

## Allelic Polymorphism in Transcriptional Regulatory Regions of HLA-DQB Genes

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### Summary

Class II genes of the human major histocompatibility complex (MHC) are highly polymorphic. Allelic variation of structural genes provides diversity in immune cell interactions, contributing to the formation of the T cell repertoire and to susceptibility to certain autoimmune diseases. We now report that allelic polymorphism also exists in the promoter and upstream regulatory regions (URR) of human histocompatibility leukocyte antigen (HLA) class II genes. Nucleotide sequencing of these regulatory regions of seven alleles of the DQB locus reveals a number of allele-specific polymorphisms, some of which lie in functionally critical consensus regions thought to be highly conserved in class II promoters. These sequence differences also correspond to allelic differences in binding of nuclear proteins to the URR. Fragments of the URR of two DQB alleles were analyzed for binding to nuclear proteins extracted from human B lymphoblastoid cell lines (B-LCL). Gel retardation assays showed substantially different banding patterns to the two promoters, including prominent variation in nuclear protein binding to the partially conserved X box regions and a novel upstream polymorphic sequence element. Comparison of these two polymorphic alleles in a transient expression system demonstrated a marked difference in their promoter strengths determined by relative abilities to initiate transcription of the chloramphenicol acetyltransferase reporter gene in human B-LCL. Shuttling of URR sequences between alleles showed that functional variation corresponded to both the X box and upstream sequence polymorphic sites. These findings identify an important source of MHC class II diversity, and suggest the possibility that such regulatory region polymorphisms may confer allelic differences in expression, inducibility, and/or tissue specificity of class II molecules.

Class II molecules of the MHC are cell surface heterodimers composed of  $\alpha$  and  $\beta$  polypeptides. Structural variation among the MHC class II molecules is key to functional differences where, as part of the trimolecular complex with peptide and T cell receptor, the class II molecule participates in immune activation events which lead to histoincompatibility, immune recognition, and disease susceptibility (1–3). Variation among class II molecules is predominantly of two types, one of which is germline genetic polymorphism, with multiple alleles existing at various loci within the MHC gene complex (4). This type of polymorphism leads to differential peptide recognition and binding by the class II molecules encoded by individual alleles (5, 6). Another source of variation arises from the differential ability of certain  $\alpha$  chains to pair with certain  $\beta$  chains to form combinatorial dimers on the cell surface (7, 8). We have examined a possible third type of polymorphism; namely, transcriptional variation between different class II alleles.

Regulated inducible and tissue-specific transcription of MHC class II genes is a complex system involving *cis*-acting sequence elements and *trans*-acting protein factors. Analysis of transcriptional control elements involved in human class II gene regulation have been reported for the nonpolymorphic HLA-DRA1 gene (9, 10) and for the HLA-DQw2 DQB1 gene (11–14). These studies have identified a number of consensus nucleotide sequences present in the proximal promoter/enhancer region conserved not only among human class II genes, but also across species barriers. Recent studies have begun to identify potential candidates for nuclear regulatory proteins which bind to these consensus nucleotide sequences and therefore presumably mediate the observed transcriptional effects (15–20). Current models for DNA–protein interactions which contribute to the function of class II promoter regions suggest that at least four or five important regulatory regions exist upstream of the class II coding sequences within the first 180 nucleotides, with additional important

tissue-specific and inducible transcriptional signals existing further upstream (21, 22). Although class II genes are often coordinately expressed (23, 24), transcriptional regulatory regions critical for constitutive and inducible class II expression differ somewhat between HLA-DRA1 and HLA-DQB1 promoter elements (11, 25). An unknown number of functionally important proteins bind these regulatory regions, and interactions between some of these proteins are essential for normal regulated gene transcription (17, 26).

To investigate potential interallelic promoter region polymorphism, we determined the nucleotide sequences for upstream regulatory regions of each of the prevalent HLA-DQB alleles. A number of allele-specific polymorphisms, even within functionally critical consensus regions, were found. Comparison of these promoter-associated elements in gel retardation assays revealed corresponding allelic differences in binding of nuclear proteins. Analysis of the same polymorphic alleles in a transient expression system demonstrated a marked difference in their promoter strengths, determined by relative abilities to initiate transcription of the chloramphenicol acetyl transferase (CAT)<sup>1</sup> reporter gene in human B lymphoblastoid cell lines (B-LCL).

## Materials and Methods

**Nucleotide Sequencing and Cell Lines Used.** Nucleotide sequences for promoter-associated regions of the DQB1 gene were derived from the following cells: DQ3.1 (DQB1\*0301), ER (DR4) and SWEIG (DR5); DQ3.2 (DQB1\*0302), BSM (DR4) and BrEh (DR4); DQ3.3 (DQB1\*0303), HIG (DR9) and 008 (DR9); DQ4 (DQB1\*0401), KT3 (DR4) and MAD (DR8); DQ2 (DQB1\*0201), VAVY and reference 27; DQ1.2 (DQB1\*0602), AMAI (DR2); and DQ1.1 (DQB1\*0601), HOM2 (DR1). 1  $\mu$ g of DNA from each cell line was amplified by PCR as described (28), annealing at 39°C for 28 cycles. The 3' primer used for amplification of all alleles was 5'-GTCCTGCAGACATAATGAGACGAAG-3', which anneals to the region starting at position -13, designating the A of ATG as +1, and includes an introduced PstI site. The 5' primer was one of the following: 5'-CAGGGATCCCTTAGGCATCAATCTTC-3', which includes an introduced BamHI site and anneals beginning at position -314 in all alleles except DQ1.1 and DQ1.2; 5'-CTC-GGATCCAATCAATATTACAAAGATGTT-3', which anneals at position -374 in DQ1.1 and DQ1.2 alleles and contains a BamHI site; or 5'-CACGGTACCTGAAACATGATCCACATT-3', which anneals at position -618 and contains an introduced KpnI site. After digestion with appropriate restriction endonucleases, amplified DNA was ligated into M13mp18 and M13mp19 vectors, transfected into JM101, and sequenced using the dideoxy method of Sanger and Coulson (29). Multiple clones were sequenced in every case.

