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The complete sequence of the MHC class II chain RT1Dα^u of the diabetic BB rat: mRNA levels of RT1.Dα in lymphocytes

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ABSTRACT

The major histocompatibility complex of the rat (RT1 complex) encodes two sets of class II molecules referred to as RT1.B and RT1.D. The complete structure of the RT1.Dα^u chain of the diabetes prone BB rat was determined by the isolation and characterization of a full size cDNA. Comparisons of the nucleotide and protein sequences of RT1.Dα with the analogous molecules, H-2 I-Eα and HLA DRα, revealed that these alpha chains have been highly conserved during evolution. Southern blot analysis indicated an association of the RT1 haplotypes, 'u' and 'l', with Bam HI DNA bands of 9.8 kb and 11.7 kb, respectively.

The BB rat develops insulin dependent diabetes as an autoimmune abnormality. Accumulating evidence suggests a cellular mediated etiology and the involvement of class II molecules. The steady state levels of RT1.Dα mRNA were measured in splenic lymphocytes of diabetes prone BB rats and age matched histocompatible normal nondiabetic WF rats by a RNase protection assay. Compared to WF rats, elevated transcripts of RT1.Dα were found in lymphocytes of young BB rats (~4x and ~2.5x greater at 20-40 d and 40-75 d, respectively). In lymphocytes of older diabetic and nondiabetic BB rats (>75 d) the levels of RT1.Dα mRNA were lower than in the young BB rats and were found at the WF control levels. The increased steady state RT1.Dα mRNA levels in the young BB rats may reflect differences in the proportion of splenic lymphocytes expressing this gene (activated lymphocytes), and thus differences in splenic lymphocyte populations. The steady state RT1.Dα mRNA levels in lymphocytes of the normal rats were found to be relatively similar at all ages examined. The increased class II gene transcripts found in lymphocytes of young BB rats indicates that they possess a highly activated immune system.

INTRODUCTION

The major histocompatibilty complex (MHC) is a multigene family which encodes various cell-surface and serum glycoproteins involved in the recognition of foreign antigens and in the generation of an immune response (1). The MHC has been highly conserved throughout vertebrate evolution and is divided into related groups of genes on the basis of similar structure and function. The class II genes

encode molecules which guide the presentation of antigen to regulatory helper T cells; the antigen-specific receptors of helper T cells recognize foreign antigen in association with class II molecules (2,3). The class II molecules are predominantly expressed on the surface of antigen-presenting cells such as macrophages, dendrictic cells, B lymphocytes, and also certain epithelial cells and activated T cells (2). Class II molecules are heterodimers consisting of a 32 kD alpha chain noncovalently associated with a 29 kD beta chain. Both alpha and beta chains each consist of two extracellular domains, an anchoring transmembrane segment and a cytoplasmic region (4). Several alleles of both alpha and beta chains have been described in mouse and man (5,6). An unusually high level of polymorphism has evolved in the extracellular membrane-distal domain of class II beta chains, whereas the alpha chains often remain constant (7). Although there is considerable homology between the gene products of the MHC region between species, the organization of the complex is somewhat different in different species.

Genetic and biochemical characterization of the rat MHC (RT1 complex) has not progressed as rapidly as the characterization of the mouse and human MHC's. This is primarily due to the scarcity of recombinants in the RT1 region. A better understanding of the RT1 complex is required not only for evolutionary studies of the MHC, but more importantly because the rat provides several experimental models of autoimmune disease. Six loci have been identified and mapped in the RT1 complex with respect to one another (8). The class II genes are located at two of these loci, RT1.B and RT1.D, which code for the molecules RT1.Bα and RT1.Bβ, plus RT1.Dα and RT1.Dβ, respectively. The RT1.B and RT1.D chains are analogous to the alpha and beta chains of the mouse H-2 molecules I-A and I-E, respectively, and of the human HLA molecules, DQ and DR, respectively.

The Wistar derived BB rat is a model of spontaneously occuring insulin dependent diabetes mellitus (IDDM) which exhibits features strikingly similar to human juvenile onset or type I diabetes (9). Approximately 50% of diabetes prone BB rats develop IDDM as a consequence of an autoimmune abnormality which is characterized by insulitis and the specific and selective destruction of the insulin producing beta cells of pancreatic islets (10). BB rats also develop other autoimmune disorders such as thyroiditis, and suffer a profound T cell lymphopenia with a virtual absense of cytotoxic and suppressor T cells (11). Many of the features of IDDM and the BB rat have been recently reviewed (12). As in humans, susceptibility to

the development of IDDM in the BB rat is genetically associated with the MHC (13); all insulin dependent diabetic rats have the $RT1^u$ haplotype (14). Genetic studies indicate that at least one other recessive autosomal gene which is not linked to the MHC is necesary for IDDM susceptibility in the BB rat (13,14). The available evidence suggests a cellular mediated etiology of IDDM in the BB rat and the involvement of class II molecules.

In studies on the possible role of class II molecules in spontaneously developing autoimmunity associated with IDDM in the BB rat, we have isolated and characterized rat class II cDNA genes (15). This communication describes the complete structure of the class II alpha chain, $RT1.D\alpha^u$, determined by the isolation and characterization of a full-size encoding cDNA clone, and measurements of $RT1.D\alpha$ gene transcripts in lymphocytes of the IDDM-prone BB rat and normal nondiabetic rats.

