

Association of the Cytotoxic T Lymphocyte-Associated Antigen 4 Gene with Type 1 Diabetes: Evidence for Independent Effects of Two Polymorphisms on the Same Haplotype Block

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A recent study mapped the known association of type 1 diabetes with the cytotoxic T lymphocyte-associated antigen 4 gene to a polymorphism at the 3' end (+6230G>A), but could not rule out additional contribution from the 5' end of the gene. To examine this possibility, we analyzed four polymorphisms at the 5'-flanking region for effects independent of +6230G>A. We confirm, by the transmission disequilibrium test, in 496 family trios overtransmission of the susceptibility allele (G) at +6230 (217/168; $P = 0.013$). Of the four promoter polymorphisms, one (-319C>T) showed overtransmission of the C allele (97/58; $P = 0.0017$). Because the undertransmitted T at the promoter is in linkage disequilibrium with the overtransmitted G at +6230G>A, the effect observed at the promoter cannot be accounted for by linkage disequilibrium with

the +6230G>A. We confirm this by showing that parents heterozygous at the promoter but homozygous at +6230 overtransmit the C promoter allele even more significantly (53/24; $P = 9 \times 10^{-4}$). *In vitro*, the T promoter allele directs higher luciferase expression in Jurkat cells by 42% ($P = 0.006$), a difference also found in lymphocyte mRNA from eight individuals heterozygous at the promoter, but homozygous at +6230 ($P = 1.3 \times 10^{-4}$). Thus, the +6230G>A cannot be the sole functional variant. Either the two polymorphisms define a haplotype carrying the (yet unexamined) functional variant or the -319C>T contributes to the genetic association independently, a possibility suggested by the functional evidence we present. (*J Clin Endocrinol Metab* 89: 6257–6265, 2004)

TYPE 1 DIABETES (T1D) is due to the autoimmune destruction of the insulin-producing pancreatic β -cells. Its etiology involves an important element of genetic susceptibility that behaves as a complex trait. Of the several putative loci identified by linkage analysis, only three have been multiply confirmed and narrowed down to specific genes by the transmission disequilibrium test (TDT). In addition to the insulin-dependent diabetes mellitus 1 (*IDDM1*) gene, which maps to the HLA region (1–3) and *IDDM2* (4, 5), which maps to the insulin gene, significant association with T1D has been found in a haplotype encompassing the cytotoxic T lymphocyte antigen-4 gene (*CTLA4*) (6, 7). This haplotype maps to a broad linkage peak that overlaps the loci previously designated *IDDM12* (8), *IDDM7* (9), and *IDDM13* (10) on chromosome 2q31 (11). This association has been extensively confirmed in both case-control and transmission-disequilibrium studies (8–17). Moreover, association of *CTLA4* polymorphisms with other autoimmune endocrinopathies, especially Graves' disease, has been repeatedly con-

firmed (18–23), although a lack of association has also been reported (24, 25).

CTLA-4 is a critical T cell surface receptor, the lack of which results in uncontrolled T cell-mediated lymphoproliferative disease in the *CTLA4*^{-/-} mouse (26, 27). *CTLA-4* down-regulates the autoimmune response in experimental animal models (28–32), and blockade of *CTLA-4* signaling in human cancer patients elicits autoimmune manifestations (33); its importance in immune regulation makes it a very strong functional candidate for association with autoimmune disease.

A recent study (7) defined the extent of the associated haplotype based on a case-control study of 840 Graves' disease patients and on TDT in 3600 T1D families as well as 210 Graves' disease families. It confirmed the previous finding (6) that the association is confined to *CTLA4* and does not extend to neighboring genes. For both diseases, the strongest association was found at +6230G>A, a newly described, single nucleotide polymorphism (SNP) in the 3'-flanking region, downstream of the previously known polyadenylation site. This polymorphism may be associated with altered levels of steady state mRNA of a soluble *CTLA4* isoform (*sCTLA4*) through a mechanism that remains unclear, but could involve allele-dependent differential alternative splicing and/or polyadenylation of *sCTLA4*. In Graves' disease, all of the association could be attributed to +6230G>A, whereas the more rigorous TDT test in 3600 T1D families failed to narrow the causative polymorphism exclusively to the 3' end, and effects from the 5' end could not be excluded

Abbreviations: *CTLA4*, Cytotoxic T lymphocyte-associated antigen 4; FP, fluorescence polarization; htSNP, haplotype tag SNP; *IDDM1*, insulin-dependent diabetes mellitus 1; LD, linkage disequilibrium; LEF1, lymphoid enhancer factor-1; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PMA, phorbol myristate acetate; *sCTLA4*, soluble *CTLA4*; SNP, single nucleotide polymorphism; T1D, type 1 diabetes; TDT, transmission disequilibrium test; TF, transcription factor.

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(7). The aim of this study was to dissect the contributions of the 5' and 3' ends of *CTLA4* to T1D susceptibility with a combination of functional and genetic studies.

The T1D-associated *CTLA4* haplotype contains several polymorphisms in tight linkage disequilibrium (LD), any one of which or a combination thereof could determine the functional effect (Fig. 1). Of these, the only nonsynonymous polymorphism is a +49A>G base substitution causing a Thr¹⁷Ala change in the signal peptide. We have recently shown that the predisposing Ala¹⁷ allele is incompletely glycosylated in the endoplasmic reticulum leading to retrograde transport of a portion of the molecules to the cytoplasm for degradation (34). This ultimately results in less mature CTLA-4 (Ala¹⁷) at the cell surface, which may explain in part the reduced inhibitory function of CTLA-4 reported in individuals with the +49G allele (35, 36). There are also several promoter polymorphisms in the LD block that could contribute to the T1D association through transcriptional effects on expression, independently of and in addition to the effect of the 3'-flanking +6230G>A on splicing or RNA stability. In this study we have found that the most proximal promoter polymorphism, -319C>T, is highly associated with T1D and that this association is independent of the effect of the 3' SNP +6230G>A. We also show through *in vitro* and *in vivo* studies, an allele-dependent effect of the promoter on *CTLA4* transcription levels.

