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BRIEF COMMUNICATION

SLA-DRB1 and -DQB1 genotyping by the PCR–SSOP–Luminex method

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Abstract

A simple and novel genotyping method was developed to detect alleles at the swine leukocyte antigen (SLA)-DRB1 and -DQB1 class II loci by using polymerase chain reaction (PCR)–fluorescently labeled sequence-specific oligonucleotide probes (SSOPs) and Luminex 100 xMAP detection. The PCR–SSOP–Luminex method exhibited accuracy of 95% for both SLA-DRB1 and -DQB1 in 6 homozygous and 16 heterozygous pig samples as confirmed by sequencing the PCR products of the same samples. In addition, 12 low-resolution SLA class II haplotypes consisting of 7 and 9 DRB1 and DQB1 alleles were identified, respectively, in one population of 283 Landrace pigs. This genotyping method facilitates the rapid and accurate identification of two- or four-digit alleles at the SLA-DRB1 and -DQB1 loci.

The class II genes of the swine major histocompatibility complex (*Mhc*:*SLA*) are involved in the genetic control of immune responses to foreign antigens. The swine leukocyte antigen (SLA)-DRB1 and -DQB1 class II genes are highly polymorphic and variable within the SLA genomic region. Namely, the IPD-MHC SLA database (http://www.ebi.ac.uk/ipd/mhc/sla) currently contains 82 DRB1, 44 DQB1, 20 DQA, and 13 DRA alleles with 21 haplotypes that have been designated by the International Animal Genetics (ISAG) SLA Nomenclature Committee (1, 2). The study of allelic variation at the SLA-DRB1 and -DQB1 loci is important for understanding the role of these loci variants in disease resistance and vaccine efficacy (3–5). To identify the SLA class I and II alleles, a number of DNA-based SLA typing methods using polymerase chain reaction (PCR)-amplified DNA were developed such as PCR-sequence-based typing (PCR-SBT), PCR-sequence-specific primers (PCR-SSP), or PCR-restriction fragment length polymorphism (PCR-RFLP) (6–8). Recently, PCR and the Luminex microbeads system for the simultaneous multiplex assay of amplicons hybridized to SSOPs in a single detection solution was described or sold commercially for high-throughput single nucleotide polymorphism typing of

human leukocyte antigens (HLAs), disease genes, or detection of microorganisms (9–18). For example, the PCR–SSOP–Luminex genotyping method was applied to analyze the association of certain HLA-A and/or -B, and -DRB1 alleles with Behcet's disease, ankylosing spondylitis or leprosy (13–15). In this paper, we describe the use of the PCR amplification–SSOP protocol with Luminex technology as a new, rapid, and simple SLA class II DNA-based typing method to genotype alleles (at least two digits and up to four digits or more in some cases) at the two highly polymorphic SLA class II loci, DRB1 and DQB1. This new PCR–SSOP–Luminex genotyping method was used to examine the SLA-DRB1 and -DQB1 allele diversity in a reference set of 22 animals representing 8 pig breeds and an outbred population of 283 Landrace pigs.

SLA genotyping and measurement of the analytes were performed according to the HLA genotyping protocol described previously for the PCR–SSOP–Luminex method (12) with slight modification to the PCR annealing temperature (60°C), reaction temperature (54°C) and hybridization time (25 min), and modification of the strepavidin–phycoerythrin (SA–PE) reaction temperature (54°C) and time (15 min). Seven allele-group-specific PCR primer pairs (four for SLA-DRB1 and three for SLA-DQB1 loci) were used for the PCR (Table S1, Supporting Information) performed on the genomic DNA samples of 22 pigs representing 8 breeds [6 Landrace pigs, 1 Landrace × Yorkshire crossbred pig, 2 Large white pigs, 3 Duroc pigs, 2 Göttingen miniature pigs, 4 Mexican hairless miniature pigs, 1 Nippon Institute for Biological Science (NIBS) miniature pig, and 3 Clawn miniature pigs]. Nucleotide sequence analysis of the PCR products identified one (homozygous) or two (heterozygous) allelic sequences in each of the DRB1 and DQB1 loci from a single individual, ensuring that each primer pair could specifically amplify the respective single locus (Tables 1 and 2).

Fourteen specific oligoprobes were designed for genotyping 11 SLA-DRB1 allelic groups from *DRB1*01* to *DRB1*11* and *DRB1*13* and 13 specific oligoprobes were designed for genotyping 9 SLA-DQB1 allelic groups from DQB1*01 to DQB1*9 (Table S2; Figures S1 and S2, Supporting Information). These oligonucleotide probes were covalently coupled to different sets of polystyrene carboxylated microbeads (MultiAnalyte Microsphere Carboxylated; Luminex, Austin, TX) using a carbodiimide method with slight modification (10, 12). Recently, three *DRB1*w12XX* alleles of the *DRB1*w12* group and two new DRB1 alleles, *DRB1*1301* and **1401* were reported and designated as novel alleles by the ISAG Nomenclature Committee (2). However, the sequence of the 5′ and 3′ end regions of exon 2 for the three *DRB1*w12XX* alleles, **w12ka02, *w12ka05,* and **w12ka12*, were not reported previously. Thus, the sequence homology between the three *DRB1*w12XX* alleles and five DRB1-specific PCR primers used for the SBT method and the PCR–SSOP–Luminex method could not be confirmed, whereas the allelic sequences of *DRB1*1301* and **1401* were 100% homologous with the exon 2 sequences of the DRB-1F/DRB-2R primer pair. Furthermore, the *DRB1*01XX* and *DRB1*1301* alleles have identical sequences for hybridization with the R01 probe. Nevertheless, the three *DRB1*w12XX* alleles, and the *DRB1*1301* and *DRB1*1401* allelic sequences were not found by the SBT method in any of the 22 samples from the 8 breeds. In addition, a single base mismatch was found between the 3′ end region of the R02 probe and the target sequences of *DRB1*02du01* or *DRB1*02ka08*. The two alleles, *DRB1*02du01* and *DRB1*02ka08*, were not detected by the R02 probe as shown in Figure S1 and their absence in the 22 samples was confirmed by PCR-SBT. Three probes, Q021, Q022, and Q023, were designed for the detection of seven *DQB1*02XX* alleles, but no specific probe was prepared for the detection of the *DQB1*0204* allele.

After PCR with biotin-labeled primers and the hybridization of PCR products with the complementary DNA probes coupled to microbeads, the hybridized amplicons with the attached microbeads were labeled with the fluorescent reporter molecule, SA–PE (12). Reactions were then analyzed on the flow cytometer Luminex 100 (Luminex) to identify the fluorescence intensity of PE on each bead. The expected reactivity of the oligobead probes with 11 and 9 allelic groups of SLA-DRB1 and -DQB1 alleles is shown in Figures S1 and S2, respectively. To assign the genotypes of SLA-DRB1 and -DQB1 alleles, the correct combinations of allele names and oligobead numbers were programmed into the GENOSEARCH HLA-typing software (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan).

Typing accuracy of the PCR–SSOP–Luminex method as a two-digit allele typing system was verified to be high at 95% for SLA-DRB1 and -DQB1 when the results of the 22 samples were compared with the SLA genotype information obtained with the same samples by the SBT method after subcloning the PCR products into a plasmid sequencing vector.

Ambiguities remained for the identification of DQB1 alleles by the PCR–SSOP–Luminex analysis of the homozygous sample D-120 (Table 2). The Q01 and Q03 probes could not differentiate between *DQB1*01XX/*0302* or *DQB1*0303* and *DQB1*0302* or *DQB1*0303*, respectively. Thus, the possibility of heterozygous alleles in sample D-120 could not be ruled out by the PCR–SSOP–Luminex method, although D-120 was assigned only as *DQB1*0303* by the PCR-SBT method. Also, the Q03 probe could not differentiate between the *DQB1*0302* and **0303* alleles, as the two DQB1 alleles showed identical exon 2 sequences located between DQB-2F and DQB-3R primers. On the other hand, the Q08 probe, which was designed for the identification of the *DQB1*08XX* allelic group, could not differentiate between *DQB1*08XX, DQB1*01Lu01* and *DQB1*01sh01*. High fluorescence detection intensity was obtained with the Q08 probe in six samples, which included two Landrace pigs, L-147 and L-d6, one Landrace/Yorkshire pig, L-7br, and three Mexican hairless pigs,

Table 1 An example of the probe-determination patterns obtained for some allele types at the SLA-DRB1 gene locus[a]