MATERIALS AND METHODS

Purification of RNA and cDNA cloning.

The following rat strains were used which expressed the serotypically defined $RT1^u$ haplotype: BB (Hospital For Sick Children, Toronto); Wistar Furth (Harlan Sprague Dawley); and Long Evans Hooded (Charles River Canada). Total RNA was extracted from suspension splenocytes into guanidine isothiocyanate and purified by centrifugation through cesium chloride (16). The samples of total RNA used for RNase protection studies were adjusted to 50 A_{260} units ml^{-1}. RNA integrity was estimated by electrophoresis on formaldehyde agarose gels (17). Poly A^+ RNA was purified by two rounds of affinity chromatography on oligo-dT cellulose (18). The synthesis of oligo-dT primed first strand cDNA with AMV reverse transciptase, and of second strand cDNA, carried out with DNA polymerase, ribonuclease H and DNA ligase, was by the method of Okayama and Berg (19), as modified by Gubler and Hoffman (20). Double standed cDNA was tailed with poly dC and cDNA's larger than 800 bp in length were purified by size fractionation on a Sepharose CL-4B column. The cDNA was annealed to pBR322 previously tailed with poly dG at the Pst 1 site and transfected into Escherichia coli strain JF1754 (21), yielding 4 x 10^6 transformants ug^{-1} cDNA.

Colony hybridization.

The cDNA clones were screened by high density colony hybridization on nitrocellulose filtres (22) with a DNA probe of 566

bp purified from Pst 1 digested pRT1-Dα^u-1 (15). Hybridization was performed at 42°C for 16 hr in the presence of 10^6 cpm ml^{-1} of nick-translated probe in a hybridization buffer (50% formamide, 5xSSC, 5x Denhardt's solution, 50 mM sodium phosphate, pH 6.5, 10 mg ml^{-1} glycine, 0.1% SDS, and 100 ug ml^{-1} sonicated and denatured salmon sperm DNA). The filters were washed four times at room temperature for 15 min in 2xSSC, 0.1%SDS, followed by two washes of 30 min at 60°C with 0.1xSSC, 0.1%SDS. The filters were autoradiographed at -70°C for 12-36 hr using intensifying screens. Positive colonies were picked and repurified.

Nucleotide sequence analysis.

Restriction site mapping was carried out with restiction enzymes individually or in combinations of two or three. DNA fragments were subcloned into M13 vectors (23) and sequenced by the chain termination method using dideoxynucleoside triphosphates (24).

Purification of DNA, Southern blot analysis.

Plasmid DNA and high molecular weight liver DNA were purified as described (25,26), respectively. Liver DNA was digested with restriction endonucleases (6-10 units ug^{-1} DNA) for 8 hr, phenol extracted and alcohol precipitated. The DNA was resolved on 1% agarose gels and transferred to nitrocellulose filters according to the method of Southern (27). The filters were prehybridized and hybridized as described above. The DNA probe used was the full-size insert from pRT1.Dα^u-11 which was [^{32}P]-labelled by nick translation. The filters were washed and autoradiographed as described above.

Analysis of RT1.Dα mRNA levels.

The riboprobe plasmid pT7/RT1.Dα^u492 was constructed by the ligation of a 492 bp Pst 1 - Sac 1 DNA fragment, exised from the cDNA clone pRT1.Dα^u-1 (15), into the similarly cleaved vector pT7-1. The insert DNA encodes amino acids 85 to 229 of the RT1.Dα^u molecule plus 49 bp of the 3' untranslated region. Plasmid pT7/RT1.Dα^u492 carries the insert DNA in antisense orientation with respect to the T7 promoter. Transcription *in vitro* with T7 RNA polymerase of the template DNA linearized with Sca 1 yielded a primary transcript of 1116 nt, of which 470 nt was complementary with RT1.Dα mRNA.

Radioactively labelled antisense RNA was transcribed *in vitro*, purified, hybridized in solution with the target RNA, and processed as described (28). The protected [^{32}P]-RNA was resolved on 8 M urea, 5% polyacrylamide gels and displayed by autoradiography with Kodak XAR x-ray film exposed for 12 hr to 3 days. Quantitation of protected [^{32}P]-RNA was performed by scanning densitometer analysis

of the autoradiograms at a fixed wavelength with a Hoefer GS300 scanning densitometer and integration of appropriate peaks.