Subjects and Methods

Subjects

Genomic DNA was obtained from 496 family trios, most of whom attended the diabetic clinic at the Montréal Children's Hospital. Ethnic backgrounds were mostly mixed European; the largest single group was Québec French-Canadian. All participants were diagnosed before the

age of 18 yr and required insulin treatment continuously from the time of diagnosis. Informed consent was obtained for all individuals involved in this study, which was approved by the research ethics boards of the hospitals where recruitment took place.

Genotyping

The +49A>G signal peptide polymorphism was genotyped by restriction digest and/or TaqMan. For the manual genotyping, the forward primer, 5'ATGGCTTGCCTTGGATTTC-3', and the reverse primer, 5'-CTTTGCAGAAGACAGGGATG-3', were used to amplify a 110-bp fragment, followed by digestion by *TseI* (New England Biolabs, Beverly, MA). The products were resolved on 10% PAGE. For genotyping using TaqMan, the primers used for amplification were: forward, 5'-ATGGCTTGCCTTGGATTTC-3'; and reverse, 5'-GCAGAAGACAGGGATGAAGA-3'. The forward probe used was 5'-tetrachloro-6-carboxy-fluorescein-CCAGTCCCTGGTAGCCAGGTTC9-tetramethylrhodamine-3', and the reverse was 5'-6-carboxyfluorescein-AGGTCCCTGGCAGCCAGGTTC9-tetramethylrhodamine-3'.

The -1722(T>C), -1661(A>G), -658(C>T) and -319(C>T) SNPs in the *CTLA4* promoter and the +6230(G>A) SNP in the 3' end were genotyped using single nucleotide primer extension with dideoxy-NTPs labeled with different fluorochromes corresponding to each allele. Primer and probe sequences for fluorescence polarization (FP) are listed in Table 1. Unincorporated PCR primers and deoxy-NTPs were removed according to the PE AcycloPrime PCR Clean-Up protocol, and primer extension was performed according to the PE AcycloPrime-FP protocol in a GeneAmp PCR system 9700 (MJ Research, Waltham, MA). Final detection of the SNP by FP used the Criterion System with Analyst HT (Molecular Devices, Sunnyvale, CA). The genotyping call rate was 97% or greater for all SNPs, and a Mendelian error rate of 0.001 was detected.

Statistics and data analysis

Functional data were compared with the two-tailed, paired *t* test. The paired test was used when comparing DNA and RNA from the same individual or *in vitro* expression of each allelic construct in each of the five experimental replications, to account for variation among experiments that affected both alleles equally.

The binomial distribution with $P = 0.05$ under the null hypothesis

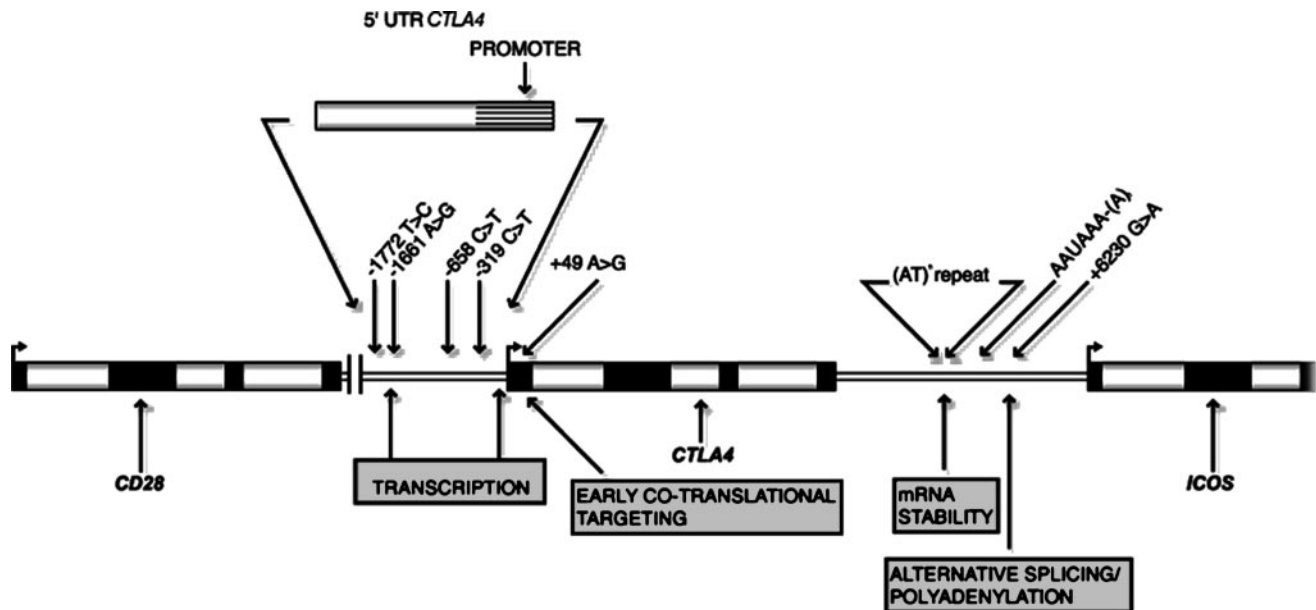


FIG. 1. Summary of genetic and functional significance of SNPs within the *CTLA4* gene. The -319 and +49 SNPs lie within a highly conserved region (overall sequence homology among mouse, rat, and human, 79%). Evolutionary sequence conservation is the best *a priori* criterion for selecting regulatory sequences to evaluate the functional importance (43, 44). The shaded area in the 5' end is the highly conserved promoter region (~400 bp), which includes the -319 SNP, but not the -658 SNP. Potential functional roles are shown in boxes, and some are addressed in this study. [Figure 1 was reproduced in part and reprinted from *Mechanisms of genetic susceptibility to type 1 diabetes: beyond the HLA*, vol. 81, no. 3, pp 187–195, 2004, with written permission from Elsevier.]

