MOLECULAR AND CELLULAR BIOLOGY, Sept. 1996, p. 4683–4690 Vol. 16, No. 9
0270-7306/96/$04.00+0

Complex Architecture of Major Histocompatibility Complex Class II Promoters: Reiterated Motifs and Conserved Protein-Protein Interactions

NABILA JABRANE-FERRAT,[1] JOSEPH D. FONTES,[1] JEREMY M. BOSS,[2] AND B. MATIJA PETERLIN[1]*

Howard Hughes Medical Institute and Departments of Medicine and of Microbiology and Immunology, University of California San Francisco, San Francisco, California 94143,[1] and Department of Microbiology, Emory University School of Medicine, Atlanta, Georgia 30322[2]

Received 15 March 1996/Returned for modification 16 May 1996/Accepted 30 May 1996

The S box (also known as at the H, W, or Z box) is the 5′-most element of the conserved upstream sequences in promoters of major histocompatibility complex class II genes. It is important for their B-cell-specific and interferon gamma-inducible expression. In this study, we demonstrate that the S box represents a duplication of the downstream X box. First, RFX, which is composed of the RFX5-p36 heterodimer that binds to the X box, also binds to the S box and its 5′-flanking sequence. Second, NF-Y, which binds to the Y box and increases interactions between RFX and the X box, also increases the binding of RFX to the S box. Third, RFXs bound to S and X boxes interact with each other in a spatially constrained manner. Finally, we confirmed these protein-protein and protein-DNA interactions by expressing a hybrid RFX5-VP16 protein in cells. We conclude that RFX binds to S and X boxes and that complex interactions between RFX and NF-Y direct B-cell-specific and interferon gamma-inducible expression of major histocompatibility complex class II genes.

Class II genes of the major histocompatibility complex (class II) code for proteins that initiate and propagate immune system responses (19, 30, 31). They present antigenic peptides to the T-cell antigen receptor and direct helper T cells to appropriate target B cells (10). The end result of these interactions is to activate and differentiate antigen-specific B cells to plasma cells (2). Expression of class II is carefully regulated at the level of transcription such that high levels of these proteins are observed on the surface of B cells, activated human T cells, and many somatic and antigen-presenting cells after their induction by the T-cell lymphokine gamma interferon (IFN-γ) (1, 4, 8, 13, 24).

Since the DRA gene is invariant and is expressed at the highest levels of all class II genes, its promoter has been studied extensively (1, 8, 24). Lessons learned with the DRA promoter hold for other class II promoters (1, 4, 8, 13, 24, 48). Mapping of *cis*-acting elements revealed that the sequences from positions −140 to −60 are sufficient for its B-cell-specific and IFN-γ-inducible expression (44). From the 5′ to the 3′ direction, these conserved upstream sequences (CUS) consist of the S box (also known as the H, W, or Z box), the X box, and the Y box (1, 4, 8, 13, 24, 48). Further downstream, the DRA promoter contains an octamer-binding site and an initiator element (Inr) (4, 13, 22, 44, 50). The X box and its flanking sequences have been subdivided into the pyrimidine tract and the X1, X2, and X3 boxes (24). For the purposes of this study, the X box refers to the X1 box. Whereas S and X boxes are most important for the B-cell-specific and IFN-γ-inducible expression (39, 43), the Y box, octamer-binding site and Inr sequences also determine the site of initiation of DRA transcription (4, 13, 22, 50).

A large number of proteins that interact with the CUS have been described. Regulatory factors X (RFX1 to RFX5 and p36) bind to the X box (25, 29, 36). Although the RFX1 homodimer binds better to the isolated X box (11, 44), RFX refers to the physiologically relevant RFX5-p36 heterodimer that binds to the X box in the presence of the Y box (11). Ets-1 binds to the pyrimidine tract (18), B-cell factor 1 (BCF1) binds to the X3 box (49), and members of the Fos/Jun (3) and ATF/CREB families of proteins (21) and X2-binding protein (X2BP) bind to the X2 box (16, 23, 27). NF-Y binds to the Y box (45) and members of the Oct family of transcription factors bind to the octamer-binding site (34, 39, 47). However, proteins that bind to the S box have not been identified (1, 4, 6, 7, 13, 24).

The S box, which was named for Servenius (32) and is also known as the heptamer, septamer, W, H, or Z box, contains seven nucleotides (GGACCCT) (6, 7, 13, 44). Some of the confusion caused by these other names is due to their inclusion of its 5′-flanking sequence, which is required for the function of the S box (44). Not only is the S box required for the B-cell-specific and IFN-γ-inducible expression of the DRA promoter, but also the X box could compete for the binding of proteins that are bound to the S box (44). This suggested that the S box is in part a duplication of the X box. In this study, we demonstrate that RFX from nuclear extracts and DNA affinity purification forms similar complexes on S and X boxes. The binding of RFX to the S box was increased by the presence of the Y box. These protein-DNA interactions were confirmed in cells by using a fusion protein between RFX5 and the activation domain of VP16. Moreover, RFX bound to the S box interacted in a spatially constrained manner with RFX bound to the X box. Taken together with previous observations, these data support and extend the importance of RFX in the B-cell-specific and IFN-γ-inducible regulation of class II promoters.

MATERIALS AND METHODS

Cell culture, transfections, and CAT assays. Raji cells (ATCC CCL86), which are human Epstein-Barr virus-positive Burkitt's lymphoma cells, express high levels of class II antigens on their cell surface. COS cells are African green monkey kidney cells. Raji and COS cells were maintained in RPMI 1640 and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, penicillin, and streptomycin.

Raji cells (10^7) were transfected with 40 μg of plasmid DNA by electropora-

* Corresponding author. Phone: (415) 476-1291. Fax: (415) 502-5081. Electronic mail address: matija@itsa.ucsf.edu.

