

# Genetic and Physical Mapping of a Type 1 Diabetes Susceptibility Gene (IDDM12) to a 100-kb Phagemid Artificial Chromosome Clone Containing D2S72-CTLA4-D2S105 on Chromosome 2q33

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Polymorphic markers within the CTLA4 gene on chromosome 2q33 have been shown to be associated with type 1 diabetes. Therefore, a gene responsible for the disease (IDDM12) most likely lies within a region of <1–2 cM of CTLA4. To define more precisely the IDDM12 interval, we genotyped a multiethnic (U.S. Caucasian, Mexican-American, French, Spanish, Korean, and Chinese) collection of 178 simplex and 350 multiplex families for 10 polymorphic markers within a genomic interval of ~300 kb, which contains the candidate genes CTLA4 and CD28. The order of these markers (D2S346, CD28, GGAA19E07, D2S307, D2S72, CTLA4, D2S105, and GATA52A04) was determined by sequence tagged site content mapping of bacterial artificial chromosome (BAC) and yeast artificial chromosome (YAC) clones. The transmission disequilibrium test (TDT) analyses of our data revealed significant association/linkage with three markers within CTLA4 and two immediate flanking markers (D2S72 and D2S105) on each side of CTLA4 but not with more distant markers including the candidate gene CD28. Tsp analyses revealed significant association only with the three polymorphic markers within the CTLA4 gene. The markers linked and associated with type 1 diabetes are contained within a phagemid artificial chromosome clone of 100 kb, suggesting that the IDDM12 locus is either CTLA4 or an unknown gene in very close proximity. *Diabetes* 49:492–499, 2000

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BAC, bacterial artificial chromosome; ETDT, extended transmission disequilibrium test; LD, linkage disequilibrium; PAC, phagemid artificial chromosome; PCR, polymerase chain reaction; STS, sequence tagged site; TDT, transmission disequilibrium test; YAC, yeast artificial chromosome.

**T**ype 1 diabetes is an autoimmune disease characterized by the T-cell-mediated destruction of the insulin-secreting  $\beta$ -cells of the pancreatic islets (1,2). It is a complex disease involving numerous susceptibility genes and environmental factors, which may act as triggers of the autoimmune response. Much research has been focused on identifying type 1 diabetes susceptibility genes in an effort to enhance the prediction of which individuals are at high risk of developing the disease, as well as to understand the pathogenesis in order to prevent the disease. Thus far, only two susceptibility regions have been well characterized. IDDM1 maps to the HLA region and actually contains multiple susceptibility genes (3–11). IDDM2 has been identified as the variable number of tandem repeats immediately 5' to the insulin gene (12–19), which appears to play a role in the level of gene expression (13,14,20–22). Many additional susceptibility intervals have been suggested by complete or partial genome scans conducted by multiple labs (23–32). Several of these intervals (IDDM4, IDDM5, IDDM8, and IDDM12) have been confirmed in additional data sets (33–35), but the exact genes responsible for increased susceptibility to type 1 diabetes have not been identified. Other proposed susceptibility intervals remain to be confirmed.

One of the confirmed type 1 diabetes susceptibility loci is IDDM12, located on chromosome 2q33. Initial studies provided evidence of linkage and association of a polymorphic marker in the CTLA4 gene with type 1 diabetes in Italian and Spanish families (35). These results were confirmed in an additional data set that included French, Spanish, Mexican-American, and U.S. Caucasian families (34). Case-control studies of a Belgian and German data set provided additional confirmation of association between CTLA4 polymorphisms and type 1 diabetes (35,36). There is also association of CTLA4 polymorphisms with Graves' disease and Hashimoto's thyroiditis, both autoimmune thyroid diseases (36–40).

Although these studies of IDDM12 provided convincing evidence for IDDM12, the precise location of the gene responsible for the disease is not well defined. Although CTLA4 is an ideal candidate gene for autoimmune diseases, the association between type 1 diabetes and CTLA4 polymorphisms could be due to linkage disequilibrium of the

TABLE 1  
Families genotyped for chromosome 2q33 polymorphisms

|                  | Multiplex | Simplex | Total |
|------------------|-----------|---------|-------|
| U.S. Caucasian   | 292       | 3       | 295   |
| French           | 32        | 12      | 44    |
| Spanish          | 9         | 9       | 18    |
| Italian          | 3         | 0       | 3     |
| Mexican-American | 11        | 88      | 99    |
| Korean           | 0         | 38      | 38    |
| Taiwanese        | 0         | 27      | 27    |
| Mainland Chinese | 3         | 1       | 4     |
| Total            | 350       | 178     | 528   |

tested markers with an etiological mutation within or outside of the *CTLA4* gene. However, this disease gene should lie within a genomic region of <1–2 cM and most likely within a few hundred kilobases of the associated *CTLA4* markers based on the degree of linkage disequilibrium observed in the human genome (41,42). Indeed, another candidate gene, *CD28*, is located only 50–150 kb from *CTLA4* (43), and it may be the gene responsible for conferring susceptibility to autoimmune diseases.