TABLE 1. Primers and probes used for FP

SNP	Sense primer	Antisense primer	Annealing temp. for PCR (C)	Probe (forward)	Probe (reverse)	Extension temp. (C)
-172T>C (rs733618)	AGCCCTTTCTGACTTCCACA	AAGCGCAACAAGCAATAAC	62	ACTCTATCATGATCATGGCTTTAGCTG	ACACAGCAGTGGCCAGGGACAG	55
-1661A>G (rs4553808)	AGCCCTTTCTGACTTCCACA	AAGCGCAACAAGCAATAAC	62.5	GCAGGAACAATTTGTTTTCACATTTTT	CAGACTGGGCAACAGAGGTTTTT	55
-658C>T rs n.a.	TCCTTCTGCAAAAACCAGAGG	AAATCCATTTAGCAATTTGGTTAAGA	60	AAACCAGAGGCAGCTTCTTTTC	AAATCACAAAGAAATAAATGAAAATAGGC	55
-319C>T (rs5742909)	GGGATTTAGGAGGACCCCTTG	AGCCGTGGGTTTAGCTGTTA	58	AAGTCTCCACTTGTATCCAGATCCT	TGAAACTGAAGCTTCACTTTCATTT	55
+6230G>A (rs3087243)	TCAATGAGTCAGCTTTTGCCACC	CTGAGAAAGCAGGCGGTAG	59	GATTTCTTCCACCACACTATTTGGGATATAAC	AAGGACTCTTTATGCTGTGTTAACCCA GGACTGCTATGCTGTGTTAACCCA	55

All sequences are in the 5'–3' direction. All primers and probes were designed using primer3 software available online at: http://genome.wi.mit.edu/genome_software/oth-er-primer3.html (46).

was used to evaluate the transmission disequilibrium test (TDT) data. The TDT is based on the distortion from the expected 50% transmission of alleles at a marker locus from heterozygous parents to affected children when the marker is both linked and associated with the disease (37). Because of strong prior evidence, the +6230G/A polymorphism was evaluated at an unadjusted α level of 0.05. For multiple testing of the four promoter SNPs, the Bonferroni-adjusted α level for each SNP was calculated as $1 - (1 - \alpha)^{1/n}$, where α is the overall significance threshold (0.05). Thus, individual results were considered significant at $P \leq 0.0127$.

Genotype odds ratios were computed using pseudocontrols generated from the untransmitted parental alleles assuming Hardy-Weinberg equilibrium. For haplotype analysis including haplotype TDT, the Family-Based Association Test software was used (<http://biostat.harvard.edu/~fbat/fbat.htm>).

Promoter constructs

Genomic DNA from a heterozygote -319C>T was used to amplify a 662-bp fragment of the CTLA4 promoter. The forward primer used was 5'-GCTGTCAAGGGACCATTAGAAGGA-3', and the reverse primer was 5'-GGCTTATGGGAGCGG TGTG-3'. PCR product was T-cloned into pCR2.1 (Invitrogen Life Technologies, Carlsbad, CA). Clones representing each allele were screened by PCR and *MseI* (New England Biolabs) restriction digestion as previously described (38). Clones were sequenced and ligated into pGL3-Basic at *KpnI* and *XbaI* sites (New England Biolabs). The constructs are denoted herein as pCTLAT⁻³¹⁹ and pCTLAC⁻³¹⁹.

Transfections and luciferase reporter assays

Jurkat T cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1 mM MEM, and 100 U/ml penicillin/streptomycin at 37 C in 5% CO₂ (cell culture reagents from Invitrogen Life Technologies). Cells (5×10^5) were transfected in triplicate with 2 μ g CTLA4 promoter constructs and 10 ng of the transfection control *Renilla* luciferase, pRL-cytomegalovirus (Promega Corp., Madison, WI) using Lipofectamine 2000 according to the manufacturer's instructions. All transfections were performed with the same plasmid DNA Maxiprep preparation. The cells were activated with 1 μ g/ml phytohemagglutinin (PHA) and 50 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich Corp., St. Louis, MO) 4 h after transfection for 48 h. Although PHA and PMA are physiologically irrelevant stimuli, they were found to be specifically required for constitutive expression of *Renilla*-luciferase under control of the cytomegalovirus promoter in the Jurkat cell line (39). Luciferase assay was performed using Promega's Dual Luciferase Reporter Assay following the manufacturer's instructions. The assay was performed on 96-well plates, with detection by a multisample, plate-reading luminometer (MicroLumat Plus; E&G Berthold, Bad Wildbad Germany).

