The Human Major Histocompatibility Complex Class II HLA-DRB1 and HLA-DQA1 Genes Are Separated by a CTCF-binding Enhancer-blocking Element*

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The human major histocompatibility complex class II (MHC-II) region encodes a cluster of polymorphic heterodimeric glycoproteins HLA-DR, -DQ, and -DP that functions in antigen presentation. Separated by ~44 kb of DNA, the HLA-DRB1 and HLA-DQA1 encode MHC-II proteins that function in separate MHC-II heterodimers and are diametrically transcribed. A region of high acetylation located in the intergenic sequences between HLA-DRB1 and HLA-DQA1 was discovered and termed XL9. The peak of acetylation coincided with sequences that bound the insulator protein CCCTC-binding factor as determined by chromatin immunoprecipitations and in vitro DNA binding studies. XL9 was also found to be associated with the nuclear matrix. The activity of the XL9 region was examined and found to be a potent enhancer-blocking element. These results suggest that the XL9 region may have evolved to separate the transriptional units of the HLA-DR and HLA-DQ genes.

The human major histocompatibility complex (MHC)⁸ encodes a dense cluster of genes that spans almost 4 megabases of human chromosome 6 (1). The MHC is divided into the following three regions: class I, II, and III. Many of the genes encoded in these subregions function in adaptive and innate immunity. The MHC-II locus consists of a group of 7–10 highly polymorphic genes that code for the α and β chains of the classical MHC-II heterodimeric molecules (reviewed in Ref. 2). In total, three MHC class II isotypes HLA-DR, HLA-DQ, and HLA-DP can be formed. MHC class II molecules function by presenting antigenic peptides to CD4⁺ T lymphocytes and are critical to the development of the T cell repertoire and the proliferation and differentiation of antigen-specific CD4⁺ T cells during adaptive immune responses (3, 4). This process is aided by two MHC class II-associated molecules, HLA-DM and DO, which are also α/β heterodimers with sequence and structural homology to MHC-II proteins (5, 6).

MHC-II genes are regulated in a cell type-specific manner and are constitutively expressed in B lymphocytes, macrophages, dendritic cells, and thymic epithelia (reviewed in Refs. 2 and 7). However, in response to interferon-γ, most other cell types can be induced to express MHC class II genes. Regulation of MHC class II genes is coordinated by a group of conserved sequence elements termed the W/Z, X1, X2, and Y boxes, located at a promoter proximal region upstream of all MHC-II genes. The factors RFX, CREB, and NF-Y bind cooperatively to the X1-X2-Y box sequences, respectively, but are not sufficient for gene expression (8–10). Expression requires the class II transactivator, CIITA, a non-DNA binding co-activator (11). CIITA mediates interactions between the DNA-bound X-Y box factors, chromatin remodeling machinery, additional co-activators, and various components of the general transcription machinery to allow for MHC class II transcription (2, 7).

Despite the requirement of the W-X-Y box conserved sequences for MHC class II gene expression, a number of observations suggest that other regulatory components may exist (12, 13). These include the discovery of a potential locus control region located upstream of the MHC class II gene HLA-DR (14). A recent search of the MHC-II locus identified additional X-Y box sequences that were not associated with functional MHC class II genes and were termed X-like (XL) boxes (15). The analysis of these sequences by chromatin immunoprecipitation (ChIP) indicated that some of them were functional as determined by their ability to bind RFX and CIITA, to drive the expression of a reporter gene, and to be associated with chromatin modifications that were similar to active MHC-II gene proximal promoter regions. Together these observations suggested that other elements are likely to function in the expression of MHC-II genes.