In an effort to fine-map the location of *IDDM12* and examine directly the possible role of *CD28* in the pathogenesis of type 1 diabetes, we have identified novel polymorphic markers within *CD28* and *CTLA4*. We performed association studies with these and several other markers in the *IDDM12* region. We observed a strong peak of association at the *CTLA4* gene and weaker but significant associations with the immediate flanking markers on either side of *CTLA4*. The other markers showed no, or only very weak, association in our data set. These results suggest that the *IDDM12* gene is either *CTLA4* or an unknown gene in close proximity.

TABLE 2  
Primers used for genotyping of polymorphic markers

| Primers                 | 5' 3' Sequences         | Temperature (°C) | Reference size (bp) |
|-------------------------|-------------------------|------------------|---------------------|
| Primers for genotyping  |                         |                  |                     |
| D2S72-1                 | ATCATTGCACTCTTGTCTGGG   | 58               | 175                 |
| D2S72-2                 | GCACTGGTGGTTCTAATTCAA   |                  |                     |
| D2S105-1                | CTCTACAGTTTATAACCAGC    | 58               | 117                 |
| D2S105-2                | TACTGACTGATTCATATTC     |                  |                     |
| D2S307-1                | CATGACCTGAAATAAACATAG   | 56               | 171                 |
| D2S307-2                | GCTGAGTATCCCATGATTGC    |                  |                     |
| 52A04-1                 | TACACGTAACATAAGAGGGC    | 58               | 204                 |
| 52A04-2                 | CAAACACAAGGGATTATCTAC   |                  |                     |
| 19E07-1                 | AGCCGACTTGAATTCTCCTT    | 58               | 180                 |
| 19E07-2                 | ATACACATCAATAAACATGAAG  |                  |                     |
| CD28-10                 | GAGAATCGCTTGAACCTGGC    | 58               | 166                 |
| CD28-15                 | TAGACAAATAATCCTTCACAGTA |                  |                     |
| D2S346-1                | AGTTTATTCAGGATGGGAGC    | 58               | 162                 |
| D2S346-2                | GTGAGAATTAATGAGTTACAA   |                  |                     |
| CTLA4-22                | CCCTGGCATTGTTGTAGAGTG   | 58               | 257                 |
| CTLA4-24                | CACTATTTTTGAGTTGATGCAG  |                  |                     |
| Primers for Bess T-Scan |                         |                  |                     |
| CD28-5                  | CTCACTCACCTGCACATCTCA   | 58               | 965                 |
| CD28-9                  | CGCTCTTTCCACAACCCACTT   |                  |                     |
| CD28-12                 | GACCTTTTAACTTCCATCATTT  | 58               | 598                 |
| CD28-13                 | TGCTCCTACCACTGTTTTTGA   |                  |                     |

## RESEARCH DESIGN AND METHODS

Subjects. Genomic DNA was obtained from members of 178 simplex and 350 multiplex (with two or more affected children) type 1 diabetes families (Table 1). The collection includes U.S. Caucasian, Mexican-American, French, Spanish, Italian, Korean, and Chinese families. All diabetic patients participating in this study were diagnosed using the criteria of the National Diabetes Data Group. The clinical information on the patients has been described in previous publications (34,44). Genotyping. Microsatellite repeats were genotyped using radioactive labeling of polymerase chain reaction (PCR) primers and denaturing polyacrylamide gel electrophoresis as previously reported (29,33,44). In each case, forward primers were end-labeled using [ $\gamma$ - $^{32}$ P]ATP and T4 polynucleotide kinase. PCR amplifications were performed on 20 ng of genomic DNA (pre-aliquoted into a 96-well microtiter plate) in 12  $\mu$ l reaction volume containing 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, 1.5 mmol/l MgCl<sub>2</sub>, 60  $\mu$ mol/l of each dNTP. Samples were subjected to 35 cycles of 30 s at 94°C for denaturing, 30 s at 58°C for annealing and 30 s at 72°C for extension, using a Perkin-Elmer-Cetus 9600 thermal cycler. After PCR amplification, 2 volumes of sequencing loading solution (0.3% xylene cyanol, 0.3% bromophenol blue, 10 mmol/l EDTA, pH 8.0, and 90% [vol/vol] formamide) were added. The samples were then heated at 95°C for 10 min to denature the DNA, and 2–4  $\mu$ l were immediately loaded onto a 6% polyacrylamide DNA sequencing gel. Primer sequences used for amplification of microsatellite markers are given in Table 2.