DNA and RNA samples

Genomic DNA was extracted from whole blood by standard phenol-chloroform methods. RNA from whole blood was extracted using a QIAamp RNA Blood Mini kit (Qiagen, Hilden, Germany). Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood by standard Ficoll gradient, and $5-8 \times 10^6$ cells were cultured in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, and 2 μ M β -mercaptoethanol and activated with 5 μ g/ml concanavalin (Sigma-Aldrich Corp.) and 10 nM PMA (Sigma-Aldrich Corp.) overnight. RNA was isolated from PBMCs using the RNeasy Mini Kit (Qiagen) with on-column deoxyribonuclease treatment.

PCR, RT-PCR, and quantification of allelic differences by dideoxy sequencing

RT was performed under standard conditions using random primers and 250-1000 ng total RNA template. Primers for exon 1 of CTLA4 (includes the +49A>G polymorphism) in DNA were: forward, 5'-ATGGCTTGCCTTGGATTCA-3'; and reverse, 5'-CTTTGCAGAAGACAGGGATG-3'. For RT-PCR an antisense primer, 5'-GCTGGCCACAGTGCATTGCT-3', spanning exons 1 and 2 was used. These

primers do not distinguish between the soluble and full-length splicing isoforms. PCR amplicons were purified by the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and were sequenced.

Allele-specific quantification by sequencing was performed by comparing ratios of peak heights of each allele in a single reaction in matching RNA and DNA samples from heterozygous individuals amplified, respectively, by RT-PCR or PCR (Fig. 2B). The peak height at a particular base is proportional to the amount present in the sample. This methodology has been validated previously (40), but we tested the principle by mixing known quantities of PCR derived from genomic DNA of individuals homozygous for each allele and sequencing the mixtures. We used the +49A>G SNP as a proxy for the -319 and +6230 SNPs due to the strong LD among the three loci ($D' = \sim 0.8$); hence, a T allele at -319 is in LD with an A at +49 and a G at +6230, a haplotype that was also found by Johnson *et al.* (41). All haplotypes of individuals used in the allelic imbalance studies were confirmed with genotypes of family members. The proportions of +49A/A:+49G/G mixed were 90:10, 70:30, 50:50, 30:70, and 10:90 (Fig. 2B). The observed ratios of A49 over G49 were plotted against the expected, and the correlation between observed and expected was strikingly good ($r^2 = 0.95$ and 0.98 for A and G, respectively), thereby validating the method for allelic-specific quantification (Fig. 2A). Ratios of peak height corresponding to each allele in DNA samples ranged between 0.8–1.2. In the corresponding RNA sam-

ples, an allelic imbalance was said to exist when this ratio deviated from the 0.8–1.2 range. All RNA and DNA peak heights are reported as a ratio of (peak height at allele A)/(average peak height of three A bases upstream and three A bases downstream of +49A) over (peak height at allele G)/(average peak height of three G bases upstream and three G bases downstream of +49G). We refer to this ratio as a standardized ratio.

Results

Genetic studies: T1D association with the -319 promoter SNP cannot be accounted for by LD with the 3' +6230 SNP

The recent study by Ueda *et al.* (7) finds a strong association between a 3'-flanking SNP, +6230G>A, and Graves' disease that cannot be explained by another SNP in the haplotype, thus strongly implicating this SNP as the causative variant in *CTLA4*. However, in the same study the association with T1D was much weaker, and an effect at the 5' end cannot be ruled out (7). To separate these effects on genetic grounds and narrow down the potential functional SNPs, we genotyped five SNPs contained within the *CTLA4* haplotype block, previously defined as haplotype tag SNPs (htSNPs) (41). htSNPs can be used to probe the haplotype diversity of an LD block without genotyping all polymorphisms within a block. In addition, we genotyped the +6230G>A 3' SNP in 496 diabetic family trios (Fig. 1). We confirmed association of +6230G>A with T1D in our dataset. We found significant overtransmission of the +6230G allele by TDT (217/168; $P = 0.013$; Table 2). However, the transmission distortion was stronger in the promoter where the C allele was significantly overtransmitted (97/58; $P = 0.0017$; Table 2). The Mendelian error rate for the -319C>T polymorphism was 1/1100, and parental genotypes for both SNPs were in Hardy-Weinberg equilibrium. The odds ratios were 1.29 (95% confidence interval, 0.9–1.73) for +6230 (AA *vs.* AG+GG) and 1.67 (95% confidence interval, 1.19–2.39) for -319 (CC *vs.* CT+TT). The other three SNPs examined in the *CTLA4* 5'-flanking region were not associated with T1D (Table 2), and the +49A>G was associated with T1D in our dataset as reported in multiple studies (8, 9, 11, 12), probably through its LD with the +6230G>A, as proposed by Ueda *et al.* (7).

To explore the full haplotype diversity at *CTLA4*, we genotyped the previously reported htSNPs (41) required to define the five haplotypes that represent 97% of European chromosomes. On haplotype-based TDT analysis, only one haplotype was significantly overtransmitted after correction for multiple tests, and it contained C at -319 and G at +6230G ($P = 0.0021$; Table 3). Two-SNP TDT analysis on the promoter (-319C>T) and 3' SNP (+6230G>A) revealed significant overtransmission of the -319C/+6230G haplotype ($P = 0.00028$; Table 4).