**Nuclear Extracts.** The human EBV-transformed B-LCL BSM used for preparation of nuclear extracts was grown in RPMI-Hepes containing 10% fetal bovine serum and harvested in logarithmic growth phase. Nuclear extracts were prepared essentially as described by Shapiro et al. (30). Briefly, cells were swollen in hypotonic buffer (10 mM Hepes, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, and 1 mM dithiothreitol) and then lysed by 5–6 strokes of a pestle B in a Dounce

homogenizer. The nuclei were isolated by centrifugation and extracted by an ammonium sulfate solution ( $\sim$ 4 M at 4°C), and nuclear proteins were precipitated with solid ammonium sulfate. All buffers contained the proteinase inhibitors phenylmethylsulfonyl fluoride (0.5 mM) and leupeptin (5  $\mu$ g/ml) (Calbiochem-Behring Corp., San Diego, CA). Protein activity was measured (Bio-Rad Laboratories, Richmond, CA) and the nuclear extracts were further purified by heparin-agarose chromatography (H6508, Type I; Sigma Chemical Co., St. Louis, MO). 1 ml of heparin-agarose was used per 30–40 mg of protein extract. The columns were equilibrated with 5 column volumes of 0.1 M TM, where the molarity refers to the molar fraction of KCl used in the TM buffer (50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 12.5 mM MgCl<sub>2</sub>, 20% glycerol). After applying the nuclear extracts, the columns were eluted successively with 2 column volumes of 0.1 M TM, 0.2 M TM, 0.4 M TM, and 1 M TM. 1-ml fractions were collected and assayed for protein activity. Protein-containing fractions from each elution were pooled, and their final protein concentration was determined; pooled fractions were then assayed for DNA-binding activity in gel retardation assays (see below). The major DNA-binding activity (85–90%) eluted with the 0.4 M TM buffer.

**Gel Retardation Assays.** Oligonucleotides were synthesized on a DNA synthesizer (model 391; Applied Biosystems, Foster City, CA). Double-stranded oligonucleotides were end-labeled with either Klenow DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN) or Terminal Transferase (Bethesda Research Laboratories, Gaithersburg, MD) (31). Fragments DQ3.1(I) and DQ3.2(I) (see Fig. 2) were end-labeled at the PpuMI restriction site with Klenow DNA polymerase. Gel retardation assays were performed as described previously (32, 33); briefly, the DNA-protein-binding reactions were prepared in a total volume of 25  $\mu$ l reaction buffer containing 12 mM Hepes, pH 7.9, 60 mM KCl, 12% glycerol, 0.12 mM EDTA, and 0.3 mM dithiothreitol. 1–1.5  $\mu$ g of nuclear protein extract was preincubated with 0.2  $\mu$ g (unless otherwise indicated in figure legends) of nonspecific competitor DNA poly(dI-dC)·poly(dI-dC) (Pharmacia Fine Chemicals, Piscataway, NJ) for 20 min at room temperature. After adding 10,000 cpm of end-labeled double-stranded DNA fragment or oligonucleotide ( $\sim$ 0.2 ng of DNA), the samples were further incubated for 10 min at room temperature (RT). For competition assays, poly(dI-dC) and specific competitor DNA were preincubated for 20 min at room temperature followed by an incubation for 10 min at room temperature after adding the labeled probe. DNA-protein complexes were separated from free probe on 4% polyacrylamide gels in 0.25  $\times$  TBE (1  $\times$  TBE is 89 mM Tris, 89 mM boric Acid, and 2 mM EDTA).

**Transcription Assays.** HincII-digested 549-bp fragments from amplified promoter elements of the DQ3.1 and DQ3.2 alleles were inserted into the polylinker of the pICAT vector at the SmaI site, 47 nucleotides upstream of the CAT gene, to form the pQ3.1 $\beta$  P1CAT and pQ3.2 $\beta$ P2CAT plasmids, respectively (see Fig. 6). The pICAT vector (kindly provided by Richard Harland, University of California at Berkeley) is a promoter test plasmid that lacks an endogenous promoter for the CAT gene. A 62-bp fragment from position -223 (AatII) to -158 (PpuMI) was removed from the DQ3.1 clone, and the corresponding DQ3.2 62-bp fragment was inserted in its place, to form the hybrid pQ3.1 $\beta$ P3CAT construct. pQ3.1 $\beta$ P2CAT represents the DQ3.1 promoter fragment inserted in the pICAT plasmid in reverse orientation. pQ3.1 $\beta$ P4CAT and pQ3.2 $\beta$ P3CAT were subcloned from the pQ3.1 $\beta$ P1CAT and pQ3.2 $\beta$ P2CAT, respectively, by removing promoter sequences 5' of the AatII site at position -223.

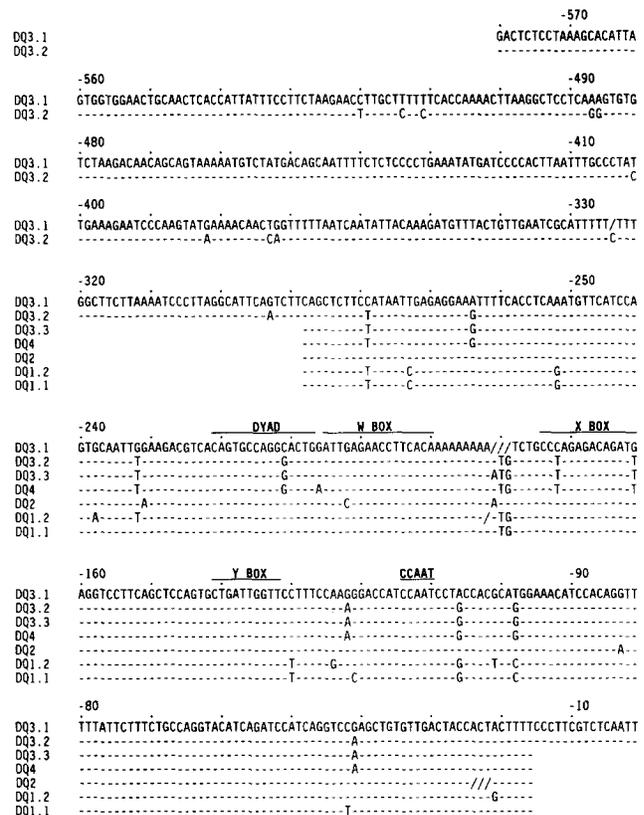
<sup>1</sup> Abbreviations used in this paper: B-LCL, B lymphoblastoid cell lines; CAT, chloramphenicol acetyl transferase; URR, upstream regulatory region.

The nonchromatographic CAT assay used was modified from that of Sleight (34). Briefly, 10  $\mu$ g of each pTCAT-derived plasmid was used to transfect  $2 \times 10^7$  BSM cells, for 30 min in 200  $\mu$ g DEAE-Dextran/ml, along with 1  $\mu$ g of a plasmid encoding the  $\beta$ -galactosidase gene driven by the CMV immediate early promoter (pEQ176, kindly provided by Adam Geballe, Fred Hutchinson Cancer Research Center, Seattle, WA) as a control for transfection efficiency. After incubation in 5% CO<sub>2</sub> at 37°C for 48 h, samples were harvested and cells lysed by three cycles of freeze-thawing. Supernatants were divided into two samples. One was used for the  $\beta$ -galactosidase assay, modified from Miller (35), where the chlorophenol red galactopyranoside color change was measured at OD<sub>574</sub>. The other sample was heated at 60°C for 10 min, centrifuged to remove subsequent precipitate, and assayed for CAT activity. 70  $\mu$ l of this sample was mixed with 20  $\mu$ l 8 mM chloramphenicol and 10  $\mu$ l <sup>14</sup>C-acetyl CoA (0.1  $\mu$ Ci), incubated for 4 h at 37°C, extracted with 1 ml ethyl acetate, vortexed, and microfuged. 800  $\mu$ l of the ethyl acetate phase was counted in a liquid scintillation counter. CAT and  $\beta$ -galactosidase values were well within the linear range of the assays.

