X Box-Like Sequences in the MHC Class II Region Maintain Regulatory Function

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**References**

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Sequences homologous to the canonical MHC class II (MHC-II) gene X box regulatory elements were identified within the HLA-DR subregion of the human MHC and termed X box-like (XL) sequences. Several XL box sequences were found to bind the MHC class II-specific transcription factors regulatory factor X and CIITA and were transcriptionally active. The histone code associated with the XL boxes and that of the HLA-DRA X box was determined. Using CIITA-positive and -negative B cell lines, CIITA-specific histone modifications were identified and found to be consistent among the active XL boxes. Although a remarkable similarity was observed for most modifications, differences in magnitude between the HLA-DRA promoter for modifications associated with the assembly of the general transcription factors, such as histone H3 lysine 9 acetylation and H3 lysine 4 trimethyl-ethylation, distinguished the very active HLA-DRA promoter from the XL box regions. In response to IFN-γ, XL box-containing histones displayed increased acetylation, coincident with CIITA expression and that observed in B cells, suggesting that the end point mechanisms of chromatin remodeling for cell type-specific MHC-II expression were similar. Lastly, an interaction between one XL box and the HLA-DRA promoter was observed in a chromatin-looping assay. Therefore, these data provide evidence that certain XL box sequences contribute to a global increase in chromatin accessibility of the HLA-DR region in B lymphocytes and in response to IFN-γ and supports the involvement of these XL sequences in the regulation of MHC-II genes. The Journal of Immunology, 2005, 175: 1030–1040.

The MHC encoded on the short arm of chromosome 6 at 6p21.31 is a gene dense region of the genome. The class II portion spans ~666,847 bp of DNA and encodes 33 genes and 26 pseudogenes (1). Of these, six to nine genes encode the α- and β-chains of the classical MHC class II (MHC-II) molecules, HLA-DR, -DQ, and -DP. The number of MHC-II genes differs between haplotypes and is dependent on the number of HLA-DRB (β-chain encoding) genes present in the region. MHC-II molecules function by presenting antigenic peptides to CD4+ T lymphocytes. This presentation is critical to the development of the T cell repertoire, as well as to proliferation and differentiation of Ag-specific CD4 T cells during adaptive immune responses. The selection and presentation of Ags by MHC-II molecules is aided by two other MHC-encoded accessory molecules, DM and DO, which are also αβ heterodimers with sequence and structural homology to MHC-II proteins.

MHC-II genes and their accessory molecules are coregulated in a cell type-specific manner. B lymphocytes, macrophages, dendritic cells, and thymic epithelia constitutively express MHC-II genes (reviewed in Refs. 2–4). Most other cell types can be induced to express MHC-II genes in response to IFN-γ. A compact, conserved upstream regulatory region composed of the W/Z, X1, X2, and Y box regulatory elements controls MHC-II gene expression. These upstream sequences are found in the S′-promoter proximal sequences upstream of all MHC-II genes and are conserved from humans to fish (5). Although the X2 and Y boxes are bound by factors (CREB and NF-Y, respectively) associated with the regulation of other genes, the X1 box is bound by regulatory factor X (RFX), a factor that is unique to the regulation of this system. RFX is a heterotrimer composed of the subunits RFX-B (RFX-ANK), RFX5, and RFXAP (6–9). Taken together, RFX, CREB, and NF-Y bind cooperatively to the X-Y box region but are not sufficient for gene expression. Expression requires CIITA, a non-DNA binding coactivator (10). CIITA mediates interactions between the DNA-bound X-Y box factors, chromatin-remodeling machinery, additional coactivators, and various components of the general transcription machinery (reviewed in Refs. 2 and 3). CIITA binding to the X-Y box region of the HLA-DRA gene results in substantial increases in acetylation of histones H3 and H4 at the promoter region (11), suggesting that the binding of CIITA influences the local nucleoprotein architecture.

The N- and C-terminal ends of histone molecules project from the core nucleosome particle and are targets of posttranslational modification. Over the last few years, the relationship between some of these histone modifications and gene expression has been realized. A “histone code” hypothesis that predicts that the sum total of these modifications will determine whether a gene is expressed or repressed has been proposed (12). Although the data supporting this hypothesis are strong, our understanding of the code is limited. The HLA-DRA gene has been characterized for some of these modifications, but the full picture has not yet emerged (11, 13–15). CIITA is responsible for the recruitment of histone acetyltransferases (16, 17) and the chromatin-remodeling Brahma-related gene 1 complex (18), but the breadth of these modifications that are made during CIITA recruitment has not been defined. It is also not known whether there are differences in the...
modifications between constitutive-expressing MHC-II genes and the IFN-γ-induced state.

Because the MHC has undergone numerous expansions and contractions over evolutionary time, it is possible that additional X-Y box regions might be present. If so, such regions could bind RFX and CIITA and contribute to changes in the accessibility of chromatin and the regulation of genes within the MHC-II locus. In the present study, we report the identification and analysis of additional X box elements within the MHC-II region that are not directly associated with a functional MHC-II gene. We have termed these sequences X box-like (XL) sequences. These data demonstrate that the CIITA-responsive XL sequences were active and, through their modified chromatin structure, bound RFX and CIITA and were found to have CIITA-immunoprecipitation (ChIP) analyses found that some of these XL sequences were active and chromatin modifications at both MHC-II promoter proximal X-Y box regions might be present. If so, such regions could bind RFX and CIITA and were found to have CIITA-dependent histone modifications. A detailed analysis of the histone modifications at both MHC-II promoter proximal X-Y box regions (HLA-DRA X box region) and at XL boxes was undertaken to determine the code for MHC-II transcription and to ascertain whether there were differences that might predict or coincide with X-Y regions associated with transcription. Cells that constitutively express and those that are induced by IFN-γ to express MHC-II were examined. Indeed, six specific modifications that were dependent on the recruitment of CIITA were identified. Although the magnitude of the modifications observed was distinct, many of the modifications were shared between the HLA-DRA X box region and the XL boxes, suggesting a conserved mechanism of action. More pronounced magnitude differences were observed between the sites in IFN-γ-induced cells. To examine the potential role of the XL sequences, a DNA looping assay was used to determine possible contributions of these sequences to the transcription of the HLA-DRA gene. Indeed, the site upstream of HLA-DRA was found to form a stable loop with the HLA-DRA promoter proximal X-Y box sequences. These data demonstrate that the CIITA-responsive XL boxes are active and, through their modified chromatin structure, maintain an open and accessible locus. The sequences also have the potential to directly impact the transcription of a MHC-II gene, thereby altering our view of the mechanism of transcriptional regulation of human MHC-II genes.