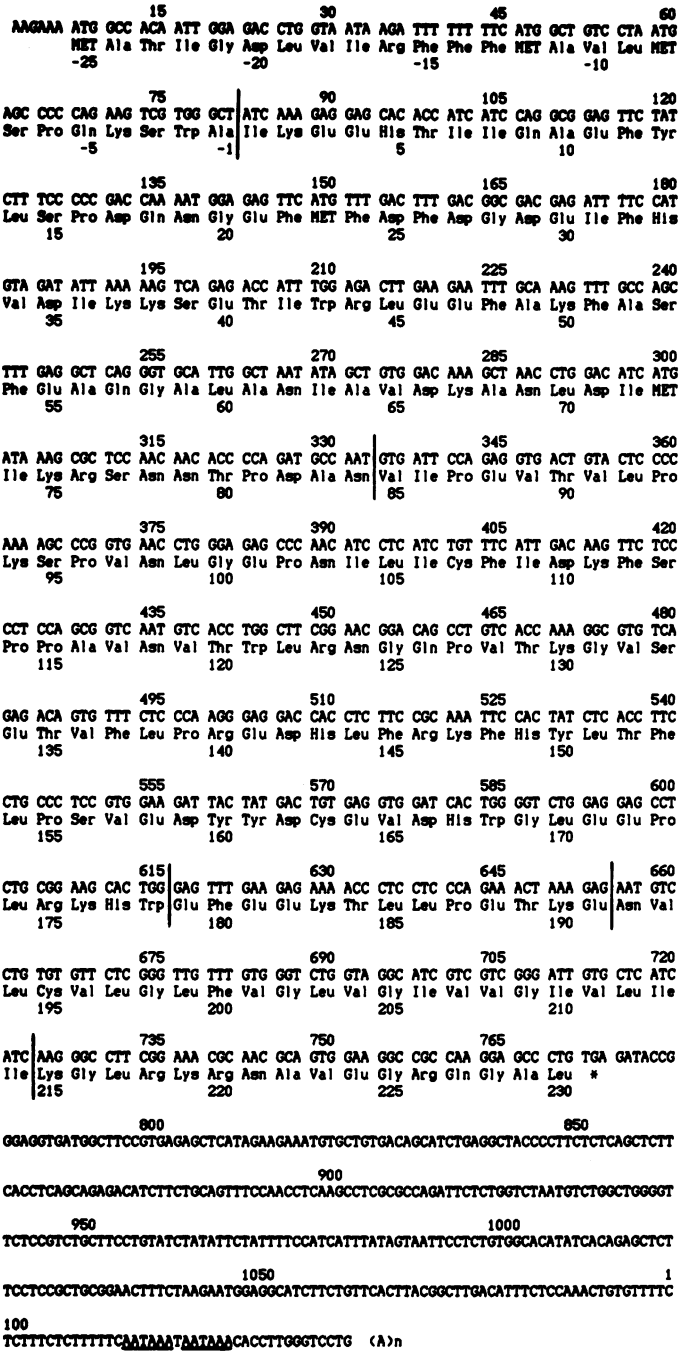
RESULTS AND DISCUSSION

Isolation and characterization of RT1.Dα cDNA.

A size selected cDNA library was constructed from poly A^+ RNA purified from suspension splenocytes of the BB rat with the aim of obtaining full-size cDNA clones. Ten thousand recombinant clones were screened by colony hybridization with a nick-translated 566 bp DNA probe purified from Pst 1 digested plasmid pRT1.Dα^u-1 DNA (15). Fourteen independent positive colonies were identified and selected for plasmid isolation. Restriction enzyme analysis showed that identical restiction maps were found with respect to internal multiple cleavage sites. This suggested that a single RT1.Dα gene is transcribed in the rat, as is the case for the analogous gene of the mouse and man (6). The plasmid pRT1.Dα^u-11 contained the largest insert and was selected for further study.

The complete nucleotide sequence of the pRT1.Dα^u-11 insert was determined and is shown with the predicted amino acid sequence of RT1.Dα^u in Figure 1. The cDNA sequence is 1138 bp in length, terminating in an 18 bp poly A tract at the 3' end, and flanked by G/C tails of 22 bp and 13 bp. Beginning at nucleotide position 7, an open reading frame of 765 nucleotides was found which terminated in the stop codon TGA and was followed by a 3' untranslated region of 364 nucleotides. Two putative canonical polyadenylation signal sequences, AATAAA (29), separated by a single T nucleotide, were found 14 and 21 nucleotides preceding the poly A sequence. The partial nucleotide sequence of RT1.Dα^u previuosly reported (15) agrees with the sequence of pRT1.Dα^u-11.

The amino acid sequence predicted for RT1.Dα^u (Figure 1) indicates that pRT1.Dα^u-11 encodes the complete precursor RT1.Dα^u molecule of 255 amino acids with a nonglycosylated calculated Mr of 29,023, which includes a signal sequence of 25 amino acids. The structure of the rat RT1.D alpha chain is similar to that reported for the analogous molecules of mouse and man (15,30-32). The mature RT1.Dα^u chain contains 230 amino acids which specify the amino terminal α1 extracellular domain (amino acids 1 to 84), the α2 extracellular domain (residues 85 to 178), a connecting peptide of 13 amino acids (positions 179 to 191), a highly hydrophobic membrane anchoring domain of 23 amino acids (positions 192 to 214), and a



cytoplasmic tail of 16 amino acids (positions 215 to 230). The nonglycosylated mature RT1.Dα^u protein has a predicted Mr of 26,182.

