

Brief Genetics Report

Analysis of the Mouse CD30 Gene

A Candidate for the NOD Mouse Type 1 Diabetes Locus *Idd9.2*

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Members of the tumor necrosis factor receptor superfamily play an important role in the initiation, expansion, and termination of an immune response. It has recently been demonstrated that one member of this family, CD30, plays a central role in maintaining peripheral tolerance by controlling the expansion of autoreactive CD8⁺ T-cells. In the present study, *Cd30* was mapped to a 5.6-cM interval on chromosome 4 containing the type 1 diabetes susceptibility locus *Idd9.2*. We determined the intron/exon structure of *Cd30* and sequenced the exons, as well as 1.8 kb of the 5' putative promoter region, from 6 different mouse strains. Remarkably, 63 sequence variants, both coding and noncoding, were found. A total of 27 sequence variants, 4 of which were nonsynonymous, were found between the diabetes susceptible NOD strain and the resistant B10 strain. Of these sequence variants, 19 are within the promoter region. However, no difference between NOD and the congenic strain NOD.B10 *Idd9R1*, which has the B10 allele of *Cd30*, was observed in CD30 expression at either the mRNA or protein level. Given its role in protecting against autoimmunity, one or more of the coding variants within CD30 is a good candidate for the *Idd9.2* etiological variant. *Diabetes* 49:1612–1616, 2000

CD30 is a member of the tumor necrosis factor receptor (TNFR) superfamily (1). It was initially described as a cell-surface antigen on Hodgkin and Reed-Sternberg cells of Hodgkin's lymphomas (2). Subsequently, CD30 has been shown to be expressed in an activation-dependent manner on both T- and B-cells (3). Mouse CD30 is a type 1 transmembrane glycosylated protein con-

sisting of 498 amino acids. Its extracellular domain contains one complete cysteine-rich domain made up of 3 cysteine-rich motifs, which is characteristic of TNFR family members. In addition, mouse CD30 also has 2 partial motifs that share homology with the regions flanking the second cysteine-rich domain found in human CD30 (3). These domains have been shown to play a role in ligand binding. The intracellular domain of CD30 has been shown to bind to the TNFR-associated factor (TRAF) proteins 1, 2, 3, and 5, which mediate CD30 activation of nuclear factor (NF)- κ B and the c-Jun NH₂-terminal kinase/stress-activated protein kinase pathway (4–8).

The biological effects of CD30 are pleiotropic (9). In T-cells, CD30 signaling stimulates proliferation (10,11) and cytokine production (3) and induces susceptibility to apoptosis (4). Further insight into the function of CD30 has recently been provided from studies that have used a transgenic model of autoimmune diabetes (12). It was observed that adoptively transferred CD30-deficient CD8⁺ T-cells are 6,000-fold more autoreactive than wild-type cells. This enhanced autoreactivity is associated with the increased proliferation of activated CD30-deficient T-cells on encountering antigens in the periphery. Thus, CD30 signaling can protect against autoimmunity by controlling the expansion of autoreactive CD8⁺ T-cells.

Cd30 has been previously mapped on distal mouse chromosome 4 (13) to a region linked to the development of type 1 diabetes in the NOD mouse (14–16). This locus, called *Idd9*, has been shown to be due to the interaction of at least 3 separate loci: *Idd9.1*, *Idd9.2*, and *Idd9.3* (17).

In this study, we have characterized the *Cd30* locus to determine whether it is a candidate for one of these loci.

Vectorette libraries constructed from whole-mouse genomic DNA were amplified by polymerase chain reaction (PCR). A 487-bp amplification product containing exon 1 of mouse *Cd30* and 414 nucleotides of intron 1 was obtained (data not shown). From this sequence data, a sequence-tagged site (STS) assay was designed for *Cd30* and used to screen the ICRF mouse P1 library. A single clone positive for *Cd30*, mP62p13, was identified.

