Autoimmune vitiligo is associated with gain-of-function by a transcriptional regulator that elevates expression of *HLA-A*02:01* in vivo

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HLA-A is a class I major histocompatibility complex receptor that presents peptide antigens on the surface of most cells. Vitiligo, an autoimmune disease in which skin melanocytes are destroyed by cognate T cells, is associated with variation in the *HLA-A* gene; specifically *HLA-A*02:01*, which presents multiple vitiligo melanocyte autoantigens. Refined genetic mapping localizes vitiligo risk in the *HLA-A* region to an SNP haplotype ∼20-kb downstream, spanning an ENCODE element with many characteristics of a transcriptional enhancer. Convergent CTCF insulator sites flanking the *HLA-A* gene promoter and the predicted transcriptional regulator, with apparent interaction between these sites, suggests this element regulates the *HLA-A* promoter. Peripheral blood mononuclear cells from healthy subjects homozygous for the high-risk haplotype expressed 39% more *HLA-A* RNA than cells from subjects carrying nonhigh-risk haplotypes ($P = 0.0048$). Similarly, RNAseq analysis of 1,000 Genomes Project data showed more *HLA-A* mRNA expressed in subjects homozygous for the high-risk allele of lead SNP rs60131261 than subjects homozygous for the low-risk allele ($P = 0.006$). Reporter plasmid transfection and genomic run-on sequence analyses confirm that the *HLA-A* transcriptional regulator contains multiple bidirectional promoters, with greatest activity on the high-risk haplotype, although it does not behave as a classic enhancer. Vitiligo risk associated with the MHC class I region thus derives from combined quantitative and qualitative phenomena: a SNP haplotype in a transcriptional regulator that induces gain-of-function, elevating expression of *HLA-A* RNA in vivo, in strong linkage disequilibrium with an *HLA-A* allele that confers **02:01* specificity.

vitiligo | autoimmune disease | HLA | transcription | enhancer

Autoimmune diseases comprise more than 80 disorders in which the immune system attacks "self" tissues and cells (1), affecting 3–5% of the United States population (2). Many different autoimmune diseases are genetically associated with variation in the major histocompatibility complex (MHC) on chromosome 6p21.3, including class I loci, class II loci, or both. MHC class I molecules present peptide antigens on the surface of almost all cells, providing targets for autoimmune sensitization and targeting by cytotoxic T cells. Extensive polymorphism of the human classic MHC genes produces great diversity in the corresponding polypeptides, enabling both diversity and specificity in the peptide antigens presented. Transcription of the classic MHC genes is also subject to complex regulation (3), which similarly may be subject to genetic variation. However, contributions of the MHC to autoimmunity have thus far largely focused on MHC antigenic specificity.

Vitiligo is associated with MHC class I region SNPs in the vicinity of the *HLA-A* gene (4), and DNA sequence analysis identified the high-risk allele as *HLA-A*02:01:01:01* (5), encoding the canonical HLA-A2 specificity. HLA-A2 can present a diversity of autoantigens, including several derived from melanocyte proteins that include tyrosinase (6), TRP2 (7), OCA2 (8), MC1R (9), gp100 (10), and MART-1/melan-A (11). In the present study we refined genetic mapping, localizing primary vitiligo risk in the MHC class I region to a SNP haplotype 20 kb downstream of the *HLA-A* gene, in strong linkage disequilibrium with *HLA-A*02:01:01:01*. This high-risk SNP haplotype is coincident with a predicted transcriptional regulator, which we find drives elevated *HLA-A* transcription in peripheral blood mononuclear cells. These findings indicate that vitiligo susceptibility in the MHC class I region involves two functional components: a primary quantitative effect of increased *HLA-A* expression, and a secondary qualitative effect of *02:01:01:01 antigenic specificity as a result of strong linkage disequilibrium through the region. Together, these features likely combine to increase cell-surface presentation of autoimmune target antigens, facilitating recognition of melanocytes by autoreactive cytotoxic T-cells.