Comparisons of the nucleotide sequence of the coding region of the rat RT1.D alpha chain with the DNA sequences of the analogous genes of the murine I-Eα^d chain (30) and the human HLA DRα chain (32) are shown in Figure 2. These analogous alpha chain genes exhibit extensive nucleotide homology, as previously reported (15). Overall DNA homologies between protein coding regions of 86% and 74% were found with the I-Eα^d and HLA DRα chains, respectively. Comparisons of the 3' untranslated regions showed 74% and 54% nucleotide homology with the mouse and human genes, respectively (15; data not shown). The nucleotide differences are found randomly throughout the length of the alpha genes, indicating that the nucleotide replacements likely represent the evolutionary distance between the three species. The rat RT1.Dα gene exhibits greater nucleotide homology with the I-Eα and HLA DRα genes, than with the other rat alpha chain gene, RT1.Bα (33). Nucleotide comparisons of the rat RT1.Dα sequence with the partial RT1.Bα sequence showed 65% homology in the coding region and less than 30% homology in the 3' untranslated region (15; data not shown). This is not unexpected for the two alpha chain genes which presumably evolved by gene duplication prior to mammalian speciation.

RT1.Dα molecule.

The predicted amino acid sequence of the RT1.Dα^u chain was compared to the sequences of the I-Eα^d and the HLA DRα chains (Figure 3). A high conservation of sequence is evident, as reported earlier (15). The levels of protein identity between the RT1.Dα^u and I-Eα^d chains, by domain, are presented in Table 1, and are as follows: signal sequence, 84%; α1, 92%; α2, 88%; connecting peptide, 100%; transmembrane region, 83%; cytoplasmic tail, 75%; and overall mature protein, 88%. Between the RT1.Dα^u and the HLA DRα: signal sequence, 60%; α1, 83%; α2, 82%; connecting peptide, 54%; transmembrane region, 65%; cytoplasmic tail, 69%; and overall mature protein, 78%. Over the length of the mature alpha chain sequence, 62% of the amino acids

Figure 1. Nucleotide sequence of the cDNA insert of pRT1.Dα^u-11 and the predicted amino acid sequence of the RT1.Dα^u chain. The vertical bars designate the putative alpha chain domains: 5' untranslated and signal sequence (nucleotides 1-81; amino acids -25 to -1); α1 (nts 82-333; aa 1-84); α2 (nts 334-615; aa 85-178); connecting peptide (nts 616-654; aa 179-191); transmembrane (nts 655-723; aa 192-214); and cytoplasmic plus 3' untranslated (nts 724-1138; aa 215-230). The underlined sequences are two tandem canonical polyadenylation sites, AATAAA (29), at positions 1112 and 1119.

```
                    10          20          30          40          50          60
RT1-Dα^u   AAGAAAATGG CCACAATTGG AGACCTGGTA ATAAGATTTT TTTTCATGGC TGTCCTAATG
H-2 I-Eα^d ---------- ---------- --C------G T---------- -C-----T-- ---T--G---
HLA-DRα    ---------- ---T--G--- --T--CT--G C--G------ -CA----A-- ---G--G---

        70          80          90         100         110         120         130
AGCCCCCAGA AGTCGTGGGC TATCAAAGAG GAGCACACCA TCATCCAGGC GGAGTTCTAT CTTTCCCCCG
---T------ ----A----- ---------- --A------- ---------- ---------- ----TA--A-
---G-T---G -A--A----- ---------A --A--TGTG- ---------- C--------- --GAAT--T-

       140         150         160         170         180         190         200
ACCAAAATGG AGAGTTCATG TTTGACTTTG ACGGCGACGA GATTTTCCAT GTAGATATTA AAAAGTCAGA
--A--CG--- ------T--- ---------- -------T-- ---------- -----C---G ----------
-----TCA-- C-----T--- ---------- -T--T--T-- ---------- --G-----GG C----AAG--

       210         220         230         240         250         260         270
GACCATTTGG AGACTTGAAG AATTTGCAAA GTTTGCCAGC TTTGAGGCTC AGGGTGCATT GGCTAATATA
------C--- ---------- ---------- ---------- ---------- --------C- ----------
---GG-C--- C-G------- ------G-CG A--------- ---------- -A-------- ---C--C---

       280         290         300         310         320         330         340
GCTGTGGACA AAGCTAACCT GGACATCATG ATAAAGCGCT CCAACAACAC CCCAGATGCC AATGTGATTC
---------- ---------- ---TG----- -A-G----T- ---------- T--------- --C---GCC-
---------- ----C----- ---A------ -C-------- -----T-T-- T--GATTA-- -----ACC--

       350         360         370         380         390         400         410
CAGAGGTGAC TGTACTCCCC AAAAGCCCGG TGAACCTGGG AGAGCCCAAC ATCCTCATCT GTTTCATTGA
---------- -------T-- -G------T- ---------- ---------- ---------- ----------
-------A-- ---G---A-G --C-----T- --G-A---A- ---------- G--------- -------C--

       420         430         440         450         460         470         480
CAAGTTCTCC CCTCCAGCGG TCAATGTCAC CTGGCTTCGG AACGGACAGC CTGTCACCAA AGGCGTGTCA
---------- -------T-- ---------- ------C--- --T----G-- --------G- ----------
-------A-- --A----T-- ---------- G---------A --T---A-A- ---------C ---A------

       490         500         510         520         530         540         550
GAGACAGTGT TTCTCCCAAG GGAGGACCAC CTCTTCCGCA AATTCCACTA TCTCACCTTC CTGCCCTCCG
---------- -------G-- ---C--T--- ---------- ---------- ---G------ ---------A
--------C- -C--G--C-- ---A------ --T------- -G-------- ----C----- --------AA

       560         570         580         590         600         610         620
TGGAAGATTA CTATGACTGT GAGGTGGATC ACTGGGGTCT GGAGGAGCCT CTGCGGAAGC ACTGGGAGTT
CA--T----T ---------- ---------- -------CT- ---------- ---------A C---------
CT--G--CGT T--C-----C AG------G- -------CT- ---T------- --T-TC---- ----------

       630         640         650         660         670         680         690
TGAAGAGAAA ACCCTCCTCC CAGAAACTAA AGAGAATGTC CTGTGTGTTC TCGGGTTGTT TGTGGGTCTG
---------- ---------- ---------- ---------- A------C-- -T-------- ----------
---T-CTCC- -G--CT---- ----G----C C-----C--G G------CC- -G--CC--AC ----------

       700         710         720         730         740         750         760
GTAGGCATCG TCGTCGGGAT TGTGCTCATC ATCAAGGGCC TTCGGAAACG CAACGCAGTG GAACGGCGCC
--G------- -T--G----- -A-C------ --G-----TA --AAA----- ---T-TT--A -----C--A-
--G------A -TA-T----C CA-CT----- --------AG -G--C---A- ---T----CA -----CA-G/

       770
AAGGAGCCCT GTGA *
---------- ---- *
//--GC-T-- ---- *
```

