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The S Box of Major Histocompatibility Complex Class II Promoters Is a Key Determinant for Recruitment of the Transcriptional Co-activator CIITA*

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Annick Muhlethaler-Mottet‡§, Michal Krawczyk‡, Krzysztof Masternak‡¶, Charalambos Spilianakis‖, Androniki Kretsovali‖, Joseph Papamatheakis‖, and Walter Reith‡ ‡‡**

From the ‡University of Geneva Medical School, Department of Pathology and Immunology, Centre Médical Universitaire, 1 rue Michel-Servet, CH-1211, Geneva, Switzerland and the ‖Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology, Vasilika Vouton, Heraklion 71110, Crete

Tightly regulated expression of major histocompatibility complex (MHC) class II genes is critical for the immune system. A conserved regulatory module consisting of four cis-acting elements, the W, X, X2 and Y boxes, controls transcription of MHC class II genes. The X, X2, and Y boxes are bound, respectively, by RFX, CREB, and NF-Y to form a MHC class II-specific enhanceosome complex. The latter constitutes a landing pad for recruitment of the transcriptional co-activator CIITA. In contrast to the well defined roles of the X, X2, and Y boxes, the role of the W region has remained controversial. *In vitro* binding studies have suggested that it might contain a second RFX-binding site. We demonstrate here by means of promoter pull-down assays that the most conserved subsequence within the W region, called the S box, is a critical determinant for tethering of CIITA to the enhanceosome complex. Binding of CIITA to the enhanceosome requires both integrity of the S box and a remarkably stringent spacing between the S and X boxes. Even a 1–2-base pair change in the native S-X distance is detrimental for CIITA recruitment and promoter function. In contrast to current models, binding of RFX to a putative duplicated binding site in the W box is thus not required for either CIITA recruitment or promoter activity. This paves the way for the identification of novel factors mediating the contribution of the S box to the activation of MHC class II promoters.

Major histocompatibility complex (MHC)[1] class II molecules are heterodimeric cell surface glycoproteins that present peptides to the antigen receptor (T cell receptor) of $CD4^+$ T cells. Engagement of MHCII-peptide complexes by the T cell receptor is essential for selection of the mature $CD4^+$ T cell repertoire during T cell development in the thymus and for the initiation, propagation, and regulation of adaptive immune responses by mature T cells in the periphery. Because of these key functions in the adaptive immune response, MHCII expression is tightly controlled in a cell type-specific and inducible manner (for reviews see Refs. 1–6). MHCII expression is generally restricted to thymic epithelial cells and professional antigen-presenting cells, namely B cells, dendritic cells, and macrophages. In most other cell types MHCII expression can be induced by interferon γ.

MHCII molecules are encoded by a family of genes that are co-regulated at the level of transcription via a conserved MHCII-specific regulatory module that is situated within the first 150–300 base pairs upstream of the transcription initiation site of each MHCII gene (4, 6). This promoter-proximal regulatory module consists of four cis-acting sequences called the W, X, X2 and Y boxes (see Fig. 1). These four boxes are present in a tightly conserved arrangement with respect to orientation and spacing, and they function together as a single composite regulatory unit (1, 2). A similar sequence arrangement has been conserved in the promoter regions of the *Ii*, *HLA-DM*, and *HLA-DO* genes (7–11), which code for proteins implicated in the intracellular traffic and peptide loading of MHCII molecules (12, 13). The promoters of MHC class I genes also contain a related regulatory module (14).

Dissection of the molecular mechanisms controlling the MHCII regulatory module has been greatly facilitated by the identification of genes that are mutated in bare lymphocyte syndrome, a hereditary immunodeficiency disease resulting from the absence of MHCII expression (2, 15, 16). Bare lymphocyte syndrome can result from mutations in four different regulatory genes, all of which code for transcription factors that are essential and highly specific for the expression of MHCII genes (2, 15, 16). These four factors are the transcriptional co-activator CIITA and the three subunits, RFX5, RFXANK (also called RFXB), and RFXAP, of the heterotrimeric DNA-binding complex called RFX (17–21).

Genetic and biochemical studies addressing the mode of action of CIITA and the RFX complex has led to a detailed understanding of the role of the X, X2, and Y elements of the MHCII regulatory module (see Fig. 1*A*) (1–3). RFX binds in a highly cooperative manner with two other factors, CREB (22) and NF-Y (23), to their respective X, X2, and Y box target sites (24–28). This generates a higher order MHCII enhanceosome complex that serves as a landing pad for CIITA (29, 30). CIITA is recruited to the enhanceosome via multiple protein-protein interactions with RFX, CREB, and NF-Y (29, 31–35). The en-

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§ Supported by the Marie Heim-Vögtlin Foundation. Present address: Onco-hematology Research Unit, Pediatrics, University Hospital of Vaud, CHUV, CH-1011, Lausanne, Switzerland.

¶ Present address: NovImmune S.A., 64 avenue de la Roseraie, CH-1211, Geneva, Switzerland.

** Present address: Section of Immunobiology, Yale School of Medicine, 300 Cedar St., New Haven, CT 06520.

‡‡ To whom correspondence should be addressed: University of Geneva Medical School, CMU, 1 rue Michel-Servet, CH-1211, Geneva, Switzerland. Tel.: 41-22-379-56-66; Fax: 41-22-379-57-46; E-mail: walter.reith@medecine.unige.ch.