Using a PCR-based approach (18), a variant microsatellite marker located within the *Cd30* locus was isolated from this clone. This marker, *DANds23*, was genotyped on 184 F2 progeny of a cross between NOD and the NOD.B10 *Idd9R28* congenic strain (Fig. 1). It maps 4.1 cM distal to *D4Mit251* (15 recombinants in 184 mice typed) and 0.5 cM proximal to *D4Mit285* (2 recombinants in 184 mice typed). No recombinants

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FITC, fluorescein isothiocyanate; IL, interleukin; NF, nuclear factor; PCR, polymerase chain reaction; PE, phycoerythrin; SAP, shrimp alkaline phosphatase; SNP, single nucleotide polymorphism; STS, sequence-tagged site; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNFR-associated factor.

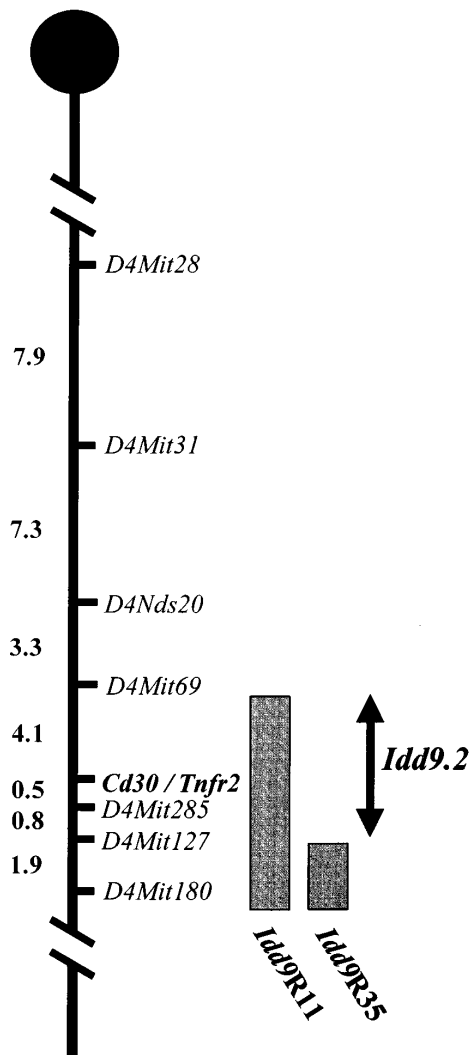


FIG. 1. Genetic map of distal mouse chromosome 4, showing the positions of *Cd30* and *Tnfr2*. Genetic markers were typed on 184 F₂ progeny of a cross between NOD and NOD.B10*Idd9* R28. The distances between the markers are given in centimorgans.

were observed between *Cd30* and *Tnfr2*, another member of the TNFR superfamily. To confirm this mapping data, *D4Nds23* was typed on the NOD.B10 *Idd9*R11 (R11) and NOD.B10 *Idd9*R35 (R35) congenic strains. The R11 strain is B10-derived at *D4Nds23*, whereas the R35 strain is NOD-derived, confirming the mapping of *Cd30* to the *Idd9.2* interval (Fig. 1). Both of these strains develop diabetes less than the NOD parental strain with R11 more protected from disease than R35. R11 has diabetes-resistant alleles at both *Idd9.2* and *Idd9.3*, whereas R35 has a resistance allele only at *Idd9.3* (17).

The complete exon structure of the CD30 gene was determined as follows. A PCR primer was designed to the *Cd30* cDNA sequence immediately downstream of the identified intron/exon boundary and used to amplify vectorette libraries constructed from the clone mP62p13. Amplification products were sequenced and aligned to the *Cd30* cDNA sequence to identify the next intron/exon boundary. This process was repeated sequentially until the entire intron/exon structure was established (Table 1). The CD30 gene comprises 13 exons ranging in size from 26 to 242 bp (Table 1); the initiation methionine is found in exon 1, and the stop codon is found in exon 13.