Insulators and enhancer-blocking elements have been discovered in a number of different organisms (16–20). The archetypical example of these elements, the gypsy enhancer-blocking insulator found in Dro sophila (21), has been shown to block the action of enhancers when placed between the enhancer and promoter, as found at the yellow locus. In addition, this element is believed to localize to the nuclear matrix where it organizes chromatin into higher order looped structures. The loops serve to prevent the promoters of one gene from the action of enhancers of another gene either directly or by blocking enhancer-promoter interactions via associations with the nuclear matrix. Insulator elements in the chicken β-globin locus are associated with high levels of histone acetylation and are bound by the protein CCCTC-binding factor (CTCF) (22, 23). CTCF has been found to be critical for function of some enhancer-blocking elements in mammalian systems (17, 18, 24). For example, at the H19/insulin-like growth factor 2 (IGF2) locus CTCF controls expression of these genes at an enhancer-blocking element that is dependent on the imprinted mark of the DNA sequence.

An XL box element located between the HLA-DRB1 and HLA-DQA1 genes was identified that displayed high levels of histone acetylation but lacked CIITA and RFX binding. Because XL9 displayed high levels of histone acetylation and was located between two distinct MHC-II subregions, we hypothesized that this sequence may contain some of the properties of an insulator element. To analyze the region more rigorously, XL9 was divided into six subregions, with the original site covered in XL9c. Histone acetylation analysis of the region revealed a peak of...
activity that centers at subsite XL9d. Specific histone marks associated with the XL9 region were indicative of an open chromatin configuration. XL9d specifically bound by CTCF in vivo and in vitro, suggesting that the element may function as an enhancer-blocking element. Moreover, sequences encompassing XL9c and XL9d were found to interact with the nuclear matrix and, most importantly, could function as a potent enhancer-blocking element. Together, these data describe a novel element within the human MHC class II locus that may function to separate and/or restrict the transcription regulatory machinery of the HLA-DRB1 and HLA-DQA1 genes.

EXPERIMENTAL PROCEDURES

Cell Culture—Raji and HS-Sultan (American Type Tissue Collection—CRL1484) cells are Burkett’s lymphoma-derived cell lines that are wild type for MHC class II expression. Cells were grown at 37 °C at a density of 0.4 × 10^6 in RPMI supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 5% bovine calf serum (HyClone), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml i-glutamine (Invitrogen). A431 epithelial cells and HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 10% bovine calf serum plus the above supplements.

Antibodies—Antibodies specific to di-acetylated H3, tetra-acetylated H4, and CTFC were purchased from Upstate Biotechnology, Inc., Lake Placid, NY. Antibodies specific for histone H3 dimethyl K4, acetylation at histone H3 K9, K14, K18, K23, and K27 and histone H4 K5, K8, and K12 were also purchased from Upstate Biotechnology, Inc. Over the course of these experiments, several lots were used with similar results.

ChIP Assays—ChIP assays were performed as described previously in Beresford and co-workers (15, 25). Cells were cross-linked with formaldehyde for 10 min at room temperature. Cell lysates were harvested at histone H3 K9, K14, K18, K23, and K27 and histone H4 K5, K8, and K12 were also purchased from Upstate Biotechnology, Inc. Over the course of these experiments, several lots were used with similar results.

Experimental procedures for the in vitro nuclear matrix attachment region assay were performed as described (28). DNA-protein complexes were incubated with 0.1% DMS for 2 min at room temperature. Reactions were terminated by the addition of 1M sodium acetate, pH 7.0, 1 mM β-mercaptoethanol, 10 mg of RNA). Samples were ethanol-precipitated and resuspended in TE, 0.1% SDS, and 1 mg/ml proteinase K. The samples were then incubated at 55 °C for 1 h; phenol/chloroform was extracted and then ethanol-precipitated. DMS-modified DNA was cleaved by incubation with piperidine as described. Following the removal of piperidine through successive lyophilization and ethanol precipitation, cleaved products were resuspended in water and analyzed on a sequencing gel.

In Vivo Nuclear Matrix Attachment Region Assay—High salt nuclear matrices were prepared as described (28).