Materials and Methods

Cell lines

Raji, a Burkitt’s lymphoma-derived cell line (19), was treated as wild type and is positive for both CIITA and MHC-II gene expression (10, 20). The human B cell line R2J 2.5 was derived from Raji via mutagenesis and is mutant for CIITA and negative for MHC-II gene expression (10, 20). Both cell types were grown in RPMI 1640 medium supplemented with 5% FBS (HyClone), 5% bovine calf serum (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml l-glutamine (Invitrogen Life Technologies). The epithelial carcinoma A-431 cell line is MHC-II and CIITA negative (21). Expression of CIITA and MHC-II genes is seen after induction with rIFN-γ (500 U/ml; PeproTech). A431 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml l-glutamine.

Computer analysis

To generate a list of potential XL sequences, the HLA-DRA, -DRB, -DQA, and -DQB gene X boxes were used to Basic Alignment Search Tool search the MHC-II region of the National Center for Biotechnology Information database. Homologous sequences were annotated and analyzed for the presence of a Y box 16–20 bp downstream of the X1 box site. XL were then aligned using the multiple sequence alignment program Clustal X. Percent similarity was determined using the Clustal X matrix algorithm.

Antibodies

All Abs specific to acetylated or methylated lysine residues on H3 and H4, as well as the general di-acetylated H3 and tetra-acetylated H4, were purchased from Upstate Biotechnology. The general H3 Ab was purchased from Abcam, and anti-GFP and anti-RNA-polymerase II Abs were purchased from Santa Cruz Biotechnology. The rabbit Abs against RFX-5 and CIITA were described previously (22, 23).

ChIP assays

ChIP assays were performed as previously described in Beresford et al. (11). Cells were harvested and treated with the cross-linking agent formaldehyde for 10 min. The chromatin was isolated and sonicated to produce an average DNA size of 600 bp. One-tenth of the final sonication volume (500 μl) was used for each immunoprecipitation. Immunoprecipitations were performed at 4°C overnight using 5 μg of the indicated Ab. Chromatin-Ab complexes were immobilized to protein-A-Sepharose beads (60 μl) and washed as described by Beresford et al. (11). The cross-links were reversed, and the DNA was purified using phenol/chloroform extraction followed by ethanol precipitation. The DNA was resuspended in water and analyzed using real-time PCR. The primers used in real-time PCR are shown in Table I. All real-time PCR were performed in duplicate, and the amount of DNA amplified was quantitated by comparison to a standard curve for that set of primers (Table I). Each ChIP experiment was performed at least three times with independent cell cultures and chromatin preparations. For specific transcription factor (RFX5 and CIITA) ChIP assays, the data were normalized to input chromatin and presented as the mean with error bars denoting the SEM. For ChIP assays investigating histone modifications, the data were normalized against the results from a ChIP performed with a general histone H3 Ab. This was performed so that the relative changes in modification could be compared between multiple loci because it was not known if a priori one region contained more nucleosomes than another. RNA polymerase II ChIP assays were performed as previously described by Fujita and Wade (24).

Table I. Real-time PCR primer sets

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Real-time PCR
A quantitative PCR analysis was performed on all samples using the iCycler, with the optical assembly unit, from Bio-Rad. In each of these ChIP assays, the incorporation of SYBR Green into the double-stranded amplified product was measured using the iCycler. Each primer set was first tested on genomic DNA to verify that only one band was produced upon amplification of genomic DNA. Because XL-7 and XL-8 were most similar to the HLA-DRB genes, primer sets were chosen to prevent cross-reactivity between the loci. Only the correct primer pairs were capable of producing a product, demonstrating that the amplicons were specific and as designed. To compare between distinct primers, standard curves of genomic DNA consisting of five points were performed for each primer set (500, 100, 20, 5, and 1 ng/reaction) during each experiment. The quantity of product for each primer set was then determined for each ChIP sample by comparison to its standard curve. PCR primer set efficiency varied by <10% between all primer sets used. All real-time PCR experiments were performed in duplicate. To correct for any differences between chromatin samples, the obtained values for each ChIP assay were adjusted to the amount of genomic DNA included in each immunoprecipitation reaction.

Constructs and transient transfections
The functional activity of each XL box was tested using the reporter construct pDRCAT consisting of a DRA minimal promoter (sequences –66 to +3) upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (25). Double-stranded oligonucleotides spanning the W-X-Y element of each XL box were then synthesized and cloned into the HindIII and XbaI sites upstream of the minimal promoter in the pDRCAT vector. Coding strand sequences were as follows: XL-1, 5′-CTAGAGGACACGAGCACTCATGCTACAGTGCAGAGATGCTGATTGGTTCTCCAT-3′; XL-2, 5′-AGCTTAGAGGACCTTCATACGACACCTCCTGACCACGGAGCTGAGATGCTGATTGGTTCTCCAT-3′; XL-3, 5′-CTAGAGGACACGAGCACTCATGCTACAGTGCAGAGATGCTGATTGGTTCTCCAT-3′; XL-4, 5′-CTAGAGGACACGAGCACTCATGCTACAGTGCAGAGATGCTGATTGGTTCTCCAT-3′; XL-5, 5′-CTAGAGGACACGAGCACTCATGCTACAGTGCAGAGATGCTGATTGGTTCTCCAT-3′; XL-6, 5′-CTAGAGGACACGAGCACTCATGCTACAGTGCAGAGATGCTGATTGGTTCTCCAT-3′; XL-7, 5′-CTAGAGGACACGAGCACTCATGCTACAGTGCAGAGATGCTGATTGGTTCTCCAT-3′; and XL-8, 5′-CTAGAGGACACGAGCACTCATGCTACAGTGCAGAGATGCTGATTGGTTCTCCAT-3′. Transfections in Raji and R2J2.2.5 were performed by electroporation using 1 × 10^7 cells/transfection as described previously (26). After electroporation, cells were then cultured for 48 h, harvested, and analyzed by ELISA for CAT protein, according to the manufacturers directions (Roche Diagnostic Systems).

