The genetic locus \textit{Idd6} confers susceptibility to the spontaneous development of type 1 diabetes in the NOD mouse. Our studies on disease resistance of the congenic mouse strain NOD.C3H 6.VIII showed that \textit{Idd6} influences T-cell activities in the peripheral immune system and suggest that a major mechanism by which the \textit{Idd6} locus modifies diabetes development is via modulation of regulatory T-cell activities. Our transfer experiments using total splenocytes and purified T-cells demonstrated that the locus specifically controls the efficiency of disease protection mediated by the regulatory CD4$^+$CD25$^+$ T-cell subset. Our data also implicate the \textit{Idd6} locus in controlling the balance between infiltrating lymphocytes and antigen-presenting cells within the pancreatic islet. \textit{Diabetes} 55: 186–192, 2006

Over 20 type 1 diabetes susceptibility loci (\textit{Idd}) have been genetically localized in the NOD mouse (1), but little information about the nature of these non–major histocompatibility complex \textit{Idd} genes has been obtained and few candidate genes have been proposed. The construction of congenic strains that differ from the NOD receiver strain by only a selected genetic region derived from a non–diabetes-prone parental donor strain (2,3) is a widely used approach to define disease-related candidate regions. All mice of a given congenic strain are genetically identical, which allows a wide variety of phenotypic studies to be applied to large groups of genetically uniform animals. A promising strategy for candidate gene identification is to combine phenotype analysis of congenic mice with expression profiling and haplotype and mutational analysis (4–7). Recently, type 1 diabetes–associated regions on distal chromosome 6, the \textit{Idd6}, \textit{Idd}19, and \textit{Idd}20 loci, have been further defined by the analysis of a series of congenic strains, carrying C3H/HeJ genomic material for distal chromosome 6 introgressed onto the NOD/Lt genetic background (8). NOD/Lt alleles at the \textit{Idd6} locus confer susceptibility to type 1 diabetes, whereas C57BL/6, C57BL/10, and C3H/HeJ alleles all confer resistance to diabetes (8–10). The NOD.C3H congenic strain described in this study carries NOD alleles at both the Natural Killer gene complex (10) and the candidate region for the islet-specific BDC-6.9 autoantigen gene (11,12), which excludes both loci from being responsible for the disease resistance.

Three observations have contributed to the idea that \textit{Idd6} might act via the control of T-cell activities: 1) that a quantitative trait locus (QTL) conferring resistance of NOD-derived immature CD4$^+$CD8$^+$ thymocytes to dexamethasone-induced apoptosis maps to within the \textit{Idd6} region in a NOD × C57BL/6 cross (13–15); 2) that \textit{Idd6} controls low rates of proliferation in immature NOD thymocytes (16); and 3) that the protective effects of C3H/HeJ alleles introgressed at the 4.5-cM \textit{Idd6} interval are completely abolished in NOD.C3H 6.VIII congenic mice after they are treated with cyclophosphamide, an alkylating agent that leads to the depletion of regulatory T-cells (17,18), leading us to speculate that the \textit{Idd6} locus controls the presence or activity of a population of suppressive T-cells in young mice (8).

In the present study, we undertook a detailed phenotypic analysis of the congenic strain NOD.C3H 6.VIII (8), which shows resistance to the spontaneous development of diabetes. We have shown that this resistance is not attributable to either the intrinsic resistance of islet $\beta$-cells to immune destruction or the defect in pathogenic T-cells. Protection of the congenic strain likely involves the control of the proportions of the different leukocyte subsets infiltrating the islet, and in particular that of CD4$^+$ T-cells. Critical to the reduced diabetes susceptibility of the \textit{Idd6} congenic mice is our finding that their regulatory CD4$^+$ CD25$^+$ T-cell subset confers enhanced disease protection.

**RESEARCH DESIGN AND METHODS**

The congenic strain NOD.C3H 6.VIII, homozygous for C3H alleles at the \textit{Idd6} locus; the control congenic strain, carrying NOD alleles at the \textit{Idd6} locus (8); and the NOD/Lt, NOD/scid, and C3H/HeJ mice were all maintained in our animal house by brother-sister mating. The NOD/scid.C3H 6.VIII strain (6.VIII/scid) was established from the congenic strain 6.VIII by crossing it to the NOD/scid strain. F1 generation mice were intercrossed, and mice homozygous for both the C3H-derived \textit{Idd6} interval (markers D6Mit14, 57, 15, 304) and the scid mutation were selected. The animal studies were approved by the institutional review boards.

