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Profiles of Epigenetic Histone Post-translational Modifications at Type 1 Diabetes Susceptible Genes*[S]

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Background: Both genetic and epigenetic factors are implicated in Type 1 diabetes (T1D).
Results: Variations in histone H3-lysine 9 acetylation are detected around the promoter/enhancer regions of key T1D susceptible genes in monocytes of T1D subjects *versus* normals.
Conclusion: The chromatin status of this key region is altered in T1D.
Significance: Epigenetic variations at T1D susceptible genes may be functionally important.

Both genetic and environmental factors are implicated in type 1 diabetes (T1D). Because environmental factors can trigger epigenetic changes, we hypothesized that variations in histone post-translational modifications (PTMs) at the promoter/enhancer regions of T1D susceptible genes may be associated with T1D. We therefore evaluated histone PTM variations at known T1D susceptible genes in blood cells from T1D patients *versus* healthy nondiabetic controls, and explored their connections to T1D. We used the chromatin immunoprecipitation-linked to microarray approach to profile key histone PTMs, including H3-lysine 4 trimethylation (H3K4me3), H3K27me3, H3K9me3, H3K9 acetylation (H3K9Ac), and H4K16Ac at genes within the T1D susceptible loci in lymphocytes, and H3K4me3, H3K9me2, H3K9Ac, and H4K16Ac at the insulin-dependent diabetes mellitus 1 region in monocytes of T1D patients and healthy controls separately. We screened for potential variations in histone PTMs using computational methods to compare datasets from T1D and controls. Interestingly, we observed marked variations in H3K9Ac levels at the upstream regions of *HLA-DRB1* and *HLA-DQB1* within the insulin-dependent diabetes mellitus 1 locus in T1D monocytes relative to controls. Additional experiments with THP-1 monocytes demonstrated increased expression of HLA-DRB1 and HLA-DQB1 in response to interferon-γ and TNF-α treatment that were accompanied by changes in H3K9Ac at the same promoter regions as that seen in the patient monocytes. These results suggest that the H3K9Ac status of *HLA-DRB1* and *HLA-DQB1*, two genes highly associated with T1D, may be relevant to their regulation and transcriptional response toward external stimuli. Thus, the promoter/enhancer architecture and chromatin status of key susceptible loci could be important determinants in their functional association to T1D susceptibility.

Type 1 diabetes (T1D)[2] is an autoimmune disease that can occur when immune tolerance in genetically susceptible individuals is disrupted by environmental or other factors (1–3). Extensive studies have provided a wealth of evidence implicating both genetic and environment factors in the etiology of T1D (4). Multiple regions of the genome, known as T1D susceptible gene loci, have been clearly linked to the risk of T1D through extensive linkage and association studies (5–7). All susceptible loci are, however, not equally important. Insulin-dependent diabetes mellitus 1 (IDDM1) in the major histocompatibility complex (MHC) region in chromosome 6 is the prime susceptibility locus, providing up to 50% of the risk of inheritable incidence (8, 9). However, it appears that only a relatively small fraction of genetically susceptible individuals progress to T1D (10) and disease incidence continues to increase, which suggests a significant contribution from environmental factors. Indeed, many studies have uncovered key links between T1D and environmental factors such as infection, diet, perinatal and prenatal nutrition (10–13). Recent clinical trials, such as TEDDY (The Environmental Determinants in Diabetes of the Young), have aimed to identify the specific environmental triggers of T1D. It is plausible that such factors may regulate T1D in a gene-environment interaction or epigenetic manner and, based on our current understanding of the disease, T1D susceptible genes and genes involved in autoimmunity are logically the best candidate genes targeted by environmental factors.

Epigenetic mechanisms in chromatin are increasingly recognized to be a molecular link between genes and the environment. Notably, in eukaryotic cells, chromatin structure can be affected by environmental factors such as diet, chemicals, and pathogens (14). Nucleosome, the basic repeat unit of chromatin, consists of two copies each of histones H2A, H2B, H3, and H4 wrapped by 147 bp of DNA (15). Post-translational modifications (PTMs) at the N-terminal amino acids of histones play

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[S] This article contains supplemental Methods, Tables S1 and S2, and Figs. S1 and S2.

All genomic and expression data can be accessed through NCBI Protein Database under NCBI accession number GSE36403.

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[2] The abbreviations used are: T1D, type 1 diabetes; PTM, post-translational modification; IP, immunoprecipitation; qPCR, quantitative PCR; TSS, transcription start site; MHC, major histocompatibility complex; SNP, single nucleotide polymorphism; HLA, human leukocyte antigen; IDDM1, insulin-dependent diabetes mellitus 1; H3K4me, H3-lysine 4 methylation; H3K9Ac, H3-lysine 9 acetylation; GWAS, genome-wide association study; FDR, false discovery rate.

an essential role in modifying chromatin structure and, along with DNA methylation, form an epigenetic layer that can modulate gene transcription (16–19). Epigenetics usually refers to heritable changes, including those conferred mitotically or meiotically, which occur without changes in the DNA sequence. More recently the definition has been refined to include the structural adaptation of chromosomal regions (20). Thus, both DNA methylation and histone PTMs can work together to control epigenetic transmission (21). Importantly, the chromatin status of gene promoters plays a role in determining how the gene responds to various stimuli. Histone PTMs have varying effects on gene expression depending on the position, type of modification (acetylation, methylation, phosphorylation, or ubiquitylation), and also the degree of methylation (mono-, di-, or tri-) through recognition by key chromatin modifying proteins (16–19). In general, histone H3-lysine 4-trimethylation (H3K4me3) and H3K9-acetylation (H3K9Ac) are associated with promoters and active genes, whereas H3K9me2, H3K9me3, and H3K27me3 are generally associated with repressed genes, and H4K16Ac may control chromatin structure through interactions with key proteins (16–19, 22).

Recent studies have shown that diabetic stimuli like high glucose can alter the levels of key histone PTMs, including H3K9Ac, H3K9me2, H3K9me3, and H3K4me1 at the promoters of inflammatory and other genes related to the pathogenesis of diabetes as well as its vascular complications and metabolic memory (23–30). Genome-wide mapping of histone PTMs has greatly accelerated the field of epigenomics mainly due to the availability of high density microarrays and next generation sequencing technologies coupled with sophisticated bioinformatics analyses methods (31–33). We recently implemented the chromatin immunoprecipitation-linked to microarray (ChIP-array) epigenomic approach to detect variations in histone PTMs in a genome-wide scale in diabetes research (25, 28). In one study (28), we compared histone H3K9me2 patterns in peripheral blood lymphocytes and monocytes obtained from T1D patients *versus* healthy control subjects. The results revealed a significant increase in H3K9me2 in a subset of genes in lymphocytes of the T1D cohort. Pathway analyses showed that these methylated genes were part of key biological networks and canonical pathways relevant to the etiology of T1D and its complications. The study provided evidence of a link between T1D and altered histone methylation of key diabetes-related genes, including those associated with inflammation and autoimmunity. Interestingly, *CTLA4*, a known T1D susceptibility gene, was found to depict differential H3K9me2 methylation in T1D lymphocytes *versus* normal (28).