Looping assay
The looping assay was performed as previously described in Refs. 27 and 28, with some modifications. A total of 1 × 10^7 cells was first resuspended in 50 ml of DMEM supplemented with 10% FBS, formaldehyde was added to a final concentration of 1%, and incubated at room temperature for 10 min. Cross-linking was terminated by the addition of glycine to a final concentration of 1%, and the nuclei were incubated for an additional 10 min at 37°C. T4 DNA ligase was added, and the samples were incubated for 4 h at 16°C, followed by 30 min at room temperature. After incubation, proteinase K (10 μg/ml) was added to the ligation mixtures, and the samples were incubated overnight at 65°C to denature the samples and reverse the cross-links. The DNA was then extracted with phenol/chloroform and ethanol precipitated. The precipitated DNA was then used for PCR (primers shown in Table I). All PCR were analyzed on a 1.5% agarose gel and stained with ethidium bromide to detect the presence or absence of the expected PCR product. The accuracy of the PCR amplicon was verified by restriction digest as indicated.

Results
XL within the human MHC
As a result of numerous expansions and contractions of the MHC locus over evolutionary time we hypothesized that in addition to the protein coding regions, this region of the human genome could also be enriched for regulatory regions, such as the X-Y regulatory motif of class II genes. Such X-Y elements could still be functional and contribute to the transcriptional activity of this region. To determine whether such X-Y sequences exist, a computer analysis of the entire MHC locus (~3.2 Mb) from the published human genome sequence (1) was performed using the sequences of the HLA-DRA, HLA-DRB, HLA-DQA, and HLA-DQB X box elements as search tools. Within the entire MHC, 115 X1-X2 sites were identified with varying degrees of similarity. When sequences downstream of the putative X box (18–35 bp) were examined for the presence of a Y box, 32 sites remained in the MHC-II and extended class II region while 13 were within the rest of the MHC (data not shown), suggesting that the class II region was enriched for these sequences. These putative sites were termed XL because they were not directly associated with a functional gene. Within the class II region, each of the eight X box-related sites was given a numerical name beginning upstream of the DRA gene and extending centromERICALLY through the MHC-II region. Because of the high density of sites within the DR region (XL-1–8), this region was chosen for detailed analysis (Fig. 1). When aligned, the sequences were most homologous at the X1 box region (68% identity), with the homology decreasing at X2 (50%) and the Y box (51%). Further analyses showed that some of the XL boxes (XL-7 and XL-8) were positioned upstream of ancient duplications bearing homology to the first exon of the HLA-DRB gene (29) and extending several kilobases downstream of exon 1. XL-4, located 2.3 kb upstream of the HLA-DRA gene, was identified previously as a X-Y box region and a potential locus control element (14).

Whereas XL-1, -2, and -3 were located further upstream of the HLA-DRA gene, was identified previously (26). After electroporation, cells were then cultured for 48 h, harvested, and analyzed by ELISA for CAT protein, according to the manufacturers directions (Roche Diagnostic Systems).

Looping assay
The looping assay was performed as previously described in Refs. 27 and 28, with some modifications. A total of 1 × 10^7 cells was first resuspended in 50 ml of DMEM supplemented with 10% FBS, formaldehyde was added to a final concentration of 1%, and incubated at room temperature for 10 min. Cross-linking was terminated by the addition of glycine to a final concentration of 0.125 M. The cells were lysed using ice-cold lysis buffer containing protease inhibitors. The nuclei were collected by centrifugation and washed with 1 ml of buffer B. Protease inhibitors were added to a final concentration of 1%, and the nuclei were incubated for an additional 10 min at 37°C to sequester the SDS. The cross-linked DNA was digested overnight with HindIII. The restriction enzyme was then heat inactivated by incubation at 65°C for 20 min. All samples were diluted 1/40 with 1× ligase buffer (NEB) containing 1% Triton X-100 and incubated for 10 min at 37°C. T4 DNA ligase was added, and the samples were incubated for 4 h at 16°C, followed by 30 min at room temperature. After incubation, proteinase K (10 μg/ml) was added to the ligation mixtures, and the samples were incubated overnight at 65°C to deproteinize the samples and reverse the cross-links. The DNA was then extracted with phenol/chloroform and ethanol precipitated. The precipitated DNA was then used for PCR (primers shown in Table I). All PCR were analyzed on a 1.5% agarose gel and stained with ethidium bromide to detect the presence or absence of the expected PCR product. The accuracy of the PCR amplicon was verified by restriction digest as indicated.

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FIGURE 1. XL sequences reside in the HLA-DR locus. XL boxes 1 through 8 are aligned and compared with the HLA-DRA, -DRB1, and -DRB3 X-Y box regions. The dotted line between the X2 and Y boxes denotes the average spacing of 13 nucleotides between these sites. The position of the XL boxes (vertical lines) is shown relative to the HLA-DR region genes. Arrows denote the orientation of the XL box with respect to the transcription of the HLA-DRA gene. Gray boxes indicate the 5′-side of HLA-DRA and -DRB genes. The dotted line between the X2 and Y boxes highlights the positions of the promoter proximal X-Y box regions.
HLA-DRA gene, they and XL-5 and -6 were not associated with an identifiable gene fragment. No sequences were identified upstream of the DRB genes at a similar position with respect to XL-4 and HLA-DRA.