[1] The abbreviations used are: MHC, major histocompatibility complex; CREB, cAMP-response element-binding protein.

hanceosome and CIITA then collaborate in the transcription activation process by interacting with co-activators, inducing histone acetylation and/or recruiting transcription initiation and elongation factors at MHCII promoters (30, 36–41).

Recruitment of CIITA constitutes a critical step because it functions as a master control factor for MHCII genes; in contrast to RFX and the other enhanceosome components, the expression of CIITA is highly regulated, which imposes a tight qualitative and quantitative control over MHCII expression (1–3, 42). Because of this absolute control over MHCII genes, CIITA has become a primary focus of research in the field of MHCII regulation.

Although the key contributions of the X, X2, and Y sequences to enhanceosome assembly and CIITA recruitment are now well established, the role of the W box has remained obscure. The W box is a 20-base pair region within which a more tightly conserved 7-base pair subelement, the S box, has been defined (4, 6). The presence of the W/S box (43–47) at a conserved distance from the X box (48–50) is required for optimal constitutive and inducible MHCII expression. DNA binding activities that show specificity for the W region in gel retardation assays have been detected in nuclear extracts (43, 44, 51), but neither the identity of these factors nor their functional relevance have been formally established. A widespread model is that the W region could be a duplicated target site for RFX (1, 52). This is based on the observation that the W region of the DRA gene contains a sequence that exhibits limited homology to the X box and can be bound weakly by RFX *in vitro* (44, 48, 49, 53). However, the functional relevance of this finding is not clear because this X box-like motif does not coincide with the most conserved core S sequence. Moreover, in sharp contrast to the S box, an X box homology is not conserved in the W regions of any other MHCII genes.

Given our limited knowledge on the function of the W/S box, we have readdressed its role in the activation of MHCII promoters. In particular we have examined its role in what is now known to be one of the most critical steps, namely the recruitment of CIITA. We demonstrate that the S box and a very precise spacing between the S and X boxes are essential for CIITA recruitment. Other sequences within the W region, notably the putative duplicated RFX-binding site, are dispensable for this function. In contrast to the prediction of current models, the factor mediating S box-dependent CIITA recruitment is thus distinct from RFX.

EXPERIMENTAL PROCEDURES

Cell Lines and Extracts—The B cell lines Raji (54), TK6, and RJ6.4 (29) were cultivated in RPMI medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine. Whole cell extracts (29) and nuclear extracts (55) were prepared as described.

Promoter Templates—Wild type and mutated HLA-DRA promoter templates (positions −150 to +6) used for promoter pull-down assays were generated by PCR on a DRsyn template (44) using a 5′-biotinylated primer. The mutations were made on the basis of previous functional studies of the DRA promoter (44). For reporter gene assays, wild type and mutated DRA promoters from −150 to +10 were amplified by PCR and inserted between the SmaI and BglII sites of the pGL3b vector (Promega).

Promoter Pull-down Assays—Promoter pull-down assays were performed as described (29). Briefly, promoter templates biotinylated at the 5′ end of the upper strand were produced by PCR and coupled to Streptavidin-coated magnetic beads (Promega). Whole cell extracts (35 μl, 1–1.2 mg) from Raji or RJ6.4 cells were incubated for 2 h. at 4 °C with the DNA template in a buffer containing 20 mM Hepes, pH 7.9, 100 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 20% glycerol, 0.01% Nonidet P-40, 0.15 mg/ml poly(dI-dC)·poly(dI-dC), 0.15 mg/ml single-stranded *Escherichia coli* DNA, and a mixture of protease inhibitors (Roche Diagnostics). The beads were washed three times with the same buffer containing 1 mg/ml bovine serum albumin, and proteins were eluted with SDS-PAGE sample buffer.

Immunoblotting—Polyclonal anti-RFX5 and anti-RFXANK antibodies and the affinity-purified anti-RFXAP and anti-CIITA-N antibodies have been described (18, 19, 29). The NF-YB antibody was a gift from Roberto Mantovani. Anti-OBF-1 antibodies were purchased from Santa Cruz Biotechnologies. The proteins were detected by immunoblotting according to standard protocols. Signals obtained with the anti-CIITA antibodies are much stronger than those obtained with the anti-RFX antibodies because the former were generated against the recombinant CIITA protein, whereas the latter were raised against short peptides derived from individual RFX subunits. Because of this difference in sensitivity, CIITA but not the RFX subunits are detected when low amounts of the input extract are analyzed (see Figs. 2–4).

Luciferase Assays—Raji cells were transfected by electroporation with a 10:1 ratio of pGL3b-DRA constructs *versus* pRL-TK (Promega). After 48 h, the cells were harvested and luciferase activity was measured using the dual luciferase reporter assay system (Promega).