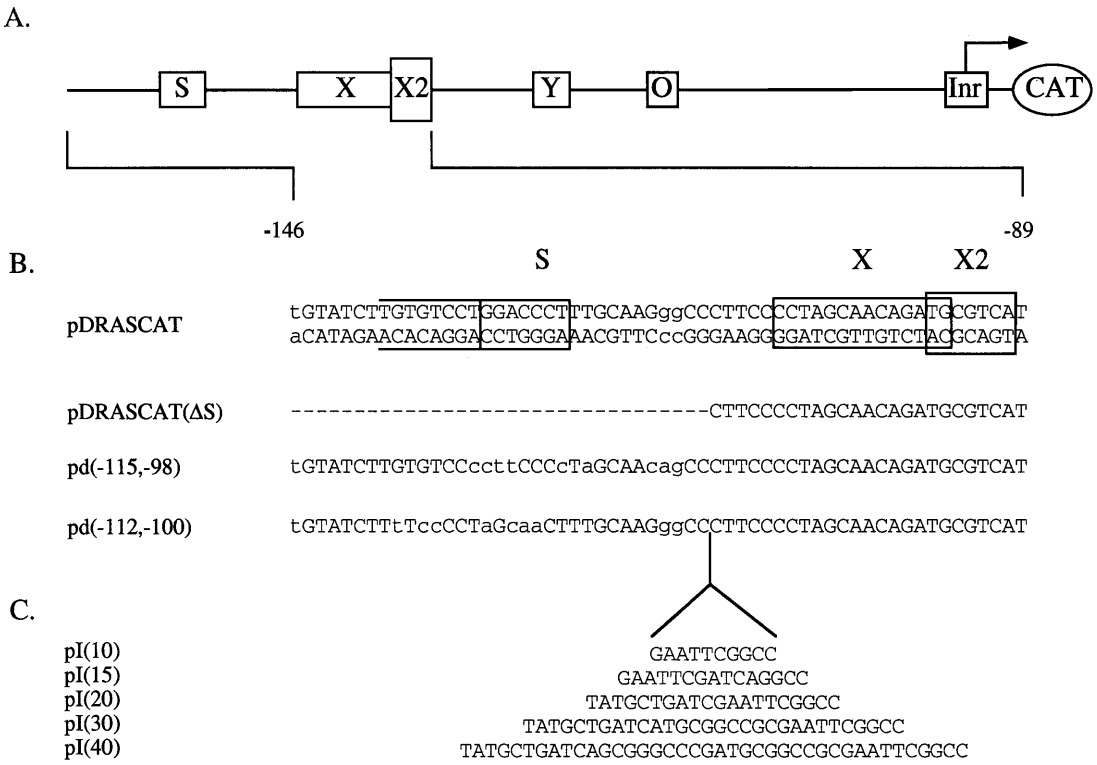

FIG. 1. Deletion and substitutions of the S box and insertions between S and X boxes in the DRA promoter. (A) Diagrammatic representation of the DRA promoter from positions −150 to +31. Boxes represent the S, X, X2, and Y boxes, the octamer-binding site (O), and the initiator sequences (Inr). Dotted lines indicate the 5′-flanking sequences of the S box. The arrow indicates the site of initiation of DRA transcription. (B) Nucleotides from positions −146 to −89 for both strands of the DRA promoter. Conserved boxes shown in panel A are diagrammed underneath the nucleotide sequences. The sequence of the deletion of the S box is placed below the wild-type sequence. Next are sequences of duplicated X boxes 3′ to the S box [pd(−115,−98)] and within the S box [pd(−112,−100)], which maintained the wild-type spacing between S and X boxes with duplicated X boxes. Nucleotides that are not found in the native DRA promoter are given in lowercase letters. (C) Into the *Apa*I site were inserted 10, 15, 20, 30 and 40 irrelevant nucleotides, some of which code for convenient restriction endonuclease sites. These plasmid constructions are called pI(10), pI(15), pI(20), pI(30), and pI(40), respectively.

tion at 300 V and 960 μF. Each transfection was done in triplicate and repeated with two separate preparations of plasmid DNA. COS cells were transfected with Lipofectamine (Gibco-BRL, Gaithersburg, Md.). The cells (5×10^5) were placed into 100-mm petri dishes 24 h before transfection. A total of 5 μg of DNA was used in each transfection. A 1-μg sample of pACTHCG was also added as an internal control to measure the efficiency of transfection. After 40 to 48 h in culture, the cells were harvested and lysed. Cell lysates were assayed for chloramphenicol acetyltransferase (CAT) activity, and levels of human chorionic gonadotropin were measured in supernatants as described previously (18).

Plasmid constructions. pDRASCAT contains the DRA promoter from positions −150 to +31 linked to the CAT reporter gene. Regions from nucleotides at positions −150 to −116 and positions −116 to −88, which contain the extended S and X boxes, respectively, are flanked by unique restriction endonuclease sites that facilitate the construction of mutated promoters (44). pDRASCAT(ΔS) was constructed by deleting the *Hin*dIII-*Apa*I (−150 to −116) fragment (Fig. 1B). pd(−115,−98) and pd(−112,−100) were constructed by substituting this *Hin*dIII-*Apa*I fragment with oligonucleotides (Fig. 1B). Insertions pI(10), pI(15), pI(20), pI(30), and pI(40) were created by placing the oligonucleotides listed in Fig. 1C into the *Apa*I site of pDRASCAT. pDRASmX CAT contains clustered point mutation at the X box of the DRA promoter and was described previously as M6 (44). The plasmid vectors pBCAT, which does not contain any DRA promoter sequence, and pREP-4CAT (pRSVCAT) were used as negative and positive controls, respectively. pSVRFX5 contains the entire RFX5 cDNA (amino acids 1 to 616) under the control of the simian virus 40 early promoter. pSVRFX5-VP16 was created by fusing the activation domain of VP16 from herpes simplex virus (amino acids 413 to 490) (9, 17) in frame 3′ to the RFX5 cDNA. This hybrid construction codes for RFX5 followed by the activation domain of VP16. pACTHCG contains the human chorionic gonadotropin gene under the control of the β-actin promoter.