A C/T polymorphism in intron 1 of *CTLA4* was amplified with a forward primer (*CTLA4*-24) that contains a mismatched base at the 3' end that introduced an *Hae*III restriction site in the C allele. PCR amplification was performed using conditions similar to those used for microsatellite markers. After amplification, products were digested with 3 units of *Hae*III for 4 h. Digested products were electrophoresed on a 3.5% agarose gel. The digested C allele yielded a fragment of 237 bp, and the undigested T allele yielded a 257-bp fragment. Screening of yeast artificial chromosome, bacterial artificial chromosome, and phagemid artificial chromosome libraries and pulsed field gel electrophoresis. The CEPH human yeast artificial chromosome (YAC) DNA pools and human bacterial artificial chromosome (BAC) DNA pools were purchased from Research Genetics (Huntsville, AL). A phagemid artificial chromosome (PAC) library was purchased from GenomeSystems (St. Louis, MO). YAC and BAC clones were identified by PCR using the *CTLA4*(AT)<sub>n</sub> marker and other markers. BAC/PAC DNA was prepared by a modified alkaline lysis method (45). Each clone was grown in 100 ml Luria-Bertani broth with appropriate antibiotics according to supplier protocols at 37°C. Bacteria were pelleted by centrifugation, then resuspended in 10 ml GTE buffer (50 mmol/l glucose, 10 mmol/l EDTA, 5 mmol/l Tris-Cl, pH 8.0) with lysozyme (180  $\mu$ l, 10 mg/ml). Bacteria were lysed (6.7 ml, 0.5 mol/l NaOH, 1% SDS) for 5 min, and then 5 ml of chilled 3 mol/l KAc was added. After centrifugation, the supernatants were treated with 200  $\mu$ l RNase

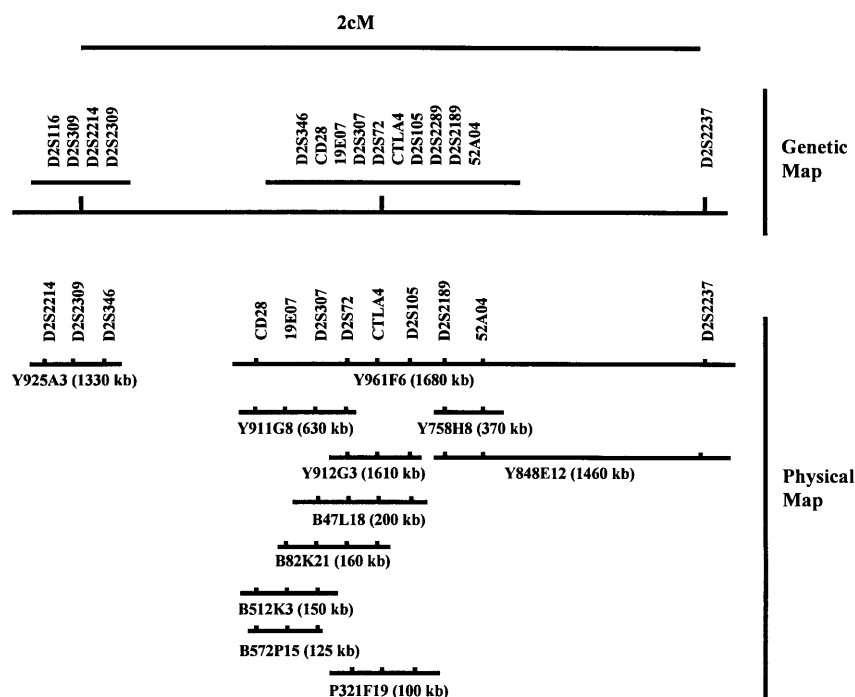


FIG. 1. Genetic and physical map of the IDDM12 region.

A (10mg/ml) for 1 h at 37°C, then extracted with phenol:chloroform:isoamyl alcohol. BAC DNA was then precipitated with isopropanol at 4°C and rinsed with 70% ethanol. After air drying, the pellet was dissolved in 500  $\mu$ l Tris-EDTA.

Pulsed field gel electrophoresis was performed on a Bio-Rad (Hercules, CA) CHEF DR II. BAC/PAC DNA (3–4  $\mu$ g) was digested overnight at 37°C with 15 U NotI (Promega, Madison, WI) in a 30- $\mu$ l volume. Three microliters of loading dye was added, and the full volume was loaded on a 1% agarose gel in 0.5 $\times$  Tris-borate/EDTA electrophoresis buffer. The switch intervals were ramped from 5 to 15 s over a total run time of 15 h. Ethidium bromide (0.5  $\mu$ g/ml) staining of the gel revealed BAC and PAC vectors of 7.3 and 15 kb, respectively, and inserts of varying size.

Identification of novel polymorphic markers. Polymorphic loci within CTLA4 were identified by cycle sequencing of PCR fragments. After amplification of a 923-bp fragment of intron 1 (CTLA4-21/CTLA4-22), purified PCR products were sequenced in the forward and reverse directions. Sequencing was performed in a 10- $\mu$ l reaction containing 1  $\mu$ l Big Dye (PE-Applied Biosystems, Foster City, CA), 3.2 pmol primer, and 1.5  $\mu$ l 5 $\times$  buffer (400 mmol/l Tris-Cl, 10 mmol/l MgCl<sub>2</sub>, pH 9). Unincorporated dyes were removed by ethanol precipitation, and sequences were analyzed on an ABI 310 automated sequencer (PE-Applied Biosystems).

Primers were designed to amplify two partially overlapping fragments of the 3' untranslated region (965 bp) of CD28 using two primer pairs (CD28-5/CD28-9 and CD28-13/CD28-12). The fragments were analyzed for polymorphisms using Bess T-Scan (Epicentre Technologies, Madison, WI). Briefly, PCR was performed using radioactively end-labeled primers and dNTPs containing dUTP. Two amplifications were performed for each primer pair, with one radioactively labeled primer in each reaction. After amplification, PCR products were digested with uracil-N-glycosylase (UNG) and Endonuclease IV, cleaving the strands wherever incorporation of dUTP had occurred. Cleavage products were denatured for 10 min at 95°C, and immediately 4–5  $\mu$ l was loaded onto a denaturing polyacrylamide denaturing gel. Products were loaded three times, with a 2-h interval between loadings to increase the reading length. Differences in cleavage patterns were confirmed by cycle sequencing with an ABI 310 automated sequencer.