Significance

Vitiligo is an autoimmune disease in which spots of white skin and hair result from destruction of melanocytes. Vitiligo is associated with *HLA-A*02:01*, which presents multiple vitiligo melanocyte autoantigens. We localize vitiligo risk to a SNP haplotype 20 kb downstream of the *HLA-A* gene, spanning a transcriptional regulatory element. Blood cells from healthy subjects carrying the high-risk haplotype expressed more *HLA-A* RNA than subjects carrying only nonhigh-risk haplotypes. Vitiligo risk in the MHC class I region thus derives from combined quantitative and qualitative phenomena: an SNP haplotype in a transcriptional regulator that induces elevated expression of *HLA-A* RNA in vivo, and strong linkage disequilibrium with an *HLA-A* allele that confers **02:01* specificity. These combine to increase HLA-A2 available to present melanocyte autoantigens.

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Results

Refined Genetic Mapping of Vitiligo Susceptibility in the MHC Class I Region to a Predicted Transcriptional Regulator. We previously showed that vitiligo is associated with MHC class I region SNP rs12206499 (4), in strong linkage disequilibrium with *HLA-A*02:01:01:01* (5). To more precisely localize causal variation in the region, we compared genotypes of 2,853 European-derived Caucasian (EUR) vitiligo cases and 37,412 controls, imputed through the extended MHC (12, 13) using data from the 1,000 Genomes Project. In the MHC class I region, greatest association was with lead SNP rs60131261 (chr6:29937336–29937339; $P = 2.15 \times 10^{-50}$, odds ratio 1.53). Logistic regression analysis conditional on rs60131261 identified 21 additional variants whose effects could not be distinguished from rs60131261, which together thus comprise the primary MHC class I vitiligo-associated haplotype (Table S1). The 22 variants span a 9.6-kb region (chr6:29928838–29938487) that almost precisely encompasses a striking ENCODE (14) transcriptional element (chr6:29,932,250–29,937,500) located ~20 kb downstream of *HLA-A*. As shown in Fig. 1, this element was observed in all cell types tested by ENCODE, and has an open hypomethylated chromatin configuration, multiple DNase I hypersensitivity sites, numerous RNA polymerase II and transcription factor binding sites, and prominent H3K4me1, H3K4me3, and H3K27ac marks. Together, these features are suggestive of an active transcriptional promoter and enhancer (16–18).

The Vitiligo High-Risk MHC Class I Haplotype Is Associated with Elevated *HLA-A* RNA Expression. Localization of primary vitiligo risk in the MHC class I region to an apparent transcriptional regulatory element downstream of the *HLA-A* gene suggested that corresponding vitiligo risk may be mediated by elevated expression of *HLA-A* RNA. To test this theory, we compared expression of *HLA-A* RNA in healthy individuals alternatively homozygous for the high-risk MHC class I region haplotype versus for nonhigh-risk haplotypes. We genotyped 81 unrelated EUR individuals without known autoimmune disease for haplotype tagSNP (Table S1) rs12193100, which is in perfect linkage disequilibrium ($r^2 = 1.0$) with the other SNPs that define the high-risk haplotype, and is in near-perfect linkage disequilibrium ($r^2 = 0.98$) with the original high-risk SNP, rs12206499 (4). Among these healthy subjects, we identified 10 homozygous for the high-risk haplotype and 27 homozygous for nonhigh-risk haplotypes.

To quantitate *HLA-A* RNA, we designed seven different quantitative RT-PCR (qPCR) assays, each agnostic to *HLA-A* subtype. The corresponding primers avoided all sequence variants in 1,000 Genomes Project data, and all amplicons spanned at least one intervening sequence (Table S2). We prepared peripheral blood cell