Affinity purification of RFX from B-cell extract. RFX was purified from B-cell nuclear extracts as described previously (11). Nuclear extracts were prepared from leukocytes obtained by leukapheresis (33). After dialysis, extracts were applied to a hydroxylapatite column and the 0.2 M and 0.3 M salt eluants were dialyzed, tested for their activity by electrophoretic mobility shift assay (EMSA), and loaded onto an xx2 oligonucleotide affinity column. After the elution, active fractions were pooled, dialyzed, and loaded onto an xmx2y oligonucleotide affinity column. Active fractions were dialyzed and stored at −80°C. The activity of the extracts was monitored by EMSA with the labelled xx2 oligonucleotide.

EMSAs and Western blotting. The following oligonucleotides were used in this study. The s, xx2, x2, and y oligonucleotides contain sequences from positions −150 to −114, −124 to −70, −95 to −82, and −87 to −56 in the DRA promoter, respectively. The xmx2y oligonucleotide, which contains X and Y sequences with a shorter spacing between X and Y and a mutated X2 box, was described previously (TGCAAGAACCCTTCCCCTAGCAACAGATGGTATC TTTTTCTGATTGGCCAAAGA) (11). The mpBR oligonucleotide contains the methylated sequence from pBR322 (CTAGATCGTCACGGCGAT). The sxy and smxy oligonucleotides contain DRA promoter sequences from positions −150 to −56, except that smxy contains clustered point mutations (by transversion) of the X box (44). These two oligonucleotides were isolated from plasmids pDRASCAT and pDRASmXCAT, respectively.

Nuclear extracts from Raji cells were prepared as described previously (14). The amounts of protein were quantified by the Bradford assay (Bio-Rad Inc., Hercules, Calif.). DNA-binding assays were as described previously (26) with slight modifications. Reactions were carried out in a final volume of 20 μl with 10 μg of crude nuclear extract or 3 μl of affinity-purified RFX and 20 fmol of γ-^{32}P-labelled oligonucleotide, 1 μg of poly(dI-dC) · poly(dI-dC), 0.5 μg of sonicated denatured *Escherichia coli* DNA, 50 ng of mpBR (see below), and 100 μg of bovine serum albumin BSA fraction V. The reaction mixtures were incubated for 30 min at 15°C and electrophoresed on 5% native polyacrylamide gels for 4 h at 200 v. Prior to the loading of samples, the gels were prerun for 1 h in the cold. Unless otherwise indicated, unlabelled oligonucleotides were added at 100-fold molar excess and the mpBR oligonucleotide was used to eliminate the binding of RFX1 to the DNA probe. All our binding-reaction mixtures also contained 50 ng of the x2 oligonucleotide to prevent the binding of X2BP to the X2 box (27).

RFX5 was synthesized in a coupled in vitro transcription and translation reaction from pSVRFX5 by using a TNT kit and T7 RNA polymerase (Promega, Madison, Wis.). RFX translated in the rabbit reticulocyte lysate (5 μl), from the hydroxylapatite column (80 μl), first DNA affinity column (20 μl), and second DNA affinity column (10 μl), was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide). Proteins were then blotted

onto nitrocellulose paper, and RFX5 was detected by Western blotting (immunoblotting) with a rabbit polyclonal antibody against a C-terminal polypeptide from RFX5.

RESULTS

Deletion and substitutions of the S box and insertions between S and X boxes inactivate the DRA promoter. To determine the functional importance of the S box in the B-cell-specific expression from the DRA promoter, a number of plasmid constructions were made. As diagrammed in Fig. 1B, the S box and its flanking sequences were deleted in the synthetic DRA promoter (pDRASCAT) resulting in the construction pDRASCAT(ΔS). Previously, we demonstrated that clustered point mutations of these flanking sequences, which were incorporated into pDRASCAT, had no effect on the constitutive or IFN-γ-inducible expression from the DRA promoter (44). Since the nucleotide sequence 3′ to the S box (TTGCAA) is similar to that found in the X box (TAGCAA), the X box was also duplicated centered on this sequence, resulting in the plasmid pd(−115,−98) (Fig. 1B). The final construction, pd(−112,−100), was identical to pd(−115,−98), but the spacing between the duplicated X boxes was changed so that it was identical to that between the S and X boxes in the wild-type DRA promoter (Fig. 1B). These plasmids were tested in Raji cells, which represent a good model for the B-cell-specific expression of class II genes.

When compared with the plasmid with the deletion of the S box [pDRASCAT(ΔS)], pDRASCAT had fivefold-higher activity in Raji cells; i.e., pDRASCAT(ΔS) was expressed at 20% of the level of the wild-type promoter (Fig. 2A). Surprisingly, the duplication of the X box 3′ to the S box [pd(−115,−98)] resulted in equally diminished levels of expression. However, transfections with pd(−112,−100), which preserved the wild-type spacing between the S and X boxes in duplicated X boxes, resulted in 1.8-fold-higher levels of expression than did transfections with pDRASCAT (Fig. 2A). Thus, although there is little similarity in sequence between them, the X box can functionally replace the S box in the DRA promoter.