Despite sharing 59% identity at the amino acid level, human and mouse CD30 differ in one key respect: Human CD30 contains 6 cysteine-rich motifs arranged into 2 distinct domains, each containing 3 repeats (1), whereas mouse CD30 contains only a single domain homologous to the most proximal domain of human CD30 (3). This cysteine-rich domain is encoded by exons 2–5, with each motif being split between 2 exons. Although they lack a second cysteine-rich domain, the amino acid residues flanking this domain in the human molecule are conserved in mouse (3). These conserved amino acids are encoded by exons 6 and 7 with the boundary occurring at the position of the intron/exon boundaries found in the first and third repeats of the first domain. The lack of a second domain in mouse may be explained by the deletion of 2 exons, equivalent to exons 3 and 4, between exons 6 and 7. Confirmation of this hypothesis will require the determination of the human CD30 gene structure.

The CD30 gene together with 1.8 kb of the putative promoter was sequenced in NOD, B10, B6, 129, NON, and *Mus*

TABLE 1
Exon structure of mouse *Cd30*

Exon no.	Exon size (bp)	Position*	5' Splice donor†	3' Splice acceptor†
1	126	1–126	ACG gtaaggga	ccctccag GAT
2	88	127–214	TCA G gtgagtat	ttccccag GG TTG
3	117	215–331	CCA G gtgagaac	tccctcag GC CTT
4	144	332–475	CCA G gtgagtgt	ttttatag GC ACA
5	91	476–566	ACT AG gtgactcc	ttctacag C CAT
6	167	567–733	CCA G gtactgtt	acctatag GT AAT
7	91	734–824	GCA AG gtaatgat	tggtatag T ACC
8	98	825–922	CCA G gtaatgat	tgtttcag GG CCC
9	99	923–1021	CAG A gtaagtgc	ttttgcag AG TTT
10	54	1022–1075	ACA G gtgagtat	ttttacag AT TCC
11	26	1076–1101	ACC gtaagtgc	gttcacag CAG
12	214	1102–1315	ATT G gtgagtcc	ccttacag AG AAA
13	242	1316–1557		

*Numbering according to Bowen et al. (3); †uppercase indicates exonic nucleotides and lowercase indicates intronic nucleotides.

TABLE 2
Sequence variants in the CD30 genes of BALB/c, NOD, B10, 129, B6, NON, and SPR mice

Exon no.	BALB/c*	NOD	B10	129	B6	NON	SPR
1	nt 24 G	A	G	A	G	A	G
2	Gly31	Gly	Gly	Gly	Gly	Gly	Arg
	Glu39	Glu	Glu	Glu	Glu	Gly	Glu
3	nt 162 G	A	G	A	G	A	A
	Gly61	Asp	Gly	Gly	Gly	Asp	Gly
	nt 222 C	T	C	T	C	T	T
4	nt 294 C	C	C	C	C	C	T
	Glu132	Glu	Glu	Glu	Glu	Glu	Lys
5	nt 453 G	G	G	G	G	G	A
	Gly161	Gly	Gly	Asp	Gly	Asp	Gly
	nt 489 C	C	C	C	C	C	A
	Thr167	Ile	Thr	Thr	Thr	Thr	Thr
6	Gln173	Arg	Gln	Arg	Gln	Arg	Arg
	Arg187	Arg	Arg	Arg	Arg	Lys	Arg
	nt 579 G	T	T	G	T	G	G
	Pro219	Pro	Pro	Ser	Pro	Pro	Ser
8	nt 786 G	G	G	G	G	A	G
9	Val296	Val	Val	Val	Val	Val	Ala
10	nt 975 C	C	C	C	C	C	T
	nt 978 C	A	A	A	A	C	A
12	Gly352	Gly	Gly	Gly	Gly	Gly	Arg
	nt 1074 A	C	C	C	C	C	T
	Thr359	Thr	Thr	Thr	Thr	Thr	Ala
	Ser364	Asn	Ser	Ser	Ser	Ser	Ser
	nt 1164 C	C	C	C	C	C	T
	nt 1203 G	G	G	G	G	G	A
	nt 1218 C	C	C	C	C	C	T
	nt 1236 C	C	C	C	C	C	T
13	nt 1287 C	T	C	C	C	C	C
	nt 1371 G	G	G	G	G	G	A
	nt 1401 G	G	G	G	G	G	A
	nt 1443 G	G	G	G	G	G	A

*Nucleotide and amino acid numbering from Bowen et al. (3).

spretus (SPR) mice to identify variants (Tables 2 and 3). Alignment of the coding *Cd30* sequences with the published BALB/c sequence (3) identified a striking amount of sequence variation. In total, 32 nucleotide variants, including 13 non-synonymous single nucleotide polymorphisms (SNPs), were identified (Table 2). A significant amount of this sequence variation occurs among the 5 inbred strains (16 SNPs including 8 nonsynonymous SNPs).