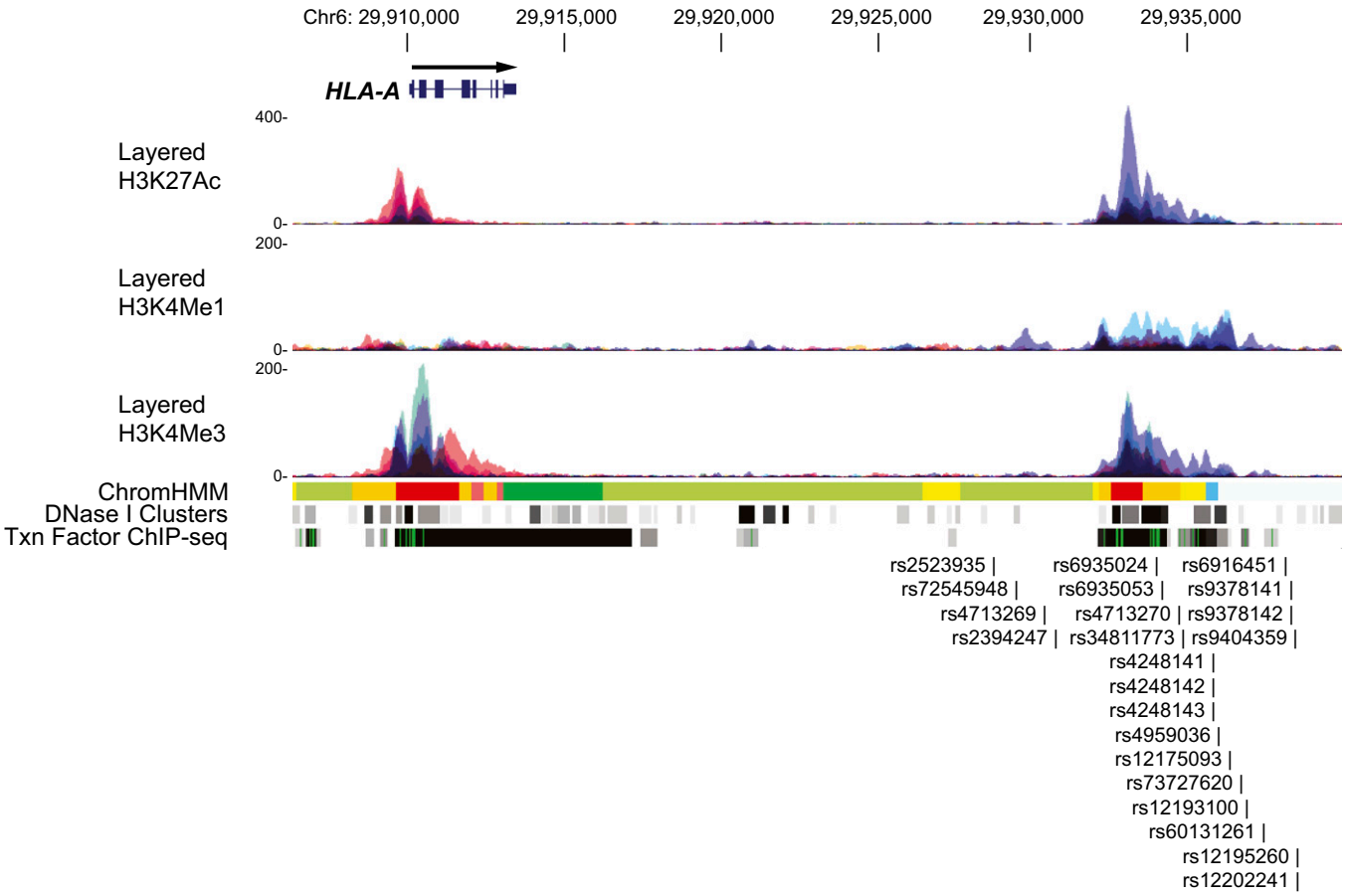

Fig. 1. Vitiligo association in the *HLA-A* region of human chromosome 6p. Nucleotide positions, *HLA-A* transcriptional orientation, and the 22 SNPs that define the vitiligo high-risk haplotype are shown. Layered H3K27Ac, H3K4Me1, and H3K4Me3 marks, hidden Markov model chromatin state segmentation (ChromHMM), DNase I hypersensitive site cluster (DNase I Clusters), and transcription factor chromatin immunoprecipitation sequencing (Txn Factor ChIP-seq) data are from ENCODE (14). For layered H3K27Ac, H3K4Me1, H3K4Me3 marks, data are shown for the seven cell lines studied by ENCODE. For ChromHMM, red indicates active promoters, orange indicates strong enhancers, and blue indicates an insulator. Data shown are for GM12878 lymphoblastoid cells. For DNase clusters, darkness indicates relative signal strength in 125 cell types from ENCODE (V3). For Txn factor ChIP-seq, darkness indicates relative signal strength of aggregate binding of 161 transcription factors, and green bars indicate ENCODE Factorbook (15) canonical motifs for specific transcription factors.