Sample name	Breed	SLA-DRB1 locus: Allele types deduced from the PCR-SBT method		Allele types deduced from the PCR–SSOP–Lumin		Detection probes/allele combinations: PE fluorescence intensity																
						R01 *01XX, *1301	R02 *02	R03 *0301	R041 *0401, *0402	R043 *0403, *0404	R05 *05XX, *02zs13	R06 *06XX	R07 *07XX	R08 *08XX	R09 *09XX	R10 *10XX	R11X *11XX	R1101 *1101	Rw11a *11ac21	R-cont 1 Except *06 or *10	R-cont 2 *06	R-cont 3 *10
C-γ 29	Clawn Hp-0.16/0.16	*DRB1*11ac21*	—	*DRB1*11ac21*	—	0	3	0	3	0	0	17	2	4	0	0	2067	84	4422	383	8	38
C-119	Clawn Hp-0.17/0.17	*DRB1*0801*	—	*DRB1*08XX*	—	0	10	9	6	21	0	27	16	1826	11	17	12	4	233	351	7	44
C-117	Clawn Hp-0.16/0.17	*DRB1*0801*	*DRB1*11ac21*	*DRB1*08XX*	*DRB1*11ac21*	0	25	21	22	10	0	52	25	1170	43	50	1064	40	3568	354	0	9
D-120	Duroc Hp-0.13/0.13	*DRB1*0403*	—	*DRB1*0403* or **0404*	—	0	0	4	9	360	0	7	5	9	0	0	0	28	6	102	5	31
D-123	Duroc Hp-0.30/0.30	*DRB1*1101*	—	*DRB1*1101*	—	0	1	0	11	0	0	7	2	0	0	0	2186	2150	0	560	20	67
D-125	Duroc Hp-0.30/0.13	*DRB1*0403*	*DRB1*1101*	*DRB1*0403* or **0404*	*DRB1*1101*	0	28	0	0	2082	0	120	0	0	0	22	2608	2342	0	592	0	30
G-12/5d	Göttingen (CSK)	*DRB1*0101*	*DRB1*0301*	*DRB1*01XX, *1301*	*DRB1*03XX*	1598	1	209	10	8	0	12	16	17	8	11	1	47	131	334	10	44
G-10/19d	Göttingen (CSK)	*DRB1*0201*	*DRB1*0801*	*DRB1*02XX*	*DRB1*08XX*	0	1444	8	1	37	0	26	11	1016	206	25	21	24	212	233	0	0
L-232	Landrace Hp-0.4/0.38	*DRB1*0101*	*DRB1*0201*	*DRB1*01XX, *1301*	*DRB1*02XX*	4907	1681	0	12	30	0	43	32	118	278	22	13	85	39	324	0	0
L-1024	Landrace Hp-0.2/0.38	*DRB1*0101*	*DRB1*0201*	*DRB1*01XX, *1301*	*DRB1*02XX*	5230	1834	0	3	35	0	20	0	119	372	45	2	97	65	362	0	1
L-1124	Landrace Hp-0.2/0.12	*DRB1*0201*	*DRB1*0602*	*DRB1*02XX*	*DRB1*06XX*	0	2206	6	3	0	0	936	10	116	301	11	4	5	0	174	50	0
L-147	Landrace Hp-0.12/0.14	*DRB1*0602*	*DRB1*0901*	*DRB1*06XX*	*DRB1*09XX*	0	295	28	14	22	0	630	3	3	1769	33	14	18	29	204	130	0
L-d6	Landrace	*DRB1*0801*	*DRB1*0901*	*DRB1*08XX*	*DRB1*09XX*	0	4	8	25	5	0	9	8	3342	1300	0	1	11	68	1043	56	117
L17-331d1	Landrace	*DRB1*1001*	—	*DRB1*10XX*	—	0	0	13	0	20	2	13	6	8	4	386	4	16	382	193	4	415
L-7br	Landrace/ Yorkshire	*DRB1*0201*	*DRB1*0901*	*DRB1*02XX*	*DRB1*09XX*	0	1683	0	2	1	0	16	18	33	1323	16	1	0	0	209	0	0
LW-20	Large white H08/H01	*DRB1*0101*	*DRB1*0201*	*DRB1*01XX, *1301*	*DRB1*02XX*	6129	2165	0	9	38	0	3	0	96	610	2	11	62	117	282	0	0
LW-18	Large white H38/H01	*DRB1*0201*	*DRB1*0402*	*DRB1*02XX*	*DRB1*0401* or **0402*	0	1678	25	1937	24	0	27	4	117	984	33	0	0	33	309	0	0
MH-8106	Mexican hairless	*DRB1*0201*	*DRB1*0501*	*DRB1*02XX/05XX, *02zs13/05XX* or **02zs13/–*		0	780	6	16	27	3506	27	15	139	6	12	1	42	80	328	10	46
MH-6	Mexican hairless	*DRB1*0201*	*DRB1*0701*	*DRB1*02XX*	*DRB1*07XX*	0	714	2	12	11	0	3	2539	101	1	10	0	5	19	249	0	27
MH-7	Mexican hairless	*DRB1*0501*	*DRB1*0701*	*DRB1*05XX*	*DRB1*07XX*	0	34	11	16	34	3222	29	2887	14	3	1	15	53	65	176	7	30
MH-9	Mexican hairless	*DRB1*0402*	*DRB1*0403*	*DRB1*0401* or **0402*	*DRB1*0403* or **0404*	0	19	9	811	257	0	29	13	6	0	11	10	30	9	128	8	29
N-j	NIBS	*DRB1*0201*	—	*DRB1*02XX*	—	0	826	0	10	0	0	14	4	169	17	0	3	6	11	387	14	36

[a]The oligo probe names and allele names are indicated in the top rows of the fifth column. The preset cutoff value for each fluorescing oligobead set was used to discriminate between positive and negative controls as described previously for HLA genotyping by the PCR–SSOP–Luminex method (21). The positive fluorescence intensities over the given cutoff values that indicate the allelic pattern are shaded as a gray box.

Table 2 An example of the probe-determination patterns obtained for some allele types at the SLA-DQB1 gene locus[a]

SLA-DQB1 locus						Detection probes/allele combinations												
Sample name	Breed	Allele types deduced from the PCR-SBT method		Allele types deduced from the PCR–SSOP–Luminex		Q01 *01XX, *0302, *0303	Q021 *02XX[b]	Q022 *02XX[c]	Q023 *0203	Q03 *03XX	Q04 *04XX	Q051 *05XX[d]	Q052 *05XX[e], *07XX	Q06 *06XX	Q07 *07XX	Q08 *08XX, *01Lu01, *01sh01	Q09 *09XX	Q-cont 1 Positive control
						PE fluorescence intensity												
C-y29	Clawn Hp-0.16/0.16	*DQB1*0601*	—	*DQB1*06XX*	—	36	0	22	11	0	3	21	3	2918	0	225	1	6300
C-119	Clawn Hp-0.17/0.17	*DQB1*0501*	—	*DQB1*05XX*[d]	—	421	1	18	22	0	15	3108	789	64	0	10	9	6550
C-117	Clawn Hp-16.16/17.17	*DQB1*0501*	*DQB1*0601*	*DQB1*05XX*[c]	*DQB1*06XX*	127	2	4	22	4	11	2560	480	1762	12	47	0	7378
D-120	Duroc Hp-0.13/0.13	*DQB1*0303*	—	*DQB1*01XX/03XX* or **03XX/–*		3353	1	16	9	340	7	40	0	28	1	78	0	4812
D-123	Duroc Hp-0.30/0.30	*DQB1*0503*	—	*DQB1*05XX*[e]*/–*	—	22	7	15	14	1	13	29	3236	56	40	21	7	6311
D-125	Duroc Hp-0.30/0.13	*DQB1*0303*	*DQB1*0503*	*DQB1*03XX*	*DQB1*05XX*[e]	4493	4	0	25	94	3	94	2902	5	21	204	2	7478
G-12/5d	Göttingen (CSK)	*DQB1*0301*	*DQB1*0501*	*DQB1*03XX*	*DQB1*05XX*[d]	604	4	1	11	36	16	3319	801	0	5	0	0	7137
G-10/19d	Göttingen (CSK)	*DQB1*0201*	*DQB1*0501*	*DQB1*02XX*[b]	*DQB1*05XX*[d]	135	297	21	10	0	0	3086	600	0	8	1	0	7054
L-232	Landrace Hp-0.4/0.38	*DQB1*0401*	*DQB1*0901*	*DQB1*04XX*	*DQB1*09XX*	4	0	0	12	0	7608	0	0	213	8	696	1783	6460
L-1024	Landrace Hp-0.2/0.38	*DQB1*0201*	*DQB1*0901*	*DQB1*02XX*[b]	*DQB1*09XX*	11	479	67	25	3	0	10	0	213	14	513	1601	7299
L-1124	Landrace Hp-0.2/0.12	*DQB1*0201*	*DQB1*0701*	*DQB1*02XX*[e]	*DQB1*07XX*	14	391	19	15	7	0	16	2922	1	72	6	0	7140
L-147	Landrace Hp-0.12/0.14	*DQB1*0801*	*DQB1*0701*	*DQB1*08XX*	*DQB1*07XX*	126	0	6	10	6	1	4	2699	298	29	2323	17	6936
L-d6	Landrace	*DQB1*0203*	*DQB1*0801*	*DQB1*0203*	*DQB1*08XX*	719	0	152	1203	3	46	62	479	616	4	3626	85	7345
L17-33d1	Landrace	*DQB1*0601*	—	*DQB1*06XX*	—	52	5	40	48	0	38	47	2	1557	0	202	7	6421
L-7br	Landrace/Yorkshire	*DQB1*0201*	*DQB1*0801*	*DQB1*02XX*[b]	*DQB1*08XX*	100	345	18	26	0	0	3	134	244	10	2330	22	6718
LW-20	Large white H08/H01	*DQB1*0101*	*DQB1*0401*	*DQB1*01XX*	*DQB1*04XX*	4777	0	12	10	0	6175	179	0	0	21	224	6	7287
LW-18	Large white H38/H01	*DQB1*0201*	*DQB1*0202*	*DQB1*02XX*[b]	*DQB1*02XX*[c]	66	393	1927	26	0	1	18	0	0	5	12	9	7227
MH-8106	Mexican hairless	*DQB1*0401*	*DQB1*0801*	*DQB1*04XX*	*DQB1*08XX*	243	4	9	30	10	7562	21	214	328	9	3076	52	8155
MH-6	Mexican hairless	*DQB1*0201*	*DQB1*0401*	*DQB1*02XX*[e]	*DQB1*04XX*	9	380	32	12	7	6371	3	0	0	20	4	0	7489
MH-7	Mexican hairless	*DQB1*0201*	*DQB1*0801*	*DQB1*02XX*[e]	*DQB1*08XX*	98	266	11	12	0	1	9	92	172	0	2222	8	6938
MH-9	Mexican hairless	*DQB1*0701*	*DQB1*0801*	*DQB1*0701*	*DQB1*08XX*	102	0	0	2	6	6	16	1968	126	70	2241	10	6417
N-j	NIBS	*DQB1*0201*	—	*DQB1*02XX*[b]	—	26	580	14	23	0	12	21	25	41	4	15	6	6428

[a]The oligo probe names and allele names are indicated in the top rows of the fifth column. The preset cutoff value for each fluorescing oligobead set was used to discriminate between positive and negative controls as described previously for HLA genotyping by the PCR–SSOP–Luminex method (12). The positive fluorescence intensities over the given cutoff values that indicate the allelic pattern are shaded as a gray box.

[b]Five DQB1 alleles, **0201, *02du01, *02kg02, *02La03,* and **02me01* are included in **02XX*[b] allele group.

[c]Two DQB1 alleles, **0202* and **02zs16* are included in **02XX*[c] allele group.

[d]Two DQB1 alleles, **0501* and **05sp06,* are included in **05XX*[d] allele group.

[e]Two DQB1 alleles, **0502* and **0503* are included in **05XX*[e] allele group.

MH-8106, MH-9, and MH-7 (Figure S2; Table S2). Because no high detection intensity was obtained in these six samples with the Q01 probe, which was designed for the detection of *DQB1*01XX* (and *DQB1*0302* or *DQB1*0303*), the possibility of finding *DQB1*01Lu01* or *DQB1*01sh01* in the six samples with the Q08 probe was ruled out. The absence of *DQB1*01Lu01* or *DQB1*01sh01* in these six samples was confirmed by PCR-SBT (Table 2).

The *DRB1*05XX* and *DRB1*02zs13* alleles have identical sequences for hybridization with the R05 probe, which could result in ambiguous typing using the PCR–SSOP–Luminex method. In fact, in the case of the MH-8106 sample, the R05 probe could not differentiate between *DRB1*02zs13/–*, *DRB1*02zs13/05XX* and *DRB1*02XX/05XX*, whereas the sample was assigned as *DRB1*0201/0501* by the PCR-SBT method (Figure S1; Table S1). Therefore, the sequences of *DRB1*02zs13* were presumed to be a mixture of the *DRB1*02* and *DRB1*05* alleles. It is possible that the exon 2 region of *DRB1*02zs13* has the sequences in the 5′ half similar to those of the *DRB1*02* subgroup and the R02 probe, whereas the sequences in the 3′ half are similar to those of the *DRB1*05* subgroup and the R05 probe. To obtain reliable results with the PCR–SSOP–Luminex method, it is important to design allele-specific probes based on known gene sequence differences. However, further sequence information will be needed for several alleles including *DRB1*02zs13*, and especially for the allele sequences for which two-digit allele group names were assigned and for the provisional alphanumeric allele names containing the two lower-case letters such as *DRB1*02ka08* and *DQB1*04sk51*. Nevertheless, as an overall result, genotypes were determined correctly for 24 samples at the SLA-DRB1 locus and for 22 samples at the SLA-DQB1 locus, as confirmed by the sequencing results for each DNA sample.