## Results

**Nucleotide Polymorphisms among HLA-DQB1 Alleles.** To investigate nucleotide polymorphism in class II upstream regulatory regions (URR), we designed oligonucleotide primers homologous to DQB consensus sequences encompassing both proximal promoter and upstream regulatory elements (27, 36) for amplification and nucleotide sequencing of a series of DQB alleles. PCR was used to amplify and clone gene segments from genomic DNA; seven different alleles were sequenced, with up to five representative cell lines sequenced for each allele. The first 285 nucleotides 5' of the ATG initiation codon for the class II polypeptide were sequenced for all alleles, with longer stretches up to 580 nucleotides sequenced for the DQ3.1 and DQ3.2 alleles; sequences are shown in Fig. 1.

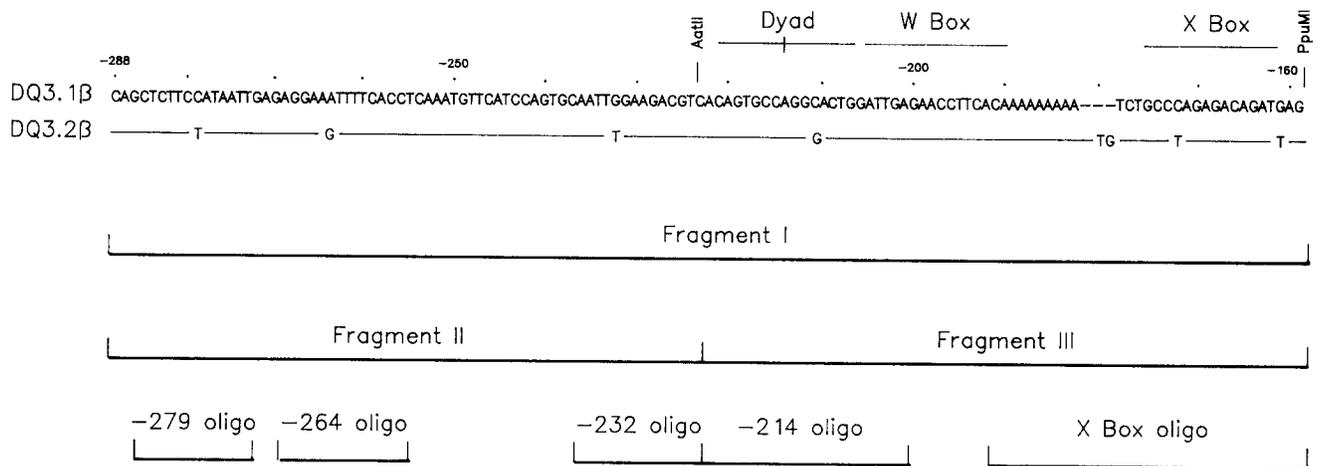
In these URR, most of the polymorphisms appear to be conserved within a haplotype. This is true regardless of the origin of the cell lines; for example, cell line MAD was derived from a DR8, DQ4 Caucasian, while KT3 was derived from a DR4, DQ4 Japanese, and their DQ URR fragments are identical. While it is perhaps not surprising that promoter sequences are maintained within clusters sharing identical DQB structural genes, a more unexpected relationship appears to exist between the different allelic groups as compared to their structural gene counterparts. The hierarchy of degree of homology which exists among the DQB structural genes is not paralleled among their promoter sequences. Within the regulatory sequences outlined in Fig. 1, the highest degree of homology (>99%) exists in a cluster including DQ3.2, DQ3.3, and DQ4, which vary by only a single nucleotide, whereas DQ3.1 and DQ3.2 have 12 differences (95.6% homology). In contrast, the structural genes of DQ3.1, DQ3.2, and DQ3.3 are the most closely related among DQB alleles, while DQ4 is much less homologous. URR from the other DQ genes, DQ1, DQ2, and DQ3.1, each vary from the "DQ3.2 cluster" and from each other by 3–7%, suggesting that the DQ URR and structural genes may have evolved independently.



**Figure 1.** Nucleotide sequences of URR of seven HLA-DQ alleles, designating the A of ATG as +1. See Materials and Methods for cells used. The dashes represent nucleotide identity to the DQ3.1 sequence, and slashes represent the absence of a nucleotide at that position. Solid lines above the sequences indicate the position of the consensus sequence elements, W, X, and Y box, and the dyad symmetry element. These sequence data are available from EMBL/GenBank/DBJ under accession numbers X55421–X55427.

While all URR sequences are >90% homologous, significant polymorphism was found, including within the MHC class II consensus sequence elements, the X and W boxes. These two sequence elements, along with the consensus Y box, are found in all murine and human class II genes so far studied, and deletion of any of the three is sufficient to drastically reduce or eliminate transcription (12, 25, 37). Among the DQB alleles, the X box consensus element is polymorphic at positions –161 and –172 (see Fig. 1). Different alleles carry either a G or T at position –161, and a C or a T at position –172. Some alleles also lack two nearby nucleotides (a TG at positions –179 and –180), while the DQ2 and DQ3.3 alleles have an A at –181. 5' of the X box, a stretch of A's varies in length from 8 to 10, depending on the allele. For the W box among the DQB URR, a single nucleotide substitution is noted, a C for a G at position –202 in the DQ2 allele only. The transcriptional control sequence known as the CCAAT box, and the Y box, which contains an inverted CCAAT sequence (13, 25), are conserved among DQ URR. However, scattered additional polymorphisms do occur in the region between these elements.