Quantitative Enhanceosome Purification—The enhanceosome complex was purified as described (18) with the following modifications. Nuclear TK6 extract (5 mg, 5 mg/ml) was incubated for 16 h at 4 °C with streptavidin-magnetic beads coupled (50 μg of DNA/mg of beads) to the wild type or mutated (M3/M4) DRA promoter fragments (from −150 to −45) in 20 mM Hepes, pH 7.9, 100 mM KCl, 6 mM $MgCl_2$, 20% glycerol, 1 mM dithiothreitol, 0.01% Nonidet P-40, 0.15 mg/ml poly(dI-dC)·poly(dI-dC), 0.15 mg/ml single-stranded *E. coli* DNA and a mixture of protease inhibitors (Roche Applied Science). The beads were washed five times with buffer D (20 mM Hepes, pH 7.9, 100 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 20% glycerol, 0.01% Nonidet P-40). Nonspecific competitor DNA (0.125 mg/ml herring sperm DNA and 0.125 mg/ml single-stranded *E. coli* DNA) was included during the last three washes, and a specific competitor (DRA fragment −150 to −45 at 12 μg/ml) was added during the last wash. The beads were then washed sequentially with buffer D containing increasing amounts of KCl (see Fig. 5) and were eluted with buffer D containing 2 M KCl for 1 h at 4 °C. Purified proteins were separated by SDS-PAGE and stained with silver nitrate (Amersham Biosciences). The amount of RFX5, RFXAP, RFXANK, and NF-YB purified on the wild type and M3/M4 promoters was quantified by Quantity One (Bio-Rad). Identity of the bands was confirmed by mass spectrometry. For the analysis of CIITA recruitment to the purified enhanceosome, recombinant CIITA, produced using a Vaccinia-T7 expression system (29), was added to the promoter-bound proteins after the indicated washing step (see Fig. 5) and incubated in buffer D containing 0.15 mg/ml poly(dI-dC)·poly(dI-dC), 0.15 mg/ml single-stranded *E. coli* DNA and a mixture of proteases inhibitors (Roche Applied Science) for 2 h at 4 °C.

RESULTS

The S Box Is Essential for Recruitment of CIITA—We have developed a promoter pull-down assay to analyze the recruitment of CIITA to the MHCII enhanceosome *in vitro* (Fig. 1*A*) (29). The assay relies on the fact that the known constituents of the MHCII enhanceosome (RFX, CREB, and NF-Y) bind together in a highly cooperative fashion, such that they assemble into a stable higher order complex on DRA promoter fragments (18, 24, 29, 30). This nucleoprotein complex generates a composite interaction surface to which CIITA binds via multiple synergistic protein-protein contacts (29, 32–34). In this assay, biotinylated DRA promoter fragments immobilized on streptavidin-coated magnetic beads are incubated with crude whole cell extracts to permit enhanceosome assembly and recruitment of CIITA (29). After isolation of the resulting nucleoprotein complex, co-purification of the various different enhanceosome components and CIITA can be analyzed by immunoblotting. A typical example is shown in Fig. 1*A*; using a wild type DRA template, the recruitment of CIITA to the MHCII enhanceosome is demonstrated by co-purification of CIITA with RFX and NF-Y. In these experiments, co-purification of the OBF1 co-activator serves as an internal control; OBF1 binds together with Oct proteins to an octamer motif situated downstream of the MHCII regulatory module in the DRA promoter (Fig. 1*A*).

The recruitment of CIITA in the promoter pull-down assay is fully dependent on integrity of the MHCII enhancer module (29). We therefore exploited this assay to examine the contri-

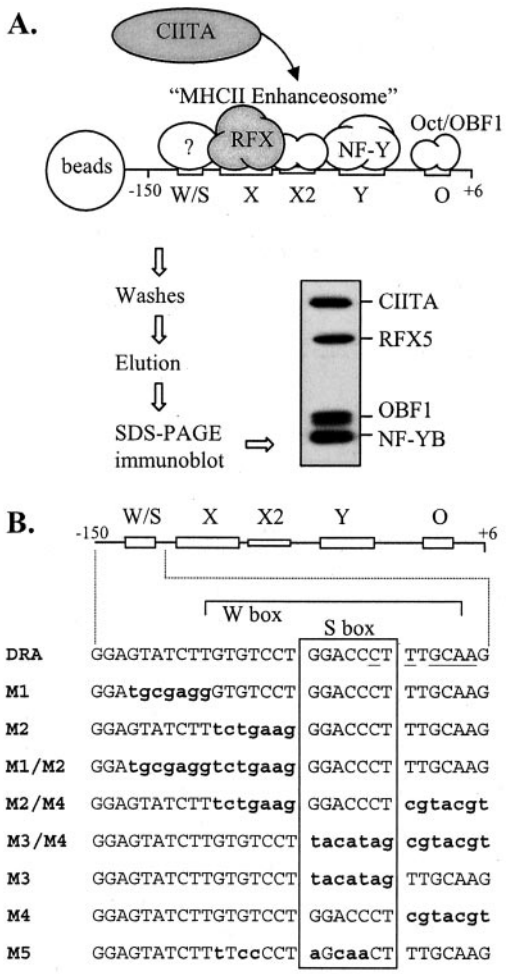

FIG. 1. **Promoter pull-down assay and DNA templates used to study the recruitment of CIITA to the MHCII enhanceosome complex assembled at the DRA promoter.** *A*, DNA fragments encompassing the W/S, X, X2, Y, and octamer (*O*) sequences of the HLA-DRA promoter (positions −150 to +6) are coupled to magnetic beads and incubated with B cell extracts under conditions that promote assembly of the MHCII enhanceosome. Incorporation of RFX, NF-Y, and CREB into the enhanceosome is strictly required to generate a platform to which CIITA can be recruited. The Oct and OBF1 factors bind together to the DRA-specific octamer (*O*) motif. Oct and OBF1 factors are not part of the MHCII enhanceosome and are not required for recruitment of CIITA (29). After extensive washes, proteins eluted from the DNA are analyzed by SDS-PAGE and immunoblotting. The representative blot demonstrates co-purification of RFX5, NF-YB, CIITA, and the control OBF1 protein. *B*, sequences of wild type and mutated templates in the region from −148 to −116 of the DRA promoter. The mutated nucleotides are shown as *bold lowercase letters*. The W and S boxes are indicated. A sequence exhibiting similarity to the core sequence of the X box is *underlined*.