To investigate further the importance of the spacing between the S and X boxes, we inserted random oligonucleotides of 10, 15, 20, 30, and 40 nucleotides between these two elements in pDRASCAT. Confirming a previous observation (44), insertions of full- and half-helical turns inactivated the DRA promoter in B cells. Interestingly, CAT activities with these plasmids were lower than those observed with the deletion of the S box alone (Fig. 2B). We conclude that the duplication of the X box at the S box is dependent on the precise spacing between these two elements. Our data also indicate that the sequences most highly conserved between 3′ to the S box and the X box (TTGCAA and TAGCAA do not represent a functional duplication of the X box. Since other point mutations further upstream of the S box and between the S and X boxes had no phenotype (reference 44 and data not presented), the 15 nucleotides 5′ to and including the S box represent the DNA-binding site for factors that interact with the S box.

Cooperative binding between RFX and NF-Y from B-cell nuclear extracts. To find which of the proteins that bind to the X box also bind to the S box, EMSAs were performed with crude and fractionated nuclear extracts from Raji cells and a variety of labelled oligonucleotides. However, before performing DNA-binding studies with the S box, we wanted to demonstrate the binding of the functionally relevant RFX complex to the X box. Previously, we and others detected only the binding of the RFX1 homodimer to the X box (25, 44). Thus, RFX1 was a good candidate for the protein that also bound to

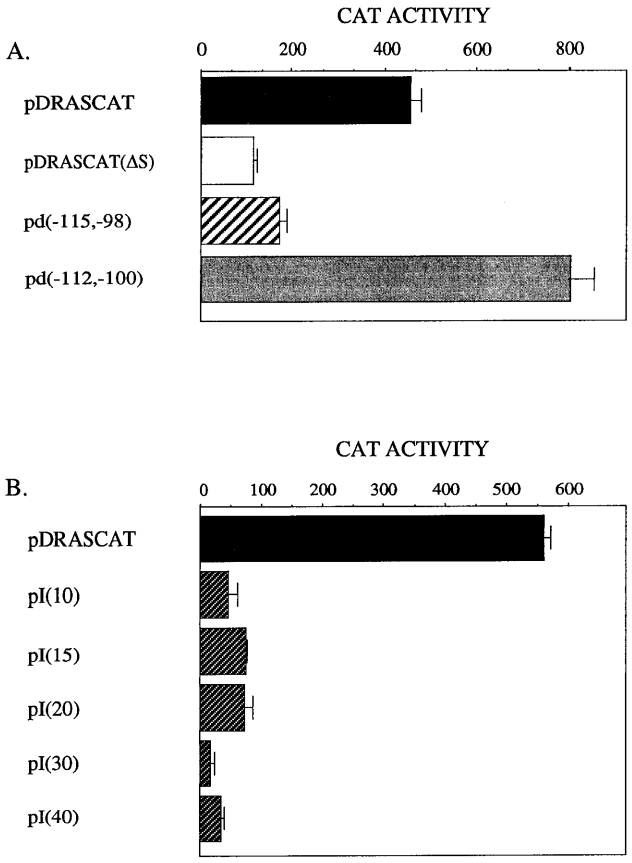

FIG. 2. Levels of expression from DRA promoters which contained the deletion and substitutions of the S box and insertions between the S and X boxes in Raji cells. (A) Names of plasmid constructions are given to the left. The activity of the wild-type DRA promoter is shown by the solid bar next to pDRASCAT. Deletion of the S box and its 5′-flanking sequence reduced the activity of pDRASCAT by 75%. The duplication of the X box in the S box pd(−115,−98) had the same phenotype, but the duplication pd(−112,−100) restored and increased the levels of expression from the DRA promoter. (B) CAT activities are given for insertions of 10 to 40 nucleotides between the S and X boxes. All insertions from pI(10) to pI(40) resulted in less than 20% of wild-type levels. Experiments are representative of three sets of transfections done in duplicate, with standard errors of the mean less than 20%.

the S box (44). However, RFX1, which is now also known as the methylated DNA-binding protein (52) and polyomavirus enhancer factor C (35), plays no role in the transcription of class II genes (29). Rather, RFX5, which contains a similar DNA-binding domain to the one that is found in RFX1 and is mutated in complementation group C of the type II bare lymphocyte syndrome (36), and p36, as yet uncloned, are the relevant regulators of class II transcription (11, 29, 36). Unlike RFX1, the RFX5-p36 heterodimer (RFX) by itself binds weakly to the X box. However, the presence of the Y box greatly increases the binding of RFX to the X box (28). NF-Y, which contains NF-YA, NF-YB, and NF-YC, binds to the Y box in all cells examined (4, 8, 13, 24, 28, 29).

By using the labelled xx2 oligonucleotide, which contained X and X2 sequences, a single faint RFX complex was observed (Fig. 3, lanes 1 to 4). Excess unlabelled xx2 but not mpBR oligonucleotides competed for this binding. In contrast, with the labelled xmx2y oligonucleotide, which contained X, mutated X2, and Y sequences, three major complexes were observed (lanes 5 to 10). The lower, strong complex corresponded to the binding of NF-Y and RFX since a 500-fold