**DNA-Protein Interactions in the DQB1 URR.** Two of these

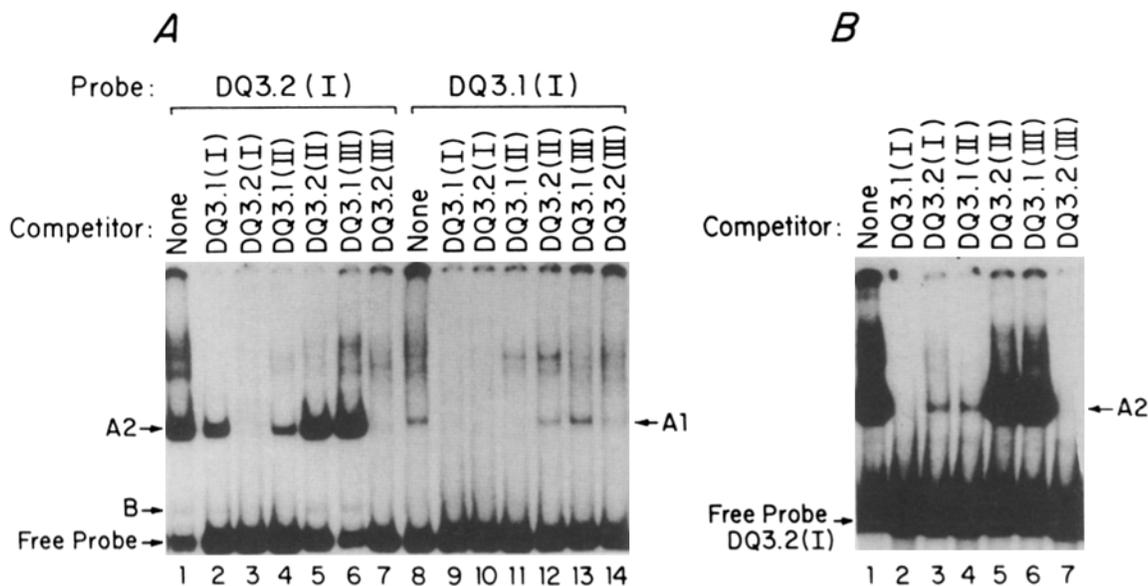


**Figure 2.** DQ URR fragments and double-stranded oligonucleotides used in gel retardation assays. The DNA sequences from -288 to -159 of the DQ3.1 and DQ3.2 URR are shown as in Fig. 1 and are termed DQ3.1(I) or DQ3.2(I), respectively. Fragment II encompasses polymorphic sequences from -223 to -288, termed DQ3.1(II) or DQ3.2(II); fragment III encompasses sequences from -223 to -159, termed DQ3.1(III) or DQ3.2(III). Oligonucleotides used in these studies span polymorphic sites which differ between URR of the DQ3.1B and DQ3.2B genes. Boundaries of the double-stranded oligonucleotides are shown.

allelic URR were evaluated for possible differential interactions with DNA binding proteins. Fragments of the DQ3.1 and DQ3.2 URR were used, encompassing sequences from -159 to -288, which contain the consensus W and X box sequence motifs, in addition to other polymorphic and non-polymorphic sequences of potential interest. These fragments are illustrated in Fig. 2. DQ3.1(I) and DQ3.2(I) fragments

encompass this entire region; DQ3.1(II) and DQ3.2(II) upstream fragments and DQ3.1(III) and DQ3.2(III) downstream fragments divide the longer fragments adjacent to the W box element, and correspond to the DQ3.1 and DQ3.2 alleles, respectively.

Nuclear proteins were extracted from the human B-LCL. BSM and the URR fragments DQ3.1(I) and DQ3.2(I) were



**Figure 3.** (A) Competition analyses for protein binding to the DQ3.1(I) and DQ3.2(I) fragment. Labeled DQ3.2(I) (lanes 1-7) and DQ3.1(I) (lanes 8-14) fragments were incubated with BSM nuclear extract. (Lanes 1 and 8) Reactions with no competitor DNA. Complex formation was tested in the presence of unlabeled competitor DNA (in ~200-fold excess): (lanes 2 and 9) DQ3.1(I); (lanes 3 and 10) DQ3.2(I); (lanes 4 and 11) DQ3.1(II); (lanes 5 and 12) DQ3.2(II); (lanes 6 and 13) DQ3.1(III); and (lanes 7 and 14) DQ3.2(III). (B) Competition analyses for protein binding to the DQ3.2(I) fragment. Labeled DQ3.2(I) fragment was incubated with BSM nuclear extract (lane 1), and complex formation was inhibited with the following nonlabeled competitor DNA (in ~300-fold molar excess): (lane 2) DQ3.1(I); (lane 3) DQ3.2(I); (lane 4) DQ3.1(II); (lane 5) DQ3.2(II); (lane 6) DQ3.1(III); and (lane 7) DQ3.2(III).

end-labeled and used as probes in gel retardation assays. Protein-DNA complexes seen after gel electrophoresis showed several bands of different intensities and mobilities (Fig. 3). Comparison of lane 1 [DQ3.2(I)] and lane 8 [DQ3.1(I)] in Fig. 3 A highlights two particular complexes of interest. Bands denoted A1 and A2, for the DQ3.1 and DQ3.2 alleles, respectively, had similar mobility patterns but markedly varied in intensity; the intensity of complex A2 was consistently much higher than that of A1 using identical nuclear extracts and probes of similar specific activity. Another complex with fast mobility was consistently observed as a unique band (B) for the DQ3.2(I) probe.

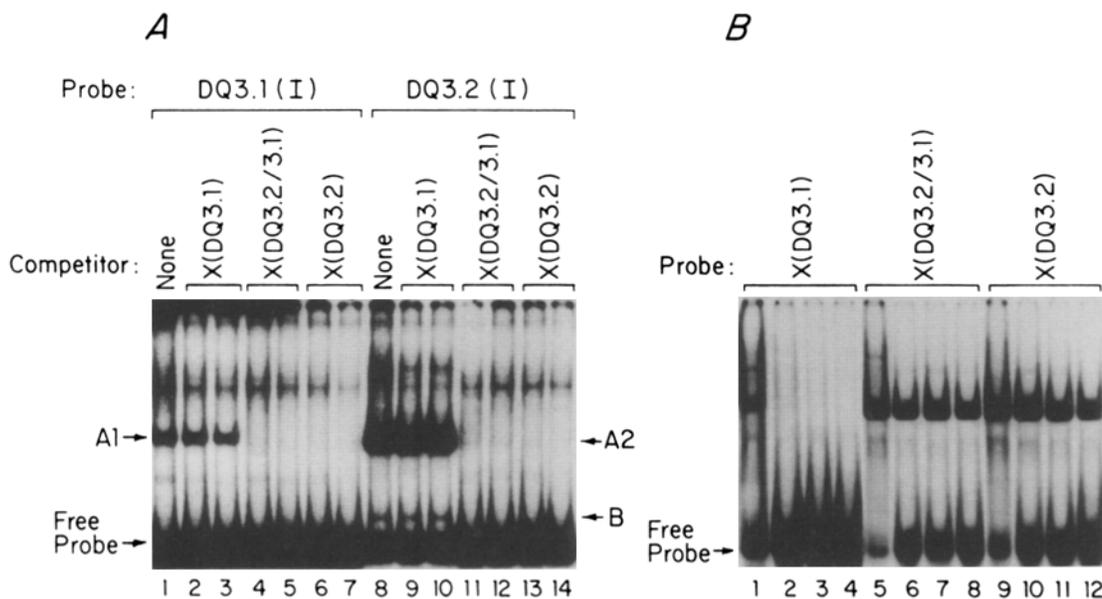
Competition experiments were performed using unlabeled DQ3.2 and DQ3.1 fragments to inhibit formation of the A1 and A2 complexes. Comparisons between the smaller competitive URR fragments revealed major differences: the downstream DQ3.2 promoter fragment DQ3.2(III) competed both complex A1 and A2 at a 200-fold molar excess (Fig. 3 A, lanes 7 and 14); however, neither of the complexes were competed away by the downstream DQ3.1 promoter fragment DQ3.1(III) (Fig. 3 A, lanes 6 and 13). The converse was seen when using the upstream DQ3.1(II) and DQ3.2(II) fragments as competitors: while the DQ3.2(II) fragment had no activity as a competitor (Fig. 3 A, lanes 5 and 12), the DQ3.1(II) fragment was an effective competitor for both complexes A1 and A2 (Fig. 3 A, lanes 4 and 11). Even with high concentrations of competitive DNA, formation of complex A2 could not be inhibited by either the upstream DQ3.2(II) fragment or the downstream DQ3.1(III) fragment (Fig. 3 B, lanes 5 and 6, respectively), but was efficiently inhibited by the up-

stream DQ3.1 fragment and the downstream DQ3.2 fragment (Fig. 3 B, lanes 4 and 7, respectively).