Examination of the haplotype frequencies suggested that the +6230G>A (which accounts for all other previously reported *CTLA4*-T1D associations) (7) cannot account for the transmission distortion at -319, because the overtransmitted G allele at +6230 was in near-perfect LD ($D' = 0.96$) with the undertransmitted -319 T allele (41) (confirmed in our dataset). The independence of the transmission distortion at -319 from the +6230 effect is more directly demonstrated by conditional TDT analysis, by which transmissions from parents heterozygous at one locus, but homozygous at the other,

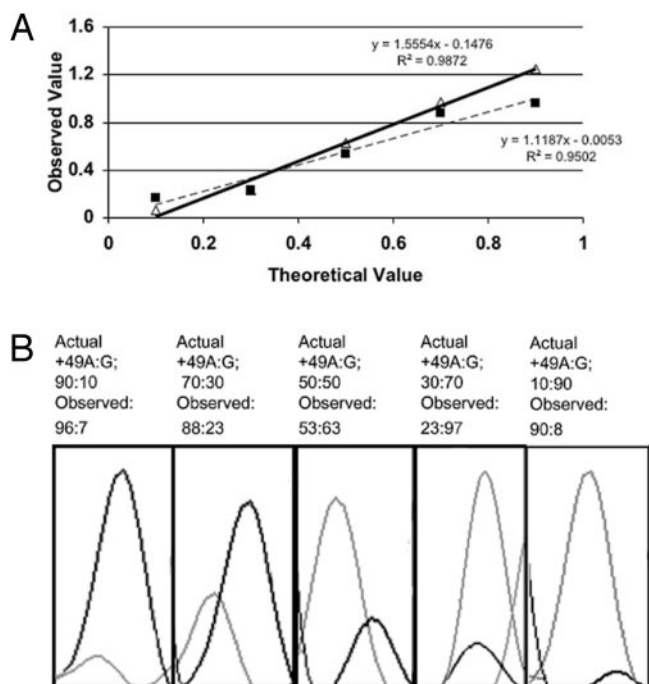


FIG. 2. Allelic imbalance in the RNA is quantified by sequencing. To quantify differential expression of the two alleles, the RT-PCR product is sequenced, and the ratio of the peak heights of the two allelic bases is used to estimate relative abundance in steady state mRNA. A, The method was validated by sequencing mixtures, in defined proportions, of PCR product derived from homozygotes for each +49 allele. The observed relative amount of each allele is plotted against the known amount. (The dashed line is the +49A allele, and the solid line is the +49G allele). The observed values in the sequencing results correlate strikingly well with those expected from the mixture. B, The peak height at the SNP is proportional to the amount sample (40) (black chromatogram, +49A; gray, +49G). Each value at the SNP base peak is corrected by dividing it by the average peak height of adjacent identical bases defined throughout the text as standardized. This accounts for variations within individual sequencing reactions independent of the relative amount of allele. Although peak heights for the same base vary within the same sequence, the nucleotide peak in any given position in relation to its same-base neighbors is remarkably stable across samples run in the same sequencing reaction.

TABLE 2. Transmission disequilibrium of SNPs within the *CTLA4* promoter and of +6230G>A in the 3' end

SNP and allele	T	UT	Total	<i>P</i>	Odds ratio	Allele frequencies
–1722 C (T>C)	50	66	116	0.13 ^a		C: 0.128 T: 0.872
–1661 A (A>G)	97	92	189	0.71 ^a		A: 0.827 G: 0.173
–658 C (C>T)	68	85	153	0.17 ^a		C: 0.896 T: 0.104
–319 T (C>T)	58	97	155	0.0017 ^b	1.70 (CI, 1.19–2.39)	C: 0.908 T: 0.092
+49 G (A>G)	194	244	433	0.024	1.26	A: 0.622 G: 0.378
+6230 G (G>A)	217	168	385	0.013 ^b	1.29 (CI, 0.9–1.73)	A: 0.435 G: 0.565

CI, 95% Confidence interval.

^a Not significant.^b The *P* value was calculated by the two-tailed binomial distribution test, significant at the 0.05 level under the null hypothesis.**TABLE 3.** htSNPs were genotyped in 496 T1D families and haplotype analysis was performed

Common haplotypes ^a					Frequency (%)	z-Score ^b	<i>P</i>
–1661	–658	–319	+49	+6230			
A	C	C	G	G	34.6	3.069	0.0021
A	C	C	A	A	33.8	–1.141	0.253 ^c
A	T	C	A	A	10.0	–0.354	0.723 ^c
G	C	T	A	G	8.6	–2.187	0.029
G	C	C	A	G	8.3	2.230	0.026

Association with T1D was tested using haplo-FBAT software. The LD block containing the entire *CTLA4* gene can be represented by five common haplotypes (frequency, >5%), which account for more than 97% of all chromosomes observed.

^a The –1722 htSNP was not included in this analysis because it is redundant with the +49A>G htSNP as reported previously (41) (confirmed in our own dataset, such that a T allele at –1722 appears exclusively on +49G chromosome, and both *D'* and *r*² values are ~1). We chose the +49 SNP due to its higher heterozygosity rate.

^b The z-score is positive when the haplotype is overtransmitted and negative when undertransmitted.

^c Not significant.

TABLE 4. Two-SNP haplotype analysis in 496 T1D families

Haplotypes		Frequency (%)	z-Score ^a	<i>P</i>
–319	+6230			
C	G	47.2	3.636	<u>0.00028</u>
C	A	43.5	–2.002	0.05
T	G	9.0	–2.204	<u>0.012</u>

^a The z-score is positive when the haplotype is overtransmitted and negative when undertransmitted. Haplotypes containing the G at +6230 and the T at –319 are *underlined*.

were counted. We first examined only transmissions from parents who were homozygous at +6230, but were heterozygous at the promoter locus (–319; –319T; +6230G *vs.* –319C; +6230G and the very few –319T; +6230A *vs.* –319C; +6230A parents), thereby eliminating any effect the 3'-flanking SNP may have on the probability of transmission. In this group, the C allele remained significantly overtransmitted (53/27; *P* = 9 × 10^{–4}; Table 5). Similarly, in parents heterozygous at +6230, but homozygous at –319, the G allele at +6230 was still significantly overtransmitted (145/99; *P* = 0.003; Table 5). In fact, removal of the –319 T effect strengthens the association at +6230 (*P* = 0.013 with the promoter *vs.* *P* = 0.003 despite the smaller number).