One population of Landrace pigs was used to evaluate the PCR–SSOP–Luminex method for the detection of SLA-DRB1 and -DQB1 allele diversity at the haplotype level. This population, designated as Miyagino L2, consisted of 283 animals that were selected by three criteria, daily weight gain from 30 to 105 kg body weight, backfat thickness measured by ultrasound technology in animals weighing 105 kg, and the area of mycoplasma pneumonic lesion measured in slaughtered sib pigs for five generations at the Miyagi Livestock Experimental Station from 2003 to 2008. Average population size of each generation was 14 boars and 39 gilts. These pigs were included in another multi-institutional study on the analyses of immunological and economical traits (19, Suzuki et al., unpublished data). Seven and nine variations of alleles in the SLA-DRB1 and -DQB1 genes were observed, respectively, making a total of 44 unique class II genotypes (Table S3, Supporting Information). There was an ambiguity in the SLA-DQB1 locus for five samples with positive signals obtained for the Q01 and Q03 probes, which could not differentiate between *DQB1*01XX/*0302* or *DQB1*0303* and *DQB1*0302* or *DQB1*0303*. However, the SLA class II genotype for the five samples was estimated to be genotype 5, consisting of *DRB1*01XX/04XX* and *DQB1*01XX/0302* or *0303*, according to an analysis of the inheritance and segregation of the alleles in their descendents (data not shown).

To evaluate the accuracy of the 44 class II genotypes assigned by the PCR–SSOP–Luminex method, the SLA-DRB1 and -DQB1 alleles of 44 individuals each representing one of the 44 class II genotypes were analyzed by the PCR-SBT method. All of the 44 class II genotypes assigned by the PCR–SSOP–Luminex method in the samples were consistent with the SLA genotype information obtained for these samples by the SBT method (data not shown). For example, in one of the five samples with genotype 5, *DRB1*0101/0403* and *DQB1*0101/0302* or *0303*, the PCR-SBT assignment was the same as the results by the PCR–SSOP–Luminex method and also by the additional analysis of the inheritance and segregation of the alleles in their descendants (data not shown).

An analysis of the inheritance and segregation of the alleles in descendants of this pig population showed the presence of 12 low-resolution SLA class II haplotypes including the novel Lr-0.38 (Table S4, Supporting Information). Six (Lr-0.1, -0.2, -0.4, -0.12, -0.13, and -0.14) of the 12 haplotypes have been reported in several breeds including miniature pigs such as the NIH, Sinclair, Hanford and Korean native pigs as high-resolution counterparts that were designated by the SLA Nomenclature Committee (2, 6, 20, 21) and were therefore numbered accordingly. The Lr-0.23 haplotype that showed the highest frequency at 33.9% of the total number of SLA class II haplotypes in the Miyagino L2 was previously detected in four breeds of outbred pig populations including the Big pig and Korean native pigs (22, 8) (Ho, personal communication), Moreover, the novel haplotype, Lr-0.38, was also detected in the Miyagino L2 population, and showed a relatively low frequency. Thus, specific SLA class II types may have been unintentionally selected in this population because of certain favorable biological traits that are linked to the SLA complex. The relationship among the SLA class II types and the traits, including selective breeding by the traits in the Miyagino L2 population, will be presented in a future publication (Suzuki et al. (19), Ando et al. unpublished data).

In this study, PCR, hybridization and detection by the PCR–SSOP–Luminex method for SLA-DRB1 and -DQB1 typing were performed under the same reaction conditions. This PCR–SSOP–Luminex method, therefore, has reduced the number of manual procedures needed for the analysis of 96 samples in a 96-well tray for the two SLA class II loci by using the GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA, USA). Furthermore, it will be important in future to apply the PCR–SSOP–Luminex technique for simple and accurate SLA class I genotyping. However, because of the high sequence homologies among SLA class I functional genes and pseudogenes, we expect that it may be more difficult to design allele-specific probes

for each defined allele of the whole class I functional genes than it was for the class II functional genes. Nevertheless, the application of the PCR–SSOP–Luminex method as a starter for gathering information on the diversity of the SLA class II alleles at two or four digits will help us to better understand the role of SLA class II genes in disease-related phenotypes.

In conclusion, we have developed and performed a rapid and reliable SLA class II typing method for the detection of SLA-DRB1 and -DQB1 alleles at the two- or four-digit level in eight different breeds and a selectively bred population of pigs by using the PCR–SSOP–Luminex genotyping system. Our results showed that this typing system is a powerful high-throughput tool for the rapid and accurate multilocus genotyping of a large number of samples.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Predicted hybridization patterns of known SLA-DRB1 alleles detected with 17 oligonucleotide probes. The predicted positive reactions between the probes and particular alleles for the 57 SLA-DRB1 alleles detected with 17 specific oligonucleotide probes in the assay are indicated by the filled black boxes, which show the expected positive reaction between the probe and known two- or four-digit allele sequences in the reaction matrix.

Figure S2. Predicted hybridization patterns of known SLA-DQB1 alleles detected with 13 oligonucleotide probes. The predicted reactions between the probes and particular alleles for the 37 SLA-DQB1 alleles detected with 13 specific oligonucleotide probes in the assay are indicated by the filled black boxes, which show the expected positive reaction between the probe and known two- or four-digit allele sequences in the reaction matrix.

Table S1. Primer pairs used for amplification of the SLA class II-DRB1 and DQB1genes.

Table S2. Locus, name and sequences for 30 single sequence oligonucleotide probes (SSOPs).

Table S3. SLA class II genotypes assigned in a population of Landrace pigs (Miyagino L2).

Table S4. Low-resolution SLA class II haplotypes identified in Miyagino L2 pigs.

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Primordial Linkage of β2-*Microglobulin* to the MHC

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Primordial Linkage of *β2-Microglobulin* to the MHC

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β2-Microglobulin (β2M) is believed to have arisen in a basal jawed vertebrate (gnathostome) and is the essential L chain that associates with most MHC class I molecules. It contains a distinctive molecular structure called a constant-1 Ig superfamily domain, which is shared with other adaptive immune molecules including MHC class I and class II. Despite its structural similarity to class I and class II and its conserved function, *β2M* is encoded outside the MHC in all examined species from bony fish to mammals, but it is assumed to have translocated from its original location within the MHC early in gnathostome evolution. We screened a nurse shark bacterial artificial chromosome library and isolated clones containing *β2M* genes. A gene present in the MHC of all other vertebrates (*ring3*) was found in the bacterial artificial chromosome clone, and the close linkage of *ring3* and *β2M* to MHC class I and class II genes was determined by single-strand conformational polymorphism and allele-specific PCR. This study satisfies the long-held conjecture that *β2M* was linked to the primordial MHC (Ur MHC); furthermore, the apparent stability of the shark genome may yield other genes predicted to have had a primordial association with the MHC specifically and with immunity in general. *The Journal of Immunology*, 2011, 186: 3563–3571.

The adaptive immune system as defined in humans arose abruptly in a jawed vertebrate (gnathostome) ancestor ~500 million years ago. The major players of adaptive immunity, the rearranging Ag receptors (Ig and TCR), the Ag-presenting molecules (MHC class I and class II), and molecules involved in Ag processing (e.g., immunoproteasomes and the TAPs) are all present in sharks as the oldest extant jawed vertebrates but absent in all invertebrates and jawless fish (1). The MHC encodes the class I and class II proteins, which present foreign peptides to T cells to initiate adaptive immune responses, as well as the Ag processing molecules and a large number of other genes involved in various immune functions. The class I and class II tertiary structures are nearly identical, composed of four external domains, the two membrane-proximal domains being members of the Ig superfamily (IgSF) and the membrane-distal domains forming a unique structure called the peptide-binding region (PBR). However, the chain composition differs between class I and class II molecules: class II molecules are heterodimers of α- and β-chains each consisting of one half of the PBR, one IgSF domain, and transmembrane/cytoplasmic regions, whereas class I molecules are composed of an H or α-chain and the requisite L chain, β2-microglobulin (β2M), the former comprising the entire PBR, one IgSF domain, and transmembrane/cytoplasmic regions, and the latter only one IgSF domain. The remarkable similarity of the class I and class II structures clearly suggests that they were generated from a common ancestor, presumably by tandem duplication; thus, it has been assumed that class I, *β2M*, and class II genes were tightly linked at one point in evolution (1), although it is debatable whether the ancestor of class I/II molecule was class I- or class II-like or an unrecognizable common ancestor (2–5).

In all jawed vertebrates except teleost fish, a taxon having a highly modified genome correlating with a genome-wide duplication early in teleost evolution (6, 7), both MHC class I and II genes are closely linked within the MHC (8). Although class I genes are encoded in a region downstream of the class II region (2–4 Mb) in the MHC of most mammals, a single or low number of class I genes are found in close proximity to class I processing and (except teleost fish) class II genes in most nonmammalian vertebrates, in what is predicted to be the primordial organization (9–15). *β2M* is encoded in diverse regions outside the MHC in all the species examined to date, including mammals (16), birds (17), amphibians (18), and bony fish (19), and therefore the lack of linkage of *β2M* to the MHC and inconsistent synteny around *β2M* have been assumed to be a result of repeated translocations out of the MHC over evolutionary time or to serial translocations after the early loss of MHC linkage (20).

In this study, we characterized the nurse shark (*Ginglymostoma cirratum*) single-copy *β2M* gene and mapped it to the MHC. The primitive synteny preserved in this extant vertebrate validates early suppositions regarding MHC evolution and further suggests that other ancient features of the MHC also may be preserved.

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Abbreviations used in this article: BAC, bacterial artificial chromosome; C1, constant-1; IgSF, Ig superfamily; LOD, log of the odds; β2M, β2-microglobulin; NJ, neighbor-joining; PBR, peptide-binding region; ssCP, single-strand conformational polymorphism; ZFP, zinc finger protein.

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Materials and Methods

Animals

Genomic DNA was isolated from RBCs for mapping analysis from the nurse shark family as previously described (21). The procedure of animal use was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Maryland.

Bacterial artificial chromosome library screening

The 17 bacterial artificial chromosome (BAC) filters with 11-fold genomic coverage (22) were screened with radiolabeled full-length *β2M* or *ring3* probes under high-stringency conditions (23). Membranes were exposed to x-ray film for various lengths of time to obtain positive signals and the desired background. Putative positive clones were then re-spotted on nylon membranes for colony hybridization and tested by Southern blotting to confirm true positives. BAC insert DNA was isolated using the PhasePrep BAC DNA kit (Sigma-Aldrich), and the sequence was determined by shotgun sequencing at the sequencing facility at Tokai University with 7.5× coverage.

Sequence alignment and phylogenetic tree

Amino acid sequences of constant-1 (C1)-IgSF domains were aligned using the ClustalX2 program with minor adjustments. A rooted neighbor-joining (NJ) bootstrapped (1000 runs) phylogenetic tree (24) was constructed, and the consensus tree was then viewed with the TreeView program (25).