**Histopathology of the pancreas.** Pancreata were excised, fixed in Bouin’s solution, and processed for paraffin embedding. Then four 5-µm sections taken at 100-µm intervals were stained using hematoxylin-eosin-safranin. At least 20 islets per specimen were analyzed.

**Immunofluorescence staining.** Islet-infiltrating leukocytes were isolated as previously described (19). Cells were pelleted in 96-well plates and stained for 30 min on ice in 20 µl of PBS supplemented with 2% FCS and 5 mM/1 sodium
azide using reagents labeled for biotin, phycoerythrin, fluorescein isothiocyanate, peridinin chlorophyll, or allophycocyanin (BD Biosciences, Le Pont de Claux, France) at optimal concentrations. Where appropriate, a secondary staining step using fluorochrome-conjugated streptavidin was performed. Cells were washed twice and resuspended in PBS containing 1% formaldehyde. Flow cytometry analysis was performed using a FACScalibur and CellQuest software (BD Biosciences, Grenoble, France). The sample size for data collection was 10,000 cells.

**Insulin autoantibody determination.** The 96-well filtration plate microassay for insulin autoantibodies (IAAs) (20) was performed as previously described (21).

**Cell purification.** To purify CD4+ cells, spleen cell suspensions were incubated on ice for 20 min with a mixture of biotin-conjugated anti-CD8, anti-Mac-1, anti-Gr1, anti-B220, and TER-119 antibodies; washed; and then incubated with streptavidin microbeads (Miltenyi Biotec, Paris, France). Cells were separated using a VarioMACS device according to the manufacturer’s protocol. The nonretained cells (90–95% CD4+ T-cells) were further sorted using a biotinylated anti-CD25 (TDo4) antibody and streptavidin microbeads. After cells were stained with fluorescein isothiocyanate–anti-CD4 and phycoerythrin–anti-CD25 (PC61) antibodies, they were analyzed by fluorescence-activated cell sorter (FACS), which reproducibly showed that the retained cells were composed of ≥80% CD4+CD25− cells. The negative fraction contained 90–95% CD4+CD25+ cells. Magnetic cell sorting was used to enrich the latter cell population for CD4+CD25−CD62L+ cells. In some experiments, CD4+CD62L− cells were directly enriched from the CD4+ cell pool. Diabetic genic CD62L+CD4+ T-cells (70–80%) (22) were enriched by the depletion of B-cells, macrophages, erythroid cells, and CD62L− and CD25+ cells by magnetic cell sorting using a pool of three to six spleens from diabetic mice or animals age ≥15 weeks.

**Adaptive transfer of diabetes.** Cells were injected intravenously into immunodeficient recipients: NOD/scid mice, irradiated mice, or thymectomized irradiated mice. Thymic ablation was performed on 6-week-old mice under anesthesia (0.01 mg/g body wt of a 2.5% solution of Avertin [Aldrich]) by suction using a Pasteur pipette. To normalize diabetes transfers, the injected cell pool. Diabeto-

**Statistical analysis.** Pooled data computed as means ± SE were compared using the Mann-Whitney test. Time-to-event distributions were calculated by Kaplan-Meier estimation and compared by log-rank tests over the period of observation.

**RESULTS**

**Protection against diabetes but not against insulitis.** The congenic strain NOD.C3H 6.VIII (6.VIII) was developed by backcrossing the C3H/HeJ-derived chromosome 6 interval distal to D6Mit57 onto the NOD/Lt genetic background. Over the 8-month test period, diabetes incidence was significantly reduced in congenic 6.VIII mice as compared with in control congenic mice in both female (P < 0.0001) and male (P = 0.0011) animals (Fig. 1A). To investigate if the decreased diabetes incidence correlated with changes in insulitis severity, we evaluated the extent of insulitis in pancreas sections obtained from pre-diabetic female strain 6.VIII and control mice (Fig. 1B). Neither 6.VIII nor control mice showed pancreatic islet abnormalities at age 4 weeks. The 8-week-old mice of both strains developed peri-islet and intraislet infiltration. Although a clear progression from peri-insulitis to invasive insulitis was observed with age, no difference in the extent of insulitis was observed between the two strains. Males of both groups developed less diabetes and generally milder insulitis than females. For example, in 16-week-old control male mice, the average percentage of peri-insulitis plus invasive insulitis was ~45% versus ~81% in female mice. The extent of sialitis development was also found to be similar in both strains (data not shown). These data indicate that C3H alleles at the Idd6 locus conferred protection against diabetes but not against pancreatic insulitis and sialitis.