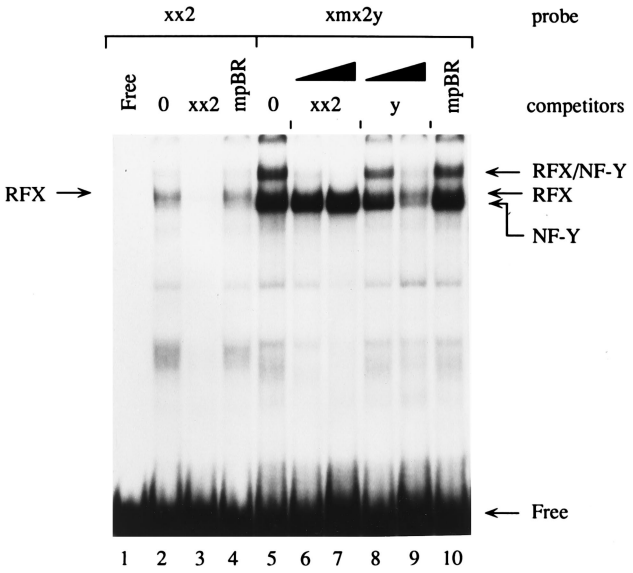

FIG. 3. Cooperative binding between RFX and NF-Y in the DRA promoter. Presented are the results of EMSAs with oligonucleotides which contained X and wild-type or mutated X2 boxes (xx2 [lanes 1 to 4] or xmx2y [lanes 5 to 10]) for the binding of RFX or RFX/NF-Y complexes, respectively, with nuclear extracts from Raji cells. Lanes 2 and 5 contain nuclear extracts alone. Lanes 3, 6, and 7 contain the same nuclear extracts with a 100-fold (lanes 3 and 6) or 500-fold (lane 7) molar excess of unlabelled xx2 oligonucleotide as the competitor. Lanes 8 and 9 contain a 100- and 1,000-fold molar excess, respectively, of the unlabelled y oligonucleotide. Lanes 4 and 10 contain a 1,000-fold molar excess of an irrelevant oligonucleotide (mpBR). Arrows denote three major complexes (NF-Y, RFX, and RFX/NF-Y) and the free probe.

molar excess of the unlabelled y oligonucleotide, which contained the Y sequence, competed for the binding of NF-Y but left a complex of similar mobility to the RFX bound to xx2 (lane 9). The xx2 oligonucleotide also competed for the remaining RFX complex (data not presented). That the upper complex contained both RFX and NF-Y was revealed by the competition with the unlabelled xx2 (lanes 6 and 7) and y (lanes 8 and 9) oligonucleotides. In our hands, RFX and NF-Y comigrated and NF-Y obscured the RFX complex, so that its binding to xmx2y was unmasked only in the presence of excess unlabelled y oligonucleotide (lane 9). Excess unlabelled mpBR did not affect the binding of RFX, NF-Y, or RFX/NF-Y to our labelled oligonucleotides (lanes 4 and 10). Thus, RFX binds specifically to the X box and RFX and NF-Y form a higher-order complex on X and Y boxes.

Binding of RFX to the S box is visible only when binding to the X box is blocked. From previous data, we knew that the binding of RFX1 to the S box is much weaker than that to the X box (44). Thus, it was not surprising that with our nuclear extracts, we could not observe any binding to the s oligonucleotide, which contained only the S box and its flanking sequences (data not presented). Conversely, no additional binding was observed with the sxx2y oligonucleotide, which contained the S, X, and Y boxes (28, 44). However, since NF-Y could increase the binding of RFX to the X box, NF-Y should also increase the binding of RFX to the S box in the absence of the X box. Because the spacing between the X and Y boxes can be increased by full-helical turns without affecting the function of the DRA promoter (46), we reasoned that the increased distance between the S and Y boxes should not impede these interactions.

To assay the binding of RFX to the S box in the presence of the Y box, we performed EMSAs with labelled sxx2y or smxx2y oligonucleotides, which contained the wild-type X or mutated X, X2, and Y sequences, respectively; excess unlabelled xx2, s, or methylated pBR oligonucleotides; and nuclear extracts from Raji cells. As noted in Fig. 4, RFX, NF-Y, and RFX/NF-Y bound to the wild-type sxx2y as well as to smxx2y oligonucleotides (Fig. 4, lanes 7 to 10 and lanes 1 to 6, respectively). A 100-fold molar excess of unlabelled xx2 (lanes 2 and 8) competed for the binding of RFX/NF-Y complexes. In the presence of the mutated X box, a 5,000-fold molar excess of unlabelled s oligonucleotide also competed for the binding of RFX/NF-Y complexes (lanes 3 to 5), which did not occur with the same molar excess of mpBR (lane 6). In the presence of the X box on the labelled sxx2y oligonucleotide, the s oligonucleotide did not compete for this binding (lane 9). Thus, RFX binds to the S and X boxes from the DRA promoter and its binding to the S box is weaker than to the X box. In both cases, the binding of RFX is stabilized by NF-Y.

DNA affinity-purified RFX binds to S and X boxes of the DRA promoter. Although our conditions mimicked those that revealed the functional RFX complex and not the binding of RFX1 to the X box, we still wanted to prove that our complexes on S and X boxes contained RFX5 and not RFX1. To this end, we prepared RFX by DNA affinity chromatography and observed complexes of identical mobilities with s and xx2 oligonucleotides (Fig. 5, lanes 4 and 11). To determine the specificity of this binding, we performed competition experiments with unlabelled xx2 and s oligonucleotides, which contained clustered point mutations in the S box (m3) and its eight 5′-flanking nucleotides (m2) (44). In Fig. 5A (lanes 1 to 6), s and xx2 but not m2 and m3 competed for the binding of RFX to the labelled s oligonucleotide. Slightly different results were obtained with the labelled xx2 oligonucleotide, for which the unlabelled s, m2, and m3 oligonucleotides did not compete (lanes 2, 9, and 10). Identical results were previously observed with RFX1 (reference 44 and data not presented), and so antibodies against RFX1 (18) were included in the binding-