Database searches

Genome synteny in various species was retrieved and analyzed from publicly available Web sites as noted. Genes from mouse, chicken, human, opossum, and zebrafish were retrieved from GenBank (http://www.ncbi.nlm.nih.gov), and information on other genomes was retrieved from the following Web sites: elephant shark genome (http://blast.fugu-sg.org/); *Anolis* genome (http://genome.ucsc.edu/cgi-bin/hgGateway?db=anoCar1); *Xenopus* genome (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html); and *Fugu* genome (http://genome.jgi-psf.org/Takru4/Takru4.home.html).

In-house EST collection

We constructed the cDNA library using the Gateway System (Invitrogen) from adult nurse shark pancreas. To eliminate Ig genes, we first hybridized with Ig H and L chain probes under high-stringency conditions. Negative colonies (~8000) were then manually picked and sequenced from the vector end. All draft sequences were blastx searched against GenBank databases, and we obtained ~1150 sequences not specific to the pancreatic enzymes (Y. Ohta and M.F. Flajnik, personal observations).

Single-strand conformation polymorphism analysis

Nurse shark *ring3* primers were designed based on the sequence obtained from BAC GC_614H19 clone. Multiple primers were tried, and we selected the primer set anchoring exons 4 and 5 for the single-strand conformation polymorphism (ssCP) analysis. The primers were exon 4 forward, 5′-GTTAACACCTGCACCAAAAT-3′; and exon 5 reverse, 5′-ATTGGGACCTGAGACACAGT-3′. PCR was performed at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 10 min using 2–500 ng genomic DNA as template. The ~1340-bp PCR product was cleaned by gel extraction. The ssCP gel (0.5× MDE gel; Cambrex Bio Science Rockland) was run at 16°C for 30 h in 0.6× Tris/borate/EDTA buffer with 1 W constant power.

Allele-specific PCR

Nurse shark *β2M* sequences were obtained from family 2 with known MHC haplotypes. PCR was performed using a forward primer in intron 2 (NSB2mint2For: 5′-TTACACATCACCACCACCTC-3′) and a reverse primer designed from the IgSF exon (exon 3) (NSB2mex3Rev: 5′-GATTGATTCAGTAGC-3′). We amplified *β2M* gene fragments from several animals carrying different maternal and paternal haplotype combinations to find allele-specific polymorphisms. After we identified a two-nucleotide deletion in intron 2 in the paternal haplotype in animals belonging to groups "i" and "j" (*p3*), allele-specific primers were designed for each gene in which deletions are positioned at the third and fourth nucleotide positions at the 3′-end of primers. PCR was performed using a combination of allele-specific and NSB2mex3Rev primers at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 10 min using 2–500 ng genomic DNA as template. We also found animals with the "CC-deletion" allele in two other families (1 and 3).

Northern blotting

Total RNA was isolated from various nurse shark tissues by using the TRIzol reagent (Invitrogen). Twenty micrograms of total RNA was electrophoresed and blotted onto Optitran Nitrocellulose membrane (Schleicher & Schuell). The membrane was hybridized with full-length shark probes and washed under high-stringency conditions (23).

Southern blotting

Genomic DNA (10 μg) was digested with various restriction enzymes to obtain useful RFLP in unrelated sharks with multiple enzymes. The IgSF exon was used to determine the number of loci for *β2m* under high-stringency conditions (23). Hybridization with MHC class I leader and α1 domain probe was performed under low-stringency conditions (23). To determine the MHC groups in the shark family 2, we digested genomic DNA with HindIII and hybridized with radiolabeled probe including the leader–α1 domains of MHC class I under high-stringency conditions.

Sequence analysis of MHC class I alleles and sire designation

MHC class I sequences were obtained from PCR amplification with primers from α1 domain forward, 5′-GGTCGGTTATGTGGATGATC-3′; and α2 domain reverse, 5′-TTGCAGCCACTCGATACA-3′. PCR amplification was performed for 4 min at 94°C, followed by 35 cycles of 94°C for 1 min, 56°C for 1~2 min, 72°C for 1 min, and a final extension at 72°C for 10 min. An ~550-bp fragment amplicon was cloned into the pCRII TA cloning vector (Invitrogen), and individual clones were sequenced. Nurse shark families 2 and 3 were genotyped using 12 DNA microsatellite markers and assigned sires (E.J. Heist, J.C. Carrier, H.L. Pratt, and T.C. Pratt, submitted for publication).

Statistical analysis of linkage

We used parametric linkage analysis to formally assess the evidence for linkage of *β2M* to the MHC region in the offspring of deletion-carrying sires. This approach assesses the odds of the likelihood of obtaining the observed data set if the two loci are linked versus if the loci are not linked, showing as a log of the odds (LOD) score. The paternal sibships were determined based on consolidated data from combination of Southern blotting, sequencing of MHC class Ia alleles, and microsatellite analyses (shown in Table I).

The LOD score is calculated as follows when parental phase (linkage status) is known: $\mathrm{LOD} = \log 10\ \{[(\theta)^{R}\ (1-\theta)^{NR}]/(0.5)^{R+NR}\}$, where θ is the recombination fraction, *NR* is the number of nonrecombinant offspring, and *R* is the number of recombinant offspring.

Because the parental phase was unknown in the current study due to a lack of grandparental genotypes, a phase ambiguous LOD score was first calculated for each family by taking the log of the average odds for the two possible phases (1 and 2 in Table I), and the resulting LOD scores were then summed over the two families to obtain the LOD score at a given recombination fraction. LOD scores were calculated at recombination fractions between 0 and 0.5 to obtain the recombination fraction where the LOD score was maximized (26). The corresponding *p* value was calculated using a one-sided χ^2 test of LOD ×2 ($\log_e 10$) (27).

Results

Characterization of nurse shark β2M

Cartilaginous fish are the oldest living vertebrates having an adaptive immune system centered upon Ig, TCR, and MHC (1). When it was suggested that class I and class II genes may have evolved in separate linkage groups from studies of teleost fish (28), we demonstrated in family studies that the two MHC classes were closely linked in two shark species, nurse shark and banded houndshark (21). To gain further insight into the primordial MHC organization, we have isolated many shark genes associated with adaptive immunity, including *β2M*. The full-length *β2M* clone was found in an in-house EST collection (GenBank accession number HM625831), as well as from a previously published genomic sequence (GenBank accession number GQ865623) (29), and the deduced amino acid sequence was aligned with β2M from other species (S1). As was noted in previous studies, evolutionarily

conserved residues are either found in all C1-IgSF (or just IgSF) domains (29, 30) or are predicted to be at class Iα-chain interaction sites (31). Some cartilaginous fish β2M have potential N-glycosylation sites that are rare in tetrapods but present in several bony fish species (32). Consistent with previous studies (33, 34), phylogenetic tree analysis revealed that cartilaginous fish β2M clustered with the orthologous proteins and to the IgSF domains of MHC class IIA/DMA, suggesting that they share the most recent common ancestor (Fig. 1*A*). Also consistent with previous studies (33), the IgSF domains of class IIB and class Ia shared the most recent common ancestor. *β2M* expression pattern seems to coincide with MHC class I expression (Fig. 1*B*).

Mapping of β2M *to the MHC in family studies*

Two families of nurse sharks previously were used to map several genes to the MHC (21, 36, 37). All of these families showed multiple paternity, at least five fathers in family 1 and seven in family 2. Southern blotting analysis using many restriction enzymes demonstrated that *β2M* is a single-copy gene (five representative digestions are shown in Fig. 1*C*); unfortunately, no RFLPs were obtained to test the linkage status, and thus we sequenced the gene from animals with different MHC haplotypes, hoping to find polymorphisms. A two-nucleotide deletion was detected in one of the paternal *β2M* alleles "*p3*" from groups "i" (p3/m2) and "j," (p3/m1) from family 2 with 39 members (Fig.