**Presence of IAAs.** Because the presence of IAAs has been correlated with the likelihood of diabetes development in the NOD mouse (20), we measured the IAA levels by radioimmunoassay in female mice of both the 6.VIII and the control strain at age 12 and 16 weeks. In 12-week-old mice, the percentage of animals positive for IAA was lower in the 6.VIII group than in the control group (P < 0.01); however, this difference was not observed at age 16 weeks (P > 0.05) (Fig. 2). These results suggest that the Idd6 locus does not control IAA levels, but that the lower IAA levels at age 12 weeks do correlate with the delay in diabetes development observed in 6.VIII mice.

**Reduced frequencies in islet-infiltrating lymphocytes.** The absence of any difference in the extent of insulitis between the two strains prompted us to search for more subtle differences in the leukocyte subsets invading the islets. We analyzed the islet infiltrate of 12-week-old female mice by FACS. The frequency of infiltrating CD4+ and B-cells (P < 0.05) was significantly lower in 6.VIII islets than in control islets. The CD8+ cell subset, however, remained unchanged. The reduction in the number of lymphocytes infiltrating the 6.VIII islets was counterbalanced by an increase in the number of infiltrating nonlymphoid cells, including macrophages (CD11b+ and F4/80+; P < 0.05) and dendritic cells (CD11c+; P < 0.05) (Fig. 3). This change in the nature of islet-infiltrating cells might modulate the aggressiveness of the autoimmune response to β-cells.

To determine whether these changes were limited to
cells in the pancreas, we performed FACS analysis of the splenic and thymic cell populations of control and 6.VIII mice using six 8-week-old females of each strain. We observed 43.8 ± 3.4 and 43 ± 3.4% B-cells, 10.7 ± 1.3 and 10.5 ± 1.8% CD8+ cells, and 29.5 ± 2.8 and 31.3 ± 3.9% CD4+ cells in the spleen of control and 6.VIII mice, respectively. The percentages of regulatory CD25+ T-cells within the CD4+ subset were also comparable: 10.6 ± 0.4 and 9.6 ± 0.4% in control and 6.VIII mice, respectively.

In the thymus, the percentages of T-cell subpopulations in the control (CD4+CD8+, 3.4 ± 0.2; CD4+CD8+, 84 ± 1.7; CD4−, 9.3 ± 1; and CD8+, 35 ± 0.3%) and the 6.VIII (CD4−CD8+, 3.8 ± 0.3; CD4+CD8+, 83.3 ± 1.5; CD4+, 9.2 ± 0.5; and CD8+, 3.8 ± 0.4%) strains were similar. The percentage of CD25+ cells within the CD4+ T-cell population was 3.9 ± 0.2 and 3.6 ± 0.4% in the control and 6.VIII strains, respectively. We concluded that the reduction in lymphocyte number in the pancreatic infiltrate of the diabetes-resistant strain 6.VIII compared with the control strain did not reflect more generalized changes in the cellular composition of the lymphoid system.

Resistance of diabetes transfer requires the immune system. To investigate the hypothesis of a resistance of β-cells to immune destruction, we tested the ability of diabetogenic NOD splenocytes to transfer diabetes to control and 6.VIII mice. The recipients were irradiated (750 rad) 1 day before 10^7 spleen cells were transferred from diabetic mice to 6.VIII (P < 0.007; n, number of recipients).

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FIG. 2. Measurement of serum IAA levels in 12- and 16-week-old pre-diabetic female mice. CO, control; % pos, frequency of positive samples; n, number of animals.

FIG. 3. FACS analysis of pancreatic islets infiltrating hematopoietic cells. The frequency of CD4+ and B-cells in 6.VIII mice is decreased and the frequency of nonlymphoid cells is increased as compared with the control strain (CO). *P < 0.05 for all markers; n = 15–25 pancreata from each strain.