To pinpoint the basis for these DNA-protein interactions, synthetic double-stranded oligonucleotides were used as unlabeled competitor DNA in similar gel retardation assays. A set of cognate oligonucleotides containing the consensus X box sequence motif and flanking sequences (see Fig. 2) was constructed. The X(DQ3.2) and X(DQ3.1) oligonucleotides correspond to the DQ3.2 and DQ3.1 allelic nucleotide sequence in this region, respectively. The X(DQ3.2/3.1) oligonucleotide is a hybrid of the DQ3.1 and DQ3.2 sequences in this region with DQ3.2-specific nucleotides TG at position -179, -180 and the DQ3.1-specific C and G nucleotides at positions -172 and -161. Gel retardation assays showed that both X(DQ3.2) and X(DQ3.2/3.1) competitor DNA efficiently inhibited formation of the mobility complexes A1 and A2 while X(DQ3.1) did not (Fig. 4 A).

Since formation of complex A2 is inhibited not only by the DQ3.2(III) but also the X(DQ3.2) DNA, we infer that proteins interacting with the X box consensus element and/or flanking sequences participate in the formation of the A2 complex. Since formation of complex A2 was not inhibited by either DQ3.1(III) or X(DQ3.1), it appears that the affinity of these proteins is less for X box-related sequences in the DQ3.1 URR compared with the DQ3.2 URR. Since X(DQ3.2/3.1) acts equally well as an inhibitor of complex formation as X(DQ3.2), nucleotides at positions -179 and/or -180 are likely important for this high affinity protein binding site present in the DQ3.2 URR.

Gel retardation analysis of nuclear proteins directly bound



**Figure 4.** (A) Competition analyses with X box oligonucleotides. Binding reactions were carried out with BSM nuclear extract and the DQ3.1(I) (lane 1-7) and DQ3.2(I) (lane 8-14) labeled fragments. (Lanes 1 and 8) No competitor DNA; (lanes 2, 3, 9, and 10) competition with X(3.1); (lanes 4, 5, 11, and 12) competition with X(DQ3.1/3.2); and (lanes 6, 7, 13, and 14) competition with X(DQ3.2). Binding reactions contained 1,000-fold molar excess competitor DNA to probe (lanes 2, 4, 6, 9, 11, and 13) or 2,500-fold molar excess to probe (lanes 3, 5, 7, 10, 12, and 14). (B) Direct binding analyses to X box oligonucleotides. Binding reactions were carried out with BSM nuclear extract and (lanes 1-4) labeled X(3.1), (lanes 5-8) labeled X(DQ3.1/3.2), or (lanes 9-12) labeled X(DQ3.2). Each X box oligonucleotide was incubated with increasing amounts of poly(dI-dC): (lanes 1, 5, and 9, 0.01  $\mu$ g; (lanes 2, 6, and 10) 0.05  $\mu$ g, (lanes 3, 7, and 11) 0.1  $\mu$ g, and (lanes 4, 8, and 12) 0.5  $\mu$ g.

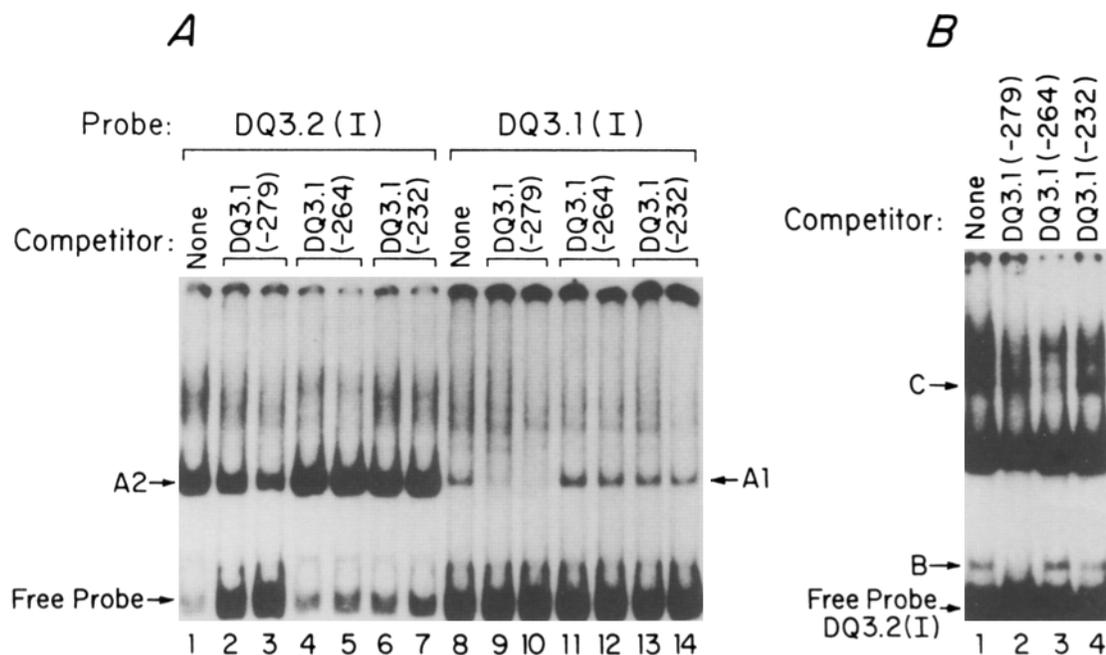
to labeled X(DQ3.2), X(DQ3.2/3.1), and X(DQ3.1) oligonucleotides showed one major complex with the same mobility for all probes (Fig. 4 B). However, the intensity of the complex bound to X(DQ3.1) was very low compared with the X(DQ3.2) and X(DQ3.2/3.1) probes, consistent with the existence of a high affinity X box binding site in the DQ3.2 URR compared with the DQ3.1 URR.

These gel retardation studies also identify another polymorphic site in the URR with consequences for DNA-protein interactions. Formation of both A1 and A2 was competed by DQ3.1(II) but not DQ3.2(II), indicating the likelihood that proteins participating in the A1 and A2 complexes also bind to polymorphic sequences in the upstream region of the DQ3.1 URR. There are only three polymorphic sites in the region encompassed by the DQ3.1(II) fragment which differ from DQ3.2(II). Oligonucleotides spanning each of these sites (Fig. 2) were used as inhibitors of DNA-protein complexes. Only the oligonucleotide corresponding to the URR site at position -279 competed with the formation of complexes A1 and A2, completely inhibiting the A1 complex, and partially competing the A2 complex (Fig. 5).