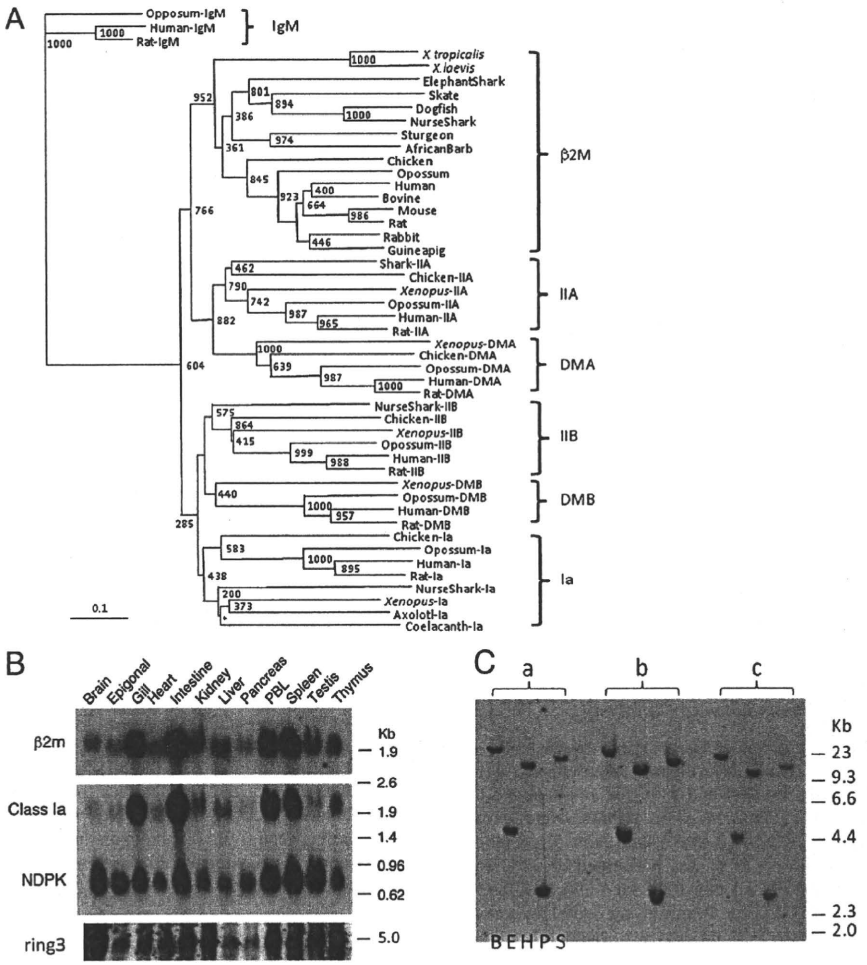

FIGURE 1. *A*, Phylogenetic tree analysis of β2M. GenBank accession numbers used for this analysis are as follows. β2M: M17987 (human), X69084 (bovine), NM_009735 (mouse), Y00441 (rat), P01885 (rabbit), P01886 (guinea pig), M84767 (chicken), P21612 (turkey), AAM98336 (opossum), BQ389924 (*X. tropicalis*), AAF37230 (*X. laevis*), L05536 (carp), NP_571238 (zebrafish), L63534 (trout), CAA10761 (cod), AAG17535 (salmon), CAB61324 (Siberian sturgeon), AAN40738 (Japanese flounder), CAD44965 (African barb), O42197 (catfish), CA330181 (*Fugu*), AAN62852 (skate), and CX197532 (dogfish). Class IIa: AAF66123 (nurse shark), AAL58430 (*X. laevis*), AAA59760 (human), AAV40625 (rat), NP_001001762 (chicken), XP_001376764 (opossum). Class IIb: AAF82681 (nurse shark), AAB86437 (human), NP_001008884 (rat), BAA02845 (*X. laevis*), NP_001038144 (chicken), AAB68822 (opossum). Class Ia: BAD92354 (human), AAC53397 (rat), AAL59857 (nurse shark), NP_001079241 (*X. laevis*), AAG28835 (chicken), NP_001165308 (opossum). IgM: AAD21191 (opossum), P01871 (human), AAH92586 (rat). DMB: ABB85336 (*X. laevis*), NP_002109 (human), NP_942035 (rat). DMA: NP_006111 (human), NP_942036 (rat), ACY01474 (chicken), XP_001377359 (opossum). The NJ tree was rooted with the fourth constant IgSF domains of IgM, and bootstrapping analysis was done after 1000 runs. Values are noted at the branch nodes, and the asterisk (*) indicates no significant value. The scale indicates divergence time (genetic distance). Teleost fish that underwent a third round of genome expansion ("3R") are omitted from this analysis because the sequences were more divergent and skewing the tree topology. DM genes have not been identified in any fish. *B*, Expression profiles of *β2M*, class Ia, and *ring3* via Northern blotting. Twenty micrograms of total RNA isolated from various nurse shark tissues was loaded onto the gel, blotted, and hybridized with full-length shark *β2M* and *ring3* probes and washed under high-stringency conditions (23). Nucleoside-diphosphate kinase (*NDPK*) (35) was used as a loading control. *C*, There is only one *β2M* locus in the nurse shark genome. Genomic Southern blot analysis was performed under low-stringency conditions (23) using the IgSF exon with three wild sharks (*a*, *b*, *c*) whose DNA was digested with five different restriction enzymes (from *left* to *right*: *Bam* HI, *Eco* RI, *Hin* dIII, *PST* I, and *Sac* I).

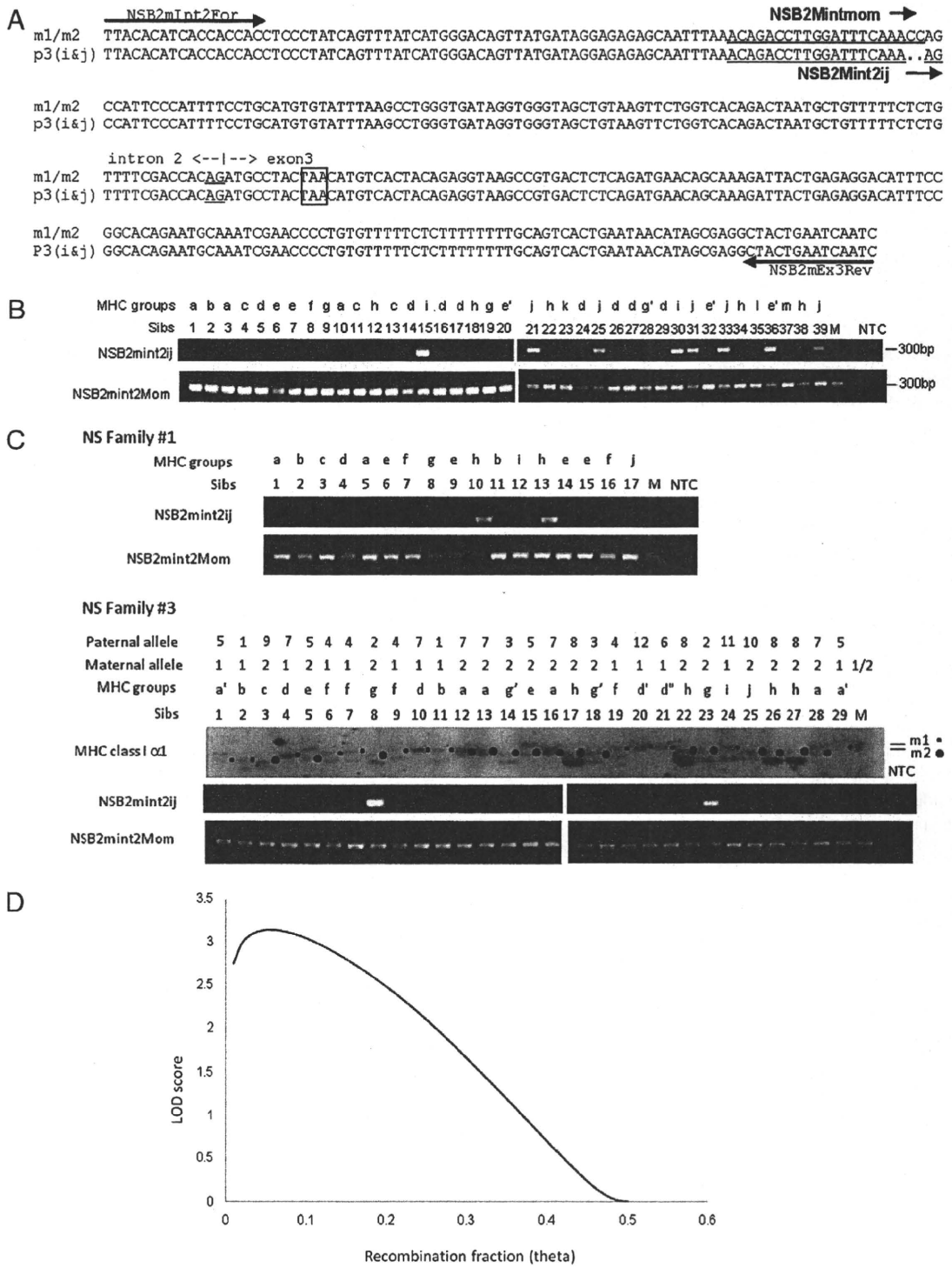

FIGURE 2. The shark *β2M* is linked to the MHC. *A*, The two-nucleotide (CC) deletion polymorphism was found in intron 2 of *β2m* sequences in "*p3*" paternal allele from siblings belonging to the groups "i" and "j." Thus, allele-specific primers were designed based on this polymorphism. All primers are underlined. The ends of coding regions are boxed. The (AG) at the end of intron 2 is underlined. *B*, PCR was carried out with a combination of allele-specific and universal NSB2mEx3Rev reverse primers. Presence or absence of the amplicon using the "*p3*"-specific primers was used for typing (*top gel*) the family 2 with 39 offsprings. Maternal primers were used for the positive control (*bottom gel*). Forward primers are indicated on the *left side* of the gels, and mother and sibling numbers are indicated above the gel along with MHC groups (36). *C*, Allele-specific PCR in the families 1 and 3. Only two animals belonging to the MHC groups "h" possessed the "CC-deletion" allele, and two animals belonging to the "g" groups had this allele in family 3. We partially typed family 3 based on the MHC groups by sequencing of the PBR of the class Ia alleles (maternal and paternal alleles are designated as numbers above the gel) and by Southern blotting with a probe containing MHC class Ia leader and α1 domains (small dot, band for maternal haplotype 1; large dot, maternal haplotype 2). The "*p2*" allele of the "g" group is the only haplotype possessing the "CC-deletion" allele of *β2M*. *D*, Plot of LOD scores at corresponding recombination fractions. The sums of the two families were used (Supplemental Table I).

2*A*), and allele-specific PCR was performed in all members of the nurse shark families in our collection (Fig. 2*B*). Family 1 had two positive members that shared the same paternal MHC haplotype (group "h") (Fig. 2*C*). In family 2, all seven members of groups "i" and "j" bearing the paternal MHC haplotype "*p3*" were positive as well as one other offspring belonging to the "e′" group. Family 3 with 29 offspring, which had not been MHC-typed previously, was tested, and two members were positive for the *β2M* polymorphism (Fig. 2*C*). Typing of this family by Southern blotting as well as sequencing of the class Ia alleles in all offspring showed that these two animals share the same paternal MHC haplotype (Fig. 2*C*). Thus, a total of 11 of 12 siblings positive for the *β2M* polymorphism in three families showed precise cosegregation with certain MHC haplotypes. In addition, 73 of 74 siblings with many other haplotypes lacked this polymorphism, further strongly indicating that *β2M* does not segregate independently of the MHC. The one discordant animal in family 2 (sib 36, group "e′") was also typed by microsatellite analysis and shown to have been sired by the same father as offspring in the "i" and "j" groups; thus this father had the MHC haplotypes "*p3*" and "*p6*" (E.J. Heist et al., submitted for publication), consistent with a paternal intra-MHC recombination event in sib 36. To quantify formally the evidence for linkage of *β2M* to the MHC, we considered all offspring of the two deletion-carrying sires (found within families 2 and 3) as assigned by Southern blotting with class I probes (Fig. 2*C*) (36), sequences of MHC class I alleles (Fig. 2*C*, Table I), and microsatellite analysis (E.J. Heist et al., submitted for publication) (Table I). Family 1 sires have not been microsatellite-characterized, and therefore family 1 was not included in the analysis. We performed a parametric linkage analysis (26) to evaluate the evidence for *β2M* and MHC synteny and obtained a maximum LOD score of 3.14 [1378:1 odds of linkage versus no linkage, equivalent to $p = 7 \times 10^{-5}$ (27)] at a θ of 0.056 (Supplemental Table I, Fig. 2*D*).

β2M *is adjacent to MHC-linked* Ring3

Ring3 (or *BRD2*) is a putative nuclear transcriptional regulator and a nuclear kinase required for early development (38–41) with no defined immune functions but nevertheless linked to the MHC of all other gnathostomes and to the "proto-MHC" in lower deuterostomes (42). A portion of *ring3* was initially cloned via degenerate PCR from nurse shark spleen cDNA, and this short fragment was used as a probe to isolate a full-length cDNA from a phage library. BLAST searches and phylogenetic tree analysis confirmed the orthology of nurse shark *ring3* to that of other species (GenBank accession number HM625830) (Fig. 3*A*). The nurse shark *ring3* is ubiquitously expressed (Fig. 1*B*). To ensure that the shark *ring3* is linked to the MHC as in all other species examined (8), we performed ssCP analysis using siblings of family 2 (Fig. 3*B*). Two distinguishing *ring3* bands corresponding with the maternal MHC allele *m2* were found in those siblings possessing this allele (groups "i" and "d" in Fig. 3) with 100% fidelity, demonstrating that *ring3* is closely linked to the MHC and further confirming the *β2M* linkage. We identified other BAC clones that were either *β2M*- or *ring3*-single-positive; unfortunately, none of them was positive for other MHC genes, again consistent with larger intergenic distances in sharks compared with those of other species (36). Chen et al. (29) drew a premature conclusion of non-MHC linkage; however, determining the linkage status of *β2M* (or almost any gene) based on a single BAC sequence is not sufficient for the shark genome, where there are large intragenic and intergenic distances. Several nurse shark BAC clones (22) were isolated with the *ring3* and *β2M* probes, and some of them were positive for both genes. As previously reported (29), the *β2M* gene contains at least three exons, having a similar genomic organization and size to other species. The shark *ring3* gene spans ~20 kb and contains 12 exons, which is approximately twice as large as mammalian *ring3* genes (e.g., 12.8 kb and 9.7 kb for human and mouse, respectively), consistent with a larger gene size found in most shark MHC genes (36). Sequencing through an entire BAC clone (GC_614H19) confirmed that the *β2M* and *ring3* genes were adjacent to each other ~45 kb apart (Fig. 4).