RNA from three subjects homozygous for the high-risk haplotype and seven homozygous for nonhigh-risk haplotypes. We then used equal amounts of RNA from each subject to assay *HLA-A* RNA by qPCR using two different primer sets, measuring 18S ribosomal RNA for normalization. As shown in Fig. 2, there was close agreement between the two *HLA-A* RNA qPCR assays. Strikingly, the average amount of *HLA-A* RNA was 1.39-fold higher in cells from subjects homozygous for the high-risk haplotype (1.16 ± 0.08; range 1.04–1.30) than in cells from subjects homozygous for various nonhigh-risk haplotypes (0.83 ± 0.05; range 0.72–0.98), with no overlap between groups. This difference was highly significant ($P = 0.0048$), confirming that the high-risk *HLA-A* region SNP haplotype is associated with elevated expression of *HLA-A* RNA.

In addition, we analyzed *HLA-A* mRNA expression data for 358 EUR subjects for whom both lymphoblastoid cell line mRNA-seq (18) and whole-genome DNA sequence (19) data were available. Subjects were classified by sequence-based genotypes of lead variant rs60131261, and mRNA-seq data were analyzed for *HLA-A* (ENSG00000206503). As shown in Fig. 3, the average amount of *HLA-A* mRNA was significantly higher in subjects homozygous for the high-risk allele of rs60131261 (1067.75 ± 20.78) than in subjects homozygous for the low-risk allele (985.60 ± 17.28) ($P = 0.006$), with heterozygotes intermediate between the two groups of homozygotes (1012.65 ± 13.56). These results thus confirm that the high-risk allele of lead *HLA-A* region variant rs60131261 is associated with elevated expression of *HLA-A* mRNA.

Convergent CTCF Sites Define a Contact Domain Between the *HLA-A* Downstream Regulatory Region and the *HLA-A* Promoter. Enhancers and other transcriptional regulatory elements modulate transcription by being brought into close proximity to their cognate promoter (20). Several approaches have been used to detect in vivo long-range enhancer–promoter spatial interactions in chromatin and identify functional domains. We analyzed in situ genome-wide chromosome conformation capture (Hi-C) sequencing data (21) in the *HLA-A* region of GM12878 lymphoblastoid cells. As shown in Fig. 4, the segment from the 5′ end of the *HLA-A* gene through the predicted downstream transcriptional regulatory element is marked by convergent CTCF insulator sites, defining an ∼22-kb contact domain (chr6:29,910,000–29,932,000). This configuration is strongly suggestive of a chromatin loop juxtaposing the downstream regulatory region and the *HLA-A* promoter, itself contained within a larger ∼170-kb chromatin loop.

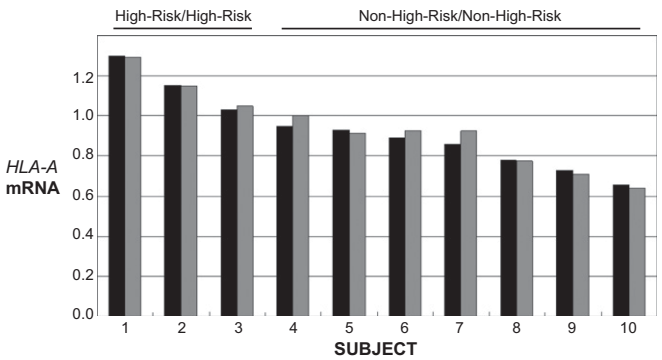

Fig. 2. *HLA-A* RNA in subjects homozygous for the high-risk and nonhigh-risk *HLA-A* region haplotypes. *HLA-A* RNA was measured in peripheral blood RNA from subjects homozygous for the high-risk MHC class I haplotype (nos. 1–3) or nonhigh-risk haplotypes (nos. 4–10) using two different qPCR assays (Table S2), and was normalized to 18S rRNA. Black bars, primer set 1; gray bars, primer set 2; each shows the mean of triplicate assays.