Several sites of DNA-protein interaction in the upstream URR did not vary between the alleles studied: Fig. 5 B shows that the DQ3.1(-264) competitor DNA specifically inhibits one of the slower mobility complexes, termed C. Included within the sequence of the DQ3.1(-264) oligonucleotide is a potential regulatory site at -265 to -270 which has exact sequence homology to the PU box, a purine-rich sequence

recognized by a macrophage and B cell-specific transcription factor PU.1 (38). Another slow mobility complex observed in the gel retardation studies (data not shown) corresponds to proteins interacting with a region of "dyad symmetry" upstream of the W box (Fig. 1). The symmetry, which in DQ3.1 extends seven nucleotides in each direction from the central nucleotide at position -214, is disrupted in DQ3.2. Two oligonucleotides were constructed: DQ3.1(-214) and DQ3.2(-214), having DQ3.1 and DQ3.2 allele-specific sequences, respectively, in this area of dyad symmetry (Fig. 2). Gel retardation assays using these double-stranded oligonucleotides as labeled probes showed one major complex with the same mobility binding to both (data not shown).

**Allelic Variation in Promoter Strength.** To compare transcriptional activity of the DQ3.1 and DQ3.2 URR, a transient expression system was used, where the ability of portions of these URR to drive transcription of the CAT reporter gene was assayed. URR fragments inserted into the pTCAT vector were used to co-transfect the human B-LCL BSM together with a plasmid (pEQ176) encoding  $\beta$ -galactosidase, serving as an internal standard control for transfection efficiency. The pTCAT vector contains no intrinsic promoter or enhancer elements adjacent to the CAT gene; transfection with the vector alone gave no detectable CAT activity above background levels in the BSM cell extracts. As a result, the CAT activity observed when transfecting the URR-pTCAT constructs into BSM cells is due to the activity of the minimal DQ promoter elements through position -210 of the



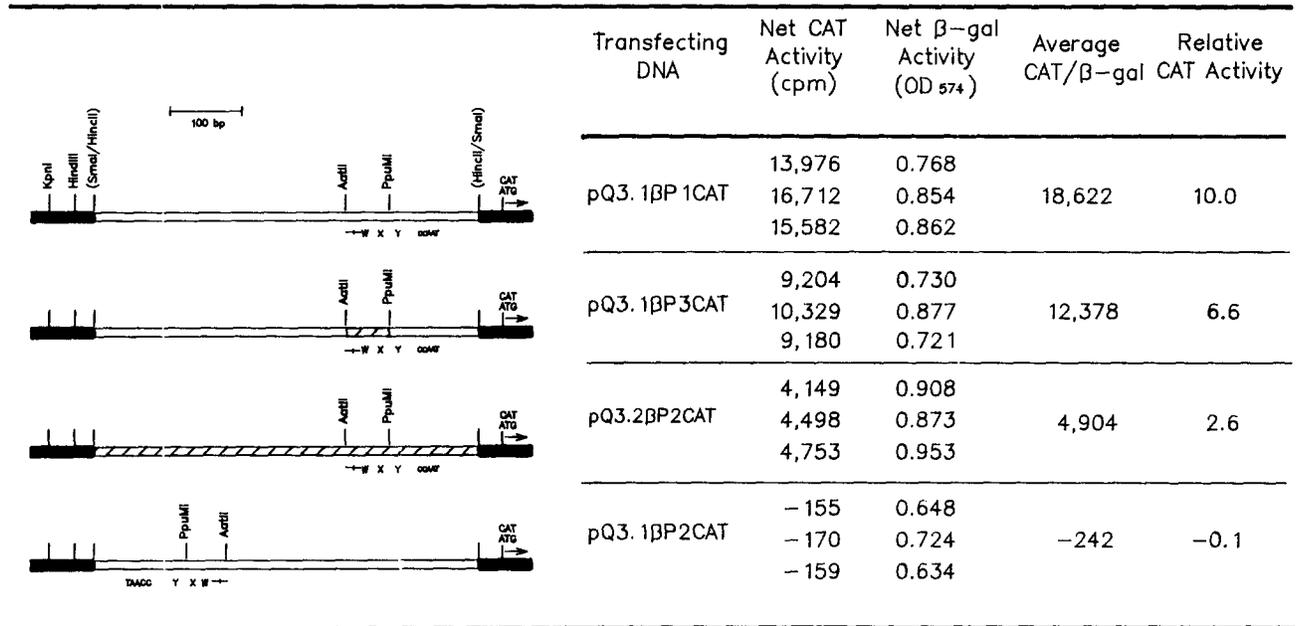
**Figure 5.** (A) Competition analyses with DQ3.1(-279), DQ3.1(-264), and DQ3.1(-232) competitor DNA. Binding reactions were carried out with BSM nuclear extract and the DQ3.2(I) (lanes 1-7) and DQ3.1(I) (lanes 8-14) probes. (Lane 1 and 8) No competitor DNA; (lanes 2, 3, 9, and 10) competition with DQ3.1(-279); (lanes 4, 5, 11, and 12) competition with DQ3.1(-264); (lanes 6, 7, 13, and 14) competition with DQ3.1(-232). Binding reactions contained 1,000 molar excess competitor DNA to probe (lanes 2, 4, 6, 9, 11, and 13) or 2,500 molar excess to probe (lanes 3, 5, 7, 10, 12, and 14). (B) Competition analyses for protein binding to the DQ3.2(I) fragment, using BSM nuclear extract and the DQ3.2(I) probe. (Lane 1) No competitor DNA; (lane 2) competition with DQ3.1(-279); (lane 3) competition with DQ3.1(-264); (lane 4) competition with DQ3.1(-232). Binding reaction contained 2,500 molar excess competitor DNA to probe.

URR and to the modulating influences of additional upstream elements.

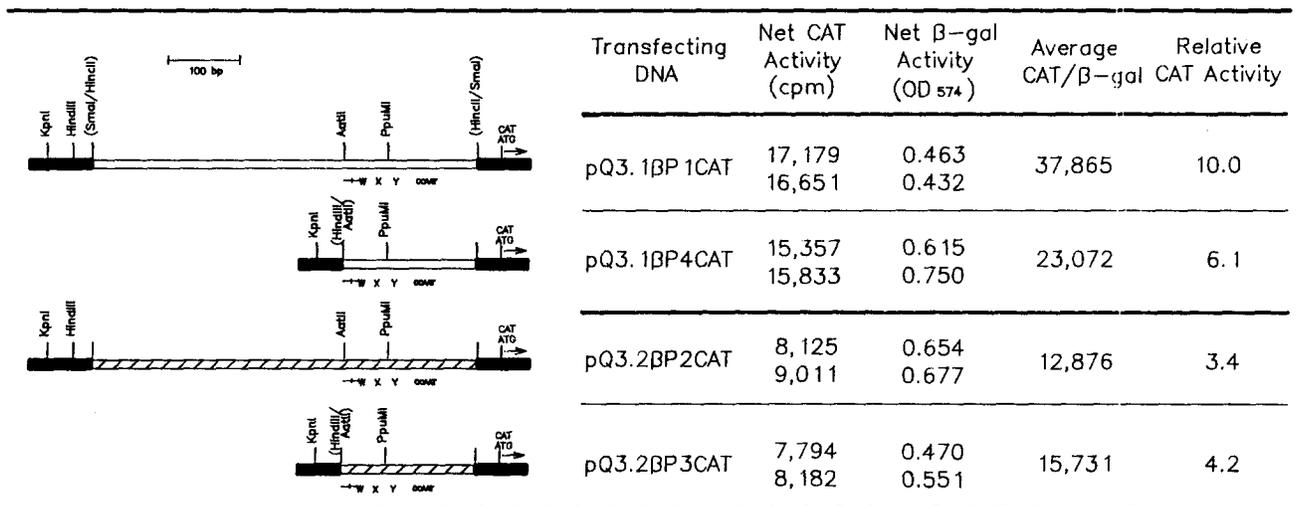
The 549-bp *HincII* fragments from the DQ3.1 and DQ3.2 URR (positions -580 to -31) were inserted into the pTCAT