The indicated DNA fragments (747 and 650 bp) were PCR-amplified from genomic DNA with the following primers: XL-9 Fwd and 747-R, 5′-GGCCACAGCACAGCATTTAG; 650-F, 5′-GAAGGATCCAGCAGCATTTAG; 650-R, 5′-GACCAGCTGCTGAGCAG; L, 5′-CTTTCCCGAGTGAAGCCAAGAAC; J, 5′-CTTTCCCGAGTGAAGCCAAGAAC; and XL-9 Fwd, 5′-CTTTCCCGAGTGAAGCCAAGAAC. For each primer set the amount of double-stranded amplicon was quantified with the aid of SYBR Green dye. Primer sets were initially tested on genomic DNA and then examined by agarose gel electrophoresis to confirm amplicon size and uniqueness. The above primer sets each produced a single specific amplicon. For each real time PCR experiment, a standard curve was generated using 500, 100, 20, 4, and 0.8 ng of genomic DNA for comparison to the DNA isolated from each ChIP assay. To correct for any differences between chromatin samples, values obtained for each ChIP assay were adjusted to the amount of chromatin, as determined by real time PCR, used in each immunoprecipitation. All ChIP assays were performed at least three times from independent cultures.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from Raji cells as described (26). Complementary, single-stranded oligonucleotides of human 5′H54 (22) were purified, annealed, and 32P-end-labeled. All DNA binding reactions were carried out in binding buffer (20 mM HEPES, pH 7.9, 150 mM KCl, 5 mM MgCl2, 5% glycerol, 1 mM dithiothreitol, and 0.5% Triton X-100). Extracts were incubated with 20–40 fmol of labeled DNA probe in the presence of 400 ng of poly(dG–dC) competitor DNA per μg of extract for 30 min on ice. Antibody supershifts were carried out with anti-CTCF (or NFκB) antibody for another 2 h on ice. DNA protein complexes were resolved on 5% (69:1 acrylamide/bisacrylamide ratio) gels in a taurine-based gel buffer system at 150 V for 2 h at 4 °C as described (10). Dried gels were exposed to PhosphorImager cassettes.
rose gels and end-labeled with [32P]ATP and T4 polynucleotide kinase (New England Biolabs).

Nuclear matrix lysates from 1 × 10^7 cells were used for each assay and were incubated with the above probes in buffer (10 mM Tris, pH 7.4, 50 mM NaCl, 0.25% sucrose, 2 mM EDTA, 0.25 mg/ml bovine serum albumin, 150 μg/ml sonicated Escherichia coli DNA) for 2 h at room temperature as described (28). Bound and unbound fractions were initially separated by centrifugation at 10,000 × g for 60 s. The pellets were washed once with assay buffer and once with assay buffer minus carrier DNA and centrifuged to collect the matrices. Bound and unbound fractions were solubilized in 10 mM Tris, 1 mM EDTA, pH 8, 0.5% SDS, and 0.4 mg/ml proteinase K and digested overnight at 55 °C. The DNA was purified and resolved on a 1.2% agarose gel, which was then dried and exposed to a PhosphorImager screen.

In Vivo Matrix Assay—Nuclear matrix extracts from Raji cells were prepared essentially as described previously (28). Following nuclear solubilization in detergents, nuclei were digested with 100 μg/ml RNase-free DNase I (Roche Applied Science) for 3 h at room temperature or 37 °C. Matrices were collected by centrifugation for 5 min at 1,500 × g, washed twice with extraction buffer and resuspended in TE, 0.1% SDS, and 1 mg/ml protease K. Extracts were incubated at 55 °C overnight. The DNA associated with these preparations was purified. Quantitative PCR was carried out on the DNA using primers/probes described above for the chromatin immunoprecipitation assays and as indicated in the figure legends.

**DNA Constructions and Transient Transfection Assays**—The pGL3 pro plasmid (Promega, Inc.) containing the firefly reporter gene was used in transient transfection transcriptional reporter assays. PCR fragments of the chicken β-globin 5′HS4 region (5′HS4 Fwd GAGCTCACGGGGACAG; 5′HS4 Rev CTCACTGACTCCGTCC), XL9 region, and a fragment of DNA were inserted upstream of the minimal promoter. The primers used to create these latter fragments are described above. To test whether the XL9 region could influence the activity of the HLA-DQA regulatory region driven chloramphenicol acetyltransferase (CAT) reporter plasmid was used as a base vector in transient transfection assays (29). The above fragments were inserted upstream of the DQA W-X-Y box region as indicated. To assay for the ability of the XL9 region to function as an enhancer block, the pGL3-promoter vector containing SV40 promoter upstream of the luciferase gene was used as the base plasmid vector (Promega). pGL3–5′HS4-SV40 was generated by inserting the SV40 enhancer at the MluI site and placing 1.2 kb of the full-length 5′HS4 insulator (22) into the SacI site. The indicated test fragments were generated by PCR using primers engineered with the appropriate restriction site at each end. The amplicons were purified