**RFX and CIITA occupy some but not all XL boxes**

To determine whether the XL sites were functional, ChIP analyses using Abs to RFX and CIITA were performed for each of the XL boxes using chromatin isolated from both Raji and RJ2.2.5 cells (Fig. 2). Raji cells are a Burkitt’s lymphoma-derived B cell line that expresses CIITA and MHC-II genes (19). RJ2.2.5 is a CIITA-deficient variant derived from Raji cells (10, 20) and is MHC-II negative. RJ2.2.5 cells serve as a matched control for the effects of CIITA in this system (11). Because of the homology between the various X-Y box regions, oligonucleotide primers were selected for their ability to only amplify the indicated XL box region DNA with their partner primer and not any other of the X box region primers. XL-4, -7, and -8 were found to strongly bind RFX5, which is the DNA binding component of the RFX complex. This binding was similar in cells lacking CIITA, demonstrating that CIITA does not affect the assembly of RFX on XL box DNA in these cells. XL-7 and -8 were found to bind CIITA strongly. When the CIITA binding data are plotted relative to the background observed at each site in RJ2.2.5 cells, XL-4 emerges as an additional site that binds CIITA. This observation is consistent with the finding that RFX also binds to XL-4. Indeed, as mentioned above, XL-4, which lies 2.3 kb upstream of the HLA-DRA X box, was previously found to bind RFX and CIITA (14).

For comparison, a similar ChIP analysis was performed for the HLA-DRA and HLA-DRB1 gene promoter proximal X box regions (termed DRA-X and DRB1-X, respectively), which were shown previously to bind these factors in vivo (11, 13). DRA-X binds approximately two and three times the amount of RFX5 and CIITA, respectively, than the DRB1-X box region (Fig. 2). The levels of DNA coprecipitated for DRB1-X by RFX5 ChIP were 150% greater than that of the XL boxes, whereas the levels of CIITA ChIP for DRB1-X were three times greater than XL-7, the highest of the XL boxes. Although this may appear to be a distinguishing feature, it should be noted that the levels of RFX and CIITA binding at other MHC-II promoter proximal X box regions, such as HLA-DQA, is within the range of XL-7 and XL-8 (data not shown). ChIP analysis of the remaining XL regions in the MHC-II locus did not identify any others that bound either RFX or CIITA (data not shown). Thus, although a large number of putative X-Y box sites can be identified by sequence homology searches, only a small subset of these sequences bind RFX and CIITA.

**RFX and CIITA-binding XL boxes can drive the expression of a reporter gene**

The above data suggest that the XL boxes that bind RFX and CIITA could be functionally active. To determine whether this was the case in a simplified system, XL-4, -6, -7, and -8 were tested directly for their ability to replace the HLA-DRA X-Y box region in a CAT reporter transient transfection assay system (30). The putative W-X-Y box sequences of XL-4, -7, and -8 were cloned 66 bp upstream of the HLA-DRA X box, was previously found to bind RFX and CIITA (14).

For comparison, a similar ChIP analysis was performed for the HLA-DRA and HLA-DRB1 gene promoter proximal X box regions (termed DRA-X and DRB1-X, respectively), which were shown previously to bind these factors in vivo (11, 13). DRA-X binds approximately two and three times the amount of RFX5 and CIITA, respectively, than the DRB1-X box region (Fig. 2). The levels of DNA coprecipitated for DRB1-X by RFX5 ChIP were 150% greater than that of the XL boxes, whereas the levels of CIITA ChIP for DRB1-X were three times greater than XL-7, the highest of the XL boxes. Although this may appear to be a distinguishing feature, it should be noted that the levels of RFX and CIITA binding at other MHC-II promoter proximal X box regions, such as HLA-DQA, is within the range of XL-7 and XL-8 (data not shown). ChIP analysis of the remaining XL regions in the MHC-II locus did not identify any others that bound either RFX or CIITA (data not shown). Thus, although a large number of putative X-Y box sites can be identified by sequence homology searches, only a small subset of these sequences bind RFX and CIITA.

**FIGURE 2.** Some XL boxes bind RFX and CIITA. ChIP assays performed using antisera to RFX (A) and CIITA (B) on chromatin prepared from Raji (wild-type (Wt)) and R2.2.5 (CIITA-/-) cells showed specific interactions at XL-4, -7, and -8. Also included were similar ChIP analysis for HLA-DRA and HLA-DRB1; however, due to scale differences, they are plotted separately. All ChIP assays were quantitated by real-time PCR. The values were normalized to input chromatin and to values obtained for the irrelevant locus GAPDH. The average of at least three separate experiments is shown with the SEM. C. The ratio of CIITA binding observed in Raji vs R2.2.5 cells was plotted for each of the indicated XL boxes.
One functional consequence of RFX and CIITA binding is the recruitment of histone acetyltransferases and subsequent acetylation of local histones (11). To determine whether this was the case for the XL boxes, ChIP assays using Abs against di-acetylated histone H3, tetra-acetylated histone H4, and unmodified histone H3 were conducted (Fig. 4). To compensate for the number of nucleosomes in a particular region between cell lines, the data were normalized against the levels of total histone H3 at each site. The results showed that XL-4, -7, and -8 displayed considerable histone H3 and H4 acetylation levels in Raji cells. These levels were reduced by 2- to 5-fold in the CIITA-deficient cell line RJ2.2.5, suggesting that increases in histone acetylation at these sites was due to the presence of CIITA or active transcription in the region. These results are similar to that observed previously for HLA-DRA (11) and are consistent with the data shown in Fig. 5.