The massive datasets generated by ChIP-array and other high throughput epigenome profiling approaches have posed enormous challenges for subsequent data analyses, bioinformatics, and statistics as well as functional correlations. In the current study, we adopted a candidate gene-set focused approach because it is well established that a set of susceptible genes or loci are involved in T1D (5–7). A recent genome-wide association study (GWAS) confirmed and extended the results from previous linkage and association studies and defined 41 genome loci as T1D susceptible regions (34). We hypothesized that the chromatin near key T1D-susceptible genes might exhibit specific histone PTM variations, and that these PTM variations may be related to the pathogenesis of T1D. Such events could uncover a cross-talk between genetics and epigenetics to significantly enhance our understanding of T1D. Thus, to gain new insights into these T1D susceptible genes (34), we used the ChIP-array approach to profile and compare histone PTMs at T1D susceptible genes in lymphocyte and monocyte cells obtained from the peripheral blood of T1D individuals *versus* normal controls. To evaluate the changes in chromatin status at these T1D relevant regions of the genome, we first mapped key histone PTMs, including histone H3K4me3, H3K27me3, H3K9me3, H3K9Ac, and H4K16Ac in lymphocytes, and H3K4me3, H3K9me2, H3K9Ac, and H4K16Ac in monocytes from T1D patients *versus* healthy controls. We then examined potential variations in these histone PTMs around some 350 genes within these T1D susceptible regions, including the well characterized IDDM1 region. Noticeable, data analyses revealed the presence of significant variations in histone H3K9Ac at the promoter (enhancer) regions of *HLA-DRB1* and *HLA-DQB1* genes within IDDM1 in monocytes between T1D patients and healthy controls. Furthermore, experiments in THP-1 monocytes demonstrated increases in H3K9Ac at the same promoter regions as that seen in the patient monocytes when HLA-DRB1 and HLA-DQB1 expressions were stimulated by interferon-γ and TNF-α treatment of the THP-1 cells, suggesting that the H3K9Ac variations observed *in vivo* at these regions might be associated with gene expression.

EXPERIMENTAL PROCEDURES

Human Subject Enrollment—All studies were performed according to a protocol approved by the City of Hope Institutional Review Board. Written informed consents were obtained from all blood donors (T1D ($n = 9$) and healthy controls ($n = 7$)). Patient demographics have been previously described (28). There were no statistically significant differences in age or gender between the two groups. Blood monocyte and lymphocyte fractions were prepared as described (28).

ChIP and ChIP-array Experiments with Human Blood Cells—Peripheral blood monocytes and lymphocytes were freshly isolated from individual normal and T1D subjects and then cross-linked in 1% formaldehyde, sonicated to shear DNA, and immunoprecipitations (IP) performed with antibodies to histone PTMs. IP with no antibody was used as the input control. DNA precipitates were washed, eluted, and cross-links were reversed. Small aliquots of DNA before IP and the ChIP-enriched DNA were saved for follow-up validations of ChIP-chip data by ChIP-qPCRs. The remaining ChIP-enriched DNA and no antibody input control were amplified and purified. The amplified DNA samples were separately pooled from nine T1D or from seven normal control samples. These two groups were designated as T1D and normals in this study, and used for microarray hybridization as described (25, 28). ChIP-array experiments were performed with human 385K RefSeq promoter tiling arrays (for lymphocyte samples) at the Roche NimbleGen Core, and with chromosome 6 tiling arrays (NimbleGen human whole genome array 15 of 38) (for monocyte samples) at our Functional Genomics Core. The following

antibodies were used for ChIPs: anti-histone H3K4me3 (Millipore 07-473), H3K9me3 (Abcam ab8898); H3K9me2 (Millipore 07-441), H3K27me3 (Millipore 07-449), H3K9Ac (Millipore 06-942), and H4K16Ac (Millipore 06-762).

Gene Expression Profiling—Human lymphocytes were prepared from four nondiabetic volunteers. RNAs were extracted using Qiagen RNeasy mini kits following the manufacturer's instructions. Biotinylated single strand cDNA was generated and subjected to expression profiling with Affymetrix Human Gene 1.0ST arrays followed by data analyses and conversion into expression measurements using Expression Console version 1.1.1 from Affymetrix.

T1D Susceptible Locus Gene Set Generation—The genes located in T1D susceptible human loci defined by Ensembl version 54 were from www.t1dbase.org. They were subsequently converted to NCBI Accession codes using BioMart. Transcripts not covered by either the Affymetrix GeneChip® Human Gene 1.0 ST Array or whose promoter regions (−2000 to +500 bp relative to TSS) were not covered by the Nimblegen 385K Refseq Promoter Array were filtered out. Redundant transcripts were finally removed by retaining only one transcript among those who shared the same gene symbol and shared the same transcription start site. The gene list is provided in supplemental Table S1.

Heatmap Generation—Gene promoter region (−2000 to 500 bp relative to transcript start site, TSS) of genes covered by both gene expression array and tiling array was divided into 25 nonoverlapping 100-bp regions (finest resolution available), each represented by a positional bin. For each mark, median-shifted $\log_2$-transformed enrichment ratios of the microarray probes falling in each promoter region of the transcript were retrieved, aligned, and assigned to the represented bin based on their positions relative to TSS. A data matrix with each row representing one transcript and 25 columns corresponding to the 25 bins was created. After removing redundant genes by retaining one transcript among those who share the same gene symbol and same TSS, the rows of the matrix are ordered by their corresponding genes' expression and visualized by Java TreeView version 1.1.3 with each row representing enrichment state of one promoter of the RefSeq gene.

Hyper- or Hypoenriched Peak Identification and False Discovery Rate (FDR) Calculation—For each histone PTM, the $\log_2$ ratio data from T1D patients or healthy controls' pooled lymphocytes were quantile normalized. The histone PTM-enriched regions (also called peaks, where $\log_2$ ratios of at least 5 continuous probes are $\geq$1.5 and peak p values are $\leq$0.05) in T1D patients and healthy individuals were then identified separately using TAMAL software (32). Hyper peaks (or T1D specific enriched regions) were subsequently selected from the peaks identified in the T1D sample by comparing the median $\log_2$ ratio of all the probes located inside the peak regions in the T1D sample with those in the healthy sample. FDR was calculated for each peak based on the difference median $\log_2$ ratio in T1D *versus* healthy samples, and the peaks with FDR $<$ 5% were considered as hyper peaks, namely specific enriched regions that can be identified in T1D lymphocytes but not found at the same genomic location in normal lymphocytes. On the other hand, hypo peak refers to the peaks specific in healthy samples, and identified using similar criteria, with FDR of the difference median $\log_2$ ratio in healthy samples *versus* T1D less than 5%. The identified hyper/hypo peaks were further screened to identify those located on the promoters (−2 kb to +500 bp relative to TSS) of genes in T1D susceptible loci for lymphocyte samples. Monocyte data sets were similarly analyzed.

Because different PTMs have different enrichment levels, the tiling-array data distribution for each PTM mark is different. Thus, it is necessary to define different cutoffs of mean $\log_2$ ratio difference between T1D and normal individuals on PTM-enriched regions for each mark based on each dataset distribution. A null dataset was therefore generated for each mark using data from nondiabetic pooled sample. Specifically, the data were randomly shuffled and split into two equal size datasets. The average difference of probe signals of every five consecutive probes between these two datasets was then calculated. The average difference of the null dataset roughly follows a normal distribution with mean of 0 and its S.D. can be empirically calculated. Based on this distribution, the p values of the $\log_2$ ratio difference between T1D and the normal sample of each peak region were calculated. The p values were adjusted to obtain the corresponding FDR. FDR less than 5% was used to identify hyper/hypo PTM-enriched regions between T1D and normal for each mark.