Test for linkage by transmission disequilibrium test and extended transmission disequilibrium test. The transmission disequilibrium test (TDT) (46) was used to analyze our data. The TDT is based on the unequal probability of transmission of two different marker alleles from heterozygous parents to affected children when the marker locus and a hypothetical disease gene are linked and in linkage disequilibrium. In a conventional TDT, the transmission of alleles is compared with the expected 50% transmission when there is no linkage disequilibrium between a marker and a hypothetical disease gene. Deviation from the 50% transmission was tested using a  $\chi^2$  test of equal expectation. For TDT with multi-allelic markers, the P values were corrected by 3 because the three most common alleles of each marker were tested.

The conventional TDT for bi-allelic markers has been extended to multi-allelic markers (47–49). These tests for multi-allelic markers are known as extended TDTs (ETDTs). All these ETDTs seek to reject the null hypothesis of no linkage and linkage disequilibrium, but they differ in the assumptions on the alternative hypothesis and computation of P values using Monte-Carlo simulation or asymptotic theory. In this study, we employed the ETDT developed by Sham and Curtis (48). Using their computer program, we have examined two tests, the G1 test under a parsimonious alternative hypothesis and the G2 test under the most general alternative known as the saturated model. In light of our large data set, the conservative G2 test may be more appropriate, and the P values from the G2 test are presented in this article. However, the G1 test also yielded very similar results. Test for association by Tsp. TDT and ETDTs use both affected siblings from multiplex families, and they test for linkage between a genetic marker and a disease locus in the presence of association. The focus of this study is to fine-map a susceptibility interval with prior evidence of linkage. Therefore, we need to test for association in the presence of linkage. The TDT is a valid  $\chi^2$  test for association only if transmission to unrelated affected individuals is used. Here we used the Tsp statistic, which is a valid  $\chi^2$  test for association in the presence of linkage using data from multiple affected sibs. This test is more powerful than the TDT with a single, randomly chosen, affected child from each sib-pair family (50). A computer program for the Tsp analysis was kindly provided by Dr. John A. Todd. Linkage disequilibrium between markers. To test for linkage disequilibrium (LD) between two marker loci, we used the methods outlined by Weir (51). Results for allele pairs with maximum LD were presented in this study. The LD values range from 1 for complete LD to 0 for no LD. A computer program has been developed and is available upon request.

## RESULTS

Physical mapping of the IDDM12 region. In an effort to identify polymorphic markers in close proximity to CTLA4, we first localized the CTLA4 locus by screening a human Mega-YAC library (Research Genetics) by PCR amplification of the CTLA4(AT)<sub>n</sub> marker. Four positive YAC clones were identified from this library. One YAC clone was discarded because of a high degree of chimerism. One YAC clone was not informative because it did not contain any other markers. The two remaining YAC clones (Y961\_F\_6 and Y912\_G\_3) contained additional microsatellite markers in the 2q33 region based on the Massachusetts Institute of Technology physical map (52) and our own sequence tagged site (STS)