vector to generate the pQ3.1 $\beta$ P1CAT and pQ3.2 $\beta$ P2CAT constructs, respectively. After transfection into BSM cells, the DQ3.1 fragment gave CAT activity that was three- to four-fold higher than the comparable DQ3.2 fragment (Fig. 6).

A



B



**Figure 6.** Relative CAT activity among allelic DQ promoter fragments 48 h after transfection into BSM. Endonuclease sites are identified with vertical bars. CCAAT, Y, X, W, and the adjacent dyad symmetry region indicated represent the locations of consensus sequences as described in the text. Open bars indicate DQ3.1 sequences, crosshatching indicates DQ3.2 sequences, and solid bars indicate the pTCAT vector. Net CAT Activity and Net  $\beta$ -gal Activity refer to total <sup>14</sup>C cpm or to raw OD<sub>574</sub> reading, respectively, minus the background values of transfected samples with no DNA added. The average "No DNA" background in experiment A was 1,958 cpm for CAT activity and an OD<sub>574</sub> reading of 0.049 for  $\beta$ -galactosidase activity, and in experiment B was 2,272 cpm for CAT activity and 0.024 for  $\beta$ -galactosidase activity. Measurements less than those for samples with no DNA added are negative net values. Average CAT/ $\beta$ -galactosidase values were derived by dividing the mean net CAT activity by the mean net  $\beta$ -galactosidase activity, which was then normalized to give relative CAT activity by assigning the pQ3.1 $\beta$ P1CAT a value of 10.

The pQ3.1 $\beta$ P2CAT construct has the same DQ3.1 URR fragment inserted in a reverse orientation, and serves as a negative control with CAT values equivalent to background CAT activity, confirming the specificity of transcription initiation by the inserted URR in "correct" orientation. Nucleotide sequencing of all of the constructs studied confirmed the sequences displayed in Fig. 1 and also showed that the flanking and joining regions in the vector were identical among the fragments.

As indicated above, sequence and protein-binding studies implicated polymorphic sites around the X box element and upstream of the W box as potential sites of important URR allelic variation. A plasmid was constructed where a portion of the DQ3.2 URR spanning the functionally important X and W boxes was shuttled into the DQ3.1 URR: restriction endonuclease sites at positions -223 (AatII) and -158 (PpuMI) were used to cut out a 62-bp fragment in DQ3.1, and the corresponding DQ3.2 fragment, which differs by five nucleotides, was inserted in its place to form the hybrid construct pQ3.1 $\beta$ P3CAT. When compared in the transfection assay to the unadulterated pQ3.1 $\beta$ P1CAT and pQ3.2 $\beta$ P2CAT plasmids, its promoter "strength" was intermediate between that of the 3.1 and the 3.2 URR alone, suggesting that this region confers some, but not all, of the variation in transcriptional activity between these allelic fragments.

To distinguish these sites of functional promoter variation from additional upstream contributions, two constructs were generated by removing promoter sequences 5' of the AatII site (-223) in the pQ3.1 $\beta$ P1CAT and pQ3.2 $\beta$ P2CAT constructs, generating pQ3.1 $\beta$ P4CAT and pQ3.2 $\beta$ P3CAT, respectively. As shown in Fig. 6 B, allelic differences in these minimal promoters were still observed, but did not account for all of the variation seen in transfections performed with longer segments of the URR. Truncation of the DQ3.2 URR to remove upstream elements did not reduce promoter strength; on the other hand, an identical deletion in the DQ3.1 URR led to a 40% reduction in promoter strength. This indicates that upstream elements in the DQ3.1 URR account for approximately half of the difference observed between the larger allelic URR fragments.

## Discussion

Polymorphism is the hallmark of the MHC. Nucleotide variation in structural genes among closely related HLA class II alleles is responsible for functionally significant amino acid variation; these differences account for variation in peptide binding properties (5, 6), allorecognition (4), and are likely critical for susceptibility to HLA-associated diseases (2, 3). This polymorphism is functionally amplified by variation in combinatorial associations of various class II  $\alpha$  and  $\beta$  polypeptides (39-41), so that allelic contributions to immune recognition are really quite complex. We now report an additional source of variation due to genetic polymorphism among HLA class II genes, namely, allelic diversity in the promoter and associated regulatory elements.

The URR of the HLA-DQB1 locus includes both the minimal promoter elements necessary for transcription and elements important for tissue specificity and inducible regulation (11-13, 42). We found up to 7% nucleotide polymorphism within these upstream elements among seven DQB1 alleles. URR sequences linked to specific structural alleles were highly conserved among different haplotypes; i.e., the DQ4 URR were identical on DR8- and DR4-associated haplotypes, suggesting that these polymorphisms are stable, and not recently derived. Sequences derived from multiple cell lines with the same haplotypes were also identical for all URR analyzed.

Several of these polymorphisms occur at sites important for transcriptional regulation. Analysis of nuclear proteins that specifically bind to the URR of the DQB genes revealed at least two sites which differ markedly in affinity for DNA binding proteins, because of the allelic nucleotide variation. One of these sites is within a recognized consensus element, the X box, which occurs in all murine and human class II genes. DNA-binding proteins from cell line BSM bind avidly to this region in the DQ3.2 URR, but much less so to the same region in the DQ3.1 URR. The other variable site lies upstream of the recognized consensus regions, and has the opposite phenotype; that is, a DNA-binding protein was identified which binds to an upstream site within the DQ3.1 URR but does not bind under similar conditions to the cognate site in the DQ3.2 URR.

Although these two polymorphic sites have different target nucleotide sequences, it is possible that the proteins which interact with these sites are related. Gel retardation studies showed that the DNA-protein complex in the upstream DQ3.1 URR was competitively inhibited by X box oligonucleotides, and vice versa. This relationship is also similar to findings in the HLA-DRA promoter, in which upstream ("Z box") elements and X box elements are thought to bind related regulatory proteins (42).