Among the coding variants, 9 are found in the extracellular portion of the protein, 1 is found in the transmembrane domain, and 3 are located in the cytoplasmic tail. X-ray crystallography studies (19) have shown that ligand binding induces trimerization of TNF receptors and that this is mediated by the extracellular cysteine-rich repeats. Thus, coding variants in the extracellular domain may alter CD30 function by interfering with ligand binding and receptor trimerization. All of the extracellular variants, except Gln173Arg and Arg187Lys, are in cysteine-rich motifs, although none occur at any of the conserved cysteine residues. The nonconservative Gly61Asp variant may be functionally important. Alignment of the CD30 and TNFR1 extracellular domains showed that this variant lies close to residues that have been identified by crystallography to be important to the binding of TNF by TNFR1 (19). Variants within the intracellular domain of CD30 may also affect CD30 trimerization or alter signaling after ligand binding. Signaling by CD30 is mediated through its interaction with the TRAF family of proteins (20), and TRAF-binding motifs have been identified in the COOH-ter-

minal region of the cytoplasmic tail of CD30 (5,21). None of the observed coding variants within the cytoplasmic tail occur at residues identified as being important for TRAF binding. Further studies are necessary to determine if they alter signaling or receptor trimerization after ligand binding.

In addition, 31 variant sites were identified in the putative promoter region, including a variant tetranucleotide microsatellite at position -448 (Table 3). None of the identified promoter variants fall in predicted transcription factor binding sites (22). Among the 6 strains, 4 distinct promoter region alleles were observed. The closely related B10 and B6 strains share a common haplotype, as do the related NOD and NON strains. Although the 129 strain has a unique haplotype, it is very closely related to that of NOD and NON, varying only in the length of the tetranucleotide microsatellite. To determine whether the different promoter alleles alter the level of *Cd30* expression, we examined the upregulation of CD30 expression on splenocytes from mice with the NOD and B10 *Cd30* promoter alleles. Upregulation of CD30 was equivalent on NOD and NOD.B10 *Idd9R1* (R1) CD4⁺ and CD8⁺ T-cells stimulated with anti-CD3 for 3 days (Fig. 2). R1 is a mouse strain that is protected from diabetes and has resistance alleles at *Idd9.1*, *Idd9.2*, and *Idd9.3* (17). In addition, *Cd30* mRNA levels were measured in the same cultures using real time PCR. No transcription was observed in resting splenocytes or at day 1 after stimulation. *Cd30* transcription was upregulated on days 2 and 3 poststimulation and then switched off at day 4 (data not shown). Equivalent max-

TABLE 3

Nucleotides at polymorphic sites within the putative promoter of the CD30 gene in NOD, B10, 129, B6, NON, and SPR mice

Position	B10*	NOD	129	B6	NON	SPR
+17	G	G	G	G	G	A
-84	G	G	G	G	G	A
-155	G	T	T	G	T	G
-281	G	A	A	G	A	G
-296	G	A	A	G	A	G
-328	C	T	T	C	T	C
-397	C	C	C	C	C	T
-426	T	T	T	T	T	C
-445	T	C	C	T	C	T
-448	(CCAT) ₉	(CCAT) ₅	(CCAT) ₆	(CCAT) ₉	(CCAT) ₅	(CCAT) ₇
-555	C	T	T	C	T	C
-612	A	G	G	A	G	A
-646	A	T	T	A	T	A
-839	G	G	G	G	G	T
-857	C	C	C	C	C	T
-858	C	A	A	C	A	C
-886	G	G	G	G	G	A
-902	A	T	T	A	T	A
-986	A	G	G	A	G	A
-993	G	G	G	G	G	A
-1003	G	A	A	G	A	G
-1041	C	C	C	C	C	T
-1103	C	T	T	C	T	C
-1114	G	A	A	G	A	G
-1158	A	G	G	A	G	A
-1186	G	G	G	G	G	A
-1218	A	G	G	A	G	A
-1266	G	T	T	G	T	G
-1470	T	C	C	T	C	T
-1472	T	T	T	T	T	C
-1500	C	T	T	C	T	C

*The major transcription start site is designated as +1 in B10 mice.