The simplest explanation for this observation is that neither of the two SNPs is responsible for the functional effect, but that the C and G alleles mark a haplotype that contains an as yet unexamined functional polymorphism. Alternatively, both polymorphisms function independently of each

TABLE 5. Testing independent effects of the 5' and 3' ends of *CTLA4* by the conditional TDT test

SNP and allele	Transmitted	Untransmitted	Total	<i>P</i>
–319 T	24	53	77	0.0009 ^a
+6230 G	145	99	244	0.0032 ^b

The *P* value was calculated by a two-tailed, binomial distribution test.

^a TDT performed on heterozygous parents at –319, who were homozygous at +6230.

^b TDT performed on +6230G>A heterozygous parents who were homozygous at –319.

other. Ueda *et al.* (7) present both functional and genetic evidence in favor of +6230 being the functional variant. To search for a possible mechanism underlying a contribution of the –319C>T promoter SNP under the second hypothesis, we examined its effects on *CTLA4* transcription. The following sections address these issues.

Functional studies

Proximal CTLA4 promoter region is highly conserved, and the –319C allele is part of the lymphoid enhancer factor-1 (LEF1) transcription consensus site. The SNPs upstream of –319 were not associated with T1D in our population (Table 2), but it is still possible that an unknown SNP in LD with –319C>T might be responsible for the independent genetic effect. Unlike coding sequences, the bounds of promoter-enhancer regions are not clearly defined. In genes such as *CTLA4*, with

highly conserved structure and function (42), evolutionary sequence conservation is a good indicator of functional importance (43, 44). Comparative sequence analysis among mouse, rat, and human, showed that the $-319\text{C}>\text{T}$ SNP lies within a 400-bp region of high conservation (overall similarity, 79%), directly upstream of the $+1$ transcriptional start site (Fig. 1). This sequence completely encompasses the 335-bp promoter shown by Lindsten *et al.* (45) to be sufficient for regulated *in vitro* expression of *CTLA4*. None of the other 5'-flanking SNPs mapped to a highly conserved sequence.

In addition, we analyzed whether any of the promoter SNPs altered transcription factor (TF) binding sites by submitting 20–30 bases flanking each SNP to MatInspector (http://genomatix.de/cgi-bin/matinspector_prof/mat_fam.pl) (46). Two potential TF elements were detected, although only one mapped to a phylogenetically conserved region, the *LEF1* that binds the consensus sequence $^{-317}\text{ttCAAAG}^{-322}$ (-319C is in bold, the core sequence is underlined, and the TF consensus sequence is in uppercase). The -319T allele did not correspond to the binding element of any known transcription factor. Although SNP -1661 also modified an *MEF2* response element, it lies outside the 5'-conserved region and is not associated with T1D. Based on these data, we chose to focus our efforts on the promoter SNP most likely to play a functional role in regulation, the $-319\text{C}>\text{T}$ SNP.

*Transcriptional effects of the -319T allele at the *CTLA4* promoter.* We investigated the effect of the C to T base change in the promoter of *CTLA4* by testing its ability to drive the expression of the luciferase reporter gene. Constructs containing 662 bp of the *CTLA4* promoter region including the SNP at -319 bp were expressed in the Jurkat T cell line. The expression of luciferase was inducible in Jurkat cells by the addition of PHA and PMA and was standardized against the transfection control plasmid *Renilla*, pRL. The results are depicted in Fig. 3 and are expressed as relative luciferase units (luciferase/pRL \times 100). There was no detectable difference between pCTLA-T $^{-319}$ and pCTLA-C $^{-319}$ in Jurkat cells when not activated. Upon activation by PHA and PMA, luciferase expression increased by 3- and 4-fold for pCTLA-C $^{-319}$ and pCTLA-T $^{-319}$, respectively (Fig. 3). The relative

luciferase levels were significantly higher in pCTLA-T $^{-319}$ transfections than pCTLA-C $^{-319}$ (21.8 ± 2.27 vs. 15.8 ± 2.05 ; $P = 0.005$, by paired *t* test; $n = 5$). All transfections were performed in triplicate, and the results shown are the means of five independent experiments. These results confirm a previous report in which greater luciferase expression was found in constructs containing the T allele in the promoter (47). We confirm similar allelic differences in a larger genomic context (662 bp of the promoter vs. 329 bp). The next section confirms that the differential transcriptional regulation of *CTLA4* promoter alleles *in vitro* was reproducible in human lymphocytes *in vivo*.