Genetic descent of β2M

The chromosomal location of the *β2M* gene varies greatly among vertebrate species (Fig. 5). Genomic synteny is well conserved in

Table I. List of sibs used for statistical analysis

	MHC[a]		Haplotypes		m-Satellite	Phase	
Sib No.	Old Group	New Group	MHC Class Ia	β2m	Sire	1	2
Family 2							
15	i	i	m2/p3	del	4	NR	R
30	i	i	m2/p3	del	4	NR	R
21	j	j	m1/p3	del	4	NR	R
25	j	j	m1/p3	del	4	NR	R
31	j	j	m1/p3	del	4	NR	R
33	j	j	m1/p3	del	4	NR	R
39	j	j	m1/p3	del	4	NR	R
20	e′	e′	m1/p6	ins	4	NR	R
32	e′	e′	m1/p6	ins	4	NR	R
36	e′	e′	m1/p6	del	4	R	NR
28	g′	g′	m2/p6[b]	ins	4	NR	R
13	c	g′	m2/p6[b]	ins	4	NR	R
Family 3							
8		g	m2/p2	del	2	NR	R
23		g	m2/p2	del	2	NR	R
6		d	m1/p4	ins	2	NR	R
7		d	m1/p4	ins	2	NR	R
9		d	m1/p4	ins	2	NR	R
19		d	m1/p4	ins	2	NR	R

[a]Old group is taken from Ref. 28, and new groups are assigned in this study.
[b]MHC class Ia sequences revealed that sib 13 is further categorized with group g′ in this study.
del, CC-deletion haplotype; NR, nonrecombinant; R, recombinant.

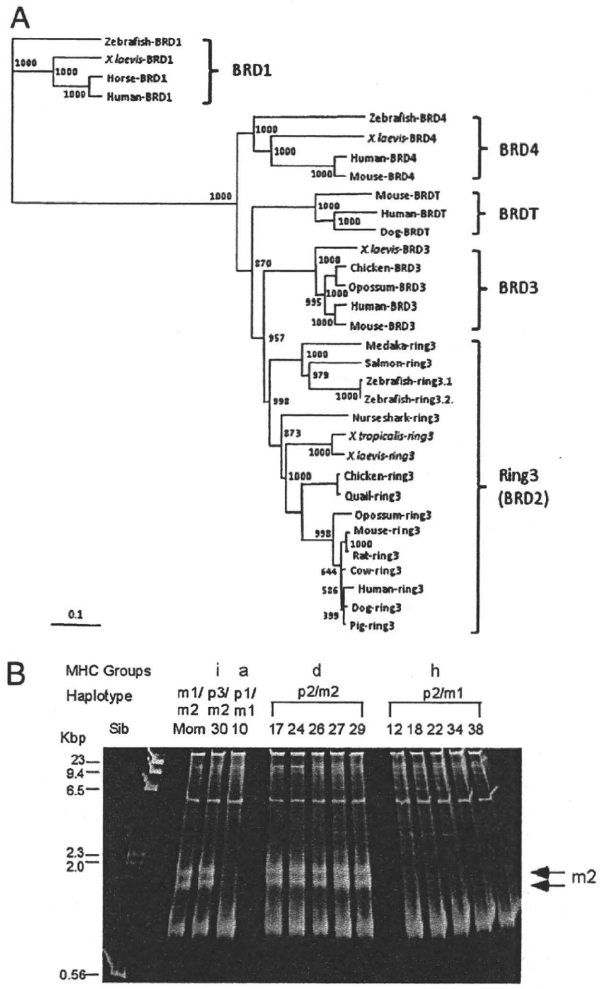

FIGURE 3. *A*, Phylogenetic tree analysis of Ring3 and homologues. GenBank accession numbers used in this analysis are as follows. Ring3 (BRD2): CAM25760 (human), AAY34703 (bovine), CAI11405 (dog), CAA15819 (mouse), CAE83937 (rat), XP_001369391 (opossum), CAN13285 (pig), CAA65449 (chicken), BAC82511 (quail), AAI68574 (*X. tropicalis*), AAI30180 (*X. laevis*), CAK04960 (zebrafish-1), CAD54663 (zebrafish-2), ABQ59684 (salmon), BAD93258 (medaka). Additional accession numbers for Ring3 homologues used for this analysis are the following: BRD3: AAI29055 (*X. laevis*), NP_031397 (human), NP_075825 (mouse), XP_001365890 (opossum), XP_425330 (Chicken). BRDT: NP_473395 (mouse), NP_997072 (human), XP_537079 (dog). BRD4: NP_490597 (human), NP_065254 (mouse), NP_001104751 (zebrafish), AAH76786 (*X. laevis*). BRD1: NP_001157300 (horse), XP_698063 (zebrafish), NP_001085846 (*X. laevis*), CAG30294 (human). Gene names are noted after species name. BRD1 does not map to an MHC paralogous region, whereas BRDT, BRD3, and BRD4 are found in the MHC paralogous regions. The tree was constructed using the NJ method, rooted with BRD1, and bootstrapping analysis was done with 1000 runs. Values are noted at the branch nodes, and an asterisk (*) indicates no significant value. The scale indicates the divergence time. *B*, The shark *ring3* maps to the MHC. Primers from exons 4 and 5 were used for PCR amplification and ssCP analysis. The ~1440-bp amplicon from the siblings along with mother shark genomic DNA were loaded on an 0.5× MDE gel. Under these conditions, "*m2*" was identified as two distinctive bands indicated as arrows. Mother and sibling numbers are indicated above the gel along with MHC groups and haplotype combinations from previous work (36).

the region of chicken *β2M* relative to humans except for deletions of certain genes (43), and the same seems to be true for the *Anolis* lizard in which the synteny near the *β2M* gene (GenBank accession number FG703784, etc.) is conserved (genomic scaffold-670, 634,364 bp) (Supplemental Table II). Mouse *β2M* is linked to the so-called minor histocompatibility complex on chromosome 2 (16) and is located within a small region syntenic to human chromosome 15 (43). Notably, a smaller syntenic block is embedded with genes mapping to human chromosome 14q11.2 in a marsupial, the opossum. Although these regions can be accounted for by block translocations or syntenic breakpoints, synteny is not conserved in species from lower vertebrate classes as *β2M* is surrounded by genes mapping to various human chromosomes. The amphibian *Xenopus β2M* is linked to the genes mapping to human chromosomes 16 and 17 (genomic scaffold-673). In zebrafish, *β2M* (chromosome 4) is surrounded by genes mapping to human chromosome 12p12, and various locations in the human genome have syntenic regions on the *Fugu* scaffold-171 (638,182 bp). As mentioned above, the teleost fish experienced a recent genome-wide duplication ("3R"), and there is another *β2M* locus in the zebrafish genome that is ~60% similar to its paralogue at the amino acid level. Notably, the second *β2M* locus is found at the telomeric region of chromosome 8 and is distantly linked to a class IIA gene and two class Ib genes of the L-lineage (44) (Supplemental Table II). Although the *β2M* linkage is not very close (i.e., 6.5 Mbp apart) in this chromosomal region (considering the rapid reorganization of syntenic regions in the teleost fish), this linkage group of class II/class I/*β2M* is likely a vestige of the primordial synteny. Combining all of the evidence, our study in nurse shark demonstrates that *β2M* was originally encoded in the MHC, and from extensive database analysis in many taxa, this gene underwent multiple translocations in gnathostomes, either stepwise or independently from the MHC (Fig. 5).

Discussion

Compared with other vertebrate models (e.g., chicken or teleost fish), the shark genome seems to be stable, first demonstrated with the linkage of MHC class I and II genes (21), which was lost in bony fish (28), and later with linkage conservation of genes found in the mammalian MHC class III region (37). These MHC linkage data are consistent with global genomic studies in the elephant shark suggesting that cartilaginous fish have greater preservation of synteny than is found in any teleost model (45, 46). The *β2M* linkage to the shark MHC demonstrated here is likely the primordial condition, thus further supporting the conservation of the cartilaginous fish genome. Furthermore, the close proximity of class I, class II, and *β2M* is consistent with the theory that they were derived from a common ancestor by tandem (*cis*) duplication. The close linkage of *β2M* and class I may have regulated their original coordinated expression and upregulation. Class I and *β2M* expression is nearly identical in the nurse shark (Fig. 1*B*), but in other vertebrates β2M is made in excess (47). Furthermore, the number of *β2M* loci is expanded in rainbow trout (48) and polyploid *Xenopus* species (18).

Unlike class II genes, class I genes are extraordinarily plastic. Besides the MHC-linked classical class Ia genes, there are also many nonclassical class Ib genes with varied functions, some encoded in the MHC and others not. The majority of class Ib proteins associates with β2M as well, and it has been speculated that there was an advantage of translocation of *β2M* out of the MHC so that it would not be subject to duplications and deletions (19), like class I genes in many vertebrates. Consistent with the idea of maintaining genomic stability, but in contrast to class I and class II genes, both *β2M* and *ring3* genes are in a very stable part of the shark MHC, with very few polymorphisms and transposable elements (Fig. 4); there was no polymorphism detected by using restriction enzymes/Southern blotting with either the *ring3* or *β2M* probe. Although there are a few bony fish species in which

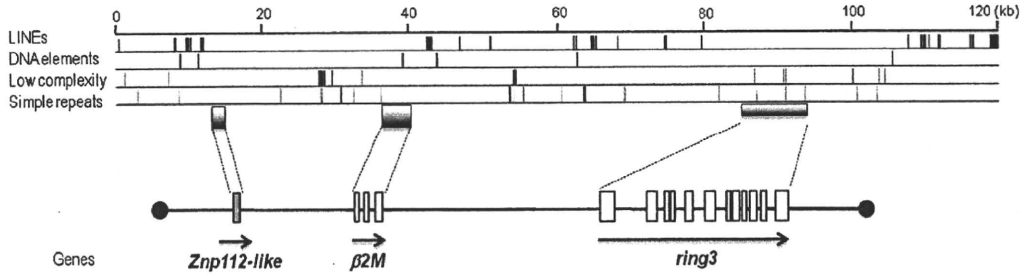

FIGURE 4. Map of BAC clone GC_614H19. Gene orientation is indicated as arrows and exons are shown in boxes. Only one exon for *ZFP112-like* gene was identified based on the similarity to other species. The positions of repetitive elements are shown above the map classified into four different categories. The total interspersed repeats are found in ~5.35% of the sequences, consisting of ~4.74% of LINEs and ~0.63% of simple repeats. Each exon is indicated as a box, and transcriptional orientations are shown with an arrow in the 5′ to 3′ direction. The sequence has been deposited in the DNA Data Bank of Japan under accession number AB571627.

the number of *β2M* loci has been expanded (49), and there are two loci in the tetraploid *Xenopus laevis* (18), generally these species are exceptions. There seems to be only one *β2M* locus in the nurse shark genome, because genomic Southern blotting with many restriction enzymes yielded a single band with an exon-specific probe (Fig. 1*C*).