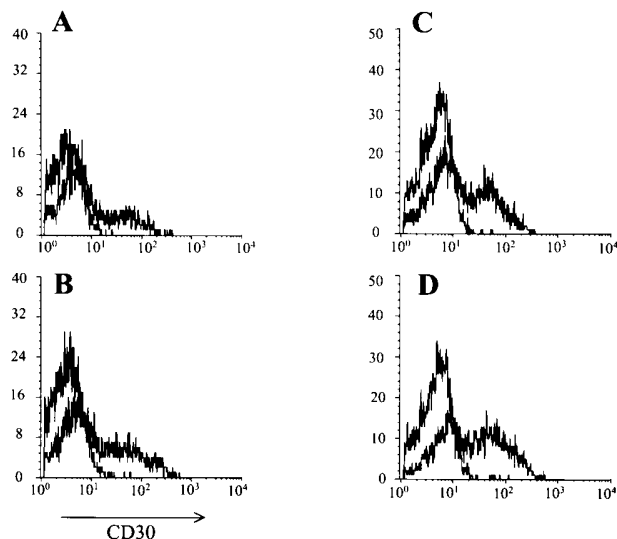


FIG. 2. CD30 expression on CD4⁺ and CD8⁺ T-cells. CD30 expression on CD4⁺ T-cells (*A* and *B*) from NOD mice (*A*) and NOD.B10*Idd9* mice (*B*) on day 3. Cells are incubated with an FITC-labeled anti-CD4 antibody and either a PE-labeled anti-CD30 antibody or a PE-labeled isotype control antibody. CD30 expression on CD8⁺ T-cells (*C* and *D*) from NOD mice (*C*) and NOD.B10*Idd9* mice (*D*) on day 3. Cells are incubated with an FITC-labeled anti-CD8 antibody and either a PE-labeled anti-CD30 antibody or a PE-labeled isotype control antibody.

imal levels of *Cd30* mRNA were observed in NOD and NOD.B10 *Idd9R1* mice (data not shown).

Variants within CD30 could have been selected by resistance to infectious disease during evolution because they alter its function and confer a selective advantage. Given the key role of CD30 in maintaining peripheral tolerance, sequence variants that alter CD30 function, thereby generating a more vigorous immune response to infection, might predispose to autoimmunity. In light of this possibility, it is interesting that sequence variation exists between the CD30 molecules of the diabetes susceptible NOD and resistant B10 mouse strains. This observation, together with their colocalization, makes *Cd30* a good candidate for the type 1 diabetes locus *Idd9.2*. Because the NOD and B10 allotypes are expressed equivalently, differences in function of all allotypes should be evaluated.

RESEARCH DESIGN AND METHODS

Mice, antibodies, and reagents. NOD/MrkTac (NOD) mice were purchased from Taconic Farms (Germantown, NY). The derivation of the NOD.B10 *Idd9R1* (N9) congenic strain has been described previously (23). The NOD.B10 *Idd9R28* (N11) and the NOD.B10 *Idd9R11* (N11) congenic strains were subsequently derived from this strain, and the NOD.B10 *Idd9R35* (N13) strain was derived from the NOD.B10 *Idd9R11*. All strains were developed as described previously (24). All mice were housed under specific pathogen-free conditions. α -CD3 (clone 145-2C11), α -CD4 (clone GK1.5), α -CD8 (clone 53-6.7), α -CD30 (clone mCD30.1), and α -TNF (clone A19-3) monoclonal antibodies and recombinant mouse interleukin (IL)-2 and IL-4 were purchased from Becton Dickinson (Oxford, U.K.).