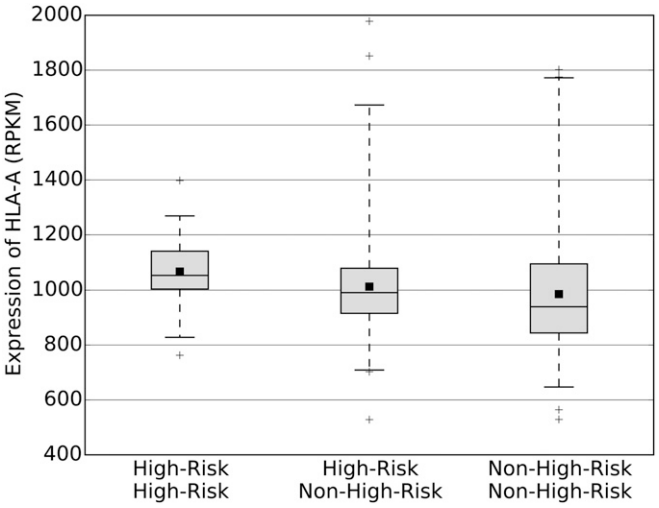

Fig. 3. Normalized *HLA-A* mRNA expression data from the 1,000 Genomes Project subjects classified by genotype of lead *HLA-A* region SNP rs60131261. RNAseq mRNA profiles for 358 EUR subjects of the 1,000 Genomes Project were obtained along with their genotypes for rs60131261 and subjected to ANOVA. RPKM, reads per kilobase of transcript per million mapped reads. The gray box denotes the first through third quartile and the horizontal line in the box denotes the median. Black squares indicate means. Short horizontal lines denote 99% confidence limits. Crosses denote outliers.

Genomic Run-on Sequence Data Identify Multiple Bidirectional Promoters in the *HLA-A* Downstream Regulatory Region. Mammalian transcriptional regulators frequently contain transcriptionally active promoters (20). To assess potential promoter modules in the *HLA-A* region, we analyzed in vivo genomic run-on sequence (GRO-seq) data generated from the human cell line HCT116 (22). GRO-seq, which provides a more sensitive and quantitative view of ongoing RNA polymerase II transcription than previous nuclear run-on assays (23), showed bidirectional transcription associated with the *HLA-A* promoter, and also detected bidirectional transcription originating from at least three distinct promoters within the downstream transcriptional regulatory region (Fig. 5). These results confirm that the *HLA-A* downstream regulatory region is transcriptionally active in vivo.

The *HLA-A* Downstream Regulatory Region on the High-Risk Haplotype Contains Multiple Transcriptional Promoters, but Does Not Act as a Classic Enhancer. To investigate differential function of the *HLA-A* downstream transcriptional regulator on high-risk and nonhigh-risk haplotypes, we compared subjects 1 and 10, who expressed the highest versus lowest amounts of *HLA-A* RNA, respectively (Fig. 2). These two subjects were homozygous for the alternative alleles of all SNPs that defined the high-risk versus nonhigh-risk haplotypes, respectively, as determined by sequencing a 6,020-bp segment of their genomic DNA (chr6:29,932,128–29,938,147) that spanned the downstream regulator (Table S3). We prepared firefly luciferase reporter constructs containing the full-length downstream regulator region from the two high-risk haplotypes (HR1 and HR2) of subject 1 and the two nonhigh-risk haplotypes (NHR1 and NHR2) of subject 10 (Table S4). For each, the element was inserted in either orientation immediately upstream of the luciferase reporter gene (*luc2*), which lacks a known promoter. Reporter constructs were transiently transfected into HeLa cells. As shown in Fig. 6*A*, both full-length high-risk haplotypes HR1 and HR2 from subject 1 exhibited significant promoter function, although in opposite orientations. Greater promoter activity was observed from haplotype HR1 than HR2. In contrast, both full-length nonhigh-risk haplotypes from subject 10 had much less promoter activity.