The primordial linkage of *β2M* to the MHC does not contribute to the debate on which gene came first, class I or class II. Among the various IgSF domains, the C1-type is a rare form, found primarily in molecules associated with adaptive immunity (50). Therefore, it is reasonable to propose that C1-type IgSF-encoding genes like *β2M* were present in the "proto-MHC," which then acquired the PBR from another gene family. Furthermore, it has been speculated that all molecules containing C1-type IgSF domains arose from a common ancestor, and thus an Ig/TCR precursor may have originated from the "proto-MHC" (20). Consistent with previous studies dating back almost 30 y (3, 5, 33, 34), our phylogenetic analysis demonstrated a common origin for the class IIA/*DMB*/*β2M* and the class Ia/*DMA*/class IIB lineages, and all of these genes share an ancestral C1 domain-encoding exon that emerged after the split between Ag receptors and MHC genes (Fig. 1*B*). Whereas *class IIA*, *β2M*, *class IIB*, and *class Ia* share an immediate common ancestor that arose by tandem duplication from the ancestral molecule, each *DM* gene was apparently generated by tandem duplications of class IIA and class IIB, perhaps early after the emergence of tetrapods, as no *DM* genes have been found in the teleost or cartilaginous fish; the maximum likelihood and Bayesian inference trees favor this scenario (S2). The NJ tree (Fig. 1*B*), however, suggests that shark class IIA and IIB genes cluster with class II genes from other species rather than at the basal position of class II/*DM*, suggesting that sharks may indeed possess *DM*.

An orthologous gene related to the ancestor of *ring3* is present in the urochordate (e.g., amphioxus) "proto-MHC" (42), and thus the MHC-linkage of *ring3* in sharks is not surprising. To determine the linkage status in other cartilaginous fish species, we examined the elephant shark genome. Current analyses of the elephant shark genome (46) has yielded only short (<1 kbp) scaffolds (AAVX01540028.1) in which we only identified the *β2M* C1 domain. Three scaffolds were found to contain some exons of the elephant shark *ring3* gene [AAVX01538535 (754 bp), AAVX01069837 (5232 bp), AAVX01012433 (4324 bp)]; however, the assembly is still in its early stages. Further progress in this genome project will reveal the synteny around *β2M* and all of the other MHC genes and likely provide insight into the natural history of the adaptive immune system by revealing other genes that have been translocated out of the MHC during vertebrate evolution. For example, there is good evidence from various vertebrates that both IgSF- and C-type lectin-containing NK cell receptor genes (in humans, they are encoded in leukocyte receptor complex and NK complex, respectively) and the

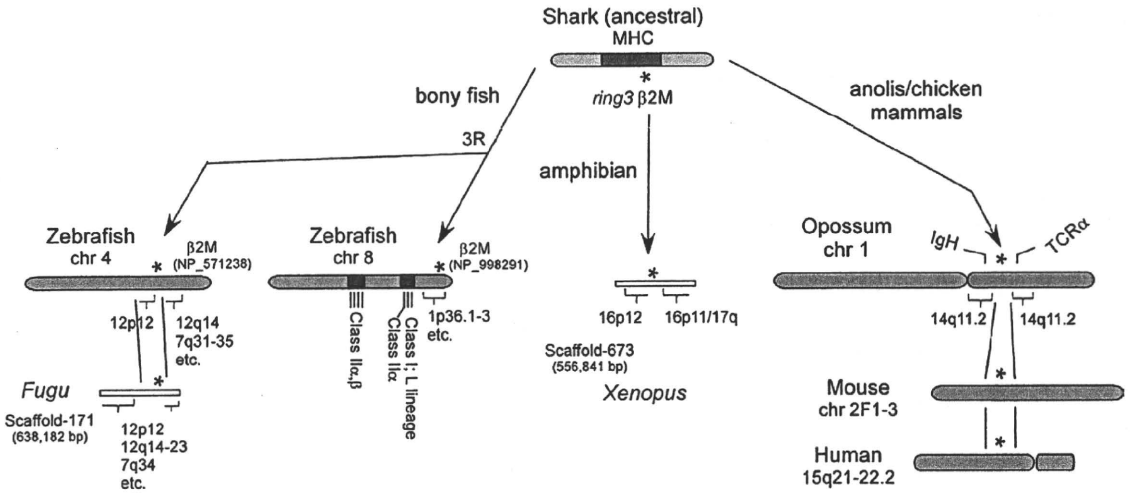

FIGURE 5. Inconsistent synteny of *β2M* among vertebrate species. Genomic synteny of *β2M* is not consistent in bony fish and *Xenopus*, suggesting that multiple translocations of *β2M* occurred over evolutionary time. An asterisk (*) indicates the location of the *β2M* gene, and brackets indicate the genomic regions corresponding with the particular human chromosome. The detailed gene assignments can be obtained in Supplemental Table II. IgH and TCRα loci are marked in opossum chromosome 1.

MHC were genetically linked at an early point in vertebrate evolution (20, 51, 52), suggesting that NK receptors co-evolved with MHC proteins. We have found a fragment of a zinc finger protein (ZFP), ZFP112-like, in BAC clone GC_614H19, adjacent to *β2M* (Fig. 5). *ZFP112* is found on human chromosome 19q13.2 near *FcRn* (19q13.3), a nonclassical class Ib molecule, and the leukocyte receptor complex (19q13.4). This region had been suggested to be an MHC paralogous region by pericentric inversion of 19p13.1. Whether the nurse shark *ZNF112* is a pseudogene or divergent from human/rodent *ZFP112* genes, the linkage of *ZFP112* suggests that the linkage of NK receptor(s) and MHC could be preserved in the shark genome. Furthermore, we found *β2M* on the same chromosome as *TCRα/δ* in horse (chromosome 1), cow (chromosome 10), and both *TCRα/δ* and *Ig* in the opossum genome (Fig. 5, Supplemental Table II). In addition, Ag receptor loci and other genes involved in immune defense (e.g., B7 ligands and Fc-like receptors) are linked to genes related to the *Xenopus* MHC (Y. Ohta and M.F. Flajnik, manuscripts in preparation), and cathepsins S and L are found on MHC paralogous regions in mammals (20). Such evidence is consistent with our hypothesis that Ag receptors (*TCR, Ig*), NK receptors, and other genes involved in Ag processing and generally in immune function might have been linked in a "pre-adaptive immune complex" in the ancestral configuration.

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Disclosures

The authors have no financial conflicts of interest.

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NFKBIL1 Confers Resistance to Experimental Autoimmune Arthritis Through the Regulation of Dendritic Cell Functions

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Abstract

We and others have reported that human NF-κB inhibitor-like-1 (NFKBIL1) was a putative susceptible gene for autoimmune diseases such as rheumatoid arthritis (RA). However, its precise role in the pathogenesis of RA is still largely unknown. In this study, we generated transgenic mice expressing human NFKBIL1 (NFKBIL1-Tg) and examined whether NFKBIL1 plays some role(s) in the development of autoimmune arthritis. In both a collagen-induced arthritis model and a collagen antibody-induced arthritis model, NFKBIL1-Tg mice showed resistance to arthritis compared to control mice, indicating that the gene product of NFKBIL1 was involved in the control of thusly induced arthritis. Total spleen cells of NFKBIL1-Tg mouse showed decreased proliferation to mitogenic stimuli, consistent with its resistance to arthritis. Unexpectedly, purified T cells of NFKBIL1-Tg mouse showed increased proliferation and cytokine production. This apparent discrepancy was accounted for by the impaired functions of antigen-presenting cells of NFKBIL1-Tg mouse; both T/B cell-depleted spleen cells and bone marrow-derived dendritic cells of the Tg mouse induced less prominent proliferation and IL-2 production of T cells. Furthermore, dendritic cells (DCs) derived from NFKBIL1-Tg mouse showed lower expression of co-stimulatory molecules and decreased production of inflammatory cytokines when they were activated by lipopolysaccharide. Taken together, these results indicated that NFKBIL1 affected the pathogenesis of RA at least in part through the regulation of DC functions.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory and joint-destroying autoimmune disease and is one of the most serious issues in the field of medicine [1]. Antigen-presenting cells (APC) such as dendritic cells (DCs) play pivotal roles for pathological setting of RA. Indeed, adoptive transfer of type II collagen-pulsed DCs is sufficient for the induction of autoimmune arthritis [2]. DCs provide cognate interaction, which is crucial for successful activation of T cells. Furthermore, DCs are main cellular source of inflammatory cytokines such as IL-6 and TNFα. As dysregulation of transcription factor nuclear factor κB (NF-κB) activity induces excessive production of inflammatory cytokines, leading to systemic and local autoimmune disorders such as systemic lupus erythematosus (SLE) and RA, the control of NF-κB seems essential for preventing such diseases [3–5].

Genetic polymorphisms are known to affect RA pathogenesis [6]. We and others revealed that single nucleotide polymorphism (SNP) in the promoter region of NFKBIL1 was associated with the pathogenesis of RA [7, 8]. NKBILL1 gene is located within the MHC class III region and encodes NFKBIL1 protein also known as IκBL. NFKBIL1 has been well conserved throughout evolution (more than 90% identical amino acids between human and mouse) and contains the ankyrin repeat domain, which exhibits high homology with the IκB family protein, suggesting that NFKBIL1 can modulate NF-κB activity, but its precise role and contribution to RA development are still to be unravelled [9, 10].

There are several experimental animal models of human autoimmune disease. As for RA, collagen-induced arthritis (CIA) and collagen antibody-induced arthritis (CAIA) are widely used for the evaluation of responsible genes. In this study, we generated NFKBIL1-Tg mice and found that they showed significant resistance to both CIA and CAIA. In addition, we demonstrated the impaired function of DCs in the Tg mice. These findings suggested that NFKBIL1 was involved in the control of RA pathogenesis via the regulation of innate immune cell functions.

Materials and methods

Generation of NFKBIL1-Tg mice. Human NFKBIL1 cDNA (BC143671) was cloned into pDRIVE-CAG vector (InvivoGen, San Diego, CA, USA), which contains CAG promoter combined with human cytomegalovirus immediate-early enhancer and a modified chicken β-actin promoter with the first intron. This construct was used as a transgene. NFKBIL1-Tg mice were generated by pronucleus microinjection into BDF1 × C57BL/6 fertilized eggs. Progeny mice were crossed with DBA/1j mice (Clea Japan, Inc., Tokyo, Japan) and germline transmission of NFKBIL1 transgene was confirmed by genomic PCR with specific primers (sense, 5′-ATGAGTAACCCC-TCCCCCCAG-3′; antisense, 5′-CACATCACCAAATCG-CCAGA-3′). Then, the mice expressing NFKBIL1 were backcrossed with DBA/1j mice over eight generations. All animals were bred in specific pathogen-free condition and used at 4–12 weeks of age. All mouse experiments were approved by the Animal Experimentation Committee, Isehara campus (Tokai University, Kanagawa, Japan).