*The -319T allele of the *CTLA4* promoter is associated with greater expression in human lymphocytes*

To determine whether this *in vitro* effect reflects *in vivo* transcription, individuals heterozygous at the promoter -319 (C/T) and at the signal peptide $+49$ (A/G) were selected ($n = 8$). Due to tight LD, the haplotypes in the vast majority of such individuals are $-319\text{T};+49\text{A};+6230\text{G}/-319\text{C};+49\text{G};+6230\text{G}$, allowing us to use the transcribed $+49\text{A}>\text{G}$ polymorphism to distinguish transcripts derived from the two promoter alleles. This was confirmed in each individual by examining genotypes of family members. We quantified relative mRNA expression levels by comparing the ratio of $+49\text{A}/\text{G}$ in RNA with that of the corresponding $+49\text{A}/\text{G}$ DNA ratio. This was accomplished by sequencing an RT-PCR product (as described in *Subjects and Methods* and Fig. 2). When steady state RNA levels of $+49\text{A}$ and $+49\text{G}$ were compared within the same individuals, statistically significant higher expression was detected from the $-319\text{T};+49\text{A};+6230\text{G}$ haplotype in activated PBMCs [0.39 ± 0.006 vs. 0.29 ± 0.005 (mean \pm SEM) for $+49\text{A}$ and $+49\text{G}$, respectively; $P = 3.34 \times 10^{-9}$, by paired *t* test; Fig. 4A, lower graph]. A similar difference was detected in activated PBMCs and is depicted in Fig. 4 (0.39 ± 0.004 vs. 0.29 ± 0.012 for $+49\text{A}$ and $+49\text{G}$, respectively; $P = 1.3 \times 10^{-4}$, by paired *t* test), almost precisely mirroring the *in vitro* effect shown in the previous section. Whereas the $+49\text{A}/\text{G}$ ratio in DNA was 1.01 ± 0.021 (\pm SEM) in the RNA of activated PBMCs, it was 1.32 ± 0.05 and 1.36 ± 0.02 in inactivated cells, clearly indicating increased transcription from the $-319\text{T};+49\text{A};+6230\text{G}$ haplotype. Because of the LDs described above, all $-319\text{C}>\text{T}$ and $49\text{A}>\text{G}$ heterozygous individuals examined in this study were G/G homozygous at the 6230 3' polymorphism; therefore, these results cannot be attributed to possible effects of 6230 on the soluble isoform.

Discussion

Due to the haplotype structure of the human genome, determination of the polymorphism(s) responsible for genetic association of a haplotype with a phenotypic trait must often rely on complementing genetic studies with functional ones. Ultimately, however, the only way to distinguish between independent functional effects of the -319 and $+6230$ SNPs vs. the functional effect of some other polymorphism for which they are merely markers can be performed only by systematic association studies of all polymorphisms in the LD block. This was actually done by Ueda *et al.* (7) in their

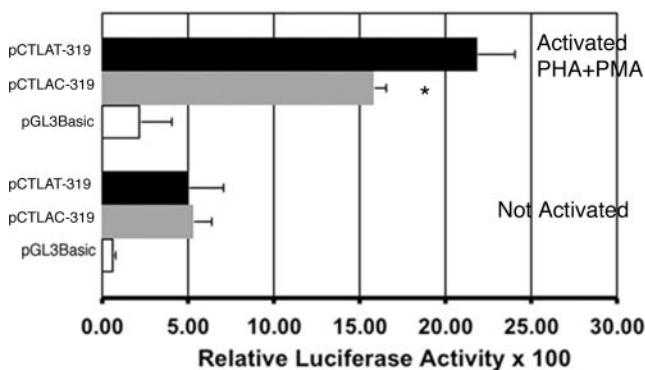


FIG. 3. Higher *CTLA4* expression from the promoter -319T allele *in vitro*. Luciferase reporter plasmids driven by the *CTLA4* 5'-flanking sequence, each corresponding to one allele of the -319 SNP, pCTLA-T $^{-319}$ and pCTLA-C $^{-319}$, were expressed in the Jurkat T cell line. pCTLA-T $^{-319}$ induces an approximately 4.4-fold increase in luciferase expression, reported as 21.8 ± 2.27 , vs. a 3-fold induction under the control of pCTLA-C $^{-319}$ (15.8 ± 1.08 ; *, $P = 0.006$, by paired *t* test).

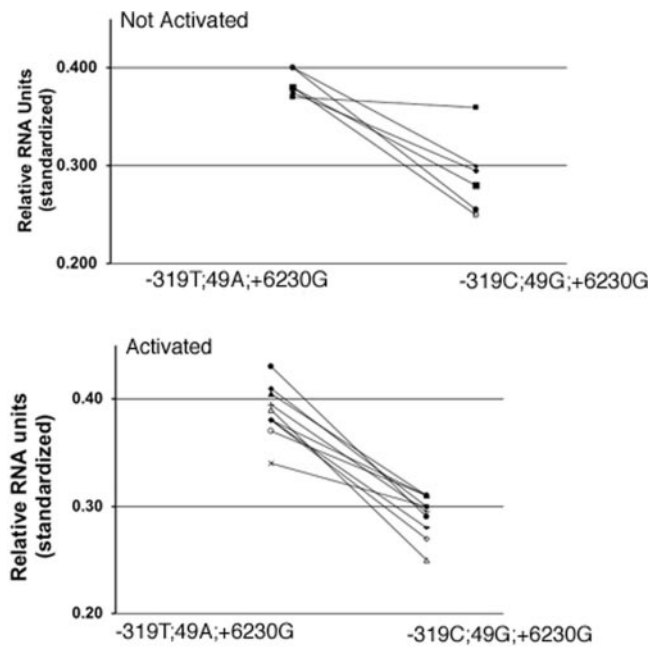


FIG. 4. Allelic imbalance at the *CTLA4* promoter locus in human lymphocytes *ex vivo* correlates with *in vitro* data; the -319 T promoter allele induces higher *CTLA4* mRNA levels. PBMCs from individuals heterozygous at the -319 locus and the $+49$ locus were used to assess allelic variation at the promoter (Fig. 2 and *Subjects and Methods*). There is consistently higher RNA levels due to the T alleles than the C alleles ($P = 1.3 \times 10^{-4}$; $n = 8$) in inactivated PBMCs. Similar data are obtained in activated PBMCs. Each data point represents an average of at least two independent experiments per individual, and the lines connect data for each individual.