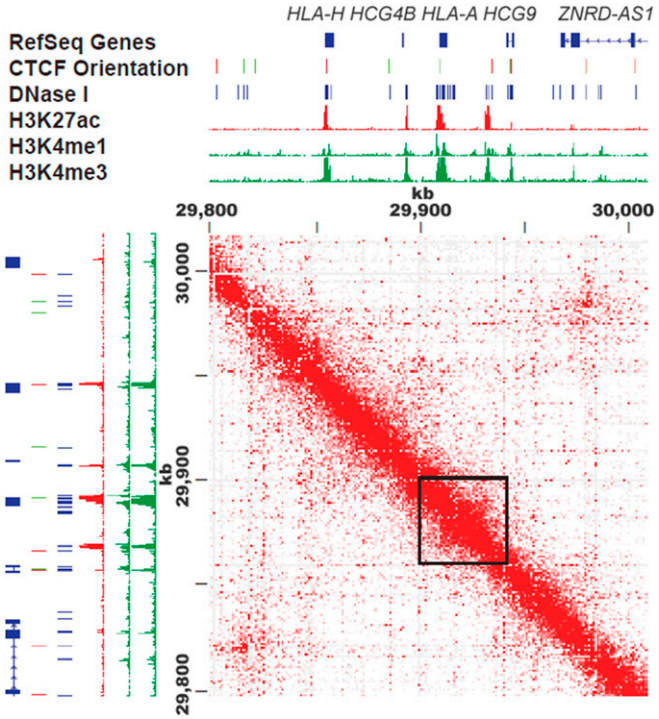

Fig. 4. Hi-C analysis of the *HLA-A* region of chromosome 6p. In situ Hi-C data for the *HLA-A* region of chromosome 6p of GM12878 lymphoblastoid cells (21) were analyzed by X-Y comparison using Juicebox (www.aidenlab.org/juicebox/). RefSeq genes, CTCF binding sites and orientation, DNase I hypersensitive sites, and H3K27ac, H3K4me1, and H3K4me3 marks are indicated. The box denotes the segment from *HLA-A* through the predicted downstream transcriptional regulatory element. *HLA-A* is the only protein coding gene in the region; *HLA-H*, *HCG4B*, *HCG9*, and *ZNRD-AS1* are all nonprotein-coding RNAs.

To map promoter function within the *HLA-A* downstream regulatory region, we prepared a series of analogous constructs containing only subfragments of the HR1 or HR2 high-risk haplotypes carried by subject 1 (Table S3). As shown in Fig. 6*B*, both high-risk haplotypes had multiple segments with promoter activity. Both similarities and differences were observed between the HR1 and HR2 haplotypes in terms of the location and functional orientation of apparent promoters, with no apparent simple pattern of promoter localization. These data are consistent with our GRO-seq findings, indicating the existence of multiple promoters within the *HLA-A* downstream regulatory region.

Comparison of the nucleotide sequences of the cloned HR1, HR2, NHR1, and NHR2 haplotypes with the human reference sequence (GRCh37/hg19) defined a remarkable pattern (Table S3). All four haplotypes shared 19 nucleotide differences from the reference sequence. Haplotypes NHR1 and NHR2 shared 16 additional differences, with 1 more difference specific to NHR1. In contrast, haplotypes HR1 and HR2 shared 45 additional differences from the reference, plus another 15 specific to HR1 and another 10 specific to HR2. Thus, the two NHR haplotypes are quite similar to each other and are generally similar to the reference, whereas the two HR haplotypes have far more base differences, both compared with the reference sequence and to each other. These sequence differences affect many different predicted transcription factor binding motifs, presumably driving the observed differences in transcriptional function among the different haplotypes.

To assess possible enhancer function of the *HLA-A* downstream regulatory element, we prepared luciferase reporter constructs containing the full-length downstream regulatory region high-risk haplotypes HR1 and HR2 and nonhigh-risk haplotypes NHR1 and NHR2, as well as corresponding subsegments, inserted in both orientations upstream of a *luc2* reporter gene with minimal promoter. In all cases, the results were similar to those obtained using a *luc2* reporter with no promoter, with no augmentation of expression (Fig. S1 *A*–*C*). Furthermore, reporter constructs containing the full-length HR1, HR2, NHR1, and NHR2 downstream regulatory regions inserted immediately downstream of the *luc2* gene yielded essentially no luciferase expression, regardless of the orientation or presence versus absence of a minimal promoter upstream of *luc2* (Fig. S1 *D* and *E*). Thus, the *HLA-A* downstream regulatory region does not act as a transcriptional enhancer for this minimal promoter in the context of a conventional assay of circular plasmids in transfected HeLa cells.

Discussion

We previously showed that vitiligo is genetically associated with variation in the MHC class I region, in close proximity with *HLA-A* (4), and specifically with *HLA-A*02:01* in both European-derived Caucasians (5) and Japanese (24). Here, we refine genomic localization of this association to an SNP haplotype ~20 kb downstream of the *HLA-A* gene itself, spanning a 5-kb ENCODE regulatory element. Primary association of vitiligo is thus with the *HLA-A* downstream regulatory region, which is secondarily in very strong linkage disequilibrium with *HLA-A*02:01:01:01*.