Figure 2. Nucleotide sequence homology of the coding regions of the analogous alpha chains which specify the rat RT1.Dα^u, mouse I-Eα^d (30), and human HLA DRα (32). Homologous DNA sequences of the I-Eα and HLA DRα chains are indicated as dashes except at positions where they differ from the RT1.Dα sequence. Deletions are indicated by an oblique slash.

```
                -25  -20        -10       -1        10        20        30
RT1-Dα^u         MATIG DLVIRFFFMA VLMSPQKSWA|IKEEHTIIQA EFYLSPDQNG EFMFDFDGDE
H-2 I-Eα^d       ----- A--L----I- ----S-----|---------- ----L--KR- ----------
HLA-DRα          --IS- VP-LG--II- ----A-E---|-----V---- ----N---S- ----------
RT1-Bα           ..... .......... ..........|.......... .......... ..........

        40        50        60        70        80        90        100
IFHVDIKKSE TIWRLEEFAK FASFEAQGAL ANIAVDKANL DIMIKRSNNT PDAN|VIPEVT VLPKSPVNLG
------E--- ---------- ---------- ---------- -V-KE----- ----|A---- --SR------
-----MA-K- -V------GR ---------- ---------- E--T----Y- -IT-|P---- --TN---E-R
.......... .......... .......... .......... .......... ....|..... ..........

        110       120       130       140       150       160       170
EPNILICFID KFSPPAVNVT WLRNGQPVTK GVSETVFLPR EDHLFRKFHY LTFLPSVEDY YDCEVDHWGL
---------- -----V---- -----R---E ---------- D--------- ------TD-F ----------
---V------ --T--V---- -----K---T ---------- ---------- -P----T--V ---R-E----
Q--T----V- NIF--VI-I- ----SK---E --Y--S--SN P--S-H-MA- ---I--ND-I ---K-E----

        180       190       200       210       220       230
EEPLRKHWEF EEKTLLPETK ENVLCVLGLF VGLVGIVVGI VLIIKGLRKR NAVERRQGAL *
------T--- ---------- --V-A---- ---------- I--M--ILL- -V-------- *
D---L----- DAPSP----T --V-A---T ------II-T IF----L--S --A---/-P- *
D--VL----P -VPAPMS-LT -T-V-A---S ---------T IF--Q---SD GPSRHP/-P- *
```

Figure 3. Amino acid sequence homology of the rat RT1.Dα^{u} chain with the analogous chains, I-Eα^{d} (30) and HLA DRα (32), and the other rat alpha chain, RT1.Bα (33). Homologous positions are indicated by dashes and deleted amino acids by an oblique slash. Unknown sequences are indicated by dots. The vertical bars indicate the putative alpha chain domains: signal sequence (position -25 to -1); α1 (position 1-84); α2 (position 85-178); connecting peptide (position 179-191); transmembrane (position 192-214); and cytoplasmic tail (position 215-230). Overlined positions indicate the cysteine amino acids (positions 107 and 163) which may form a disulfide bridge, and two putative asparagine-linked glycosylation sequences (positions 78 and 118).

(143 of 230 positions) are conserved. Figure 3 also indicates several structural features common to the three alpha chains. The two cysteine residues at positions 107 and 163 likely bridge a disulfide loop of 55 amino acids in the α2 domain, resulting in an immunoglobulin-like fold of alternating beta-pleated sheets and bends (31). This immunoglobulin-like structure is recognized by the presence of several highly conserved amino acids that are characteristic of immunoglobulin constant region domains (31,34). In addition, the α1 and α2 domains contain the conserved potential asparagine-linked glycosylation peptide sequence, asn-x-thr, in positions 78 and 118, respectively. A comparison of 26 MHC class II alpha chain sequences has recently been reported which shows in detail the distribution of species specific and allele specific differences found in the alpha chains of the rat, mouse, rabbit and human (35). The comparisons of

Table 1. Amino acid sequence homology among class II alpha chain domains[a]