CIITA directs specific histone modifications

Although the above activity assays and survey of bulk histone H3 and H4 acetylation marks at the XL boxes suggests that these regions are more accessible to transcription factors, they do not allow differentiation between chromatin marks that might have a direct functional consequence on transcription nor allow the prediction of which factors might induce these marks. Therefore, to better understand the differences between sites that showed RFX/CIITA binding (XL-4, -7, and -8) and those that did not (XL-1), the promoter proximal X-box region of the HLA-DRA gene (referred to as DRA-X) and XL-1, -4, -7, and -8 were assayed by ChIP for specific modifications of their H3 and H4 histones (Fig. 5). ChIP assays were conducted using Abs specific to histone H3 acetylation at K9, K14, K18, K23, and K27 and the di-acetylated form (Fig. 5, A and B). The prevalence of histones bearing H3 dimethyl K4 and trimethyl K4 modifications was also examined. The data were normalized to bulk histone H3, which was determined by ChIP for each site in both Raji and RJ2.2.5. With the exception of XL-1, all sites showed enhanced levels of H3 K9 acetylation in Raji cells as compared with RJ2.2.5, with DRA-X displaying ~2-fold higher levels. Interestingly, acetylation of histone H3 K14 was observed at all sites tested in both CIITA+ Raji and CIITA− RJ2.2.5 cells, with the values in Raji being approximately twice those in RJ2.2.5. Histone H3 K18 acetylation was observed only at sites that bind CIITA (DRA-X, XL-4, -7, and -8) in Raji cells and was clearly absent in RJ2.2.5 cells, indicating that this specific mark is dependent on CIITA recruitment. Histone H3 K27 acetylation was observed at DRA-X, XL-4, and XL-7 and was absent or near absent at XL-8 and XL-1, respectively. This mark was also CIITA dependent. Its absence at the XL-8 site may reflect some threshold effect as XL-8 showed the lowest levels of CIITA binding when compared with RJ2.2.5 cells (Fig. 2C). DRA-X showed the greatest overall level of histone H3 di-acetylation, a value consistent with each of the modifications analyzed. A greater than 2-fold difference in CIITA-dependent histone modification was noted for DRA-X, XL-4, -7, and -8. This is consistent with the fact that in RJ2.2.5 cells the gene specific transcription factors are assembled (11, 31) and poised for activation.

For histone H4, Abs to acetylated K5, K8, and K16 and the tetra-acetylated form were also used. Analysis of histone H4-specific modifications showed strong K8 acetylation at DRA-X, XL-4, -7, and -8 but not XL-1 (Fig. 5, C and D). K8 acetylation at DRA-X, XL-4, -7, and -8 was reduced substantially in RJ2.2.5 cells, whereas K8 acetylation at XL-1 remained at background levels, indicating that this modification is mediated by the recruitment of CIITA. These results are in agreement with those that we previously reported for the DRA-X region (11).

In contrast to the above CIITA-dependent marks, DRA-X, XL-4, -7, and -8 were found to contain histones bearing the H3 dimethyl K4 mark. This modification is indicative of accessible chromatin, a result that is consistent with the assembly of RFX at these sites. Interestingly, the levels of this modification in RJ2.2.5 cells was 30–40% less than that observed in Raji cells, suggesting that the assembly of CIITA and other recruited factors can lead to the enhancement of this modification. XL-1 displayed background levels of this modification that were unchanged between
Raji and RJ2.2.5 and may represent the basal level of this modification in the DR region.

Trimethylation of histone H3 at lysine 4 is restricted

Histone H3 K4 trimethylation is one of the few histone modifications that is clearly associated with transcriptionally active genes (32, 33). ChIP analysis for this mark was performed as above (Fig. 5). The results showed a robust CIITA-dependent increase in H3 K4 trimethylation at the DRA-X box region and modest levels at XL-7. The lack of K4 trimethylation at the other sites, suggests that functionally these sites are distinct.

To determine whether the H3 trimethyl K4 modification is limited to the promoter region or can be found downstream of the HLA-DRA gene, ChIP assays were performed at sites located at 1-kb intervals downstream of DRA-X. The results showed that the H3 trimethyl K4 mark is maintained within the first kilobase of DNA from the promoter region but is substantially decreased at sites located further than 2 kb downstream of the transcription start site (Fig. 6). Thus, at least for HLA-DRA, K4 trimethylation represents a modification that is associated with transcription initiation and the promoter region.

Histone H3 K36 dimethylation is associated with the HLA-DRA gene

Because both robust H3 K9 acetylation and K4 trimethylation were found at the DRA-X but not at the XL boxes, another transcription-associated histone modification was analyzed: histone H3 K36 dimethylation. Histone H3 K36 dimethylation is primarily localized to the transcribed regions of genes and is created by the histone methyltransferase Suvar39 enhancer of trithorax (SET2), which is recruited by RNA polymerase II during transcription elongation (32, 33). To determine a possible role of a SET2-like factor in this system, ChIP analysis was performed in 1-kb increments downstream of the HLA-DRA promoter using Abs to H3 dimethyl K36 in both Raji and RJ2.2.5 cells (Fig. 6). The results showed substantial differences between Raji and RJ2.2.5 along the length of the gene. This is in sharp contrast to the H3 trimethyl K4 modification, which was localized to the promoter proximal sequences. Interestingly, H3 dimethyl K36 modifications were observed at the DRA-X site; however, these modifications were not dependent on CIITA. These results are consistent with the notion that H3 dimethyl K36 marks the transcribed regions of MHC-II genes and implicate a role for SET2 or a SET2-like enzyme in MHC-II-specific transcription complexes. Additionally, the finding of a CIITA-independent dimethyl K36 modification at the promoter suggests that SET2 or a SET2-like factor is recruited to the promoter in the absence of CIITA.