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**FIGURE 1.** XL9 chromatin is acetylated. A, scaled diagram depicting the HLA-DRB1 to HLA-DQA1 subregion of the human MHC, with an enlarged section representing the XL9 region. **Shaded boxes** labeled a–f indicate the locations of the primer set amplicons used in this study. The black box labeled c represents the amplicon that contains the X-Y homologous sequence. B, ChIP analyses for RFX5 and CIITA were quantitated for each of the indicated primer sets by real time PCR as described under “Experimental Procedures.” Positive control ChIP assays for RFX5 and CIITA at the HLA-DRA promoter region are shown separately because of the scale. C, ChIP analyses for diacetyl histone H3 and tetra-acetyl histone H4 are shown with the indicated primer sets. The data represent the average of three independent ChIP assays and chromatin preparations. Standard error of the mean is shown.
and inserted into the Xhol or SmaI site of this plasmid, located between the SV40 enhancer and SV40 promoter.

In all assays, an expression vector constitutively expressing the Renilla luciferase (pRLTK) was co-transfected into cells along with the test vectors using firefly luciferase or CAT reporter vectors. As a negative control the vectors pRLTK and pGL3-pro were co-transfected into Raji cells. All transfections were carried out by electroporation into Raji cells as described previously (29). After a 48-h incubation, cell lysates were prepared according to the manufacturer’s instructions (dual-luciferase reporter assay system; Promega, Inc.), and luciferase activity for both firefly and Renilla was determined with a luminometer, as outlined in the manufacturer’s protocol. CAT activity for each sample was measured by ELISA. All values (firefly luciferase and CAT) were normalized to the activity of the co-transfected Renilla luciferase reporter. All assays were carried out at least three times, and the data are presented as the average with the standard error of the mean.

RESULTS

Analysis of the MHC Reveals a Region of Significant Histone Acetylation—XL9 was identified through a DNA sequence search for homologous X-Y box regions within the MHC-II locus (15). XL9 is located in the 43.8-kb intergenic region between the HLA-DRB1 and HLA-DQA1 genes (Fig. 1A). Because XL9 had homology to an X-box region, ChIP assays were performed to determine whether XL9 (primer set XL9c) was bound by RFX and CIITA and to determine whether there were specific modifications in this region that would provide additional insight into its function, antibodies against nine specific histone modifications were used. The histone H3 dimethyl K4 modification was observed. A peak of histone H3 and H4 acetylation was observed using primer set XL9d at the site located ~235 bp away from XL9c. The most 5′ site (XL9a) examined had low levels of H3 and modest levels of H4 acetylation, whereas the site examined 4 kb downstream of XL9 (XL9f) still retained high levels of acetylated H3 and H4 nucleosomes. Examination of these sequences for RXF5 and CIITA binding was negative (Fig. 1B).

XL9c and XL9d Are Associated with Histone Modifications Indicative of an Open Chromatin Structure—Specific histone modifications are thought to dictate the overall accessibility of a region (30). To determine whether there were specific modifications in this region that would provide additional insight into its function, antibodies against nine modifications associated with open chromatin structure were analyzed for their ability to immunoprecipitate XL9 chromatin (Fig. 2). Although XL9c and XL9d displayed high levels of acetylation at histone H3 K9, K18, and K27, XL9a displayed near background levels. All three sites displayed high levels of histone H3 K14 acetylation and histone H4 K5 and K8 acetylation. No histone H3 K23 or H4 K12 acetylation modifications were observed. The histone H3 dimethyl K4 modification was also observed at XL9c and XL9d but was substantially lower at XL9a. This modification is also an indication of accessible chromatin. Most of these modifications are often associated with the histone acetyltransferase activity of co-activators such as GCN5, CBP, p300, and P300 (31–35), suggesting a potential role for these factors at XL9.