Experiments with THP-1 Cells—THP1 cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, HEPES (10 mM), glutamine (2 mM), streptomycin (50 g/ml), penicillin (50 units/ml), β-mercaptoethanol (50 μM), and glucose (5.5 mM) at 37 °C in a 5% CO_2 incubator. Cells were treated with 20 μM IFNγ and 10 μM TNFα for 4, 16, and 24 h and total RNA was prepared from these samples and from untreated THP1 cells (0 h) using RNA-STAT-60 (Tel-Test). cDNAs were prepared according standard procedures and used for RT quantitative (q)PCR. For ChIP assays, THP1 cells (IFNγ and TNFα treated and untreated) were cross-linked in 1% formaldehyde, then sonicated to shear DNA, and IPs were performed with anti-histone H3K9Ac (Active Motif 39585) (24). A ChIP without antibody was used as control for enrichments and a small fraction of total chromatin saved as input control. The ChIP-enriched DNA and the controls were used in ChIP-qPCRs. ChIPs were also performed with untreated THP-1 cells using antibodies to H3K4me3 (Millipore 07-473), H3K4me1 (ab8895), and p300 (ab14984). These ChIP samples were amplified and used in ChIP-array experiments with human chromosome 6 tiling arrays at the Roche NimbleGen Core and the City of Hope Functional Genomics Core.

Real-time qPCR—ChIP-qPCRs were performed using the histone H3K9Ac ChIP-enriched DNA from each individual or from experiments with THP-1 cells and normalized to the input DNA as described (28). RT-qPCRs were performed as described (25). Student's t tests were used for statistical analyses of the data. Sequences of primers used in this study are provided in supplemental Table S2.

Data Deposition—All genomic data reported in this study have be deposited in the NCBI Gene Expression Omnibus (GEO) data base (accession number GSE36403).

TABLE 1

Summary of the ChIP-array experiments in this study

Summary of Chip-array experiments performed in this study. Antibodies to histone PTM marks used are indicated. The lymphocyte experiments utilized human Ref- Seq promoter tiling arrays (Roche Nimblegen 385K RefSeq), while the monocyte experiments utilized chromosome 6 tiling arrays (Roche Nimblegen Human whole genome array 15 of 38). Details are described under "Experimental Procedures."

	Lymphocytes	Monocytes
Histone marks	H3K9Ac H3K9me3 H3K4me3 H4K16Ac H3K27me3	H3K9Ac H3K9me2 H3K4me3 H4K16Ac
Microarray used	Human promoter tiling array	Human Ch. 6 tiling array
Genome regions	T1D susceptible loci[a]	IDDM1

[a] T1D susceptible loci are from Ref. 34.

RESULTS

Experimental Design—The T1D and control volunteer cohort used is the same as that previously reported (28). Primary lymphocytes and monocytes were isolated from the peripheral blood of these T1D patients ($n = 9$) and nondiabetic healthy controls ($n = 7$), respectively. ChIP assays with the indicated antibodies were performed and ChIP-enriched DNA samples were obtained from monocytes and lymphocytes of each volunteer separately, and then amplified. Here, amplified samples from each individual in T1D or healthy control cohorts were pooled and ChIP-array experiments were performed on each cell type. The study is summarized in Table 1. We used human promoter tiling arrays to map the histone PTMs H3K4me3, H3K27me3, H3K9me3, H3K9Ac, and H4K16Ac at promoters of genes located within the T1D susceptible loci in lymphocytes of T1D patients *versus* controls (Table 1). For monocytes from the same volunteers, we mapped H3K4me3, H3K9me2, H3K9Ac, and H4K16Ac marks at the IDDM1 locus within the MHC region using the chromosome 6 tiling arrays covering the MHC locus (Table 1).

Profiling Histone PTMs in Blood Lymphocytes and Monocytes from Normal Healthy Volunteers—Increasing evidence links histone PTMs with disease states, including diabetes and its complications. However, very little is known about histone PTM profiles at key disease susceptible genetic regions. In our previous study, we observed that key diabetes-related genes displayed increased promoter histone H3K9me2 in T1D patient lymphocytes (28). Based on the fact that many T1D susceptible genes have been shown to play key roles in T-lymphocyte cell differentiation, proliferation, and signal transduction (35), we first mapped several key histone PTMs (H3K4me3, H3K27me3, H3K9me3, H3K9Ac, and H4K16Ac) at 24,659 gene promoter regions using promoter tiling arrays in lymphocytes obtained from the recruited seven healthy volunteers (28). To verify the functional association of these PTM marks to gene expression in general, we also performed expression profiling (with Affymetrix arrays) using RNA extracted from lymphocytes of another four separate normal healthy volunteers. These gene expression data were integrated into the ChIP-array data to evaluate the association between histone PTMs and gene expression.

Heatmap results of the lymphocyte ChIP-array experiments are shown in Fig. 1*A*. Rows represent the enrichment status of H3K4me3, H3K27me3, H3K9me3, H3K9Ac, and H4K16Ac levels genome-wide at gene promoter regions from −2000 to 500 bp relative to TSS. *Red* indicates the relative enrichment of the corresponding mark, whereas *green* indicates depletion. Genes are ordered and sorted by expression level (low to high) from top to bottom based on the Affymetrix mRNA profiling data. As expected, genes depicting high expression levels in lymphocytes had higher enrichment of H3K4me3 and H3K9Ac, two chromatin marks generally associated with active genes. Reciprocally, RefSeq genes with low expression levels had the highest content of H3K27me3 and H3K9me3, marks usually associated with repressed genes (Fig. 1*A*). The compos-

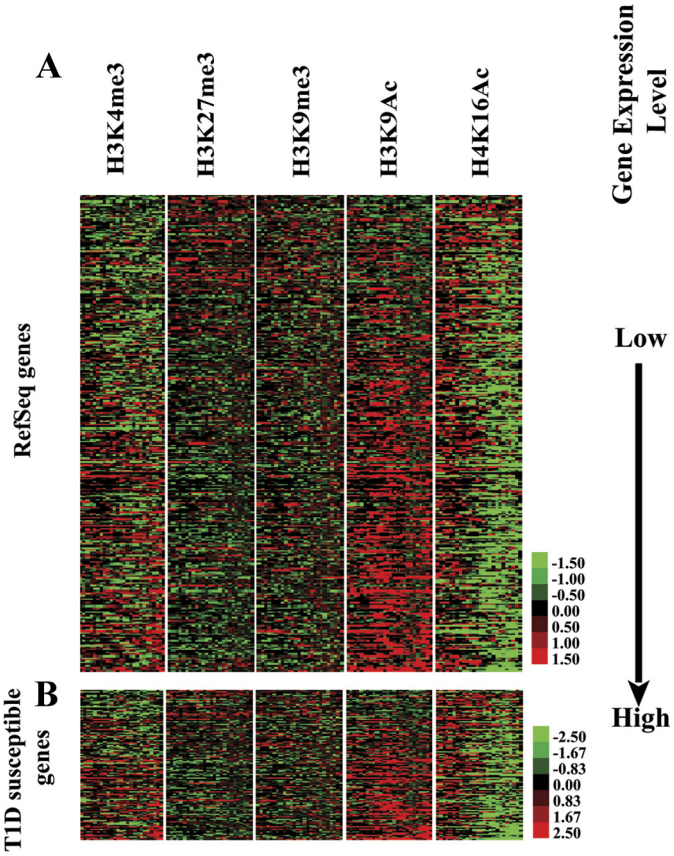

FIGURE 1. **Heatmap of various histone PTMs obtained from ChIP-promoter array profiling of human blood lymphocytes from normal healthy volunteers.** *A*, heatmap of key histone PTMs at RefSeq gene promoters. *Red* represents enrichment, *green* represents depletion, and *black* represents no enrichment or missing data due to lack of probes. The enrichment level of each PTM at gene promoter regions is shown in the heatmap side-by-side with genes sorted by expression from the lowest (*top*) to highest (*bottom*). Details of heatmap generation are provided under "Experimental Procedures." *B*, heatmap of histone PTMs at the promoter regions of 350 T1D genes within 41 T1D susceptibility loci. (The gene list is provided in supplemental Table S1).