FIG. 4. Cumulative diabetes incidence after transfer of diabetogenic cells. Total spleen cells (10^7) from diabetic mice were transferred to irradiated congenic mice (A), thymectomized irradiated mice (B) and NOD/scid.C3H 6.VIII and NOD/scid mice (C). Significant differences were found only when irradiated 6.VIII (•) and control (CO, □) recipients were used (P = 0.007); n, number of recipients.

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To determine if this protection was actually related to the susceptibility of β-cells to destruction, we performed two additional experiments. First, mice were thymectomized at age 6 weeks, irradiated 1 week later, and injected with diabetogenic spleen cells the next day. As shown in Fig. 4B, thymectomized, irradiated 6.VIII recipients lost their protection against diabetes transfer. This suggests that the thymus is required to protect irradiated 6.VIII mice against diabetes transfer. In a second experiment, we constructed a novel congenic strain, NOD/scid.C3H 6.VIII
(6.VIII/scid), that is homozygous for both the C3H/HeJ-derived Idd6 interval and the Prkdc<sup>-/+</sup> mutation (23). Homozygous scid mice have been found to lack functional T- and B-cells. Splenocytes from diabetic NOD mice were transferred to 6-week-old male or female 6.VIII/scid and NOD/scid mice. All the scid recipients developed diabetes within 6–7 weeks after the transfer, irrespective of whether they carried the C3H alleles at the Idd6 locus or not (Fig. 4C). At 4 weeks after the transfer, the extent of insulitis was comparable in each strain, with 35% of the islets showing peri-insulitis and 18% showing intra-insulitis (n = 4 recipients per strain). These results indicated that the genetic transfer of the C3H-derived Idd6 interval did not result the islets becoming resistant to autoimmune attack in the absence of the immune system.

**Presence and activity of diabetogenic T-cells.** In light of the above experiments, the immune system, the thymus, and particularly T-cells appear to be crucial to the protection afforded by genes at the Idd6 locus. We then tested whether diabetogenic T-cells from 6.VIII mice have reduced activity as compared with those from control mice. In transfer experiments we used both total spleen cells and splenocytes enriched for CD25<sup>-/+</sup>CD62L<sup>-/+/+</sup> T-cells from aged mice. Both cell populations have been shown to concentrate diabetogenic activity (24,25), and CD25<sup>-/+</sup>CD62L<sup>-/+/+</sup> T-cells have been observed to be devoid of regulatory T-cells (22, F.L., M.-G., unpublished observations).

Adoptive transfer of splenocytes to NOD/scid mice was performed using pre-diabetic 15-week-old male and female control and 6.VIII donors. Splenocytes from aged NOD mice are expected to induce diabetes rapidly in immunodeficient recipients due to the progressive decrease in the activity of regulatory T-cells and the enhanced pathogenicity of CD25<sup>-/+</sup> T-cells with age (25,26). Aged pre-diabetic mice allow a better estimation of the diabetogenic T-cell pool size than diabetic mice because the latter most likely harbor equivalent numbers of effector T-cells independent of their genotype. Splenocytes from diabetic NOD mice were used as positive controls and allowed a rapid induction of diabetes within 3–5 weeks after transfer. Diabetes developed with similar kinetics when donor cells of male and female pre-diabetic control and 6.VIII mice were used, with diabetes being observed from 6 weeks onwards (Fig. 5A). Our results suggest that splenocytes from aged 6.VIII mice induce diabetes as efficiently as cells from aged control mice, with both showing a delay of at least 3 weeks compared with splenocytes from diabetic NOD mice. This delay was likely associated with the use of nonpurified splenocytes in the transfer experiment, which contain both effector and persistent regulatory T-cells.