TABLE 3  
TDT, ETDT, and Tsp analyses

|                   | Marker name (heterozygosity) |                           |                            |                             |                            |                                |                                |   |                             |                            |
|-------------------|------------------------------|---------------------------|----------------------------|-----------------------------|----------------------------|--------------------------------|--------------------------------|---|-----------------------------|----------------------------|
|                   | D2S346<br>(63%)<br>Allele 5  | CD28<br>(33%)<br>Allele 2 | 19E07<br>(40%)<br>Allele 3 | D2S307<br>(73%)<br>Allele 5 | D2S72<br>(76%)<br>Allele 8 | CTLA4-A/G<br>(48%)<br>Allele G | CTLA4-C/T<br>(48%)<br>Allele T | CTLA4(AT) <sub>n</sub><br>(75%)<br>Allele 8 | D2S105<br>(68%)<br>Allele 8 | 52A04<br>(79%)<br>Allele 6 |
| Conventional TDT  |                              |                           |                            |                             |                            |                                |                                |   |                             |                            |
| U.S. Caucasians   |                              |                           |                            |                             |                            |                                |                                |   |                             |                            |
| %T                | 49.5                         | 45.8                      | 47.2                       | 48.5                        | 55.1*                      | 53.6                           | 55.2*                          | 43.0†                                       | 42.0*                       | 52.8                       |
| T/NT              | 274/279                      | 170/201                   | 216/242                    | 269/286                     | 306/249                    | 297/257                        | 307/249                        | 230/305                                     | 124/171                     | 291/260                    |
| Europeans         |                              |                           |                            |                             |                            |                                |                                |   |                             |                            |
| %T                | 54.0                         | 42.0                      | 48.4                       | 48.2                        | 56.1                       | 58.1                           | 57.0                           | 44.4  | 44.6                        | 54.3                       |
| T/NT              | 34/29                        | 29/40                     | 30/32                      | 41/44                       | 46/36                      | 61/44                          | 49/37                          | 52/65                                       | 25/31                       | 38/32                      |
| Mexican Americans |                              |                           |                            |                             |                            |                                |                                |   |                             |                            |
| %T                | 55.3                         | 53.1                      | 54.9                       | 54.3                        | 61.9                       | 61.8                           | 62.5*                          | 41.1  | 57.1                        | 41.5                       |
| T/NT              | 42/34                        | 17/15                     | 28/23                      | 38/32                       | 52/32                      | 42/26                          | 45/27                          | 30/43                                       | 24/18                       | 22/31                      |
| EUR/MA            |                              |                           |                            |                             |                            |                                |                                |   |                             |                            |
| %T                | 54.7                         | 45.5                      | 51.3                       | 51.0                        | 59.0                       | 59.5*                          | 59.5*                          | 43.6  | 50.0                        | 48.8                       |
| T/NT              | 76/63                        | 46/55                     | 58/55                      | 79/76                       | 98/68                      | 103/70                         | 94/64                          | 82/106                                      | 49/49                       | 60/63                      |
| Asian             |                              |                           |                            |                             |                            |                                |                                |   |                             |                            |
| %T                | 45.5                         | 33.3                      | 36.4                       | 61.3                        | 45.1                       | 52.9                           | 54.3                           | 55.2  | 40.7                        | 48.6                       |
| T/NT              | 10/12                        | 7/14                      | 8/14                       | 19/12                       | 23/28                      | 18/16                          | 19/16                          | 16/13                                       | 11/16                       | 17/18                      |
| Total             |                              |                           |                            |                             |                            |                                |                                |   |                             |                            |
| %T                | 50.4                         | 45.2                      | 47.6                       | 49.5                        | 55.3*                      | 54.9*                          | 56.1†                          | 43.6†                                       | 43.8*                       | 51.9                       |
| T/NT              | 360/354                      | 223/270                   | 282/311                    | 367/374                     | 427/345                    | 418/343                        | 420/329                        | 328/424                                     | 184/236                     | 368/341                    |
| ETDT              |                              |                           |                            |                             |                            |                                |                                |   |                             |                            |
| U.S.              | NS                           | NS                        | NS                         | NS                          | NS                         | NS                             | 0.02                           | 0.00003                                     | NS                          | 0.05                       |
| EUR/MA            | 0.01                         | NS                        | NS                         | NS                          | NS                         | 0.01                           | 0.02                           | 0.002                                       | 0.007                       | NS                         |
| Total             | NS                           | NS                        | NS                         | NS                          | 0.05                       | 0.007                          | 0.001                          | 0.001                                       | 0.02                        | 0.03                       |
| Tsp               |                              |                           |                            |                             |                            |                                |                                |   |                             |                            |
| U.S.              | NS                           | NS                        | NS                         | NS                          | NS                         | NS                             | 0.02                           | 0.002                                       | NS                          | NS                         |
| EUR/MA            | 0.03                         | NS                        | NS                         | NS                          | NS                         | 0.02                           | 0.02                           | 0.02  | 0.04                        | NS                         |
| Total             | NS                           | 0.02                      | NS                         | NS                          | NS                         | 0.01                           | 0.0009                         | 0.03  | NS                          | NS                         |

EUR/MA, Europeans/Mexican Americans; P<sub>c</sub>, P values corrected for multiple tests per marker; %T, percent transmission; T/NT, transmitted/not transmitted to affected offspring. \*P<sub>c</sub> < 0.05; †P<sub>c</sub> < 0.005.

content mapping results (Fig. 1). All these markers except one (D2S2237) clustered at one mapping position on the genetic maps (53,54) (Fig. 1). The YAC contig and the published genetic maps did not allow us to determine the relative order of the markers. Therefore, a BAC library from Research Genetics and a PAC library from GenomeSystems were screened by PCR. Four BAC clones and one PAC clone overlapping the CTLA4/CD28 gene region were selected and further analyzed (Fig. 1). A physical map for the IDDM12 region was generated by detecting the presence or absence of the microsatellite markers in the BAC/PAC clones. We also estimated the sizes of the BAC/PAC clones by NotI digestion and pulsed field gel electrophoresis (Fig. 1).

Identification of a trinucleotide repeat in CD28. To directly assess association/linkage of CD28 with type 1 diabetes, it was necessary to identify a polymorphic marker within the gene. This was accomplished using the Bess T-Scan system (Epicentre Technologies) to identify sequence variations in DNA from eight individuals. Two nonoverlapping fragments from the 3' region of the gene were amplified. After cleavage and electrophoresis on a denaturing polyacrylamide gel, a polymorphism amplified by primers CD28-5 and CD28-9 was apparent. The result was confirmed by direct sequencing, which revealed a polymorphic CAA repeat in the 3' untranslated region of the gene. Two new primers (CD28-10 and CD28-15) were designed for microsatellite genotyping of this polymorphism.