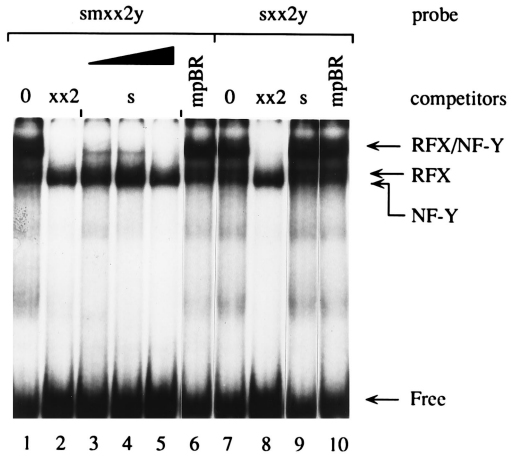

FIG. 4. RFX binds to the S box in the presence of the Y box. Presented are the results of EMSAs with oligonucleotides which contained S, wild-type, or mutated X, X2, and Y sequences (sxx2y and smxx2y) and Raji nuclear extracts. Lanes 1 to 6 and lanes 7 to 10 contain oligonucleotide probes which had a mutation in the X box (smxx2y) and the wild-type X box (sxx2y), respectively. Lanes 1 and 7 also contain nuclear extracts alone, and lanes 2 and 8 additionally contain a 100-fold molar excess of the unlabelled xx2 oligonucleotide as the competitor. Lanes 3 to 5 contain a 100-, 1,000- and 5,000-fold molar excess, respectively, of the unlabelled s oligonucleotide. Lane 9 contains a 5,000-fold molar excess of the s oligonucleotide. Lanes 6 and 10 additionally contain a 5,000-fold molar excess of an irrelevant oligonucleotide (mpBR). Arrows denote the three major complexes (NF-Y, RFX, and RFX/NF-Y) and the free probe.

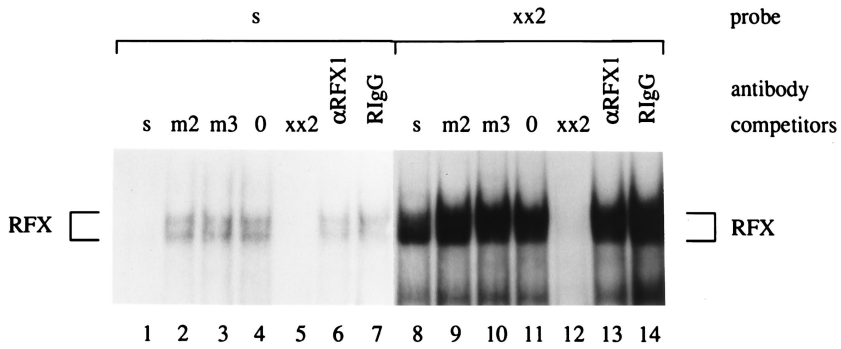

FIG. 5. DNA affinity-purified RFX binds to the S and X boxes in the DRA promoter. RFX binds to the S box and its 5′-flanking sequence (lanes 1 to 7) and to the X box (lanes 8 to 14). Above the lanes are indicated unlabelled oligonucleotides used as the competitor. They were added at a 100-fold molar excess over the labelled probe. In lanes 4 and 11, no competitor was added. Competitors were as follows: s (lanes 1 and 8); m2 and m3, which contained mutations in seven 5′-flanking nucleotides and the s box, respectively (lanes 2, 3, 9, and 10); and xx2 (lanes 5 and 12). In lanes 6 and 13, the same extracts were incubated with an anti-RFX1 antibody raised against the N-terminal 20 amino acids of RFX1 (αRFX1). This antibody causes a supershift of the RFX1 complex. In lanes 7 and 14, an irrelevant rabbit antiserum (RIgG) was used. Arrows denote RFX complexes.

reaction mixture as a control. No modification of the binding was observed in the presence of anti-RFX1 or normal rabbit immunoglobulin antisera (lanes 6 and 7 or 13 and 14). From this analysis, we conclude that RFX binds to the S box and its 5′-flanking sequence.

DNA affinity-purified RFX restores the binding to the X box by SJO nuclear extracts. To prove conclusively that RFX5 was present in our complexes, we also complemented nuclear extracts from SJO cells with DNA affinity-purified RFX fractions. SJO cells belong to complementation group C of the class II bare lymphocyte syndrome, in which the RFX5 gene is mutated and the RFX5 protein is not expressed (36). Therefore, class II promoters are bare in in vivo footprinting analyses and no RFX complexes are observed in EMSAs (20, 28). However, the introduction of the wild-type RFX5 gene into SJO cells and exogenously added RFX5 protein to nuclear extracts restores class II expression and the binding of RFX, respectively (11, 28, 36).

Since cooperative binding was observed between RFX and NF-Y (28) (data in Fig. 3 and 4), we performed EMSAs with the xmx2y oligonucleotide and nuclear extracts from SJO cells. A single shifted complex, corresponding to NF-Y, was observed with these extracts. However, the addition of the DNA affinity-purified RFX to the SJO nuclear extracts resulted in the appearance of a lower-mobility complex, which was identical to that observed with nuclear extracts from Raji cells, in which it was identified as RFX/NF-Y (Fig. 6A, compare lane 2 with lane 5). Thus, our DNA affinity-purified fractions also restored the binding of RFX to nuclear extracts from SJO cells. We also confirmed the presence of RFX5 in DNA affinity-purified fractions by using an antibody against a C-terminal synthetic peptide from RFX5 and Western blotting (Fig. 6B). This antibody reacted with a 75-kDa protein that comigrated with the RFX5 protein translated in the rabbit reticulocyte lysate (Fig. 6B, lanes 2 to 4). From these data and Fig. 5, we conclude that our DNA affinity-purified fraction contains RFX5 and that RFX binds to both S and X boxes.