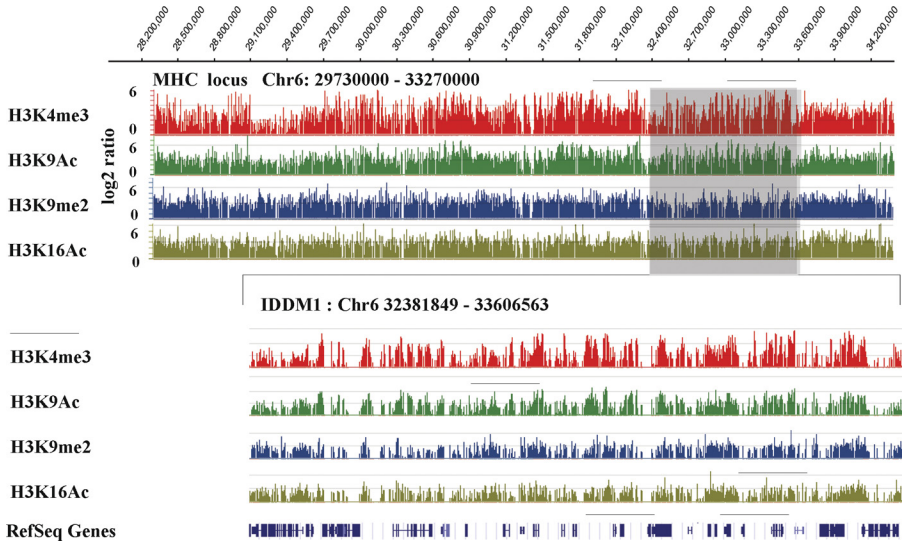

FIGURE 2. **Mapping the chromatin architecture of the human MHC locus and IDDM1 in blood monocytes from normal healthy volunteers.** Histone H3K4me3, H3K9Ac, H3K9me2, and H3K16Ac maps of the genome region at the human MHC and IDDM1 locus. ChIP-chips were performed with chromosome 6 (*Chr6*) tiling arrays and the indicated antibodies are as described under "Experimental Procedures." The enrichment level is presented in $\log_2$ ratio. *Upper panel*, the landscape of histone PTMs patterns (H3K4me3, H3K9me2, H3K9Ac, and H4K16Ac) of the MHC locus (Chr6: 29730000–33270000, human NCBI build 36) in normal human monocytes. *Lower panel*, IDDM1 (Chr6: 32381849–33606563), which contains about 43 genes including HLA genes located within the MHC locus. RefSeq genes are denoted in the lowest lane. The data shown is generated from SignalMap software (Roche Nimblegen).

ite files in lymphocytes also showed similar relationships between histone PTMs and the various gene expression groups from high, moderate, and low expression to silent (supplemental Fig. S1 and supplemental methods). These results confirmed the correlations between histone PTMs and gene expression as demonstrated in other cell types (32) and also verified the quality of the promoter array data sets generated in this study.

Next, to gain insights into the potential role of histone modifications at T1D susceptible genes, we adopted a candidate gene-set survey approach by focusing on 41 T1D susceptible loci defined by recent GWAS. Fig. 1*B* shows the heatmap of the histone modification patterns at the promoter regions of some 350 T1D susceptible genes within these 41 loci in normal human lymphocytes (the gene list provided in supplemental Table S1). Notably, most of the expressed genes also appear to have high levels of histone H3K9Ac activity near their promoter regions (Fig. 1*B*) indicating that the H3K9Ac mark might be indispensable for the "open" chromatin status required for active or induced expression of these genes.

We next focused specifically on the human MHC locus, which contains the major T1D susceptible IDDM1 locus (5–7). MHC is a gene dense locus in the human genome that displays the highest density of single nucleotide polymorphism (SNP) variations (36). Its short form spans 3.6 Mb and contains ~140 genes between flanking genetic marks *MOG* and *COL11A2* on chromosome 6p21.3 (Fig. 2) and plays a vital role in immune system functions (37). MHC locus is tightly associated with many human autoimmune diseases including T1D, inflammatory disorders, and cancers (36). Innumerable reports have described the associations between MHC SNP variations and various human diseases. The IDDM1 region is located within MHC, and its SNPs are highly associated with T1D, providing up to 50% of the inheritable risk of incidence (9). Furthermore, compelling evidence shows that haplotypes of the human leukocyte antigen (HLA) class II genes *HLA-DRB1* and *HLA-DQB1* in IDDM1 within the MHC are among the major determinants of T1D risk (5–7). We hypothesized that there could be a correlation between the chromatin status (histone PTMs) of the IDDM1 locus in monocytes and T1D. Monocytes were assessed here because they are the source of macrophages and dendritic cells, potent antigen presenting cells (38), which express HLA class II genes and have well documented roles in inflammation and immune responses.

We used the same ChIP-array approach to evaluate several key histone PTMs at the MHC locus and its chromatin status in blood monocytes of the normal controls but using chromosome 6 genomic tiling arrays, which allowed us to map the entire MHC locus. Fig. 2 illustrates the patterns of H3K4me3, H3K9me2, H3K9Ac, and H4K16Ac at the MHC locus (*upper panel*) and IDDM1 (*lower panel*) in monocytes from control volunteers. Similar to that observed in lymphocytes from these volunteers, composite files showed that active histone PTM marks (H3K4me3, H3K9Ac) in the normal monocytes correlated well with highly expressed genes, whereas inactive marks (H3K9me2) were associated with silenced or low expression genes (supplemental Fig. S2 and supplemental methods). These data also provide for the first time a snapshot of the epigenome of this important genomic region and a resource for studying the chromatin status of MHC locus in human monocytes. We used these data as a reference baseline for the next step to identify potential specific variations in these histone PTMs at the T1D susceptible regions in the blood lymphocytes and monocytes obtained from nondiabetic *versus* T1D volunteers.

Comparing Epigenomes of T1D Susceptible Genes between T1D and Nondiabetic Controls—In parallel, we profiled histone PTMs using ChIP-enriched DNA from lymphocytes and monocytes of a group of nine T1D patients. As discussed above, our approach was to map the histone PTMs at T1D susceptible genes in T1D patients and nondiabetic controls separately, and then scan for potential variations between these two cohorts

TABLE 2

Summary of histone PTM variations at T1D susceptible gene promoters between T1D patients and normals (lymphocytes)

The T1D susceptible genes are defined as genes located within 41 T1D susceptible loci. The gene list is provided in supplemental Table S1. Detailed methods for hyper- and hypo-enriched regions at T1D susceptible gene promoters are provided under "Experimental Procedures."

Histone PTMs	Hypo peaks	Hyper peaks
H3K4me3	0	0
H3K9me3	1	1
H3K9Ac	0	0
H3K27me3	0	0
H4K16Ac	0	0

TABLE 3

Summary of histone PTMs variations at the IDDM1 locus between T1D patients and normals (monocytes)

The definitions of the enriched region, hyper-peak and hypo-peaks are provided under "Experimental Procedures." IDDM1 locus spans from 32381849 to 33606563 on chromosome 6, based on human NCBI build 36. Computation indicates the key variations in chromatin marks histone H3K9Ac and H4K16Ac at key loci, but not other marks in monocytes of TID *versus* normals (in the IDDM1 locus).