Linkage evidence in the presence of association. In addition to the two CTLA4 markers previously analyzed (34), we identified a novel C/T polymorphism in intron 1, at 819 bp upstream of the exon 2 start site (position 1358 of Genbank M37243). We genotyped this new marker and seven additional microsatellite markers in the region surrounding CTLA4 for all 528 diabetic families. The TDT was then used to examine the transmission of the three most frequent alleles at each marker locus. The transmissions for alleles showing the greatest degree of deviation for each marker and the corrected P values (P<sub>c</sub>) are given in Table 3. Because the families are from various regions/ethnic groups, we first performed TDT analyses in individual data sets: U.S. Caucasians, Europeans, Mexican Americans, and Asians. We also analyzed the combined European and Mexican-American data set because this combined data set showed the greatest deviation in our previous studies, and the diabetes genes in Mexican Americans may have an European origin (34). The results for individual data sets are presented in Table 3. As this study focuses on the fine-mapping of IDDM12, we will only discuss the results for the total data set in details. It should be pointed out that the results from individual subsets are generally consistent with those from the total data sets. The TDT revealed statistically significant deviation from the expected 50% transmission for five markers in the total data set: D2S72, CTLA4-A/G, CTLA4-C/T, CTLA4-(AT)<sub>n</sub>, and D2S105 (P<sub>c</sub> = 0.009, 0.007, 0.0009, 0.002, and 0.03, respectively). The remaining mark-

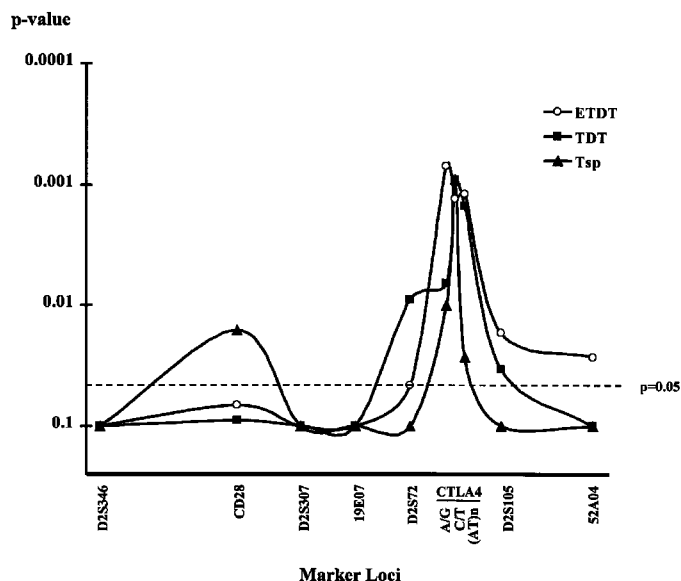


FIG. 2. Association of markers on chromosome 2q33 with IDDM. The P values for the TDT, ETDT, and Tsp analyses of each marker are plotted against the approximate distance of the markers. When the P values are  $>0.1$ , they are plotted at the 0.1 position. A peak of linkage and association was observed in the CTLA4 region.

ers, D2S346, CD28, CHLC.GGAA19E07, D2S307, and CHLC.GATA52A04 showed no statistically significant deviation in transmission (Table 3). Data analyses by an ETDT (48) also revealed statistically significant differences in transmission of the same five markers [ $P = 0.05, 0.007, 0.001, 0.001$ , and  $0.02$  for D2S72, CTLA4-(AT) $_n$ , CTLA4-C/T, CTLA4-A/G, and D2S105 respectively] and 52A04 ( $P = 0.03$ ) (Table 3 and Fig. 2). The TDT and ETDT analyses used both siblings, and the results indicate linkage in the presence of association. These analyses, therefore, provided further linkage evidence for IDDM12 with five markers in the D2S72–D2S105 interval but not the outside markers.

Test for association in the presence of linkage. To test for association in the IDDM12 interval, the data were further analyzed by the Tsp method, a statistic for association in the presence of linkage using sib-pair families (50). This analysis revealed significant associations with the three markers within the CTLA4 gene [ $P = 0.01, 0.0009$ , and  $0.03$  for CTLA4-A/G, CTLA4-C/T, and CTLA4-(AT) $_n$ , respectively] and some evidence for association with the CD28 marker, as well ( $P = 0.02$ ). Since there was no evidence for linkage with the CD28 marker in the TDT and ETDT analyses, the weak association with CD28 detected by Tsp is most likely due to random chance. Therefore, only the CTLA4 markers showed significant linkage and association with type 1 diabetes in the IDDM12 interval.

We plotted the P values for the conventional TDT, ETDT, and Tsp analyses for each marker against the approximate physical distance for the markers in Fig. 2. The plot graphically demonstrates that a region encompassing D2S72–CTLA4–D2S105 is linked to type 1 diabetes in both TDT and ETDT analyses and that only the CTLA4 markers are both linked and associated with type 1 diabetes. The D2S72–CTLA4–D2S105 interval is contained within one PAC clone of 100 kb. The markers flanking this region did not show convincing evidence for linkage or

TABLE 4  
ETDT analyses of haplotypes

| Haplotyped loci         | P value    |
|-------------------------|------------|
| CTLA4 (AT)-D2S105       | $<10^{-6}$ |
| CTLA4 (AT)-D2S72        | 0.001      |
| CTLA4 (AT)-A/G          | 0.0002     |
| CTLA4 (AT)-C/T          | 0.0002     |
| CTLA4 C/T-D2S105        | 0.00004    |
| CTLA4 C/T-D2S72         | NS         |
| CTLA4 C/T-A/G           | 0.0003     |
| CTLA4 A/G-D2S105        | 0.0003     |
| CTLA4 A/G-D2S72         | NS         |
| CTLA4 A/G-C/T-(AT) $_n$ | 0.000005   |
| D2S72-D2S105            | NS         |

P values for these analyses were not corrected.

association, suggesting that IDDM12 is most likely contained in the 100-kb PAC clone.