RFX5-VP16 fusion protein activates expression of the DRA promoter from S and X boxes. To extend our observations on the binding of RFX5 to the S box and to study the relevance of this binding in cells, we expressed a fusion protein between the full-length RFX5 and the activation domain (amino acids 413 to 490) of VP16 from herpes simplex virus (pSVRFX5-VP16) in COS cells (9, 17). The activation domain of VP16 had to be added because RFX5 does not itself contain an activation

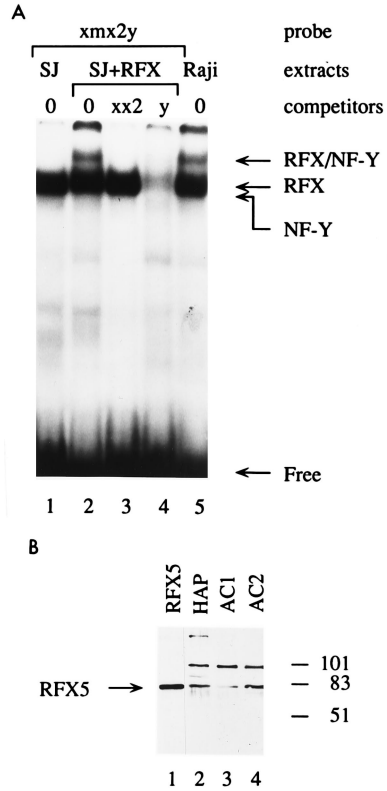

FIG. 6. (A) Complementation of nuclear extracts from SJO cells (an RFX-deficient cell line) with the DNA-affinity purified RFX. EMSAs were performed with the oligonucleotide, which contained a mutation in the X2 box (xmx2y), and nuclear extracts from SJO cells (lane 1), extracts complemented with the affinity-purified RFX (lanes 2 to 4), or nuclear extracts from Raji cells (lane 5). Unlabelled oligonucleotides used as competitors are indicated above the lanes. Arrows indicate RFX, NF-Y, RFX/NF-Y complexes, and the free probe. (B) Western blot analysis of proteins obtained at different steps of RFX purification. Lane 1 represents RFX5 translated in the rabbit reticulocyte system. Lanes 2, 3, and 4 contain 80 μl of 0.2 M salt elution from the hydroxylapatite column (HAP), 20 μl of the first affinity column eluant (AC1), and 8 μl of the second affinity column eluant (AC2), respectively. The arrow denotes the 75-kDa protein that corresponds to RFX5. Molecular weight standards are given to the right of the blot (in thousands).

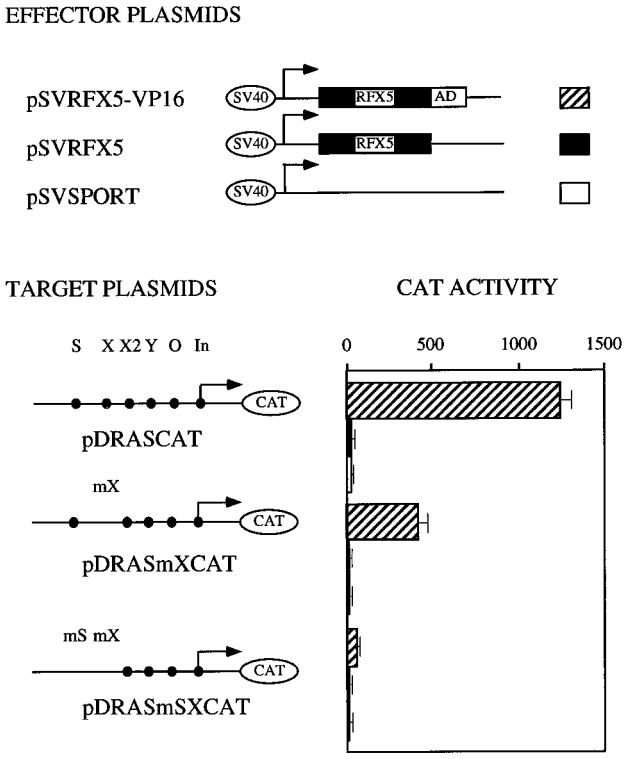

FIG. 7. RFX5-VP16 fusion protein activates expression from the S box of the DRA promoter. A schematic representation of the plasmid effectors used in the assay is shown at the top. pSVRFX5 was created by inserting the full-length RFX5 (amino acids 1 to 616) with the addition of the *myc* epitope tag at the 5′ end into pSVSport-1. pSVRFX5-VP16 is similar to pSVRFX5 except that the activation domain of VP16 (amino acids 413 to 490) was linked to the 3′ end of the RFX5 cDNA. The RFX5-VP16 fusion protein was tested on three targets, the wild-type DRA promoter (pDRASCAT)-mutated X box (pDRASmXCAT) and mutated S and X boxes (pDRAmSXCAT). The absolute CAT values are representative of three transfections done in triplicate, and the standard error of the mean was less than 20%. Bar patterns for each plasmid effector are given at the top right-hand panel.

domain (36) (see below). Effector plasmids coding for RFX5 or the RFX5-VP16 fusion protein were cotransfected into COS cells with target plasmids pDRASCAT, pDRASmXCAT, or pDRASmSXCAT, which contained the wild-type DRA promoter, mutated X box, or mutated S and X boxes, respectively. The ratio of effector to target plasmids was 4:1. Figure 7 depicts the effector plasmids used in this study.

pSVRFX5 had no effect on the expression from any of these plasmids. However, the expression of the RFX5-VP16 fusion protein resulted in 43-fold *trans*-activation of the DRA promoter. Mutation of the X box (pDRASmXCAT) resulted in 70%-decreased effects of pSVRFX-VP16, and the chimera had less than 5% of the wild-type activity on the target plasmid lacking both S and X boxes (pDRASmSXCAT). Since RFX could not bind to the mutated X box (Fig. 5) and since the chimera could not activate a promoter lacking both S and X boxes, the residual activity of the RFX5-VP16 fusion protein on pDRASmXCAT was due to its binding to the S box. Thus, RFX5 binds to the S and X boxes in vitro and in vivo. Finally, since this chimera had no effect on the expression from an irrelevant promoter (pRSVCAT), the effect of the RFX5-VP16 fusion protein was specific to the DRA promoter (data not presented).