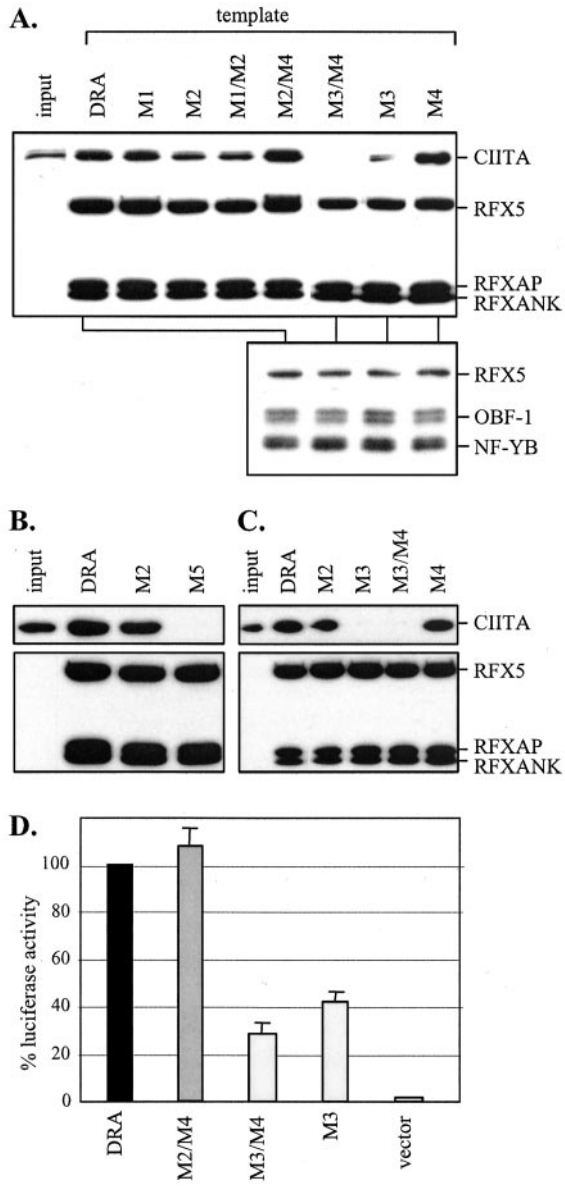

FIG. 2. **The S box is required for recruitment of CIITA.** *A–C*, the wild type DRA promoter and promoters containing the indicated mutations in the W and S boxes were tested for their ability to sustain CIITA recruitment in promoter pull-down assays. Three independent experiments are shown. Purified proteins were analyzed by immunoblotting for the presence of CIITA, the RFX complex (RFX5, RFXAP, and RFXANK), the NF-Y complex (NF-YB), and OBF1. 1.5% (*A*) or 0.75% (*B* and *C*) of the input extract was loaded in the first lanes. *D*, the indicated wild type and mutated DRA promoters were tested for their activity in Raji cells by a luciferase reporter gene assay. The values are given as percentages of luciferase activity obtained with the wild type promoter. The empty pGL3b vector was used as negative control. The results represent the means of two independent experiments.

bution of the W/S region to CIITA recruitment. A series of clustered point mutations were introduced into the W/S region of the DRA promoter (Fig. 1*B*). These mutations lie upstream, within or downstream of the S box. The repercussion of these mutations on the recruitment of CIITA was then assessed by promoter pull-down assays. Recruitment of CIITA is strongly impaired by mutations of the S box (Fig. 2, *A–C*, *mutations M3*, *M3/M4*, and *M5*). The loss of CIITA recruitment is specific, as demonstrated by the fact that these mutations do not affect purification of the enhanceosome complex containing all subunits of RFX (RFX5, RFXAP, and RFXANK) and NF-Y. Binding of the control OBF1 protein is also not affected. In sharp contrast to mutations of the S box, mutations of the W region situated upstream and/or downstream of the S box have little or no effect on the recruitment of CIITA (Fig. 2, *A–C*, *M1*, *M2*, *M1/M2*, *M2/M4*, and *M4*). A modest reduction in CIITA recruitment is evident in certain experiments with templates containing the M2 mutation (Fig. 2*A*). However, this reduction is probably not significant because it is not observed reproducibly in all experiments (compare *M2* in Figs. 2, *A–C*) and is not discerned for all templates containing the M2 mutation (compare *M2* and *M2/M4* in Fig. 2*A*). It is moreover very weak compared with the dramatic loss of CIITA recruitment to templates containing a mutated S box.