Histone PTMs	H3K4me3	H3K9Ac	H3K9me2	H4K16Ac
Hyper- peaks	0	6	0	3
Hypo- peaks	0	3	0	0

Details of chr6 loci depicting H3K9Ac variations in Monocytes of T1D vs normals

Chrom	Start	End	Log2ratio Difference	FDR	Location
chr6	32745105	32745854	2.55	0.00143	HLA-DQB1, 3kb upstream
chr6	33497406	33497955	1.99	0.00802	SYNGAP1 transcript
chr6	32911482	32912031	1.74	0.01723	TAP2 transcript
chr6	33506196	33506645	1.68	0.02086	SYNGAP1 transcript
chr6	33206671	33207120	1.64	0.02321	HLA-DPB2, 12KB downstream
chr6	33362982	33363631	1.4	0.04433	WDR46 transcript
chr6	32669021	32669470	-2.37	0.00173	HLA-DRB1, 4KB upstream
chr6	33435913	33436506	-2.25	0.00269	LOC653639, 5KB downstream
chr6	33315248	33315697	-2.06	0.005	VPS52, 10KB downstream

Details of chr6 loci depicting H4K16Ac variations in Monocytes of T1D vs normals

Chrom	Start	End	Log2ratio Difference	FDR	Location
chr6	32911482	32912031	2.3	0.00266	TAP2 transcript
chr6	33508756	33509205	1.96	0.00969	SYNGAP1 transcript
chr6	33163862	33164311	1.8	0.01436	HLA-DPB1, 1KB downstream

through computational methods (25, 28). Five PTMs (histone H3K4me3, H3K27me3, H3K9me3, H3K9Ac, and H4K16Ac) were examined in lymphocytes, and four (H3K4me3, H3K9Ac, H2K9me2, and H4K16Ac) in monocytes. The H3K9me2 profile in lymphocytes was not evaluated in this study because we studied it earlier, albeit not specifically at T1D susceptible genes (28). The data obtained from profiling each PTM from the T1D or lymphocytes of monocytes from healthy individuals were quantile normalized and the TAMAL software (39) was used to identify promoter regions enriched by each PTM (peak) in T1D and control individuals separately (detailed analysis procedure is described under "Experimental Procedures"). Subsequently, we screened the data to identify those PTMs located specifically in the T1D susceptible gene loci for lymphocyte samples, or in the IDDMI locus (chromosome 6: 32381849–33606563, human NCBI build 36) for monocyte samples. As mentioned earlier, the reason for this different comparison is because many T1D susceptible genes have functions in T lymphocytes, whereas monocytes are more likely to show changes at the IDDM1 regions because they are precursors of potent antigen presenting cell cells.

Tables 2 and 3 summarize the numbers of differentially methylated/acetylated regions between T1D *versus* controls in these specific regions as identified by computation in lymphocytes and monocytes, respectively. Our results revealed that most of these known T1D susceptible genes (34) show relatively few variations in their promoter regions of the key histone PTMs evaluated in this study in either lymphocytes or monocytes between T1D and healthy volunteers (Tables 2 and 3). However, the exception was that marked variations in histone H3K9Ac levels were detected at several regions in the IDDM1 locus in monocytes (Table 3, *middle panel*). Interestingly, two variations were located in the upstream region of *HLA-DRB1* and *HLA-DQB1* (Table 3, Fig. 3, *A* and *B*). Compared with controls, T1D patients had lower histone H3K9Ac levels at the *HLA-DRB1* upstream promoter region (Fig. 3*A*), and higher H3K9Ac levels at the *HLA-DQB1* upstream promoter region (Fig. 3*B*). This is noteworthy because *HLA-DRB1* and *HLA-DQB1* are known to be two top T1D susceptible genes and their SNPs have the highest association with T1D (5, 6). We also observed hyperacetylation at H4K16 at three regions within transcripts of *TAP2*, *SYNGAP1*, and downstream (1 kb) of *HLA-DPB1* (Table 3, bottom panel).

To validate the microarray data showing variations in H3K9Ac around the *HLA-DRB1* and *HLA-DQB1* promoters, we next performed follow-up ChIP-qPCR validations utilizing separate H3K9Ac antibody-enriched ChIP-DNA samples from each individual in the two cohorts. Fig. 3, *C* and *D*, respectively, confirm the decreases in H3K9Ac in T1D noted at the upstream promoter regions of *HLA-DRB1* and the increases in H3K9Ac in T1D at the upstream promoter/enhancer region of *HLA-DQB1* ($p < 0.001$).

It is interesting to determine whether the variations noted in the H3K9Ac levels are associated with any changes in gene expression. However, because all the human volunteer blood cells were used to isolate chromatin to perform multiple ChIP assays, there was insufficient cell material to isolate total RNA as well. Thus, further gene expression as well as additional ChIP and ChIP-array experiments were performed in THP-1 monocytes.

Histone H3K9Ac Variations within the Region Containing the Majority of Top T1D SNPs Are Associated with Up-regulation of HLA-DRB1 and HLA-DQB1—The transcription of MHC class II molecules is tightly regulated (40). The binding of key transcription factors, RFX, X2BP, and NF-Y, to the promoter region of MHC class II molecules is highly cooperative and results in the formation of a stable nucleoprotein complex, called the MHC-II enhanceosome. In addition, the master controller of MHC class II genes is class II transactivator (41). A more detailed picture emerged recently in which the insulator CCCTC binding factor was shown to interact with class II transactivator and the enhanceosome complex to form long-distance chromatin loop structures that co-regulate MHC-II genes (42, 43). Interferon-γ (IFN-γ) and TNF-α have been shown to increase the expression of class II molecules including HLA-DQB1 (44). Interestingly, the acetylation state of the upstream region of *HLA-DRA* has been linked to gene expres-