Promoter strength was evaluated for two of the allelic URR that differ at these two polymorphic sites, using CAT as a reporter gene for transient transfection assays in human B cell lines. Consistent 3-5-fold differences in CAT activity were found; URR constructions designed to separate the X box from upstream elements demonstrated that both regions contribute to these allelic differences. Patterns of protein binding and hierarchy of CAT activity between the alleles were observed to be similar with other B-LCLs.

These findings of allelic promoter variation can be related to previous studies indicating interlocus variation among HLA class II promoter elements. Regulatory regions of class II  $\alpha$  genes for the DR, DQ, and DP loci have been previously compared for ability to bind selected nuclear proteins: The X box binding protein, RF-X, does not recognize all X boxes equally well and shows a gradient in X box binding affinity with the profile DRA>DPA>DQA (18). A different protein, NF-S, binds downstream of the X box motif and shows binding characteristics that are the reverse: DQA>DPA>DRA (19). Differences in promoter sequences with varying affinities for X box binding proteins thus appear to account for both inter- and intralocus variation. In our studies, com-

petition and direct binding analyses indicate that one or both of the nucleotides TG at positions -179 and -180 are required for the high affinity X box binding site in DQ3.2. Two different X box binding proteins have previously been described, termed NF-X and RF-X, which have been found in a variety of cell types and both contact the center of the X box motif and bases upstream (18, 43, 44). Whether these proteins are related to the specific allelic interaction described here is not yet known.

In studies of the DQ2 class II promoter, Sakurai and Strominger (13) demonstrated that a 95-bp fragment from -223 to -132 was sufficient to confer cell type-specific enhancer activity on a heterologous promoter. Our study shows that even small nucleotide variation between alleles in this region significantly alters the activity of the URR. Shuttling of a 62-bp promoter fragment from position -223 to -158 containing the conserved X and W box sequences from the DQ3.2 URR into the DQ3.1 URR fragment gave intermediate levels of transcriptional activity. However, since truncation of the promoter sequences to -223 in the DQ3.1 construct gave promoter activity that was 40% lower than the construct containing 549 nucleotides of URR, it is clear that additional upstream sequences contribute to transcriptional regulation as well. These results correlate with the findings that polymorphic sites differ in protein interactions not only within the X box, but also at a more upstream polymorphic site.

Transcriptional regulation of the DQB1 gene is undoubtedly a composite of multiple protein-DNA interactions, involving both polymorphic and nonpolymorphic sites within the URR. The principle nonpolymorphic sites include (a) Potential binding sites for regulatory proteins AP4 and AP1, from -40 to -27, which are nonpolymorphic between alleles; these are analogous to similar sites found in the SV40 late enhancer element, as well as in several cellular promoters, where the juxtaposition of these elements contribute to inducible transcriptional control (45, 46). (b) Two nonpolymorphic CCAAT motifs, one of which is called the "Y box" in class II promoters and contains the CCAAT motif in reverse orientation, and an additional polymorphic CCAAT site which occurs only in the DQ3.1 and DQ1.1 URR at position -232. A large variety of CCAAT-binding proteins are recognized as ubiquitous transcriptional activators in many systems (20, 47-49). (c) The nonpolymorphic potential regulatory site at -270 to -265, which has sequence homology to the PU box, a purine-rich sequence recognized by a macrophage and B cell-specific transcription factor PU.1 (38); and (d) a nonpolymorphic ATF site at -227 to -222, which may be important for regulation by cAMP (50-52).

It is likely that these nonpolymorphic sites contribute to regulatory properties common to all DQB1 genes. In contrast, among the polymorphic sites, two were found to lead to functional allele-specific consequences while the others, as yet, have not. Sites with both sequence polymorphism and marked variation in DNA-protein interaction were identified at -179 and at -279. The location of the -179 site in the critical class II consensus X box region, and the large differ-

ences seen in protein binding between the DQ3.1 and DQ3.2 URR at this site suggest that this polymorphism may account for the transcriptional variation seen between the truncated URR constructions shown in Fig. 6 B, and thus account for some of the overall differences between these two alleles. In HLA-DQ, as in other class II genes, the X and Y boxes are separated by a conserved number of nucleotides, which however frequently varies in sequence (27, 53). Both the X and Y box have been shown to be essential for transcription and both have been implicated in IFN- $\gamma$  inducibility, as has the W box area (11, 12, 54). A complex of regulatory proteins appear to coordinately recognize the X box and nearby sequences; the precise role of each element in this interaction remains unknown.

The nature of the protein binding to the polymorphic site at -279 is similarly unknown. A possibility is that the polymorphic site at -279 creates in the DQ3.1 URR an additional positive transcriptional recognition sequence. This is a candidate site which may contribute to the increased transcriptional activity of the DQ3.1 URR relative to DQ3.2, specifically to the portion of the observed activity which is due to elements upstream from the previously recognized consensus regions.

Two additional polymorphic sites are of potential interest. A polymorphic region of extensive dyad symmetry occurs just upstream of the W box. With the A at position -214 forming the center, this symmetry stretches seven nucleotides in each direction in the DQ3.1, DQ1, and DQ2 alleles. However, a substituted G at position -211 disrupts the complementarity in the DQ3.2, DQ3.3, and DQ4 alleles. A similar dyad symmetry element has been described in the URR of the HLA-DRA and IFN- $\beta$  genes (25, 55). In the IFN- $\beta$  URR, this site was implicated in negative regulation, whereas disruption of the dyad symmetry element in the DRA URR results in a decrease in promoter activity in B cells and an increase in other cell types. In the DQB1 URR, DNA-protein interactions were found which corresponded to this dyad symmetry element, but they were similar for the alleles tested, in spite of the nucleotide polymorphism. Similarly, the DQ3.1, DQ2, DQ1.2, and DQ1.1 alleles, but not the DQ3.2, DQ3.3, and DQ4 alleles, also have recognition sequences at the 3' end of the X box related to sites commonly associated with a class of developmentally regulated DNA-binding proteins (56).

MHC class II genes are primarily expressed on cells of the lymphoid lineage; i.e., on mature B cells, activated T cells, macrophages/monocytes, and other antigen-presenting cells (57, 58). Additionally, cell surface expression of class II molecules can be induced in many cell types of nonlymphoid origin by exposure to cytokines such as IFN- $\gamma$ , leading to the acquisition of antigen-presenting properties (59). Other lymphokines and cellular factors have been shown to stimulate (60-62) or repress class II gene expression (63, 64). Our data, which indicate the presence of significant polymorphisms within the transcriptional regulatory regions of different HLA-DQ alleles, point to the need to relate such allelic differences to the control of DQ expression in B cells, other lymphoid

cells, and tissues capable of expressing inducible HLA class II molecules. In particular, since HLA class II expression in specific organs is associated with triggering of autoimmunity, transcriptional variation due to allele-specific promoter

polymorphism, which affects inducibility or tissue specificity, may play a role in susceptibility to HLA-associated autoimmune diseases.

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