Enhanceosome components such as RFX are purified very efficiently by the pull-down assay, as evidenced by the strong enrichment observed when comparing the input and purified

fractions (Fig. 2, *A–C*). CIITA is enriched less efficiently (compare ratios between the input extract and purified fractions for RFX and CIITA in Fig. 2, *A–C*). Only a few percent of the input CIITA is typically recruited to the enhanceosome complex. This is entirely consistent with our earlier results and is believed to reflect low affinity of CIITA for the enhanceosome complex (29).

Selected mutations were tested in a luciferase reporter gene assay to confirm that there is a correlation between the promoter activity of the mutated templates and their ability to recruit CIITA in our *in vitro* recruitment assay. Wild type and mutated DRA promoters were inserted into a luciferase reporter gene vector, and luciferase activity was measured after transfection of these constructs into the B cell line Raji. As expected, the mutation that alters the W region on both sides of the S box (M2/M4), which does not affect CIITA recruitment (Fig. 2*A*), has no effect on promoter activity (Fig. 2*D*). This is in full agreement with previous studies showing that neither the individual M2 or M4 mutations (44) nor a distinct mutation affecting exactly the same nucleotides modified in M2 (43) impair DRA promoter strength in Raji B cells. On the other hand, the mutations in the S box (M3 and M3/M4), which impair CIITA recruitment (Fig. 2, *A* and *C*), reduce promoter activity to 30–40% of wild type levels (Fig. 2*D*). The extent of this reduction is consistent with that observed in previous studies addressing the function of the S box (43, 44, 47). The detrimental effect of the S box mutations on CIITA recruitment is stronger than the reduction in promoter activity. The pull-down assay is thus more stringent than transient transfection assays. It is currently not possible to determine which reflects the *in vivo* situation more faithfully because S box mutations have not been targeted into the endogenous MHCII loci.

Recruitment of CIITA Requires Precise Spacing between the S and X Boxes—Correct spacing between the S and X boxes is important for the activity of MHCII promoters (48–50, 56). We therefore determined whether the S-X spacing constraint could determine the efficiency of CIITA recruitment. We generated a series of DRA promoters having modified S-X spacing (Fig. 3*A*). We either removed 1 or 2 base pairs or inserted 1, 2, or 10 base pairs at the same position used in previous functional studies (49). These spacing mutants were then tested in the promoter pull-down assay (Fig. 3*B*). Remarkably, all mutants interfere with the recruitment of CIITA. Impairment of CIITA recruitment is strong with the −1, −2, +2, and +10 templates. With the +1 template CIITA recruitment is only partially affected. In contrast to recruitment of CIITA, none of the mutants affect enhanceosome formation as detected by binding of RFX (Fig. 3*B*), NF-Y or OBF1 (data not shown). Taken together the results demonstrate that CIITA recruitment exhibits a very strict dependence on the distance between the S and X boxes. A deviation of even 1–2 nucleotides from the wild type distance (16 base pairs) has a negative effect.

The CIITA recruitment results suggest that the S-X spacing constraint is considerably tighter than previously reported. Earlier functional studies had examined this spacing constraint by increasing the S-X distance of the DRA promoter by a minimum of 5 base pairs (48–50). Our data imply that even a 1–2-base pair insertion or deletion should be deleterious for promoter activity. To confirm this we tested the activity of our spacing mutants in the luciferase reporter gene assay (Fig. 3*C*). In agreement with our CIITA recruitment results, the −1, −2, +2, and +10 promoters have significantly reduced activity. On the other hand the mutated +1 promoter, which retains its ability to recruit CIITA, albeit with reduced efficiency, exhibits an activity that is nearly wild type. As mentioned above, these results again emphasize the fact that the *in vitro* CIITA recruitment assay is more stringent than the reporter gene assay. This is particularly evident for the +1 mutation, which partially inhibits CIITA recruitment yet has very little effect on promoter strength.