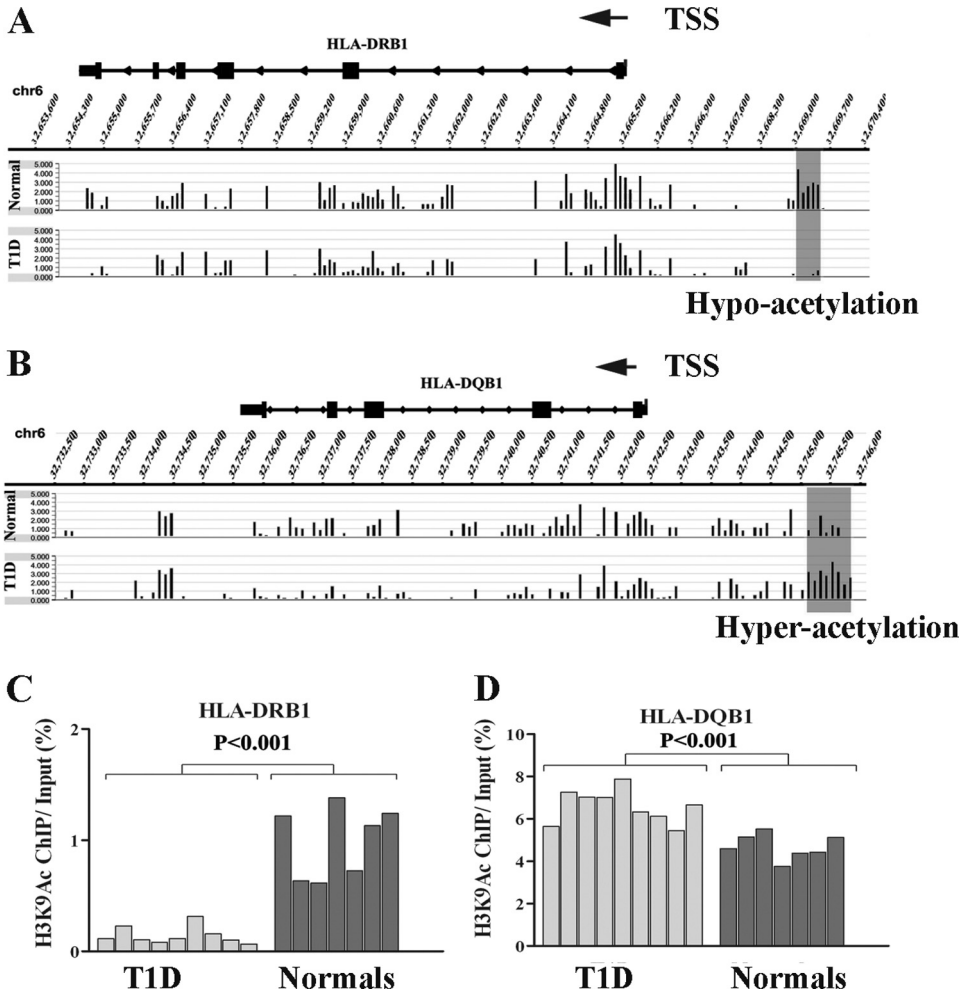

FIGURE 3. **Histone H3K9Ac variations at *HLA-DQB1* and *HLA-DRB1* promoter (enhancer) regions in monocytes from T1D *versus* nondiabetic control volunteers.** Snapshots of histone H3K9Ac status of *HLA-DQB1* and *HLA-DRB1* gene regions in T1D patients and normals. Pooled histone ChIP-enriched DNA samples from monocytes of T1D and normals were hybridized to human chromosome 6 (*Chr6*) tiling arrays and the data were extracted according to standard operating procedures of Nimblegen-Roche. Results were visualized by SignalMap software (Roche Nimblegen). Hyper- and hypo-histone H3K9Ac regions are indicated. *A, HLA-DRB1*; *B, HLA-DQB1*. For ChIP-qPCR validation of histone H3K9Ac variations at *HLA-DQB1* and *HLA-DRB1* promoter/enhancer regions in monocytes, histone H3K9Ac ChIPs were prepared from each individual volunteer (nine T1D and seven normal control samples). Standardized ChIP-qPCRs were used to quantify the amount of specific modified ChIP-enriched DNA in monocyte samples from individual T1D patients and normal controls. *C,* ChIP-qPCR validation of H3K9Ac at *HLA-DRB1*. Results shown are from one set of PCR primers ($p < 0.001$ T1D *versus* normal controls, by Student's *t* tests). Similar ChIP-qPCR results were obtained with two other sets of PCR primers amplifying nearby regions (data not shown). *D*, ChIP-qPCR validation of H3K9Ac at the *HLA-DQB1* promoter region, which was done in triplicates ($p < 0.001$ T1D *versus* normal controls, by Student's *t* tests).

sion (45), suggesting that variations in histone acetylation observed in this study might be relevant to the expression of HLA-DRB1 and HLA-DQB1.

To further explore this in the context of the current results, we used THP1 cells, a human monocyte cell line, as a model because we noticed that HLA-DRB1 and HLA-DQB1 were up-regulated in response to IFNγ/TNFα treatment in these cells (Fig. 4*left*). Importantly, Fig. 4*right* shows for the first time that, along with this up-regulation of gene expression in these treatments, there was an increase in histone H3K9Ac levels at the same promoter regions of *HLA-DRB1* and *HLA-DQB1* that showed variations between T1D patients and normal controls in Fig. 3, *C* and *D*. H3K9Ac levels started to increase by 4 h after addition of IFNγ/TNFα, whereas gene expression was significantly increased by 16 h, indicating a time lag between an increase in gene expression and chromatin H3K9Ac changes, suggesting that chromatin remodeling is required for the gene stimulation. To ascertain specificity, we evaluated interleukin-2 (*IL2*), as a control gene that is not induced by IFNγ/TNFα. We observed that neither the expression of IL2 nor levels of histone H3K9Ac levels at its promoter region are altered upon IFNγ/TNFα treatment (Fig. 4, *left* and *right*). IL4 also behaved similarly (results not shown). These results suggest that specific changes in H3K9Ac levels at the genome region between *HLA-DRB1* and *HLA-DQB1* could reflect changes in the chromatin configuration that would alter accessibility to transcription factors, co-activators/repressors, which in turn could affect gene expression or the response to various stimuli relevant to T1D pathology. We speculate that the dysregulated expression of key T1D-associated genes can occur by at least two steps. First, the chromatin of these genes attains a poised state through alterations in the chromatin configuration marks such as histone PTMs, without affecting gene expression. Next, in response to a second

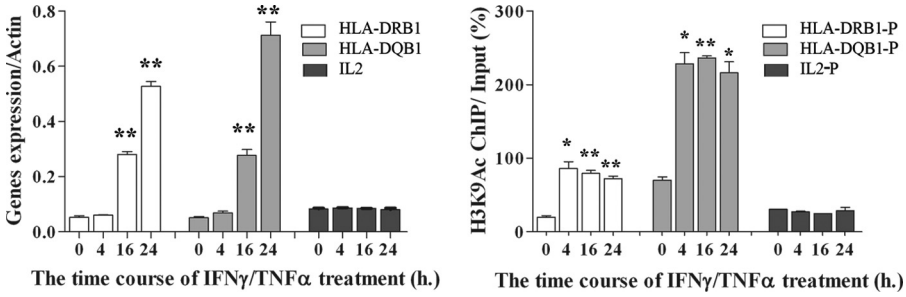

FIGURE 4. **Increased histone H3K9Ac at the promoter/enhancer regions of *HLA-DRB1* and *HLA-DQB1* correlates with gene expression in response to IFNγ/TNFα in THP1 cells.** *Left,* time course of IFNγ/TNFα-induced HLA-DRB1 and HLA-DQB1 mRNA expression in THP1 cells. IL2 was included as a negative control gene, which was not induced under these conditions. THP1 cells were treated with 20 μM IFNγ and 10 μM TNFα and collected at 0 (no treatment), 4, 16, and 24 h. Total RNAs were prepared and RT-qPCRs were performed in triplicate. **, $p < 0.0001$ *versus* 0 h. *Right*, ChIP-qPCR data showing histone H3K9Ac enrichment levels at *HLA-DRB1* and *HLA-DQB1* promoter/enhancer regions in THP1 cells treated with IFNγ/TNFα. *HLA-DRB1-P* and *HLA-DQB1-P* refer to the same genomic regions where variations in acetylation were noted in the T1D patient monocytes (Fig. 3, *C* and *D*). The IL2 promoter (*IL2-P*) was examined as a negative control, which did not show any changes in H3K9Ac under these conditions. The ChIPs were performed as described under "Experimental Procedures." ChIP-qPCRs were preformed in triplicates. Data shown are mean ± S.E. from triplicates. *, $p < 0.002$ *versus* 0 h; **, $p < 0.0001$ *versus* 0 h.