XL9c/d Acts neither as an Enhancer nor as a Repressor—Because high levels of acetylation are typically associated with gene regulatory sequences, we hypothesized that this element may serve a regulatory function. As a regulatory element, XL9 could function to regulate either a novel gene within the region or the adjacent HLA genes HLA-DRB1 or HLA-DQA1. However, DNA searches against EST data bases using the University California, Santa Cruz, genome browser programs failed to identify a novel gene or transcripts in the region between HLA-DRB1 and HLA-DQA1, suggesting that the first possibility was unlikely.

To establish if XL9 could act as an enhancer for the MHC class II genes in the region, the 747 bp of DNA encompassed by the 5′ and 3′ primers of XL9c and XL9d, respectively, was cloned upstream of a minimal promoter driven luciferase reporter construct. This fragment was termed XL9c/d. As a negative control, a fragment of equal size corresponding to λ DNA and the 5′HS4 chicken β-globin insulator (22) were
FIGURE 3. The XL9 region does not function as an enhancer or as a repressor of transcription. A, chicken 5′HS4, XL9c/d, and 747 bp of λ DNA were cloned downstream of a minimal promoter (pGL3) and assayed for their ability to drive expression of a luciferase reporter gene. A fragment corresponding to the SV40 enhancer was used as a positive control, and a minimal promoter alone served as a baseline. B, XL9d and XL9c/d region fragments of either 153 or 747 bp, respectively, as well as size matching fragments of λ DNA, were cloned upstream of an HLA-DQA1 W-X-Y controlled CAT reporter gene. The indicated reporter constructs were transiently transfected into MHC-II-positive Raji cells and assayed for their ability to express the CAT reporter gene. The basal HLA-DQA1 promoter, lacking the W-X-Y region, provided the base line of expression for this assay system. The average of three independent experiments is shown with their respective standard errors.

also cloned into the same site of the reporter vector (Fig. 3A). An SV40 enhancer-containing reporter served as a positive control for these experiments. The constructs were transiently transfected into Raji cells, and the lysates were assayed for luciferase activity. Analysis of the data revealed that XL9c/d, the 5′HS4, λ DNA, and the empty vector were unable to drive expression of the reporter gene (Fig. 3A). The SV40 enhancer, however, provided high levels of expression of the reporter.

To determine whether the XL9 region could potentially function as a repressor, constructs were designed to measure its effects on the transcription of a reporter gene driven by the W-X-Y box regulatory region of the HLA-DQA1 gene (Fig. 3B). Fragments corresponding to XL9c/d (747 bp), XL9d (153 bp), or λ DNA (747 or 153 bp) were cloned upstream of the HLA-DQA1 W-X-Y sequences and transiently transfected into Raji cells. By comparison to the HLA-DQA1 upstream regulatory region alone, constructs containing any XL9 region DNA sequences showed no change in expression of the reporter gene (Fig. 3B). Constructs containing λ DNA also had no effect on expression of the reporter. These experiments demonstrated that despite the high levels of histone acetylation observed for the XL9 region, sequences corresponding to the peak of histone acetylation do not function as an enhancer or repressor of a transcription reporter gene.

CTCF Binds XL9d in Vivo and in Vitro—Some regions of high acetylation have been found to be associated with locus control regions, genomic insulators, and enhancer-blocking elements (as reviewed in Ref. 36). CTCF is one of a few well described vertebrate factors that have been associated with such elements (22, 37). If the XL9 region functioned as one of these elements, then it is formally possible that CTCF would be bound to XL9 DNA. To test the possibility that XL9 sequences were bound by CTCF, a ChIP assay was performed with antibodies against CTCF using chromatin prepared from Raji cells. The results showed that strong CTCF binding was coincident with the peak of acetylation observed at XL9d (Fig. 4A). No significant CTCF binding was observed at any of the other six sites tested, including the HLA-DRA X-box region. Additionally, HS Sultan B cells were also found to display CTCF binding activity to XL9d (Fig. 4B).