Domain (amino acid position)		% amino acid sequence homology with RT1.Dα^u		
		H-2 I-Eα^d	HLA DRα	RT1.Bα
SS	(-25 to -1)	84% (21/25)	60% (15/25)	---
α1	(1 to 84)	92% (77/84)	83% (70/84)	---
α2	(85 to 178)	88% (83/94)	82% (77/94)	62% (48/78)
CP	(179 to 191)	100% (13/13)	54% (7/13)	31% (4/13)
TM	(192 to 214)	83% (19/23)	65% (15/23)	70% (16/23)
Cyt	(215 to 230)	75% (12/16)	69% (11/16)	31% (5/16)
Overall	(1 to 230)	88% (204/230)	78% (180/230)	56% (73/130)

a. Sequences of H-2 I-Eα^d, HLA DRα, and RT1.Bα were from ref. 30, 32, and 33, respectively. Abreviations: SS, signal sequence; CP, connecting peptide; TM, transmembrane; Cyt, cytoplasmic.

intra-species alleles indicate that the RT1.Dα, I-Eα and HLA DRα chains exhibit little if any polymorphism and that they represent the most invarient class II molecules.

The rat RT1.D alpha chain is more homologous with the analogous mouse and human alpha chains than with the rat RT1.B alpha chain, as shown in Figure 3 and Table 1. This supports the hypothesis that the two alpha chain genes evolved by gene duplication prior to mammalian speciation. Comparisons of the RT1.Dα and partial RT1.Bα chains indicated 56% (73 of 129 amino acids) overall protein homology (Table 1). The most highly conserved sequences were in the transmembrane domain (70%; 16 of 23 positions) and the α2 domain (62%; 48 of 78 positions). Little of the sequence was conserved in the connecting peptide (31%) or in the cytoplasmic tail (33%). The highly conserved transmembrane domain of the RT1.D and RT1.B alpha chains may reflect the importance of this region not only in membrane-anchoring, but in associating with the invariant chain or the beta chain during intracellular assembly of the class II complex, as previously suggested for the mouse alpha chains (36). The conservation of peptide sequence in the α2 domain of these rat alpha chains suggests a similar structure and function. The α2 domains of the murine I-E and I-A alpha chains are also highly conserved, with 68% homology (36). The high conservation of the α2 domain found in alpha chain sequences indicates strong selective pressure for sequence constraint, likely due to the preservation of its immunoglobulin-fold structure. Although no defined function has been demonstrated for the α2 domain, it may be involved in the noncovalent association of

the alpha chain with the beta chain, an interaction analogous with the dimeric association of the immunoglobulin constant regions.

Southern blot analysis of RT1.Dα

Southern blot analysis near the RT1.Dα gene of rat chromosome 14 with 11 different restriction endonucleases (Bam H1, Bgl 2, Eco R1, Eco RV, Hind 3, Kpn 1, Pst 1, Pvu 2, Sal 1, Xho 1, Xma 3) has revealed no detectable polymorphic restriction fragment associated with susceptibility or resistance to IDDM in BB or WF rats, which have the serologically indistinguishable $RT1^u$ haplotype (unpublished, data not shown). Such studies, however, may identify haplotype specific polymorphic restriction fragments and provide genetic markers of chromosome 14 carried by various inbred strains. Figure 4 shows a Southern blot of BB and Lewis rat DNA which was probed with full-size RT1.Dα cDNA. The 'u' haplotype of the BB rat and the WF rat (not shown), and the 'l' haplotype of the Lewis rat, were associated with Bam H1 DNA bands of 9.8 kb and 11.7 kb, respectively (both haplotypes also had a 7.1 kb band). Although these results indicate a haplotype-specific chromosomal polymorphism, the identical hybridization patterns were found with all other restriction enzymes used, which suggests that the RT1.Dα region of chromosome 14 is relatively nonpolymorphic. For example, Figure 4 also shows the hybridization of Pvu 2 or Pst 1 digested BB and Lewis rat DNAs probed with the full size RT1.Dα probe. The identical pattern was found, Pvu 2 bands of 6.8 kb, 4.4 kb and 1.9 kb, and Pst 1 bands of 7.4 kb and 1.3 kb, in size.

mRNA levels of RT1.Dα in lymphocytes.

It is now apparent that the expression of class II genes is not a constant but a variable phenomenon which may depend on the immune status of the individual. Membrane expression of class II molecules has been correlated with mRNA content of cells, indicating that the primary regulation of class II gene expression is at the level of transcription (37). The autoimmune abnormality of the BB rat leads to the development of IDDM in ~50% of BB rats at 60-120 d of age. This suggests that diabetes prone rats possess a highly acvtivated immune system prior to and during the onset of IDDM. It seemed reasonable that the class II genes would be transcribed and expressed at a greater level in lymphocytes of a highly activated immune system compared with a normally active immune system. One approach which we have taken to test this hypothesis was to measure class II gene transcripts purified from lymphocytes of age matched diabetes prone BB rats and nondiabetic normal rats. In order to measure RT1.Dα gene transcripts, a riboprobe plasmid was constucted which specified the α2, transmembrane and cytoplasmic