Analysis of IFN-γ-induced histone modifications

The B cell lymphoblastoid cells used in the above experiments represent a cell type with a high steady-state level of MHC-II expression. In non-MHC-II-expressing cells, the occupancy of the HLA-DRA promoter by RFX, CREB, and NF-Y, as determined by in vivo footprinting, was not robust (25). This could be due to a small number of cells having stably occupied X-Y boxes or reflect a transient association of the factors with these sequences. Upon IFN-γ stimulation, CIITA is expressed and interacts with the X-Y box factors, resulting in a clear/stable footprint. This is in contrast to the RJ2.2.5 cell line, which has a clear footprint, although MHC-II genes are not expressed (31). One possibility for these differences could reflect the local chromatin environment and histone modifications that preexist in each of these cell types. The analysis of histone modifications at the XL boxes in non-MHC-II-expressing cells will also provide a ground state for the local chromatin structure/modifications. Therefore, a time course of IFN-γ stimulation was performed in A431 cells, an epithelial line that induces MHC-II genes in response to IFN-γ. ChIP analysis was conducted with chromatin from IFN-γ-treated and untreated...
HLA-DRB1 genes as determined by ChIP, it is within the range of RFX and CIITA binding were less than that of the functionally bind both RFX and CIITA. Although the levels of the data collected demonstrate that some of the XL boxes can recruit RNA polymerase II.

FIGURE 6. Histone modifications associated with transcription initiation and elongation are observed in a CIITA-dependent fashion. H3 K4 trimethylation is typically associated with active gene promoters, whereas H3 K36 dimethylation is associated with nucleosomes during transcription elongation. Real-time PCR coupled with ChIP was performed using Abs to these modifications at the DRA-X box region and at ~1-kb intervals downstream of that site to determine the extent of these modifications. As in Figs. 4 and 5, chromatin was prepared from Raji (CIITA wild-type (Wt)) and RJ2.2.5 (CIITA−/−) cells, and the results were normalized to unmodified histone H3 at each site assayed.

cells. As above, DRA-X, XL-1, XL-4, and XL-7 were chosen. For DRA-X, increased histone H3 acetylation at K9, K18, and K27, as well as the overall acetylation of H3 (diacetylation), was observed over the 48-h time course (Fig. 7). Dimethyl and trimethyl K4 also increased over the time course for DRA-X, indicating the chromatin becoming more accessible and being transcribed. In contrast to the B cell-derived data, XL-4 showed only slight increases in histone H3 acetylation over the time course. XL-7 displayed significant increases in K14 and K18 acetylation. No increases were observed for XL-1 in response to IFN-γ. Interestingly, with the exception of XL-1, which is upstream of the DR subregion, H3 dimethyl K4 increased at all sites (suggesting that the HLA-DR subregion is becoming globally accessible in response to CIITA binding and poised for transcription). In contrast, trimethyl K4 increases were minimal, achieving a 2-fold increase at XL-7 and no increase at XL-4 or XL-1. This suggests that transcription at XL-7 may be very low.

ChIP analysis of XL-1, -4, and -7 and DRA-X with H4 Abs to acetylated K5, K8, K12, and K16 provided similar results by comparison (Fig. 7). Small but possibly meaningful increases in acetylation at K8 and K12 were seen at DRA-X over the course of 48 h. XL-4 and XL-7 displayed a 2-fold increase in H4 K5 and K8 acetylation. In contrast to the levels of histone modifications in CIITA-positive B cells, the weak increases in histone modification at the XL boxes following IFN-γ treatment suggests that these regions respond in a cell type-specific manner.

XL sequences can recruit RNA polymerase II

The data collected demonstrate that some of the XL boxes can functionally bind both RFX and CIITA. Although the levels of RFX and CIITA binding were less than that of the HLA-DR and HLA-DRB1 genes as determined by ChIP, it is within the range of other MHC-II genes, such as the HLA-DQA gene (data not shown). Moreover, some of the XL boxes display increases in histone modifications that are predictive of accessible chromatin structures. Thus, the possibility exists that the XL boxes that bind CIITA could recruit RNA polymerase II. The ability of the XL box to recruit RNA polymerase II was determined using a modified ChIP assay for enhanced detection (Fig. 8A). The results found that in addition to DRA, RNA polymerase II could be found at XL-4 and XL-7 but not at XL-1. The recruitment of RNA polymerase II was dependent on the presence of CIITA. Taken together, these data suggest that XL boxes that assemble RFX and CIITA are functional and may either contribute to the global accessibility of the MHC or transcribe sterile/transcripts.

XL-4 directly interacts with the X box region of the HLA-DRA gene

To explore the possibility that the XL boxes may work in conjunction with a promoter proximal X box, the ability of XL-4 to directly interact with the X box region of HLA-DRA was examined by a chromatin-looping assay (Fig. 9A; Refs. 27 and 28). In this assay, cross-linked chromatin isolated from cells was digested to completion with HindIII, which cuts multiple times between the sites of interest. The digested DNA is ligated, and the cross-links are reversed. If a loop is formed, the divergent PCR primers can amplify the newly created sequence. As shown, a loop was specifically formed between sequences associated with XL-4 and DRA-X (Fig. 9B). The loop was dependent on cross-linking of the sample and on the inclusion of DNA ligase in the assay. Restriction analysis of the PCR product for existing sites (EcoRI and AvalII) and absent sites (BamHI) confirms that the appropriate amplicon is being generated (Fig. 9C). No loops between XL-1 or XL-5 and DRA-X were observed under similar conditions (data not shown). In addition, formation of the loop between XL-4 and DRA-X is dependent on the presence of RFX5, as evident by comparison of the loop assay performed in both Raji (RFX5+) and SJO (RFX5-deficient) cell lines. These data demonstrate that XL-4 interacts directly with the HLA-DRA transcription regulatory complex and implies that the XL-4 element aids in the expression/regulation of the HLA-DRA gene (Fig. 9D). The result also implies a function for the other XL boxes in contributing to the regulation of MHC-II genes through similar mechanisms.