The *HLA-A* promoter and downstream regulatory region are flanked by convergent CTCF sites, with an apparent 22-kb chromatin loop juxtaposing the downstream regulatory region and the *HLA-A* promoter. This configuration suggests that the downstream regulatory region modulates function of the *HLA-A* promoter. Consistent with this finding, RT-PCR analysis of *HLA-A* RNA in peripheral blood cells from normal healthy subjects showed that subjects homozygous for the high-risk SNP haplotype spanning the *HLA-A* downstream regulatory region express significantly percent more *HLA-A* RNA than subjects homozygous for nonhigh-risk haplotypes. Similarly, mRNA-seq analysis of lymphoblastoid cells from 1,000 Genomes Project subjects showed that subjects homozygous for the high-risk allele of lead variant rs60131261 express significantly more *HLA-A* mRNA than subjects homozygous for the low-risk allele.

Nevertheless, the specific function of the *HLA-A* downstream regulatory region is not yet clear. The downstream regulatory region has an open hypomethylated chromatin configuration in all cell types tested by ENCODE, multiple DNase I hypersensitivity sites, RNA polymerase II and transcription factor binding sites, active bidirectional promoters, and prominent H3K4me1, H3K4me3, and H3K27ac marks, and contains multiple sites of active bidirectional transcription mapped by GRO-seq. These

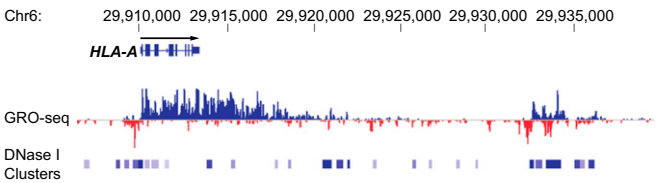

Fig. 5. GRO-seq data in the *HLA-A* region of chromosome 6p. Histogram of reads from HCT116 GRO-seq data shows transcription of both the *HLA-A* gene and a region 20 kb downstream of the gene coincident with the predicted downstream transcriptional regulatory element. Blue are reads on forward strand and red are reads on reverse strand. Reads from two 1-h replicates were summed from cells treated with control DMSO alone (Gene Expression Omnibus GSE53964). File is a Bedgraph with reads mapped and normalized to millions (22). DNase I clusters track (GM12878) is from ENCODE.