Because the ChIP assay has low resolution with respect to identifying a binding site and the fact that CTCF sites display poor homology (38), EMSAs were used to verify and to localize the binding region within the XL9d region. To begin these experiments, the 72-bp sequence encoding the 5′HS4 (chicken β-globin) CTCF-binding site was used as a probe in EMSAs with nuclear extracts obtained from Raji cells. DNA competitors spanning XL9c and XL9d were able to compete for factor binding and narrowed down the binding site activity to a 747-bp fragment (Fig. 5). The EMSA gel demonstrates that three bands are present, corresponding to factors binding to the 5′HS4 CTCF-binding site. EMSA competition assays (Fig. 5, A and B). Nonspecific DNA fragments were unable to compete for binding, demonstrating the specificity of the interaction. To delineate the site further, a series of DNA fragments was generated by PCR and used to compete for factor binding to the 5′HS4 site (Fig. 5A). In these DNA competition assays (Fig. 5, B and C), the 747-bp segment and fragments A, C, D–G, I, and J competed for binding, whereas fragments B, H, and K–N did not. Thus, the smallest region able to compete for the DNA binding activity of the 5′HS4 CTCF site was fragment J, which is 153 bp in length. To further demonstrate the specificity of this interaction and to show that the binding was dependent on CTCF, additional EMSAs were carried out (Fig. 5D). Using the 5′HS4 CTCF-binding site as a probe, nonspecific DNA corresponding to an NF-κB-binding site was not able to compete for binding activity of CTCF. Additionally,
antibodies to CTCF but not antibodies to RFX5 were able to supershift the complex (Fig. 5D, lanes 5 and 6, respectively). EMSAs carried out using the 153-bp PCR product corresponding to the CTCF-binding site within XL9d and nuclear extract from Raji cells demonstrated the formation of a single complex (Fig. 5E, lane 2). This interaction was shown to be specific by the reduction of protein-DNA complex formation when incubated with either unlabeled 153-bp fragment or the "cold" 5′HS4 oligonucleotide competitor DNAs (Fig. 5E, lanes 3 and 4). Non-specific competitor DNA, corresponding to an NF-κB site, did not have any effect on complex formation (Fig. 5E, lane 5). Antibody supershift analysis using anti-CTCF antibodies but not NF-κB p65 antibodies disrupted or altered the migration of the complex (Fig. 5E, lanes 6 and 7, respectively). Together, these data demonstrate that the 153-bp region of XL9d contained a CTCF-binding site.

To further define this potential CTCF-binding site, an in vitro methylation protection DNA footprinting assay was carried out over the 153-bp region of XL9d detected to bind CTCF by EMSA (Fig. 6). The results revealed a footprint of 70 bp that was seen on both strands only.
MHC-II Enhancer-blocking Element

FIGURE 6. In vitro footprint identifies a putative CTCF-binding site. Dimethyl sulfate protection footprinting was performed using XL9d sequences represented in fragment J (Fig. 4), which is 153 bp in length. DNA labeled on the upper or lower strands was incubated with or without Raji cell nuclear extract (NE) as indicated. The vertical lines indicate the footprint region. The 153-bp sequence is shown adjacent to the footprint with the horizontal lines representing the footprint sequence.

FIGURE 7. The XL9 region functions as an enhancer-blocking element. DNA sequences encoding the 5’HS4 chicken β-globin CTCF-binding site, a 747-bp fragment of λ DNA, XL9c/d region (A), and the SV40 transcriptional enhancer were placed upstream of a minimal promoter-luciferase reporter gene in the configurations shown (B and C). The reporter constructs were transiently cotransfected into Raji cells (B) and into A431, HeLa, or HS Sultan B cells (C) with a Renilla luciferase normalizing control vector, and assayed for luciferase activity. The data from three independent transfections are shown as the average of the mean and plotted with their standard error.

in the presence of nuclear extract. The footprint was only partially contained within the EMSA fragment H and therefore explains why this fragment could not compete for binding. CTCF sites are complex because of the fact that CTCF has 11 zinc fingers, and it only uses a portion of these to bind to its targets, providing a high degree of flexibility in its binding sites (38, 39).