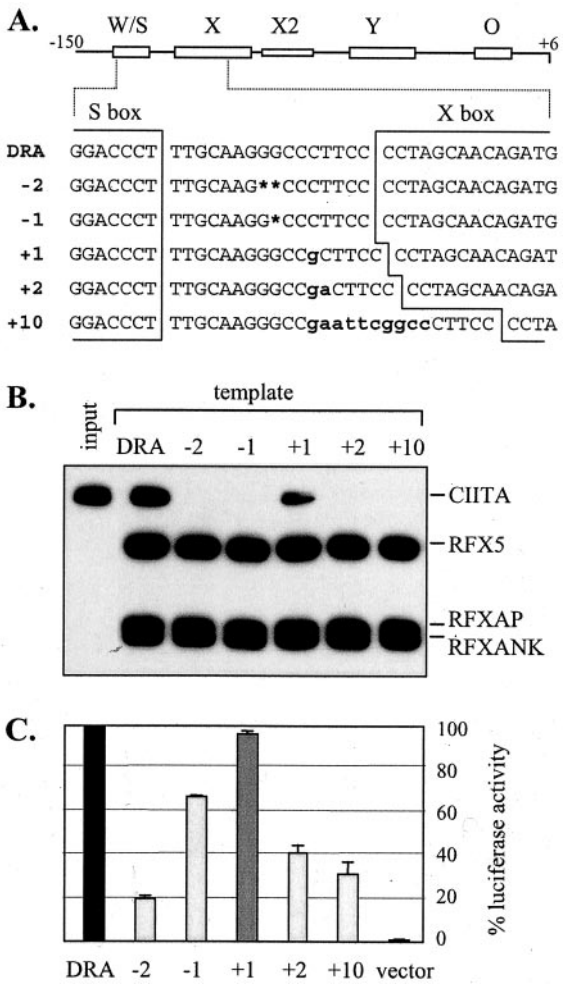

FIG. 3. **A fixed distance between the S and X boxes is critical for CIITA recruitment.** *A*, sequences of wild type and mutated templates in the region from −131 to −95 of the DRA promoter. The S and the X boxes are indicated. Deleted and inserted nucleotides are indicated, respectively, as *stars* and *bold lowercase letters*. *B*, the intact wild type DRA promoter and promoters containing the indicated S-X spacing mutations were tested for their ability to sustain CIITA recruitment in the promoter pull-down assay. Purified proteins were analyzed by immunoblotting for the presence of CIITA and the RFX complex (RFX5, RFXAP, and RFXANK). 1.5% of the input extract was loaded in the first lane. *C*, the indicated wild type and mutated DRA promoters were tested for their activity as in Fig. 2.

The S Box Does Not Constitute a Duplicated RFX-binding Site—The W region of the DRA promoter contains a motif that exhibits partial homology to the X box (Fig. 1*B*) and RFX can bind weakly to this motif in gel retardation assays (48, 49). Binding of two RFX molecules to this motif and the X box was found to be cooperative in these studies (48, 49). Finally, it was reported that the W box can be converted into a perfect X box sequence without compromising promoter function (49). These observations led to the hypothesis that the W region constitutes a duplicated binding site for RFX and that the requirement for a precise W-X spacing reflects cooperative binding between two RFX molecules (48, 49). We therefore used several complementary approaches to examine the possibility that the W box is required for CIITA recruitment because it provides a second binding site for RFX.

We first introduced mutations that either remove the X box homology within the W box (mutation M4) or are known to

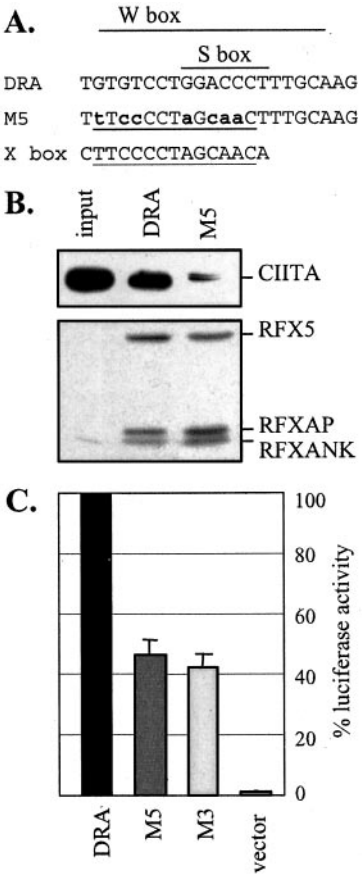

FIG. 4. **Replacement of the S box by a perfect RFX binding site is detrimental for both CIITA recruitment and promoter strength.** *A*, sequences of the W regions of the wild type promoter (DRA) and the mutated template (M5) in which the S box is converted to a perfect X box (*underlined*). Mutated nucleotides are indicated with *bold lowercase letters*. The DRA X box sequence is aligned below. *B*, the wild type and M5 promoters were tested for their ability to sustain CIITA recruitment in the promoter pull-down assay. Purified proteins were analyzed by immunoblotting for the presence of CIITA and the RFX complex (RFX5, RFXAP, and RFXANK). 3% of the input extract was loaded in the first lane. *C*, the wild type, M5, and M3 promoters were tested for their activity as in Fig. 2.