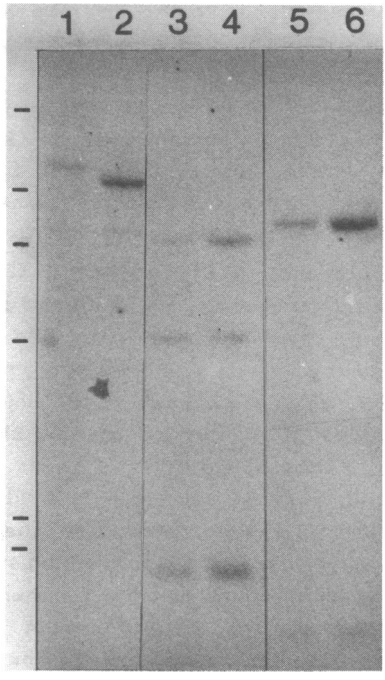


Figure 4. Southern blot analysis of chromosome 14 of the rat near the RT1.Dα gene. DNA of the BB rat (lanes 2,4,6) and the Lewis rat (lanes 1,3,5) was digested with Bam H1 (lanes 1,2), Pvu 2 (lanes 3,4), or Pst 1 (lanes 5,6), electrophoresed on agarose gels, and processed as described (26). Each lane contained 10 ug DNA. The radioactively labelled probe used was the full size coding region of RT1.Dα, exised from pRT1.Dα^{u}-11 with Pst 1. Indicated size markers in kb are: 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0.

domains plus 49 nt of the 3' untranslated region, of the RT1.Dα mRNA. Highly radioactive antisense RNA was transcribed in vitro from Sca 1 linearized pT7RT1.Dα^{u}492 template DNA. Of the 1116 nt primary RNA transcript, 470 nt was complementary with RT1.Dα mRNA. RNase protection studies with poly A^{+} target RNA indicated that RT1.Dα mRNA was quantitatively detected and that the assay was directly proportional to input target RNA over at least a 200-fold range in concentration (from 0.05ug to 10.0 ug; data not shown). Similar results were demonstrable with 0.5 ug to 20 ug of total cellular RNA purified from suspension splenocytes (not shown). The use of total RNA as target was preferred for comparisons of the relative amounts of RT1.Dα mRNA found in lymphocytes of individual rats, as this circumvented the problems associated with the degree of purity

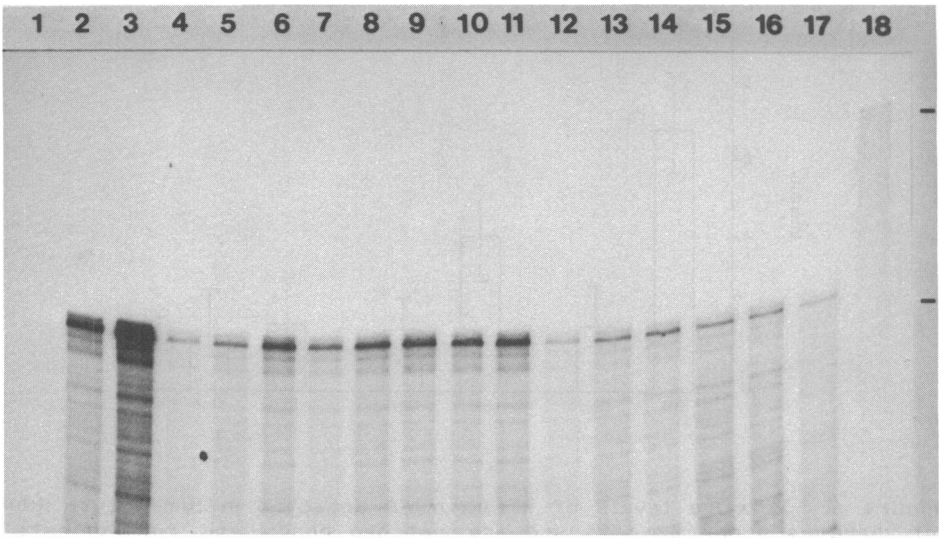


Figure 5. Analysis of RT1.Dα mRNA levels in lymphocyte RNA. Radioactive antisense RNA was synthesized in vitro, purified, and hybridized in solution with a known amount of target RNA purified from suspension splenocytes of individual rats. The riboprobe is homologous with 470 nt of the RT1.Dα mRNA. Nonhybridized RNA was digested with RNases A and T1, the products were fractionated on a 5% denaturing acrylamide gel, the gel was dried and exposed to x-ray film for 24 hr. The autoradiogram displays the RNase protected 470 nt riboprobe fragment for the following samples: (1) 4 ug tRNA; (2,3) 1 ug, 2 ug BB rat poly A^+ RNA; (4,5) 4 ug total RNA, newly diabetic BB rats of 112, 111 d; (6-11) 4 ug total RNA, prediabetic BB rats of 48 d; (12-14) 4 ug total RNA, nondiabetic Wistar Furth rats of 45 d; (15-17) 4 ug total RNA, nondiabetic Long Evans Hooded rats of 45 d; and (18) primary transcript. The two markers indicate the 1116 nt primary transcript and the protected 470 nt probe.

of poly A^+ RNAs. The relative RT1.Dα mRNA levels were quantified by scanning densitometer analysis of the RNase protection autoradiograms and integration of the protected 470 nt peak. In order to compare results and to eliminate experiment-experiment variable signal intensity, each assay included the same poly A^+ control sample which was utilized to normalize the results.