Isolation of a genomic clone carrying the mouse CD30 gene. C57BL/6 (B6) (1 µg) genomic DNA was digested with the restriction enzymes *DraI*, *EcoRV*, *PvuII*, and *ScaI* at 37°C for 1 h in a volume of 30 µl. After heat inactivation, 100 ng of annealed bubble oligos (25) were ligated to the restricted DNA in a final volume of 100 µl. The ligation reaction was terminated with the addition of 400 µl of 1× TE_{0.1}. A 5-µl sample of each genomic vectorette library was used as the template for PCR amplification with the primers CD30 outer 5'-TTTCACCGCGGAGAGATC-3' and NotIA (25). PCR reactions contained 1× TaqGold buffer, 0.2 mmol/l dNTPs, 2 mmol/l MgCl₂, 62.5 ng of each primer, and 1 U AmpliTaqGold polymerase (PE Biosystems, Warrington, U.K.) in a volume of 25 µl. The thermal cycling conditions of 94°C for 15 min followed by 32 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s were performed on a PTC-225 thermal cycler (MJ Research, Boston, MA).

The primary PCR product was diluted 1:5, and 5 µl used as template for a second round of PCR amplification using the primers CD30 inner 5'-CGTCGGAGAGTCGGGAAG-3' and NotIA nested 5'-GCATTTCTGCTCCTCCTTC-3' as described previously. Secondary PCR products were gel purified and sequenced directly using the BigDye Terminator Cycle Sequencing Kit (PE Biosystems). Sequencing reactions were analyzed on an ABI 377 automated sequencer. An STS assay for *Cd30* was developed from the obtained genomic sequence and used to identify a *Cd30*-positive clone from the ICRF mouse P1 library as previously described (17).

Microsatellite marker development and genotyping. The microsatellite marker *D4Nds23* (forward 5'-TTGTAGACCAGTCAACCAGGG-3'; reverse 5'-GATCTCTTGACTGCCTGGCT-3') was isolated from the clone mp62p13 using a PCR-based approach as previously described (18). Genotyping was performed as described elsewhere (26).

Sequencing of the CD30 gene and putative promoter. The *Cd30* genomic sequence was determined by sequencing vectorette PCR products generated using primers designed to the *Cd30* cDNA sequence as described previously. Nested PCR primer pairs flanking each exon and overlapping primer pairs covering the putative promoter region were designed from the *Cd30* genomic sequence. Nested PCR products were cleaned up enzymatically with shrimp alkaline phosphatase (SAP) and ExoI (Amersham, Little Chalfont, U.K.) before direct sequencing using the BigDye Terminator Cycle Sequencing Kit. Before gel electrophoresis, excess dye terminators were removed using SAP. Sequencing reactions were analyzed as described previously. DNA sequences were assembled using the GAP4 program (27) and aligned using the clustalx program.

Splenocyte cultures and flow cytometry. Splenocytes (6×10^5 /ml) were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 10 mmol/l Hepes, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 0.1 mmol/l nonessential amino acids, 50 µg/ml gentamicin, and 50 µmol/l 2-mercaptoethanol. T-cells were activated with soluble α-CD3 (0.5 µg/ml) in the presence of IL-2 (2 ng/ml) and IL-4 (20 ng/ml). At various time points after activation, cells (1×10^6) were stained with phycoerythrin (PE)-conjugated α-CD30 or isotype control in combination with fluorescein isothiocyanate (FITC)-labeled α-CD4 or α-CD8, and were analyzed on a FACSort (Becton Dickinson, Oxford, U.K.). At least 10,000 viable cells were analyzed at each time point.

Cd30 mRNA analysis. Total RNA was extracted from $\sim 1 \times 10^7$ splenocytes using the Trizol reagent (Life Technologies, Paisley, U.K.) according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA, and *Cd30* mRNA was measured by real-time PCR using an ABI Prism 7700 sequence detection system kit (PE Biosystems) (*Cd30* forward 5'-ATGAAGA CCGGAAGTGCACAG-3', *Cd30* reverse 5'-CGGAACACGGAGCCTTCTC-3', *Cd30* probe 5'-CGTGACCTGTTGCCAGGCCTTG-3').

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