Haplotype analyses. We also constructed two-marker and three-marker haplotypes and evaluated their transmission from heterozygous parents to affected offspring using ETDT. The haplotypes were constructed using genotype information from parents and multiple siblings. In some families, haplotypes could not be definitively assigned, and such information was discarded from haplotype analyses. As shown in Table 4, highly significant deviation from expected transmission was observed for haplotypes involving seven marker pairs [CTLA4(AT) $_n$ -D2S105; CTLA4(AT) $_n$ -D2S72; CTLA4(AT) $_n$ -CTLA4-A/G; CTLA4(AT) $_n$ -CTLA4-C/T; CTLA4-C/T-D2S105; CTLA4-C/T-CTLA4-A/G; and CTLA4-A/G-D2S105]. All of these marker-pairs involve at least one CTLA4 marker. There was also highly significant deviation in transmission of the three marker haplotype CTLA4-A/G-CTLA4-C/T-CTLA4(AT) $_n$  ( $P = 5 \times 10^{-6}$ ). No haplotypes outside of the D2S72–CTLA4–D2S105 region showed any evidence for linkage. These data provide further evidence for a type 1 diabetes gene in the D2S72–CTLA4–D2S105 region.

LD between markers. We evaluated the degree of LD between each marker pair of all 10 genotyped markers in the IDDM12 region using the method discussed by Weir (51). The results for all marker pairs with one of the three CTLA4 markers were graphically presented in Fig. 3. Several important points are revealed by these analyses. First, high degrees of LD ( $>0.95$ ) were observed between all three CTLA4 markers. Second, there is some degree of LD (0.3–0.7) between the CTLA4 markers and the two immediate flanking markers (D2S72 and D2S105), which also showed significant but weaker evidence of linkage with type 1 diabetes. Third, the LDs between the CTLA4 markers and the other, more distant markers were very low or not present and tended to decrease over the physical and genetic distances. These analyses (Fig. 3), together with the linkage and association results between these markers and type 1 diabetes (Fig. 2), suggest that the most logical explanation for all our data is that the IDDM12 locus is located within the D2S72–CTLA4–D2S105 interval.

#### DISCUSSION

The susceptibility gene IDDM12 was revealed by TDT analyses using two polymorphic markers within the CTLA4 gene (34,35). Evidence for linkage/association has now been

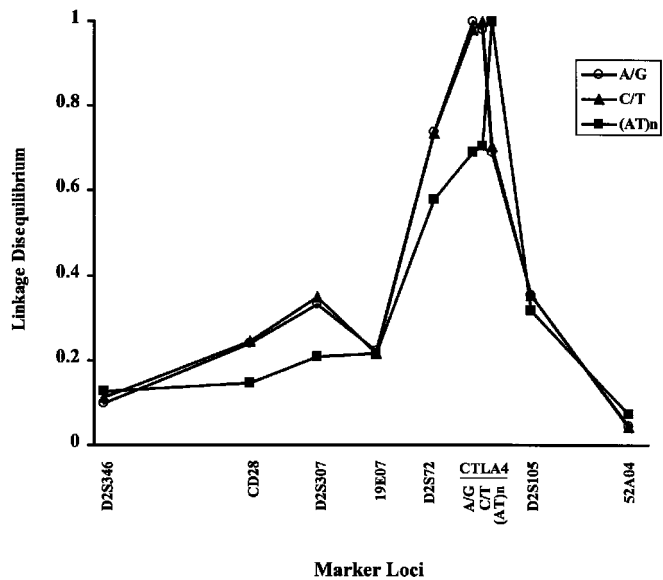


FIG. 3. LD of markers in the IDDM12 region. LD values are plotted against the approximate location of the markers. Each of the three curves represents the LD values between one of the three CTLA4 markers [A/G, C/T, and (AT)<sub>n</sub>] and all other markers. Note that each curve reaches the maximum LD value of 1.0 (complete LD) when the marker is compared against itself [i.e., at the locations for A/G, C/T, and (AT)<sub>n</sub> for the A/G, C/T, and (AT)<sub>n</sub> curves, respectively]. The curves indicate high degrees of LD between the CTLA4 markers and the gradual decrease of LD between these CTLA4 markers and other flanking markers.

reported in diabetic families from six different populations (Italian, Spanish, French, Mexican-American, Caucasian American, and Korean), although significant linkage/association was not found in a large data set of 284 U.K. families (35) or in the small Chinese and Sardinian data sets. In addition to the family-based studies, highly significant evidence for association was also reported in four independent case-control studies in Belgian ( $P = 0.00001$ ) (35), German ( $P = 0.00001$ ) (36), Swedish ( $P = 0.001$ ) (55), and Japanese ( $P = 0.01$ ) (37) populations. These data together provide strong evidence for a diabetes susceptibility locus in close proximity to the CTLA4 gene.

