from both the NOD sequence and the conserved sequence of H-$2^{b,d,k,q,u,s}$ haplotypes at around amino acid residue 57. This is consistent with the results of restriction site analysis (Fig. 7) of PCR products. The CTS and ICR No. 9 sequences hybridized with the NOD probe but not with the BALB/c-probe, suggesting that these mice have the same sequence as that of NOD mice at around amino acid residue 57.

Direct sequencing of amplified DNA. On direct sequencing by the amplified DNA method, 59 bases (nucleotides 222–280) and 54 bases (nucleotides 228–281) of CTS mice and the ICR mouse No. 9, respectively, could be resolved. The first readable bases of CTS mice and the ICR mouse No. 9 were three and nine nucleotides, respectively, from the 3′ end of the sequencing primer. The results confirmed that these mice have

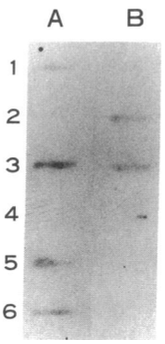

Figure 9. Allele-specific oligonucleotide slot blot analysis of amplified sequences. Amplified sequences of NOD (sample *1*), BALB/c (sample *2*), F1(NOD × BALB/c) (sample *3*), NON (sample *4*), CTS (sample *5*), and ICR No. 9 mice (sample *6*) were hybridized with ASO probes for the NOD-specific sequence (EA261-21NOD, lane *A*) and BALB/c-specific sequence (EA261-21BALB, lane *B*). The NOD sequence (sample *1*) hybridized with the NOD-specific probe, but not with the BALB/c probe, while the BALB/c sequence (sample *2*) hybridized with the BALB/c probe but not with the NOD probe. The F1 sequence (sample *3*) hybridized with both probes. The sequences of CTS and ICR No. 9 (samples *5* and *6*, respectively) showed the same hybridization pattern as that of a NOD mouse. The NON sequence (sample *4*) did not hybridize with either probe.

the same I-A_β sequence, including the unique 5-nucleotide substitution at nucleotides 248–252, as that in NOD mice.

Discussion

The developments of IDDM in man, the Biobreeding rat, and the NOD mouse are closely linked to the MHC (4–6). Previous studies have suggested that the class II MHC gene, especially HLA-DQB1 in humans (7–11) and I-A_β in mice (6, 16–18), is more closely associated with IDDM than other MHC genes, but the precise localization of the MHC-linked diabetogenic gene is unknown. The recombination frequency within MHC is too low to allow fine mapping of the MHC-linked diabetogenic gene by usual breeding studies. Thus, recombinant animals with NOD type MHC in a certain region and non–NOD type MHC in other regions are essential for further localization of the MHC-linked diabetogenic gene.

Our previous study (16) suggested that CTS mice are naturally occurring recombinant animals with the same unique class II MHC molecules as the NOD mouse, but different class I MHC at the K and D loci from those in NOD mice. In the present study we confirmed these observations at the genomic level. As the I-A_β sequences of NOD mice and control strains have been reported (22–25), we used the PCR technique to analyze the I-A_β sequences of NOD-related strains. Because the specificity and yield of amplified products were high, we could examine amplified sequences by simple restriction site analysis without using radioactive probes. As shown in Fig. 7, CTS mice were found to have the same unique I-A_β sequence as NOD mice. These results were further confirmed by hybridization with ASO probes (Fig. 9) and direct sequencing. Since CTS mice showed the same restriction map in the A_β region as NOD mice (Fig. 3) and the same reactivity with a panel of MAbs against the I-A_β chain (16), CTS mice probably have the same A_β gene as NOD mice. In addition, CTS mice, like NOD mice, do not have surface I-E molecules (16) and appear to have the same I-A_α as NOD mice, as demonstrated by MAb typing (16) and RFLP analysis (16, 32, 33). These data suggest that CTS mice have the same class II MHC as NOD mice. Since one of the ICR mice studied also had the same class II MHC as NOD mice, the unique class II MHC of NOD mice is probably derived from the original strain, ICR mice, and did not arise by specific mutation.

In this work, however, we analyzed only the sequence of the β_1 domain of the I-A_β chain. CTS mice and ICR mouse No. 9 may have different sequences from that of NOD mice in the rest of the I-A_β chain or in the I-A_α chain, and examinations of the relationships of the rest of the I-A_β chain and the I-A_α chain to diabetogenesis seem important. However, the present study clearly indicated that the unique β_1 sequence of NOD mice was also present in the sister strain, CTS mice, and in one mouse of the original strain, ICR mice. Similar results have recently been reported for human IDDM (12) and BB rats (34, 35). In all species, the class II MHC sequences associated with IDDM are also found in nondiabetic controls.

The PCR procedure is very useful for amplification and analysis of a sequence of interest, but it has several limitations. First, only relatively short sequences can be analyzed successfully. Judging from our experience and previous reports (21), sequences of up to 2 kb can be amplified efficiently, but the efficiency of amplification and yield of products decreases with the length of the sequence. Second, misincorporation of bases occurs during the PCR reaction, although at very low frequency (2×10^{-4}/nucleotide per cycle) (21). The small fraction of misincorporated bases does not affect the analysis by restriction site analysis, hybridization with ASO probes, or direct sequencing because a large amount of PCR products is used for the analysis. However, these errors may be critical when individual amplification products are cloned and sequenced. Therefore, several independent clones should be sequenced.

CTS mice and the ICR mouse No. 9 had the same potentially diabetogenic class II MHC as NOD mice, but they did not have diabetes. These data suggest the following three possibilities with regard to the MHC-linked diabetogenic gene of NOD mice. First, CTS mice and the ICR mouse No. 9 did not develop diabetes because they lacked a non-MHC gene(s) that is also necessary for diabetes. Second, the class II region may not be the only MHC-linked region determining susceptibility to diabetes, whole MHC of the NOD mouse being necessary for the development of diabetes. Third, the MHC-linked diabetogenic gene may not be a histocompatibility gene, but another gene or gene complex closely linked to the MHC region. These possibilities can be tested by breeding studies in crosses of NOD with CTS mice and selected ICR mice.

In summary, the unique class II MHC of NOD mice was found in a sister strain, CTS mice, and one mouse in the original ICR strain. These data suggest that the unique class II MHC of NOD mice is not the result of a recent mutation, but was derived from the original ICR strain. Class I MHC of CTS mice differs from that of NOD mice in both the K and D regions, so CTS mice are a naturally occurring recombinant strain with NOD type class II MHC and non–NOD type class I MHC. This strain should, therefore, be useful in breeding studies for determination of the MHC-linked diabetogenic gene.

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