**Evidence for the control of regulatory T-cells by Idd6.** Splenocytes from young, 8 week-old mice contain few diabetogenic cells and efficiently inhibit diabetes in cotransfer with diabetogenic cells (27,28). A total of 2 × 10<sup>5</sup> splenocytes isolated from mice of each strain were injected together with 10<sup>7</sup> splenocytes from diabetic NOD mice into recipient mice. Diabetes incidence was observed weekly for 10 weeks after the adoptive transfer. Compared with the transfer of diabetogenic cells alone, which induced diabetes in 90% of the recipients at 10 weeks, coinjection of splenocytes from 6.VIII mice conferred an almost complete protection against diabetes, with only 1 of 16 recipients becoming diabetic within 10 weeks (P < 0.0001). Co-injection of splenocytes from young control mice was partially protective (P = 0.005). The difference in the protection conferred by spleen cells from strain 6.VIII versus that from the control strain was statistically significant (P = 0.0014) (Fig. 6A). We concluded that both 6.VIII and control splenocytes contain suppressor cells that are able to control the development of diabetes, but that 6.VIII spleen cells exhibit significantly higher protective activity.
Suppressor activity of strain 6.VIII regulatory T-cells. To better define the regulatory T-cells involved in the Idd6-dependent protective mechanism, we performed cotransfer experiments using purified T-cell subsets. In young pre-diabetic NOD mice, the CD4+ T-cell subset, originally reported as being able to control diabetes development (27), contains at least two populations of regulatory T-cells that express CD25+ and/or CD62L+ (28–30). We first tested the regulatory potential of the CD4+CD62L+ T-cell population, which contains regulatory T-cells belonging to both the CD25+ and CD25− subsets (F.L., M.-C.G. unpublished observations). Young NOD/scid mice were injected with 0.5 × 10^6 CD62L+ diabetogenic cells together with 10^6 CD4+CD62L+ T-cells from 6- to 8-week-old mice. In full agreement with the results obtained when total splenocytes were injected (Fig. 6A), none of the five cotransfer recipients of 6.VIII CD4+CD62L+ splenocytes became diabetic. Less efficient protection was again observed when cells from control mice were used (P = 0.002 vs. the diabetogenic cell group) (Fig. 6B).

In view of this result, the regulatory potential of CD4+CD25+ and CD4+CD25−CD62L+ T-cell populations was tested independently. Young NOD/scid mice were injected with purified diabetogenic cells together with previously determined optimal cell numbers of either CD4+CD25+ or CD4+CD25−CD62L+ T-cells from 6- to 8-week-old congenic mice (28,29). Our experiments showed that 2 × 10^6 CD4+CD25+ cells from strain 6.VIII protected the recipients from diabetes transfer more efficiently than did the equivalent cells from the control strain (P < 0.05), whereas no differences were found comparing the two strains in cotransfer experiments using either 9 × 10^6 (Fig. 6C) or 2 × 10^6 CD4+CD25−CD62L+ T-cells (data not shown).

To determine whether this difference in inhibitory activity persists over time, we performed a cotransfer experiment using purified CD4+CD25+ and CD4+CD25−CD62L+ regulatory T-cells from 15-week-old donors. Again, all four cell populations were found to be protective (P < 0.0005 for each cotransferred population vs. diabetogenic cells). CD4+CD25+ T-cells from both the 6.VIII and control strains provided efficient protection from diabetes transfer (P < 0.0001), with the greatest suppressive activity associated with 6.VIII cells (Fig. 6D). Again, no difference in protection was observed between 6.VIII CD4+CD25+CD62L+ T-cells and control CD4+CD25−CD62L+ T-cells (Fig. 6C and D). Although the suppressive activity of CD4+CD25−CD62L+ cells from both strains persisted over time, only cells from 6.VIII mice were able to ensure complete protection over a 10-week period (P = 0.05).

DISCUSSION
The mechanisms that trigger the activation of autoreactive lymphocytes directed against insulin-producing β-cells are still largely unknown. Alteration of known diabetes-implicated genes, such as the MHC class II, the insulin, and the CTLA4 genes cannot, by itself, account for the development of type 1 diabetes. An association of subphenotypes, each controlled by a unique or several QTLs, that imprint a functional specificity on the key steps in the development of immune reactions or β-cell functions likely underlies the development of diabetes.

The present study focused on the analysis of the congenic NOD.C3H 6.VIII mouse strain. Both male and female congenic 6.VIII mice developed insulitis, although they were protected from diabetes. The 6.VIII strain exhibited reduced levels of anti-IAAs at age 12 weeks, which correlated with its degree of protection from spontaneous diabetes (20). The efficient transfer of diabetes and insulitis by splenocytes from diabetic NOD donors into 6.VIII/scid recipients, however, excludes the possibility that the diabetes resistance of this strain is due to intrinsic β-cell modifications conferring major resistance to immune destruction. Moreover, the resistance of pre-irradiated 6.VIII recipients to the transfer suggests that the protection...
relies on a radio-resistant process that requires the host immune system.