Because IDDM12 was detected through TDT analysis of markers within CTLA4, the disease gene may be in a small region surrounding CTLA4. However, the precise location of the gene was unknown because LD can occur between markers spaced several million base pairs apart, even though it most often occurs between markers separated by < 200–300 kb (41,42,56). To define the interval for IDDM12, we have constructed a physical map of the CTLA4/CD28 interval using STS content mapping of YAC, PAC, and BAC clones. The physical maps have allowed us to determine the microsatellite markers in closest proximity to CTLA4. Using TDT analyses with these markers, we have now shown that five markers—three within the CTLA4 gene and two others flanking CTLA4 (D2S72 and D2S105)—are linked with type 1 diabetes. The markers outside of the D2S72-CTLA4-D2S105 region did not show significant evidence for association. In addition, when the data were analyzed by Tsp, only the three CTLA4 markers showed significant evidence for association. These observations do not seem to be due to low degree of polymorphism

of the markers, because D2S307 and 52A04 are more polymorphic than the CTLA4-A/G marker. The heterozygosity for 19E07 is also very similar to that of CTLA4-A/G. Furthermore, haplotype analyses, which increase the informativeness of the families, did not reveal any evidence of linkage or association with the markers outside of the D2S72-CTLA4-D2S105 region. Since linkage was observed for all markers in the D2S72-CTLA4-D2S105 region but not with the flanking markers and association was observed with all three CTLA4 markers but not others, our data together provided strong evidence that the boundaries for IDDM12 are defined by the markers D2S72 and D2S105. This interval is contained within one 100-kb PAC clone, suggesting that the IDDM12 gene is most likely located within this PAC clone.

The initial IDDM12 interval contained two candidate genes: CTLA4 and CD28, which are located only 50–150 kb apart (43). CD28 is a costimulatory molecule constitutively expressed on T-cells. Interaction of the T-cell receptor with the antigen-HLA complex of antigen-presenting cells will lead to anergy of the T-cell in the absence of costimulation (57). When there is simultaneous engagement of CD28 by its ligands B7-1 or B7-2, a productive immune response is initiated, and the T-cells will proliferate and differentiate into mature T-cells expressing cytokines (57,58). Stimulation of the T-cells also leads to decreased expression of CD28 (59) and increased expression of CTLA4 on the cell surface (60,61). Extensive research has been performed on CTLA4 in recent years. Much of the recent research has focused on elucidating the regulatory role of CTLA4 (62–65). CTLA4 knockout mice experience a severe lymphoproliferative disorder and die within 3–4 weeks of birth (66). Further studies have shown that CTLA4 may act to downregulate expression of interleukin-2 (67), an autocrine and paracrine growth factor responsible for continued T-cell proliferation and survival (57,58,68–70). Clearly, dysregulation of either CD28 or CTLA4 could lead to autoimmune disease, either through inappropriate activation of T-cells through CD28, or through a failure to control and discontinue an immune response by CTLA4.

To test directly the possible role of CD28 in type 1 diabetes, we have identified a trinucleotide repeat marker at the 3' end of the CD28 gene. Although the CD28 marker showed some weak evidence of association by Tsp analyses ( $P = 0.02$ ), this marker did not show any evidence for linkage by either conventional TDT or ETDT analyses. Therefore, CD28 is located outside of the IDDM12 interval and is unlikely to be the IDDM12 gene. CTLA4 is the only other gene that has been identified in the interval, and although other genes may be located in the interval, CTLA4 remains the most likely candidate for IDDM12. However, our preliminary sequencing and expression analyses (data not shown) have not identified an etiological mutation. Because of the small contribution of IDDM12 to the overall susceptibility to type 1 diabetes, there may be difficulty in identifying an etiological mutation within the gene. Presumably any mutation in CTLA4 that has a profound effect on its structure or function would lead to a more severe phenotype in the individuals possessing it. Therefore, it is most likely that the defect is of consequence only in the context of the full array of susceptibility loci possessed by diabetes patients. A phenotypic effect may be difficult to detect without knowing the full genetic background, and the exact etiological mutation may be extremely difficult to identify with certainty. This study narrowed the susceptibility interval to

a 100-kb genomic region and most likely a very small number of genes. It should be very helpful to the final identification of the *IDDM12* locus by genomic sequencing, mutation analyses, and functional studies of all candidate genes within the region.

Fine-mapping of complex disease gene intervals is an extremely challenging task. This study is one of the few successful attempts. Our fine-mapping analyses of the *IDDM12* susceptibility interval demonstrate both the feasibility and difficulties associated with the search for susceptibility genes implicated in complex diseases. It appears that LD mapping in candidate intervals is a feasible strategy to fine-map the many intervals showing linkage with type 1 diabetes. By narrowing the region to only a few hundred kilobases, the number of candidate genes can be dramatically reduced. This approach should also be applicable to the studies of other complex diseases.

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