"hit," gene expression might be altered in response to an external triggering stimulus.

Recent studies show that active gene enhancer regions are typically marked by histone H3K4me1 and occupancy of the histone acetyltransferase p300, whereas promoter regions and TSS are marked by H3K4me3 (46, 47). To further explore such features of the chromatin structure in the *HLA-DRB1* and *HLA-DQB1* genome regions, we also mapped histone H3K4me3, H3K4me1, and p300 occupancy using the chromosome 6 tiling arrays in untreated THP1 cells. The *top panel* of Fig. 5 shows a snapshot of the profiles of these marks at this genome region, which reveals that histone H3K4me3 localizes around TSS of *HLA-DRB1* and *HLA-DQB1*, whereas H3K4me1 co-localizes with p300 at regions that could potentially represent locations of enhancers within this key region. Notably, a cluster of top T1D SNPs are located within the loop and enhancer regions (Fig. 5). In particular, we found for the first time that two SNPs highly associated with T1D, rs9273363 and HLA-DQB1 (34), co-localize with H3K4me1 and p300 peaks (Fig. 5, *lower panel*, indicated by *arrows*), which suggests that both rs9273363 and HLA-DQB1 SNPs are located within potential enhancer regions of key T1D susceptibility genes.

Because the majority of top T1D SNPs are located around the *HLA-DRB1* and *HLA-DQB1* genome regions (34), we next used ChIP assays to determine whether there are changes in histone H3K9Ac levels around these SNPs during IFNγ/TNFα-induced gene up-regulation in THP-1 cells. Results shown in Fig. 6 demonstrate that there is a significant increase in histone H3K9Ac levels at several of these T1D SNP loci at a time period when HLA-DRB1 and HLA-DQB1 are also up-regulated by IFNγ/TNFα, although to varying degrees. Interestingly, the highest increase of H3K9Ac was observed at the same promoter region of *HLA-DQB1* (Fig. 6, DQB1-P) that displayed hyperacetylation in T1D patients, which might explain why acetylation at this region was detected in our study with T1D patients (Fig. 3*D*). As controls, we also examined two T1D un-related SNPs, rs28747031 and rs28747037, which are located about 20 kb upstream of the *HLA-DQB1* locus. Results show that H3K9Ac levels at both of these unrelated SNPs are unchanged in response to IFNγ/TNFα treatment (Fig. 6, *last four bars*) unlike the increases seen at the T1D SNPs. Collectively, these results indicate an association between gene expression and the increased level of H3K9Ac at the genome region between *HLA-DRB1* to *HLA-DQB1*, and reveal novel correlations between T1D-associated SNPs, histone PTMs, and the chromatin configuration of this key IDDM1 region. They further support our hypothesis that variations in histone PTMs can be found between T1D and normal controls at T1D susceptible loci, and may further enhance the risk of T1D susceptibility.

DISCUSSION

The recent GWAS in T1D have confirmed and extended the results from previous studies and defined 41 distinct genome locations as T1D susceptible regions (34). The huge datasets generated by GWAS have provided a rich resource of invaluable information, enormous challenges, and exciting opportunities for subsequent diabetes research. There is significant interest in exploring how the susceptible loci identified by GWAS are related to T1D. As such, in our current study, we focused on these 41 T1D susceptible regions to determine whether an epigenetic layer over these genetic loci can have additional correlations between these regions and T1D disease phenotype and pathogenesis. We profiled and mapped the key histone PTM status of genes located within these 41 genomic loci in lymphocytes, and more specifically, within the IDDM1 region in monocytes of patients with T1D *versus* healthy controls. Notably, we observed for the first time that there were marked variations in H3K9Ac levels at upstream regions of *HLA-DRB1* and *HLA-DQB1* in monocytes of this cohort (T1D *versus* control). On the other hand, the levels of the other tested histone PTMs at most of the T1D susceptible gene promoters were relatively quite similar between T1D and control volunteers in their lymphocytes or monocytes. It should be underscored that there were also variations noted in many of the histone PTMs beyond T1D susceptible regions in this cohort (data not shown). For reasons stated above, we focused only on T1D-related loci and adopted a T1D candidate gene-set profiling approach due to recent advances in GWAS of T1D (34).

It is well known that *HLA-DRB1* and *HLA-DQB1* are two major candidates associated with T1D incidence, with lesser contributions from the remaining T1D susceptible genes (5–6, 48, 49). This may explain why we detected variations at only