Graves' disease dataset and failed to identify a polymorphism with an effect stronger than that of $+6230$ after a resequencing effort that is unlikely to have missed an SNP responsible for the effect (7, 41).

The alternative explanation is that $-319C>T$ polymorphism has an independent functional effect. Both the genetic and the functional effects described in *Results* are independent of LD with the 3'-flanking SNP, because both were observed in individuals homozygous at $+6230$. In this study we confirm the approximately 30% increase in expression at the -319 T allele described in previous *in vitro* studies and directly demonstrate that this expression difference is reproducible in human lymphocytes, the cells involved in diabetes autoimmunity. It is conceivable that a single base substitution in a critical region for transcription factor binding, such as that of the *LEF1* factor, may affect transcriptional efficiency, the regulation of which is an important determinant in the pathogenesis of disease. Such is the case for the *TNF* promoter, where allelic-specific binding of the transcription factor *OCT1* determines survival outcome in malaria infections (48).

A study comparing lymphocyte expression levels in 44 individuals heterozygous at the promoter *vs.* 186 homozygotes (C/C) found an effect in a small number of myasthenia gravis patients, but not in normal controls or in patients with multiple sclerosis (49). This inconsistent result is not surprising, because between-individual comparisons may easily miss subtle differences in the noise introduced by intersub-

ject variation due to genetic loci *in trans* and the individual's immune and other environmental experience. Our approach solves the problem by eliminating biological (as opposed to technical) noise by comparing transcriptional effects within the same cell sample from the same heterozygous individual and is becoming the standard for evaluating the subtle transcriptional effects responsible for complex phenotypes (50, 51).

The assumption that a disease marker association must be reduced to LD with a single functional polymorphism is often implicit in discussions of complex traits, but under the common variant/common disease hypothesis, it is quite plausible that more than one SNP may be contributing functional effects, which, each in isolation, might not have been substantial enough to detect. Ueda *et al.* (7) addressed this possibility and, in the case of T1D, could not rule out an effect from the 5' end of *CTLA4*. They did not, however, examine the $-319C>T$ polymorphism.

The possibility of two independent effects at the *CTLA4* locus suggests yet another complexity that must be considered when dissecting association haplotypes. In the presence of two functional polymorphisms on the same LD block, it is possible that the predisposing allele of one is in LD with the protective allele of the other, in which case the genetic effects of individual SNPs will be blunted by an increase in the frequency of haplotypes that carry one predisposing and one protective allele. In the case of *CTLA4*, under the two-effect hypothesis, the predisposing CG haplotype exerts its genetic effect against a background of partially predisposing haplotypes TG and CA, because the nonpredisposing TA is virtually absent because of the LD. This diminishes the genetic effect of each SNP examined separately.

Although $-319C>T$ appears to confer higher risk than $+6230G>A$ (odds ratio, 1.67 *vs.* 1.29, respectively), it is overshadowed in the haplotype association because of the low frequency of the minor T allele. Nevertheless, for individuals carrying that allele, it must be taken into account in any future algorithms used to determine T1D risk from a panel of genotypes.

The recent association of the $+6230G>A$ 3' flanking SNP with Graves' disease and T1D reported in the largest and most comprehensive SNP mapping and association study to date raises some important issues (7). The researchers found increased susceptibility to autoimmune disease associated with the $+6230$ G allele. They propose that the predisposing $+6230$ G allele mediates the lower expression of a soluble *CTLA4* isoform, *sCTLA4*. A molecular mechanism to explain such effects remains unclear. Although Ueda *et al.* (7) show evidence of the presence of the 3' SNP in Northern blot transcripts, no clear molecular model was proposed. If an alternative polyadenylation site indeed exists that includes $+6230G>A$ in the mRNA, this SNP might affect RNA stability and/or the efficiency of the alternative splicing that creates *sCTLA4*. The possibility of an effect through a 3' enhancer cannot be eliminated either, because we are not aware of any conclusive evidence that *sCTLA4* is transcribed from the same promoter as the full-length form.

Ueda *et al.* (7) reported highest association in the 3' UTR of *CTLA4* with Graves' disease and autoimmune thyroiditis after testing 108 SNPs. They subsequently tested only nine

SNPs in the 3600 T1D families, not including the –319 promoter SNP. Its effect partially explains the weaker association they found in the 3' end (odds ratio, 1.15). Our data clearly point to independent effects of the promoter and the 3' end. After accounting for the promoter SNP, the odds ratio at 3' becomes substantially stronger. It is worth noting that Ueda *et al.* (7) found no association with the –319C>T in Graves' disease, a disorder of humoral immunity whose pathogenesis is probably quite different from the T cell-mediated destruction of the β -cells involved in T1D. If the role of the soluble form is more important in humoral immune dysregulation, it may overshadow the promoter effect. This scenario also provides an attractive explanation for the much weaker effect of +6230G>A in T1D than in Graves' disease (7).

As in all disease association studies with candidate polymorphisms, our findings will need to be replicated in independent DNA sets. A poor reproducibility record in association reports is the result of multiple hypothesis testing without statistical correction. Unlike those reports, this study focused on only four SNPs after genetic evidence of an effect from the 5' end of the gene in addition to the 3' end.

To summarize, taken together with previous work, the results presented here raise the possibility of a model in which disease susceptibility is modulated through nonmutually exclusive, allele-specific transcriptional and, possibly, posttranslational mechanisms. These new, *Homo sapiens*-specific alleles may have arisen in evolution because of the need for better protection of humans against infection, an advantage that would have easily counterbalanced a small increase in the risk of autoimmunity.

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