Our finding that the frequency of islet-infiltrating CD4+ T-cells and B-cells decreases in 6.VIII mice whereas the percentage of macrophages and dendritic cells increases underscores the possible role of the local infiltrate in disease protection. It can be hypothesized that a switch in the representation of lymphoid subsets within the islets is directly involved in diabetes protection and may be critical in determining whether the initial infiltrate proceeds to diabetes or whether steady-state insulitis can be maintained. In contrast to the infiltrate, no major change was found in the distribution of T-cell subsets in primary and secondary lymphoid organs in 6.VIII mice, suggesting that the QTL associated with the CD4-to-CD8 T-cell ratio between C57BL/6 and DBA/2 mice (Tmql) (31) is unlikely to be controlled by NOD or C3H alleles at Idd6.

The putative role of the immune system in the diabetes protection afforded by C3H alleles at Idd6 suggests two different hypotheses: decreased activity of effector cells or increased efficiency of immune regulatory mechanisms. The possibility of reduced activity of effector T-cells has been excluded, as diabetes transfer using diabetogenic cells from 6.VIII and control congenics gave comparable results. In contrast, we obtained clear evidence to support the hypothesis that regulatory T-cells are involved in the diabetes protection of 6.VIII mice. The first indication was provided by results showing that thymectomy abrogated the resistance of irradiated 6.VIII congenics to diabetes transfer. The thymus is known to be the major site for the generation of regulatory T-cells, and impairment of the regeneration of thymocytes has been shown to rapidly induce diabetes in the NOD model (27,32–34). Co-transfer experiments using splenocytes from young 6.VIII mice have shown that protection from diabetes can be transferred by splenocytes from the disease-protected mice. Both purified CD4+CD25+ and purified CD4+CD62L+ T-cell subsets showed suppressive activity against the transfer of diabetes by effector T-cells from diabetic donors. In all the experiments that we have undertaken, the highest protective activity was associated with the CD4+CD25+ T-cell subset, with 6.VIII CD4+CD25+ T-cells showing significantly higher suppressive activity than control CD4+CD25+ T-cells. This phenotypic observation leads us to draw the qualitative conclusion that Idd6 alleles modulate the efficiency of CD4+CD25+ regulatory T-cells. The interactions of regulatory T-cells with other cell types likely play an important role in the protection conferred by C3H/HeJ alleles at the Idd6 locus. The local changes in the islet infiltrate suggest such interactions may take place in either the islet itself or the draining lymph nodes. Previous studies have shown that pancreatic lymph nodes are a major crossroads in which the immune response to B-cells occurs in the NOD model (35,36). At present, it is not clear whether one or a combination of genes in the Idd6 interval control the different phenotypes that have been observed.

The importance of regulatory T-cells in the control of autoimmunity, including the well-characterized CD4+ T-cells expressing the interleukin-2 receptor (CD25) (37,38) and l-selectin (CD62L) (28), has been known for a long time (27,39,40), and their inhibitory function on the activation and proliferation of pathogenic T-cells is well characterized (41). Recent work has clearly demonstrated the role of CD4+CD25+ T-cells in the development of type 1 diabetes (42). Defining a cell type whose activity is controlled by Idd6 in the NOD mouse has led us one step further toward identifying the molecular mechanisms underlying Idd6 action and the relevant disease genes lying within its candidate region. Phenotypes associated with regulatory T-cell activity may also contribute to the other genetic traits that localize to the Idd6 region, such as the susceptibility to systemic lupus erythematosus (Lbw4) (43) or to lung and skin cancer (44–46). Because none of the genes known to be implicated in T-cell regulatory activity, such as the transcription factor forkhead box P3 (47–49) or the tumor growth factor β1 (50), localize to the Idd6 interval, the definition of the Idd6 genes is likely to contribute in novel ways to our understanding of the genetic pathways that underpin regulatory T-cell action in controlling immune responses. The definition of a cellular phenotype correlating with Idd6 spontaneous resistance provides an important source of information for the transcriptional profiling approaches that, with sequence and polymorphism evaluation, underlie our current efforts to define and functionally test Idd6 candidate genes.

ACKNOWLEDGMENTS

This study was financed by research grants from the Juvenile Diabetes Research Foundation International (1-2000-600) and the Ministère de la Recherche et Technologie (03246) and by funds from INSERM, the CNRS, and the Pasteur Institute.

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