The XL9 Functions as an Enhancer-blocking Element—One property of some insulator regions is the ability to block the action of an enhancer at a downstream promoter (17–19, 22, 40, 41). This is also a property of CTCF (18). To determine whether the XL9 region encoded such properties, an enhancer-blocking assay was performed by using a transient transfection system. In each assay, a test fragment was inserted between an SV40 enhancer and a luciferase reporter gene. Because these test sequences were inserted into circular plasmids, the known 5’HS4 chicken β-globin insulator was placed upstream of the SV40 enhancer to block enhancement from the other direction. As shown, placement of the 5’HS4 insulator upstream of the SV40 enhancer had minimal effect on its function (Fig. 7). XL9c/d was tested and found to ablate completely the function of the SV40 enhancer, although an equal size fragment of λ DNA had no effect (Fig. 7). The XL9c/d element marked a 1,500-fold reduction in activity from the SV40 enhancer. The 5’HS4 β-globin fragment placed between the SV40 enhancer and the promoter displayed similar activity to XL9c/d. The enhancer blocking activity of XL9c/d was tested in other cell lines, including HS-Sultan B cells, A431 epithelial cells, and HeLa cells. Although not as robust in Raji cells, the results show that XL9c/d has the same enhancer blocking activity as the 5’HS4 insulator element in each cell line tested. These data demonstrate that the XL9 region contains potent enhancer-blocking activity that is independent of cell types.

XL9 Interacts with Nuclear Matrix—Insulators and CTCF sites have been associated with regions that are attached to the nuclear matrix (28, 42). Given that XL9 was found to bind CTCF and function as an enhancer block element, the ability of this region to interact with the nuclear matrix both in vivo and in vitro was examined. In vitro γ-32P-radiolabeled probes corresponding to the 747-bp region spanning XL9c/d (Fig. 8A, Probe A), which bound CTCF and acted as an enhancer-blocking element as described above, and a 650-bp sequence (Probe B) situated ~2 kb away from XL9d were generated and tested. Probe B, which is close in proximity to XL9c, did not bind CTCF and had reduced (~50%) levels of histone acetylation. A third probe constructed of λ DNA was included as an additional negative control. Nuclear matrix containing extracts (28) were prepared from Raji cells and incubated with each of the probes. Samples were then fractionated into input and nuclear matrix-bound and -unbound and resolved by agarose gel electrophoresis and autoradiography (Fig. 8B). Of the three probes, only the XL9c/d probe was substantially bound by the nuclear matrix preparation. Probes B and λ were only found in the unbound fraction.

To determine whether XL9 region sequences interacted with the nuclear matrix in vivo, an in vivo nuclear matrix attachment region assay was performed. In this assay, nuclei obtained from Raji cells were washed with a mild detergent and then digested with DNase I. After digestion, the nuclear matrix was prepared, and the co-purifying DNA was precipitated and quantitated by real time PCR. Primer sets used in
the ChIP assays were employed to determine the relative enrichment of XL9 sequences within the nuclear matrix preparation. Of these primer sets, only XL9c and XL9d displayed substantial increases over the background (Fig. 8C). Primer sets XL9a, -b, and -e were at background levels. This enrichment suggests that sequences encoded within the amplicons of XL9c and XL9d interact with the nuclear matrix.

DISCUSSION

In this report we identified an intergenic region located between two HLA isotype genes that exhibits high histone acetylation and features of an open chromatin architecture, binds the insulator factor CTCF in vitro and in vivo, and encodes potent enhancer blocking activity. EMSA, DNA footprinting, and ChIP assays carried out on the XL9 region narrowed the CTCF-binding portion of this region to ~70 bp adjacent to the original site examined. Like other elements that bind CTCF, the XL9 region also functions as an enhancer-blocking element and is capable of interacting with the nuclear matrix (28, 42).