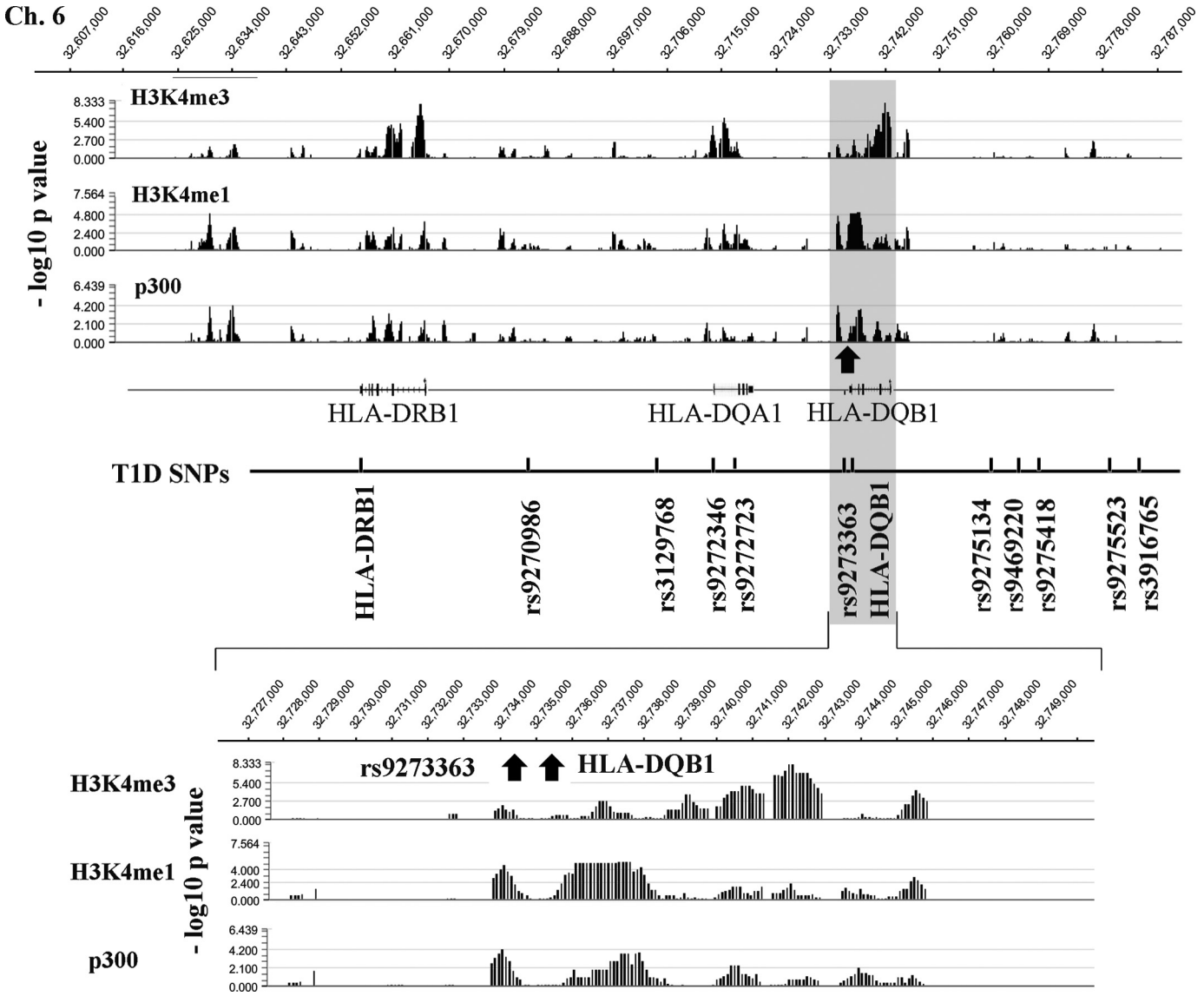

FIGURE 5. **Mapping the chromatin conformation of the genome region between *HLA-DRB1* and *HLA-DQB1* in THP1 cells.** Histone H3K4me3, H3K4me1, and p300 maps of the genome region between *HLA-DRB1* and *HLA-DQB1* in untreated THP1 cells. ChIP-chips were performed with chromosome 6 (*Chr6*) tiling arrays and the indicated antibodies as described under "Experimental Procedures." *Top panel*, histone H3K4me3, H3K4me1, and p300 enrichment levels at genome region between *HLA-DRB1* and *HLA-DQB1* (Chr.6: 32607000–32787000). The enrichment level is presented in smoothed-data format $-\log_{10}$ (*p* value). *p* value at each probe was generated by Nimblescan using a nonparametric, one-sided Kolmogorov-Smirnov test of the probes located within the 750-bp sliding window centered by the probe. Genes and SNPs located in the regions are marked based on their genomic location in the region. *Lower panel*, zoom-in view of histone H3K4me3, H3K4me1, and p300 levels near rs9273363 and HLA-DQB1 with 100-bp resolution.

HLA-DRB1 and *HLA-DQB1* in the samples, although it could also be due to the level of sensitivity of detection in the ChIP-array approach, and that only limited genome regions and histone marks were assessed. It is possible that there are variations at T1D regions in other histone PTMs not examined in this study, or in DNA methylation, all of which are the focus of future endeavors. Interestingly, a recent study using next generation sequencing revealed that high glucose could induce genome-wide variations in histone H3K9Ac and DNA methylation in human aortic endothelial cells, and furthermore, the regions with histone H3K9Ac variations contained many common human disease SNPs including T1D (23).

Although GWAS have confirmed and cataloged a large number of SNP-associated T1D (34), most of these SNPs are intronic and intergenic and few SNPs have been linked to T1D at the functional or mechanistic level, a key step for a better understanding of T1D. Concerted efforts are ongoing to catalog additional SNPs with recent advances in sequencing technologies. Our results indicate the presence of marked variations in the levels of a key chromatin mark H3K9Ac at the upstream regions of two top T1D genes, *HLA-DRB1* and *HLA-DQB1*, suggesting a link between the chromatin status of their promoter/enhancer region and T1D. We also observed that the epigenome or status of several histone PTMs at the MHC locus, when viewed globally, is quite similar between T1D and nondiabetic controls (Fig. 2), implying that, overall, most T1D-related SNPs do not affect the tested histone PTMs to a great extent. Hence, the variations we observed are most likely not due to SNPs but may be due to environmental factors that are known to affect epigenetic processes.

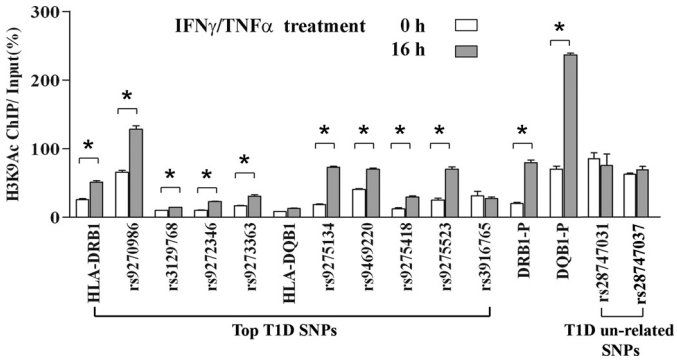

FIGURE 6. **ChIP-qPCR analysis of histone H3K9Ac-enrichment levels at key T1D SNPs loci in THP1 cells treated with and without IFNγ/TNFα.** THP-1 cells were cultured in RPMI 1640 medium containing 5 mM glucose and treated with 20 μM IFNγ and 10 μM TNFα for 16 h. The ChIP was performed with histone H3K9Ac antibody, and ChIP-qPCRs with primers spanning the top T1D SNPs region shown, as well as HLA-DRB1-P and HLA-DQB1-P (same regions showing H3K9Ac changes in Figs. 3 and 4) were performed as described under "Experimental Procedures." Two T1D unrelated SNPs, rs28747031 and rs28747037, were also included as controls and did not show any increases in H3K9Ac. Primer sequences are provided in supplemental Table S2. Data are shown as mean ± S.E. from triplicates. *, $p < 0.001$ treated *versus* untreated (0 h).

We are aware that our results are derived from a small number of T1D patients and controls, and therefore the exact similar type and intensity of variation might or might not be the same in all T1D patients. Disease duration and extent of complications may also play a role. However, based on the current data, we speculate that the chromatin architecture of the genome region near *HLA-DRB1* and *HLA-DQB1* within IDDM1 is quite sensitive to histone modifications and relevant to the expression of HLA-DRB1 and HLA-DQB1. This notion is further supported by our data with THP-1 cells showing increased gene expression along with increases in histone H3K9Ac in response to IFNγ/TNFα treatment at the same promoter/enhancer regions of *HLA-DRB1* and *HLA-DQB1* as that seen in the T1D patient monocytes. Moreover, we also noticed increases in H3K9Ac at several well documented T1D SNPs in the vicinity of *HLA-DRB1*/*DQB1*. Therefore, a combination of genetic and epigenetic changes at these regions could potentially further enhance the risk of disease incidence. Thus, the susceptibility to T1D might require not only the presence of disease-related SNPs, but also disease poised chromatin states such as hyperacetylation at the enhancer regions of these SNPs.

Finally, it is still an enigma why only a small fraction of genetically susceptible individuals appear to acquire T1D later in their lifetime. A general explanation is that there are protective SNPs that prevent T1D acquisition, whereas on the other hand, certain environmental factors might also influence T1D incidence. The role of epigenetics in this process is largely unknown. Based on various reports (4, 12, 23, 25, 28, 50), we are gradually gaining more details of epigenetic changes in T1D. Our current study links variations in histone H3K9Ac in monocytes at *HLA-DQB1* and *HLA-DRB1* promoter/enhancer regions to T1D, although it is limited to a small cohort and also cannot determine whether the variations are early triggers or late events. Parallel studies with THP-1 cells support the data with T1D patients and demonstrate novel connections between H3K9Ac at *HLA-DQB1* and *HLA-DRB1* especially at their enhancer regions and their expression. The pathological implications and significance of the findings need further investigation and reinforcement through similar studies in the future with larger well characterized cohorts as well as with T1D discordant twins and siblings. Notwithstanding, based on the current results, we speculate that the susceptibility to the development of T1D could require not only SNPs as documented by extensive genetic studies, but also potential "chromatin hits" from accompanying epigenetic events that could confer a functional context to the SNPs. Identifying such events and contributions will lead to a greater understanding of T1D susceptibility and, hopefully to better predictions or early detection of disease onset.

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