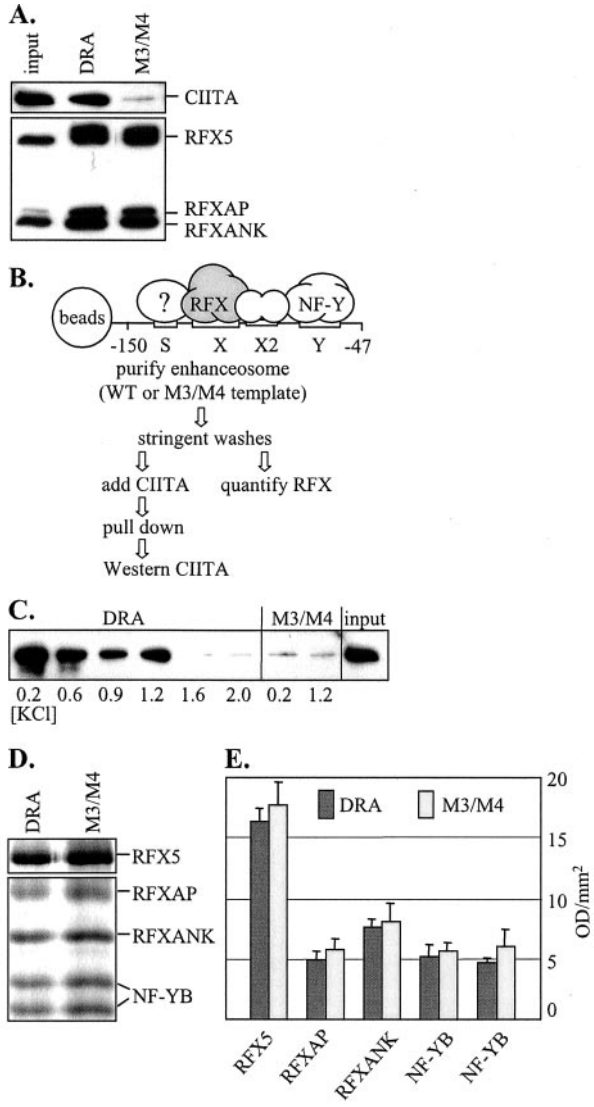

FIG. 5. **The amount of RFX purified on DRA templates does not depend on integrity of the S box.** *A*, enhanceosome complexes assembled on the wild type (DRA) and mutated (M3/M4) DRA promoters were analyzed by immunoblotting for incorporation of the three RFX subunits and for the recruitment of CIITA. 10% of the input was loaded in the *left lane*. *B*, the enhanceosome complex was assembled in parallel on the wild type (DRA) and mutated M3/M4 templates. After washes of variable stringency, the enhanceosome was tested for its ability to recruit CIITA (*C*) and its composition was analyzed (*D* and *E*). *C*, enhanceosomes were assembled on the wild type and mutated templates, subjected to washes containing the indicated KCl concentration, and then analyzed for their ability to recruit recombinant CIITA. *D*, enhanceosomes assembled on the wild type and mutated templates were subjected to washes containing 1.2 M KCl, and their composition was then analyzed by SDS-PAGE and silver staining. Portions of the gel containing the three RFX subunits and the two isoforms of NF-YB are shown. Identity of the bands was confirmed by mass spectrometry. *E*, the amounts of RFX5, RFXAP, RFXANK, and NF-YB purified on the wild type and mutated promoters were quantified. The results represent the means of two independent experiments.

eliminate binding of RFX in gel retardation assays (mutation M2) (49). These mutations did not impair incorporation of RFX into the enhanceosome, CIITA recruitment, or promoter activity (Fig. 2, *templates M2*, *M1/M2*, *M4*, and *M2/M4*).

We then examined the effect of spacing mutants that would be anticipated to disrupt cooperative binding of two RFX complexes to the W and X boxes (49). The reduction in CIITA recruitment and promoter activity observed with the spacing mutants did not correlate with a diminution in the amount of RFX that is incorporated into the enhanceosome complex (Fig. 3, *templates −2*, *−1*, *+2*, and *+10*).

We next introduced a mutation that converts the W box into a perfect X box (Fig. 4*A*, M5 mutant). This mutant is identical to the one used previously to support the notion that the W region constitutes a second RFX-binding site (49). In contrast to what is predicted by the model that the W box is a duplicated RFX binding site, we found that the M5 mutation strongly inhibits CIITA recruitment (Figs. 2*B* and 4*B*) and reduces promoter activity (Fig. 4*C*). Moreover, the amount of RFX bound to the promoter in the pull-down assay was not increased, indicating that the introduction of a second optimal X box sequence into the W box does not increase incorporation of RFX into the enhanceosome complex.

Finally, we performed promoter pull-down assays to rigorously compare the amounts of RFX that bind to the wild type and mutated M3/M4 DRA templates. The amount of RFX pulled down with the two templates was first examined by Western blotting using antibodies directed against all three RFX subunits (Fig. 5*A*). No difference in the level of RFX binding was observed between the wild type and mutated template. We next modified the pull-down assay to obtain a more rigorous quantification (Fig. 5*B*). To optimize purification, washing conditions of increasing stringency (ranging from 0.2 to 2.0 M KCl) were tested. The ability of the DNA-bound pro-

teins to sustain the recruitment of CIITA was verified after each wash. Because association of endogenous CIITA with the enhanceosome is disrupted under the more stringent conditions used, the recruitment of CIITA was analyzed after the addition of exogenous recombinant CIITA to the purified enhanceosome complex. CIITA can still be recruited efficiently to the wild type DRA template after washes containing up to 1.2 M KCl (Fig. 5*C*). At higher concentrations the ability to recruit CIITA is eliminated, indicating the loss of enhanceosome components critical for binding of CIITA. A 1.2 M KCl wash was therefore chosen to purify in parallel the enhanceosome complexes that assemble on the wild type and M3/M4 templates. The proteins recovered by this purification procedure were then analyzed by SDS-PAGE (Fig. 5*D*) and quantified (Fig. 5*E*). The amounts of RFX5, RFXAP, and RFXANK purified on the wild type and mutated DRA templates are rigorously identical. The same is observed for a control enhanceosome component, namely the two isoforms of NF-YB. This indicates that the M3/M4 mutation does not reduce binding of RFX to the promoter, although it has a drastic effect on CIITA recruitment. Taken together our results thus provide compelling evidence that the requirement of the S box for the recruitment of CIITA cannot be accounted for by binding of a second RFX complex to the W region. In fact, there is no evidence that the W box can function as a second binding site for RFX under the conditions used for the pull-down assays.