Discussion

We have identified functionally active regulatory sequences in the human MHC-II HLA-DR region that are not directly associated with a functional MHC-II gene. Although a relatively large number of these sequences can be found in the human MHC, few were found to be functional, as defined by their transcription factor binding and ability to drive transient expression of a reporter gene. One consequence of RFX and CIITA binding is an increase in the local chromatin structure, a process that allows additional factors and the general transcription machinery to assemble. Remarkably, sequences that bound RFX and CIITA were found to be associated with histone modifications that were similar to those identified in the highly active HLA-DRA regulatory region. Additionally, at least one of the XL box elements can interact directly with a MHC-II proximal promoter region, suggesting that these sequences indeed function to regulate MHC-II genes. This interaction was dependent on the binding of the X1 box factor RFX5. The data implicate coordination between upstream and proximal promoter elements and a more complex series of the events leading to transcription initiation of MHC-II genes than was suggested previously by the analysis of only the proximal W-X-Y regulatory elements.
From the data presented here, we suggest that the events that govern MHC-II expression begin with the assembly of the RFX-CREB-NF-Y protein DNA complex at both MHC-II promoter proximal W-X-Y box regions and functional XL boxes. This assembly is then associated with changes in the local chromatin structure (H3 K14 acetylation and H3 K4 dimethylation) because cells lacking any one of the RFX subunits have decreased overall histone acetylation at the promoter proximal X boxes (11). The RFX-CREB-NF-Y DNA complex also serves as a scaffold for CIITA binding, which further stabilizes the nucleoprotein complex (34). In addition to the multiple protein-protein interactions that CIITA makes with DNA-bound factors, CIITA binding is clearly required for further chromatin modifications, which promote open/accessible DNA and are favorable to the recruitment of coactivators and the general transcription machinery. This change in chromatin structure likely allows interactions to occur between the proximal WXY elements and those at functional XL boxes. We speculate that this interaction then increases stability of the preinitiation complexes and allows for efficient transcription initiation.

The histone code hypothesis posits that posttranslational modifications of histone molecules controls the accessibility of the local chromatin to transcription factors by either altering DNA/nucleosome contacts or by serving as recognition targets for chromatin-remodeling proteins or the components of the basal transcription machinery (12). Thus, once the code is known, potential coactivators/repressors that either create the modifications or recognize them can be predicted to participate in the regulation of a gene. The availability of multiple tested reagents has allowed this characterization. To begin to understand the relationship between chromatin and CIITA, the histone H3 and H4 code associated with CIITA binding was determined for the HLA-DRA proximal promoter region and the XL boxes. The histone H4 code for acetylation of HLA-DRA was previously found to be limited to acetylation of K8 in B cells. H4 K8 acetylation can be catalyzed by CREB-binding protein (CBP)/p300 and P300/CBP-associated factor (35), coactivators shown to interact with CIITA and are necessary for its maximal activity (16, 17, 36). In IFN-γ-stimulated fibroblasts, additional time-dependent increases observed for histone H4 acetylation at DRA-X included K12 and possibly K5. However, XL-4 and XL-7 showed only slight increases for all H4 modifications, which were at best 2-fold above the control and background. Although 2-fold differences could be considered important, the fact that the levels were much higher in B cells suggests that there is a cell type-specific variation in the extent of the modifications and that these H4 modifications may not be as important as others. Alternatively, the extent of the modifications may correlate directly to the level of transcripts from that region, which at least for HLA-DRA, is substantially lower.

**FIGURE 7.** IFN-γ induction of MHC-II genes displays similarities and differences from the B cell-expressed H3 and H4 modifications. As above, real-time ChIP assays using histone H3 and H4 modification-specific Abs were performed on DRA-X, XL-4, XL-7, and XL-1 and were analyzed in a MHC-II-inducible system using A431 epithelial cells. Chromatin for these experiments were isolated from cells treated with IFN-γ for 0, 16, 24, and 48 h. All values obtained were normalized to input chromatin and plotted as fold increase over the 0 time point. The average of at least three independent experiments is shown with their SEM.
The CIITA-dependent code for histone H3 modifications at HLA-DRA was more complex and included acetylation modifications at K9, 18, and K27 and trimethylation of K4. Acetylation of K9 can be catalyzed by the coactivator GCN5 and is ultimately thought to aid in the recruitment of the TATA-binding protein to promoters (37, 38). In addition, this modification is associated with gene activation and was shown here to be most robust at DRA-X. Acetylation at K18 and K27 were constitutively present in CIITA-positive B cells at DRA-X, XL4, and XL-7. K18 acetylation can be mediated by CBP and p300 (35). This modification can impact the recruitment of the coactivator-associated arginine methyltransferase (39), which is known to modify histone H3 R17, a modification associated with transcriptional activation. Thus, the possibility that CARM1 is recruited to MHC-II genes and the XL boxes exists. At this time, it is not known what histone acetyltransferase acetylates K27. One intriguing possibility is that CIITA, which was found to have histone acetyltransferase activity (40), may be directly responsible for some of the observed modifications. Other modifications, including acetylation of K14 and dimethylation of K4, were present in RJ2.2.5 cells, indicating that the X-Y box factors—RFX, CREB and NF-Y—may recruit their own histone modification enzyme complexes to MHC-II promoters. K14 can be acetylated by CBP, p300, and p300/CBP-associated factor (35) and may be recruited to MHC-II X box regions through interactions with phospho-CREB, which interacts directly with CBP/p300. The enhancement of these latter two modifications in CIITA-positive cells B cells may be due to the stabilization of the DNA-bound factors provided by CIITA association (25).

Analysis of other transcription modifications such as trimethylation of K4 led to the observation that this modification was restricted to a region of 1 kb that spanned the HLA-DRA promoter. Because K4 trimethylation has been found to be associated with actively transcribing genes and can be catalyzed by the recruitment of the trithorax complex homologue mixed lineage leukemia (37) or SET7/9 (41), this implicates a SET-like factor in the regulation of MHC-II genes. The elongation-specific modification dimethyl K36 on H3, which is catalyzed by the histone methyltransferase SET2, was also observed over the coding region of the HLA-DRA gene. SET2, which associates with the C-terminal domain of RNA polymerase II (42), places this modification on the tails of H3 as it passes over the transcribed gene. At the HLA-DRA locus, it was found that this mark was CIITA dependent and in the region downstream of the promoter. The placement of this modification on a

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**FIGURE 8.** RNA polymerase II associates with RFX/CIITA-bound X box regions. Real-time ChIP was performed with anti-RNA polymerase II using chromatin from RJ2.2.5 (CIITA−/−) and Raji (wild-type (Wt)) cells. The values obtained for each locus were plotted relative to the amount of RNA polymerase II determined for DRA-X in Raji cells. Three independent experiments were performed. The data were normalized to the level of input DNA and averaged in each set of experiments, and the mean of these data are shown with their SE. 