BLAST searches for XL9 revealed that it is conserved in primates, dogs, and bovine. It is interesting that the XL9 sequence could not be found in the mouse. The failure to identify the sequence in the mouse could be due to the fact that CTCF sites are extremely divergent, allowing a CTCF functional sequence to be present although not detectable by in silico means.

CTCF-binding regions function as locus control regions, insulators, and/or enhancer-blocking elements. Many of these functions are associated with high levels of local histone acetylation (43). For example, insula-

tors, which prevent the encroachment of heterochromatin, are suggested to function by recruiting histone acetyltransferases, which keep key histone lysine residues in local nucleosomes in the acetylated state thereby preventing lysine methylation modifications associated with heterochromatin formation and maintenance (43). Similarly, a locus control region would use histone acetylation to sustain accessibility to tissue or developmental specific transcription factors under situations in which the neighboring genes needed to be activated.

Because CTCF binding is CpG methylation-sensitive, CTCF-binding sites can regulate the expression of imprinted genes (24, 44, 45). This is the case for the H19/IGF2 imprinted region, where CTCF binding is determined by the methylation status and parental origin of the allele. Microdeletion of this region leads to aberrant regulation of the IGF2 gene and correlates with Beckwith-Wiedemann syndrome and Wilms tumor formation (46–48). Currently, there is no evidence that genomic imprinting regulates the binding of CTCF to XL9d.

CTCF regions may also associate with the nuclear matrix and bind proteins such as nucleophosmin, a major component of the nuclear matrix (28). Although the full function of the nuclear matrix has yet to be realized (42), it is suggested that the nuclear matrix provides an ordered structure to the nucleus. Although not exclusive of other mechanisms, it has been proposed that the association of CTCF sites with the nuclear matrix allows such regions to form large chromatin loops and function as enhancer-blocking elements. By analogy to the *Drosophila* system in which gypsy, suppressor of hairy wing, and modifier are tethered to the nuclear periphery, genes on one side of a chromatin loop organized by these factors cannot be regulated by elements on the opposite side of these binding sites (42, 49). Thus, CTCF sites may function to help organize chromatin into large regulatory loops that limit the effects of enhancers. This may be the case for the HLA-DRB1 and DQA1 genes, which are oppositely transcribed.

Combining the data from the results presented, we propose that the XL9 region functions as a regulatory region for the HLA-DR and HLA-DQ regions. Such a regulatory region could manifest itself in several forms. The first of these possibilities is that this site functions as a tissue-specific or cell type-specific LCR. In this scenario, the XL9 would function much like the chicken β-globin LCR/enhancers described by Felsenfeld and co-workers (22), maintaining an open chromatin state for the MHC class II subregion to allow expression during distinct stages of B cell development and differentiation. A region located upstream of the HLA-DRA gene was proposed as a locus control region (14). This was based largely on the fact that the region exhibited high histone acetylation when compared with the surrounding DNA and produced low levels of transcripts. The increased histone acetylation in that region was because of the fact that RXF and CITIA assembled at this site (14, 15). If this is an HLA-DRA locus control region as proposed, then it is possible that it marks the 5′ boundary for the HLA-DR region with the XL9 region marking the 3′ boundary.

XL9 could also function to separate the regulation of the HLA-DR genes from the HLA-DQ genes. Although for the most part HLA-DR and DQ gene expression is co-regulated, the enhancer blocking activity of the XL9 region may have revealed itself in the rare instances of non-Hodgkin lymphomas where discordant expression of HLA-DR and HLA-DQ isoforms has been observed (50–52). These tumor cells may have captured an unusual regulatory feature associated with this gene system. An additional possibility is that the XL9 region serves as an architectural structure for the chromatin organizing these genes. If this is the case, we speculate that the XL9 region is not functionally unique within the MHC and that there are likely to be other such organizing structures in the intergenic regions of the other MHC-II genes.
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