Antibodies and reagents. Fluorescein isothiocyanate (FITC)-conjugated anti-CD19 (1D3), FITC-conjugated anti-CD44 (IM7), PerCP-Cy5.5-conjugated anti-CD4 antibody (RM4-5) and APC-conjugated anti-CD11c antibody (HL3) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). FITC-conjugated anti-CD80 (16-10A1) and FITC-conjugated Thy1.2 (53-2.1), PE-labelled anti-CD25 (PC61.5), PE-labelled anti-CD86 (PO.3), APC-labelled anti-CD8 (53-6.7), APC-labelled anti-IL-2 (JES6-5H4) and purified anti-CD28 antibody (37.51) were purchased from eBioscience (San Diego, CA, USA). Anti-mouse CD3ε antibody (145-2C11) was prepared in our laboratory. Concanavalin A (ConA) (C5275), phorbol 12-myristate 13-acetate (PMA) (P1585), ionomycin (I0634) and brefeldin A (BFA) (B6542) were purchased from Sigma-Aldrich (St Louis, MO, USA). Lipopolysaccharide (LPS) for DC stimulation was purchased from Santa Cruz Biotechnology (sc-3535; Santa Cruz, CA, USA).

Collagen-induced arthritis and collagen antibody-induced arthritis. NFKBIL1-Tg mice and littermate control mice with DBA/1j background were immunized with 200 μg of chicken type II collagen (CII; Chondrex, Seattle, WA, USA) emulsified with complete Freund's adjuvant (CFA; Chondrex) intradermally at the base of the tail on day 0. Three weeks later, mice were immunized again with CII without CFA. Mice were examined for up to 100 days. Clinical signs of arthritis were assessed daily and graded: 0, no swelling; 1, paw with single joint; 2, paw with two joints; 3, paw with multiple joints; 4, severe swelling and joint rigidity. Each limb was graded, giving a maximum possible score of 12 per mouse. For CAIA induction, a mixture of anti-CII monoclonal antibodies (2 mg/500 μl; Chondrex) was administered intraperitoneally on day 0. Three days later, LPS (50 μg/100 μl; Chondrex) was injected intraperitoneally. Clinical signs were assessed daily and graded similarly to CIA, with brief modification.

Joint histology. Joints were fixed in 10% formaldehyde and decalcified with 5% formic acid. Fixed joints were embedded in paraffin, sectioned into 4 μm thickness, stained with haematoxylin and eosin, and examined for collagen disruption, pannus formation, synovial space infiltrates and cartilage/bone erosion.

Cell isolation and culture. All cells used in this study were maintained in RPMI1640 medium supplemented with 10%FCS, 2 mM L-glutamine, 50 mM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM sodium pyruvate. Spleen $CD4^+$ T cells were isolated by using mouse CD4 dynabeads and Detacha CD4 according to the manufacturer's instructions (Invitrogen/Dynal; Carlsbad, CA, USA). For naïve and memory-phenotype T cell preparation, enriched $CD4^+$ cells were incubated with FITC-labelled anti-CD44 antibody plus PE-labelled anti-CD25 and fractionated by FACSAria (BD Biosciences) according to the CD44/CD25 expressions. Spleen $CD8^+$ T cells were isolated by AutoMACS system (Miltenyi Biotec; Bergisch Gladbach, Germany) after staining with anti-CD8 and anti-rat IgG MACS beads. $CD4^+$ cell-depleted spleen cells were incubated with FITC-labelled anti-Thy1.2 and CD19, and then, the $Thy1.2^-$, $CD19^-$ fraction was purified by FACSAria and used as non-T and non-B cells. Bone marrow cells were cultured in 10%FCS/RPMI with 5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, AF-315-03; PeproTech, Rocky Hill, NJ, USA). Half of the culture medium was replaced by fresh 5 ng/ml GM-CSF in 10%FCS/RPMI after 3 days. Cells were used at 10 or 11 days of culture as bone marrow-derived dendritic cells (BMDC). In some cases, $CD4^+$ T cells were cultured with APC at a ratio of 10:1 in the presence of 10 μg/ml anti-CD3ε antibody. Splenic $CD11c^+$ cells were isolated by using mouse Pan DC microbeads according to manufacturer's procedure (Miltenyi Biotec).

{^{3}H}-thymidine incorporation. Whole splenocytes, $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+$ naïve T cells and memory-phenotype T cells were cultured in 96-well plates for 48–

72 h, with 1 μCi [^{3}H]-thymidine added to each well for the last 18 h. Cells were harvested and [^{3}H]-thymidine uptake was measured by scintillation counter.

Measurement of cytokine production. The amounts of mouse IL-1β, IL-6, IL-12p70 and TNFα in culture supernatants were measured by flow cytometric beads array (CBA; BD Biosciences) according to manufacturer's instructions. The data were analysed by FACSCalibur (BD Biosciences) and FCAP array software (BD Biosciences).

Intracellular cytokine staining and flow cytometric analysis. Purified CD4$^+$ T cells were stimulated with 10 μg/ml anti-CD3 and 10 μg/ml anti-CD28 for 48 h. Cells were collected and stimulated again with 50 ng/ml PMA and 750 nM ionomycin for 4 h in the presence of 10 μg/ml BFA. Cells were fixed and permeabilized by Cytofix/Cytoperm Fixation/Permeabilization solution (BD Biosciences; No. 554722) according to the manufacturer's instructions and then stained with APC-anti-IL-2. BMDC were stimulated with or without 1 μg/ml LPS for 24 h and stained with FITC-anti-CD80, PE-anti-CD86 and APC-anti-CD11c. Cells were analysed by FACSCalibur and Cell Quest software (BD Biosciences).

Quantitative real-time PCR. RNAs form total spleen cells, CD4$^+$ T cells and CD11c$^+$ DCs of hNFKBIL1-Tg and littermate control mice were prepared using TRIzol reagent (Invitrogen). Complementary DNA was synthesized by SuperscriptIII reverse transcriptase (Invitrogen) with random hexamer primer. Quantitative real-time PCR was performed using Fast SYBR Green Master Mix on an ABI Fast 7500 machine (Applied Biosystems, Carlsbad, CA, USA). Primers for hNFKBIL1 forward, 5′-TGGAGACAGAAGCTCCAGGGTGA-3′ and reverse, 5′- CGGGATCCCTCTGCTTCTCGC-3′ and mouse β-actin forward, 5′- GACGGCCAGGTCATCACTATTG-3′ and reverse, 5′- AGGAAGGCTGGAAAAGAGCC-3′ were used to evaluate the relative gene expression. The data were analysed by $\Delta\Delta$C method.

Statistical analyses. Difference between wild-type (WT) and Tg mice in CIA and CAIA experiments were analysed by Mann–Whitney *U*-test. Cell proliferation and cytokine production were compared with Student's *t*-test. All data are represented as mean ± SEM or SD where indicated. Values of $P < 0.05$ were considered statistically significant.

Results

NFKBIL1 suppressed the development of collagen-induced arthritis

To evaluate the role of NFKBIL1, we generated transgenic mouse lines expressing human NFKBIL gene under the control of CMV promoter. We detected transgene-derived transcripts in total spleen cells, CD4$^+$ T cells and CD11c$^+$ DCs of transgenic (Tg) mouse, but not of WT mouse (Fig. 1A). Endogenous NFKBIL1 was expressed in all tissues examined (data not shown). We noticed that

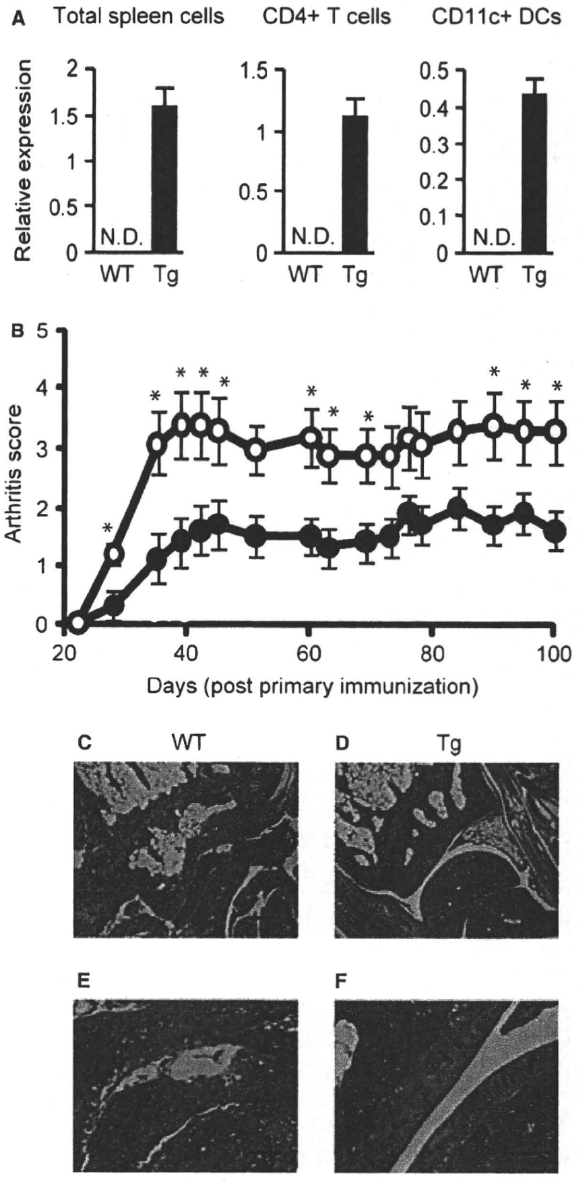

Figure 1 Reduced susceptibility of collagen-induced arthritis in NFKBIL1-Tg mice. (A) Expression of NFKBIL1 transgene in total spleen cells, CD4$^+$ T cells and CD11c$^+$ dendritic cells of NFKBIL1-Tg (n = 3) and WT littermates (n = 3) was analysed by quantitative RT-PCR. N.D., not detected. (B) NFKBIL1-Tg mice (closed circles) (n = 26) and WT littermates (open circles) (n = 83) with DBA/1j background were administered chicken type II collagen emulsified with CFA intradermally at the base of the tail on day 0. Three weeks later, mice were immunized again with CII/IFA. Severity of arthritis is shown. Similar results were obtained in two independent experiments, and one of them is demonstrated. (C–F) Joints of front limb of NFKBIL1-Tg (right) and WT littermate (left) mice at day 60 after primary immunization. Sections were stained with H&E. Scale bars represent 200 μm in (C, D) and 50 μm in (E, F). *$P < 0.05$.