**FIGURE 9.** XL-4 interacts directly with the HLA-DRA promoter region. A, A schematic of the DNA looping assay and the region are shown. B, Stained agarose gel of PCR products from the looping assay. Minus and plus signs indicate whether the samples were derived from cross-linked chromatin and/or processed with HindIII and DNA ligase as described in the Materials and Methods. Lane M, The DNA marker. Two concentrations of DNA were used in each as indicated by the inclined plane. Experiments were conducted in Raji (RFX5+/−) and SJO (deficient for RFX-5) cell lines. C, Restriction digest analysis of the PCR product created in the looping assay. The PCR amplicon obtained from the looping assay was validated by restriction digest and visualized by agarose gel electrophoresis: lane 1, undigested; lane 2, EcoRI; lane 3, AvaII; and lane 4, BamHI (absent site). D, Schematic model of the loop between XL-4 and DRA-X is shown.
transcribed gene would then mark this gene as having been transcribed and potentially allow easier passage of the next RNA polymerase complex through the gene. However, the appearance of this modification over the HLA-DRA X box region in both CIITA-positive and -negative cells suggests that a second mechanism may be present.

As with the DRA-X, the XL boxes contained both CIITA-dependent and independent histone modifications. For the most part, these modifications were similar in nature to the DRA-X box but differed in magnitude, implying that the initial function of these regions is similar. The largest difference was observed for histone H3 K9 acetylation. This may be related to the increased levels of CIITA associated with the DRA-X region as seen by ChIP assay. An example of how some of these modifications were restricted to unique sites includes the trimethylation of lysine 4 of histone H3. This modification was specifically associated with the DRA-X and moderately with XL-7 but not the other sequences. As stated above, this modification is associated with transcribing genes, suggesting that XL-7 may be transcribed to some extent. Attempts to quantify transcripts downstream of XL-7 and XL-8 were not successful due to the cross-reactivity of the PCR primers with each other and HLA-DRB genes. This is likely a consequence of the numerous duplications that formed these regions.

Treatment of cells with IFN-γ produced a similar set of CIITA-dependent modifications at the HLA-DRA promoter as observed in the constitutively expressing B cell line. However, for the most part, these modifications were small in magnitude if observed at all at XL-4 and XL-7. The one exception to this was H3 dimethyl K4. This modification showed a similar increase to DRA-X for both XL-4 and XL-7. One interpretation of this is that the XL boxes serve to enhance the accessibility of the region via the recruitment of MHC-II transcription factors RFX, CREB, and NF-Y. Although the first time point in the IFN-γ time course presented here was at 16 h, a shorter term time course was performed (0.5, 1, 2, 4, 8, and 16 h; data not shown). The results showed that only H3 K18 acetylation appeared at 4 h, whereas all the other modifications were only observed after 8 h, a time point associated with strong CIITA binding. Thus, with the exception of K18 acetylation, which occurs first, the other histone modifications assayed appear to occur in a similar time frame. The early appearance of H3 K18 acetylation could be attributed to the direct association of CBP/p300 with CIITA because both CBP and p300 are able to generate this modification in vitro (35). Although an increase in K14 was observed at XL-7 in response to IFN-γ, a similar increase at K14 at DRA-X was not observed, highlighting slight differences between the XL-7 and DRA-X.

The presence of functional XL elements in the DR region suggests several possibilities for their role. The least interesting of these possibilities is that these elements are just the remnants of defunct genes and have no role. Although this remains a possibility, it is the most unlikely as the sequences still bind RFX/CIITA, whereas other XL box sequences with similar homology do not. Moreover, the ability of XL-4 to interact with the HLA-DRA promoter would suggest a direct involvement for these sites. A second possibility is that the XL boxes serve to enhance local gene expression by making the chromatin accessible to transcription factors and RNA polymerase. The fact that following IFN-γ induction, dimethyl K4 is increased within the region suggests that the regions are in fact contributing to the opening of the local chromatin structure. This would imply that additional X-Y box elements or other sequences that aid in increasing chromatin accessibility are critical to MHC-II expression. Such a mechanism would provide an evolutionary advantage to maintain these sequences in the genome.

A third possibility is that such sequences serve as distal enhancer-like elements for MHC-II genes. This possibility is supported from the chromatin-looping experiments presented here as well as several additional data sets reported previously. The first of these reports (43) appeared >10 years ago, where deletion of an upstream-inverted X-Y element in an Eox transgene produced a phenotype in which cell-specific expression profiles were altered. A more recent report (14) identified the orthologous Eox X-Y sequence upstream of HLA-DRA and suggested that it functioned as a locus control region. In the present study, we identified this sequence as XL-4. This could suggest that all MHC-II genes have an upstream element. Although our search did not identify other XL boxes in close (2–4 kb) proximity to the other MHC-II genes, a recent report (44) identified one upstream of the HLA-DRB1 gene that bound RFX and CIITA. This report did not pick up the any of the XL boxes that we found, pointing out the limitations associated with DNA search parameters. Nonetheless, together these results suggest the possibility that these sequences will be found at other MHC-II loci.

With the advent of ChIP and the development of chromatin-looping assays, distal regulatory regions and locus control regions have been shown to interact with transcriptionally active promoter regions, such as the IL-5, Rad50, IL-13, and IL-4 locus (27). Additional experimentation in the MHC with respect to chromatin loops and in other gene systems sets the stage for understanding the mechanistic action of distant enhancers through chromatin loops. Therefore, the concept that the XL boxes found here may form chromatin-mediated loops with other MHC-II gene regulatory sequences remains an open and viable possibility.

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Disclosures

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