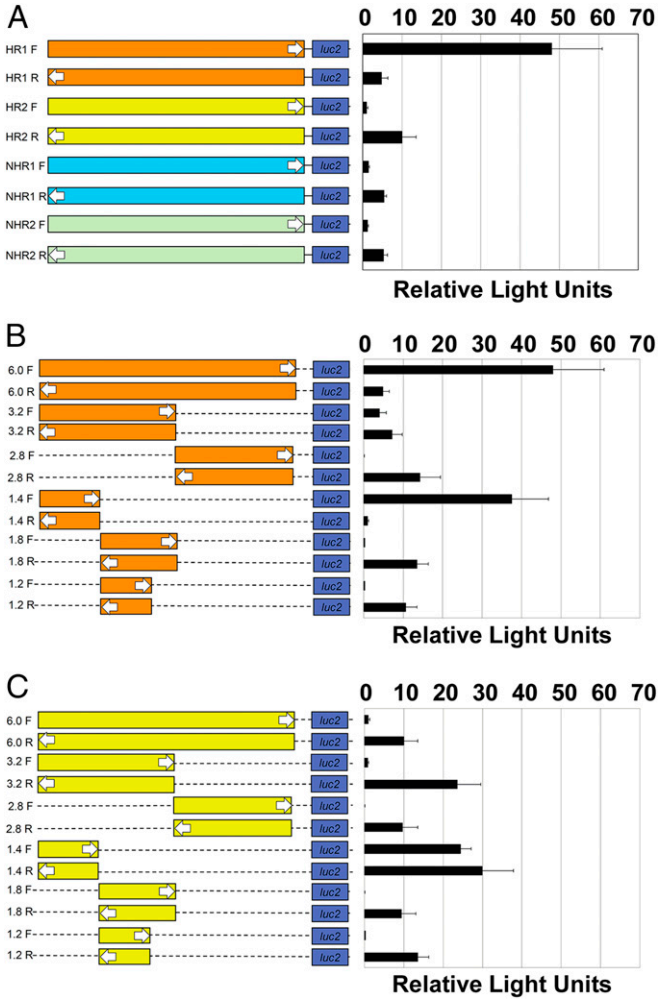

Fig. 6. Transient transfection assay of promoter activities in the *HLA-A* downstream transcriptional regulatory element. Luciferase reporter constructs containing segments of the *HLA-A* downstream regulatory element inserted immediately upstream of the *luc2* gene. (*A*) Full-length high-risk haplotypes (HR1, HR2) from subject 1 (orange and yellow) or full-length nonhigh-risk haplotypes (NHR1, NHR2) from subject 10. (*B*) Subfragments of the high-risk HR1 haplotype. (*C*) Subfragments of the high-risk HR2 haplotype. Arrowheads denote forward (F) and reverse (R) orientations relative to genomic orientation in chromosome 6. Relative light units denote fold-change of transcriptional activity relative to the pGL4.10 backbone plasmid. SEMs are indicated.

data suggested the presence of multiple promoters, which we confirmed in luciferase reporter assays of transfected cells. These features are all suggestive of an active transcriptional enhancer (16, 17, 20). However, the *HLA-A* downstream regulatory region did not act as an enhancer in a conventional transfection assay driving a minimal promoter. This finding may reflect specificity for the native *HLA-A* promoter in a linear chromosome, rather than the minimal promoter in the context of a circular plasmid. Alternatively, bidirectional promoters often serve specialized functions (25), and it may be that the in vivo biological function of the *HLA-A* downstream regulatory region is more complex, perhaps acting as a superenhancer (26), locus control region (27), or other higher-order transcriptional regulatory element in the context of a locus that is expressed in almost all cell types.

DNA sequence analysis of the *HLA-A* downstream regulatory region identified a large number of predicted transcription factor binding motifs. Moreover, the DNA sequences of the two nonhigh-risk haplotypes analyzed are generally similar to the GRCh37/hg19 reference sequence, whereas the two high-risk haplotypes analyzed differ far more, both from the reference sequence and from each other. These sequence differences affect many different predicted transcription factor binding motifs, which together presumably account for the differences in transcriptional activity observed among the different haplotypes.

Most studies of HLA autoimmune disease associations have focused on HLA-type specificity, which governs antigen binding and presentation as a result of amino acid sequence differences among alleles. However, genomewide association studies, including those of autoimmune diseases, have implicated transcriptional regulatory elements at many disease loci, accounting for an estimated 79% of total heritability across multiple common complex diseases (28). Our findings show that causal variation underlying genetic association of vitiligo with the *HLA-A* region affects both *HLA-A*–type specificity and transcriptional activity, resulting in a combination of qualitative and quantitative consequences. Primary association of vitiligo with the MHC class I region association is with a 9.6-kb SNP haplotype spanning a transcriptional regulatory region downstream of *HLA-A*. The high-risk haplotype induces gain-of-function, up-regulating expression of *HLA-A* mRNA in vivo, in strong linkage disequilibrium with *HLA-A*02:01:01:01*-type specificity. Expression of HLA class I protein molecules corresponds closely with RNA level (3); thus, the vitiligo high-risk haplotype likely causes elevated expression of HLA-A*02:01:01:01 protein. Because HLA-A*02:01 presents a number of melanocyte-derived peptides that constitute vitiligo autoimmune antigens (6–11), its elevated expression would facilitate recognition and immune targeting of melanocytes by cognate autoreactive T cells. Our findings thus highlight the pathogenic importance of quantitative functional effects of variation in the classic MHC genes, beyond just antigenic specificity.

Materials and Methods

Genotypes were imputed through the extended MHC (11, 12) in 2,853 vitiligo patients and 37,412 controls, and we used logistic regression analysis to determine which variants represent the strongest association signal in the MHC class I region. Healthy adult controls were genotyped for SNPs in the MHC class I region, and *HLA-A* RNA was quantitated by RNA-seq and RT-PCR analyses. Luciferase reporter constructs containing segments of the downstream regulatory region representing high-risk and low-risk haplotypes were transfected into HeLa cells and relative light units were assayed. Full experimental details can be found in *SI Materials and Methods*. This project was approved by the Colorado Multiple Institutional Review Board (COMIRB), and written informed consent was obtained from all subjects.

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