RNase protection experiments were carried out with total RNA purified from suspension splenocytes of diabetes prone BB rats and age- and haplotype-matched ('u') normal nondiabetic rats. Three age groups were used: group 1, 20-40 d; group 2, 45-75 d; and group 3, >80 d. The group 3 BB rats included at least six each of diabetic and

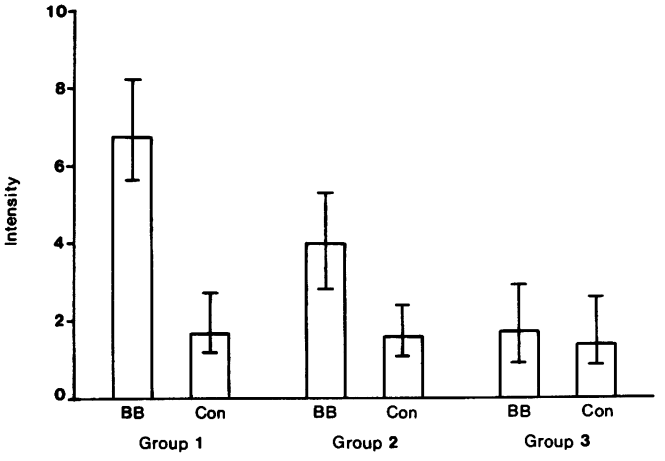


Figure 6. Relative levels of RT1.Dα mRNA detected in lymphocyte RNA of diabetes prone BB rats and age matched nondiabetic control rats. The relative mRNA levels were quantified by scanning densitometry of the RNase protection autoradiograms. The intensity of each target signal was comparable with others after normalization on each autoradiogram with the intensity found for control samples of 1 ug and 2 ug poly A^+ RNA. The average for each group is shown and the vertical lines indicate the range of intensity. The sample size for each group included a minimum of 6 rats. The samples were divided into groups 1, 2 and 3 on the basis of age: 20-40 d; 45-75 d; and >80 d, respectively.

nondiabetic rats. A minimum of six BB or normal rats were used for the other groups. The normal rats used were haplotype-matched ('u') WF rats and LEH rats; RT1.Dα steady-state mRNA levels were not significantly different between them at all ages examined (not shown). Figure 5 shows a representative autoradiogram of an RNase protection assay and Figure 6 indicates a summary of the RNase protection studies. Comparisons of the youngest rats (group 1) indicate that the levels of RT1.Dα transcripts in BB rat lymphocytes were approximately 4x greater than the levels found in the normal rats. The RT1.Dα transcripts in the group 2 BB rats were about 2.5x greater than the levels found in the normal rats. In the group 3 rats, little difference in the levels of RT1.Dα mRNA was found between the BB and normal rats. The group 3 BB rats included diabetic and nondiabetic rats, and no significant differences were found between them. The relative level of RT1.Dα mRNA found in lymphocytes of the nondiabetic normal rats was approximately the same at all ages examined. In contrast, the relative level of RT1.Dα

transcripts in lymphocytes of diabetes prone BB rats was greater in the youngest animals; the steady-state levels of RT1.Dα mRNA decreased with increased age to the levels seen in normal rats >75 d of age. The reduction of RT1.Dα gene transcripts to a relatively "normal" level coincided with the approximate time or age at which the BB rat develops insulitis and diabetes (10). Approximately 40-60% of BB rats develop overt diabetes (10). However, the elevated steady-state levels of RT1.Dα mRNA in lymphocytes of young diabetes prone BB rats does not correlate with the development of overt diabetes. Figure 6 clearly indicates that the range of RT1.Dα mRNA levels found in the group 1 BB rats is greater than that found in any of the group 3 BB rats. Nevertheless, the increased RT1.Dα mRNA levels correlate with the age at which the autoimmune effector mechanisms are thought to be activated. The use of the incidence of diabetes as a measure of immune abnormalities leading to IDDM development is deceptive; all of the diabetes prone BB rats undergo autoimmune/pathological events to various degrees of severity, which lead to the development of overt IDDM in a fraction of the animals.

The differences found in the steady-state levels of RT1.Dα mRNA in lymphocytes of BB and normal rats is likely due to variations in the percentage of splenic lymphocytes expressing this gene, and thus, reflects changes in lymphocyte populations. These results support the hypothesis that the diabetes-prone BB rat has a highly activated immune system, as reflected by increased transcripts of RT1.Dα in lymphocytes. The increased transcripts of the RT1.Dα gene in lymphocytes of the BB rat may be considered representative of other class II genes as the expression of class II genes is coordinate (37), but this remains to be determined. For example, other studies have found increased numbers of class II positive circulating T cells in BB rats a few weeks prior to, and coincident with, diabetes diagnosis (38). These studies used the monoclonal antibody OX6 which detects RT1.B determinants (39).

We have looked at RT1.Dα gene transcripts in BB rats from two additional colonies (Ottawa, Canada; Dr. J. Logothetopolous, Toronto, Canada) and have found similar results, which suggests that the increased transcripts of RT1.Dα are not likely due to induction by infectious agents in the colony.

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