DISCUSSION

Although it has been established for some time that the W/S box and its distance from the X box are important parameters determining the activation of MHCII promoters, the precise mode of action of this regulatory element has remained largely unresolved. We demonstrate here that the S sequence within the W box is essential for a pivotal step in the activation of MHCII promoters, namely the physical recruitment of CIITA to the MHCII enhanceosome complex. This is entirely consistent with the fact that the S box is required for optimal transactivation by CIITA in transient transfection experiments (34, 57). CIITA recruitment is dependent on the integrity of the S box and on a remarkably precise spacing between the S and X boxes. In the absence of a functional S box, or when S-X spacing is modified by even only 1–2 nucleotides, the enhanceosome complex containing RFX, CREB, and NF-Y can assemble but cannot sustain CIITA recruitment, despite the fact that it provides multiple contacts with CIITA (29, 32–35). The contribution of the S box is thus absolutely critical.

The S-X spacing constraint is remarkably tight. Previous experiments with the DRA promoter had demonstrated that 5- or 10-base pair insertions between the S and X boxes reduce promoter activity (48–50). We now show that even a 1–2-base pair divergence from the natural 16-base pair spacing found within the DRA gene has a deleterious effect on both CIITA recruitment and promoter activity. This is consistent with the fact that the distance between the S and X boxes is tightly conserved, between 15 and 17 base pairs, in all of the MHCII promoters in mouse, man, and other species (4, 6). In the DQB1 gene there is a natural polymorphism affecting the S-X spacing (56, 58, 59). In certain DQB1 alleles this distance is increased up to 19 base pairs. However, it has been shown that this increased length reduces promoter activity and that the optimal spacing for DQB1 is in fact also 16 base pairs (56, 58). The almost invariant S-X spacing requirement is very different from the constraint observed for spacing between the X and Y boxes. In the latter case, spacing can be modified as long as it remains a multiple of 10 base pairs such that proteins bound to the X and Y boxes are aligned on the same face of the DNA helix (28, 50, 60). This suggests that the S-X and X-Y spacing constraints reflect quite different functions and types of interactions.

It has been observed that the W region contains a putative X box duplication, and it was therefore proposed that the W box might represent a second binding site for RFX (48, 49). Our results challenge this hypothesis. We find that the amount of RFX that is incorporated into the enhanceosome complex assembled on the DRA promoter is independent of the presence of the second putative RFX target site in the W region. Moreover, we find that there is no correlation between the presence of a second RFX target site and either the recruitment of CIITA or the activity of the promoter. Finally, S box-dependent CIITA recruitment cannot be obtained using enhanceosome complexes containing only recombinant RFX, CREB, and NF-Y (data not shown). The reasons for the discrepancy between our results and the earlier experiments probably reside in the fact that different experimental approaches were used. The previous studies used gel retardation assays to show that RFX could bind to the W box, albeit with much lower efficiency than to the X box (48, 49). In contrast, we have used a more stringent DNA binding assay relying on the cooperative assembly of an enhanceosome complex containing all components required for the recruitment of CIITA.

Despite extensive efforts, we have been unable to reproduce S box-dependent CIITA recruitment using enhanceosome complexes assembled from recombinant RFX, CREB and NF-Y (data not shown). The inability to obtain efficient CIITA recruitment using exclusively recombinant proteins was also reported by others (31). This suggests that cell extracts supply an additional activity that is required for mediating the influence of the S box on CIITA recruitment. In an attempt to identify a putative S box-binding factor we have co-purified, to near homogeneity, all proteins capable of assembling into a stable complex on the DRA promoter.[2] These highly purified enhanceosome fractions contain all known components of the MHCII enhanceosome complex. Surprisingly, however, we observed no differences in composition between the enhanceosome complex that assembles on the wild type DRA promoter and the one that forms on a mutated promoter lacking a functional S box.[2] This suggests that the role of the S box is different from that of the X, X2, and Y boxes. In contrast to the latter, the S box does not function as a binding site for a stable enhanceosome component that can be co-purified quantitatively with RFX, CREB, and NF-Y. This is consistent with the fact that no S-box-specific factor has yet been reliably identified by DNA binding studies or affinity purification procedures. It is also in agreement with *in vivo* footprint experiments, which have demonstrated that there is no clear footprint on the W/S box, whereas there is stable occupation of the X, X2, and Y boxes (61, 62). Moreover, disruption of the S box in stably transfected reporter gene constructs does not alter the *in vivo* footprint pattern at the X, X2, and Y boxes (26). Finally, the precise spacing constraint also suggests that the S box serves a function distinct from the other cis-acting elements of MHCII promoters. One intriguing possibility is that the S box is a docking site for a protein that modifies one of the other DNA-bound complexes, thereby rendering them competent for interacting with CIITA. The requirement for a modifying activity present in cell extracts was recently also put forward by others to account for their inability to obtain CIITA recruitment using pull-down assays with recombinant RFX and NF-Y proteins (31).

In conclusion, our results demonstrate that the S box promotes physical recruitment of the non-DNA-binding co-activa-

[2] A. Muhlethaler-Mottet, K. Masternak, and W. Reith, unpublished data.

tor CIITA and thus provides the first biochemical and functional characterization of the mode of action of this unique cis-acting sequence of MHCII promoters. However, how the S box mediates CIITA recruitment remains to be defined. In contrast to a widely accepted model (1, 52), this function of the S box cannot be ascribed to a second binding site for RFX. Our results thus pave the way for the identification of novel factors implicated in the activation of MHCII promoters.

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