DISCUSSION

The S box is required for the optimal expression of class II in B cells and antigen-presenting cells following the administration of IFN-γ. In this study, we demonstrated that the S box is a duplication of the X box and that it binds RFX. Since the spacing between these two boxes could not be changed, RFXs at S and X boxes must also interact directly with each other. Additionally, NF-Y stabilizes the binding of RFX to both the X and S boxes. This finding was demonstrated first in vitro and confirmed in an in vivo binding assay, in which a fusion protein between RFX5 and the activation domain of VP16 could activate expression via the S and X boxes of the DRA promoter in cells. We conclude that RFX also binds to the S box and that complex assembly on CUS requires direct interactions between proteins bound to the S, X, and Y boxes (see Fig. 8).

In the DRA promoter, RFX binds to the sequence TGTG TCCTGGACCCT, which contains the S box and its eight 5′-flanking nucleotides (44) (Fig. 1 and 5). Centered on the thymidine, 5 of 7 nucleotides form a perfect palindromic sequence. However, the conserved sequence of the S box [GGACCT(C/T)], has little similarity to the conserved sequence of the X box [CC(C/T)AG(C/A)(A/G)ACAGATG] (4). Moreover, the 5′-flanking sequence of the S box, which is also required for the binding of RFX to the S box, is even less well conserved among all class II genes. Thus, it is not surprising that RFX binds to the S box much more weakly than to the X box and that both require NF-Y for efficient interactions with DNA. Recently, we obtained evidence that NF-Y not only selects RFX for the binding to the S and X boxes but also prevents the binding of RFX1 homodimers to the same sites (12). However, since p36 is the primary determinant for the binding of RFX, the assignment of exact contact points of RFX on the S box and its 5′-flanking sequence must avait the cloning of p36.

Thus, the S and X boxes represent reiterated motifs in class II promoters (Fig. 8). Optimal binding of RFX and NF-Y to DNA and spacing constraints between the S, X, and Y boxes constitute conserved interactions between RFX, NF-Y, and

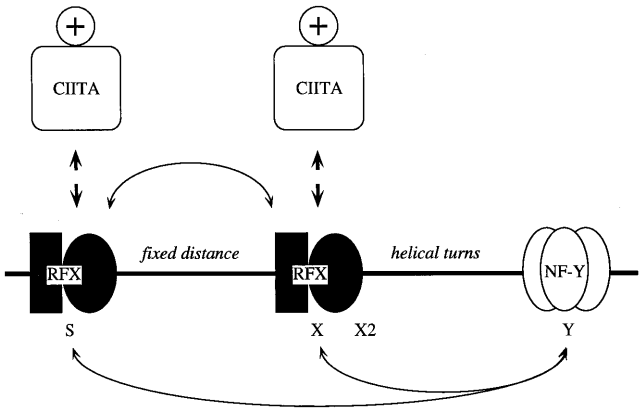

FIG. 8. A model of protein-protein and protein-DNA interactions with CUS from class II promoters. The S, X, and Y boxes are spaced two helical turns from each other. RFX (RFX5-p36 heterodimer) binds to the S and X boxes. NF-Y binds to the Y box. X2BP was not studied and is not depicted. NF-Y selects and stabilizes the binding of RFX to S and X boxes (arrows). RFXs bound to S and X boxes also interact with each other on the DNA (arrows). Presumably, these protein-protein and protein-DNA interactions with CUS increase the subsequent binding of CIITA, which activates the transcription of class II genes. Since binding between CIITA and RFX has not been demonstrated, these contacts are denoted by broken arrows. Circles above CIITA represent the N-terminal activation domain of CIITA. Fixed distance and helical turns refer to invariant spacing between the S and X boxes and the ability of the X and Y boxes to be moved full helical turns away from each other, respectively.

CUS. Placing all three regulatory proteins on the same face of the double helix facilitates their protein-protein and protein-DNA interactions (Fig. 8). In this configuration, RFXs bound to S and X boxes attract the class II transactivator (CIITA), which is required for the subsequent transcriptional activity of class II promoters (5, 38). Interestingly, in this and related studies (2, 44), we found little effect of the X2 box on the binding of RFX to CUS in cells.

Interactions between NF-Y and RFX with CUS in class II promoters represent a new model in eukaryotic transcription. Unlike other systems, in which promoter architecture is required for DNA bending and subsequent precise alignment of regulatory proteins (15, 41), in which multiple proteins juxtaposed to each other either help or prevent the binding of the complex of proteins to DNA (40, 42, 51), or in which cooperative interactions between proteins bound to DNA strengthen interactions with cooactivators and general transcription factors (15, 41, 42), in class II promoters, proteins bound to separated but precisely aligned sequences increase their binding to those sites. In these promoters, NF-Y increases the binding of RFX to the S and X boxes, and they are two and four helical turns removed from the Y box, respectively. NF-Y also selects against the binding of RFX1 and for the binding of RFX to these sequences in cells (12). Thus, the duplication of RFXs on class II promoters promotes interactions with CIITA, which is required for the transcription of class II genes (Fig. 8). The end result of these interactions is the exquisite control of the expression of class II determinants on the cell surface, which is sufficient for antigen processing and presentation on antigen-presenting cells and for T-cell help on B cells bearing low-affinity immunoglobulin M receptors for the foreign protein (1, 2, 4, 8, 10, 13, 24).

ACKNOWLEDGMENTS

We acknowledge Michael Armanini for his expert secretarial assistance and Charles Toth for many helpful suggestions and comments on the manuscript.

Nabila Jabrane-Ferrat has been funded in part by a fellowship from American Heart Association and the Treadwell Foundation. Jeremy Boss is funded by grants from the NIH (AI34000 and GM47310).

REFERENCES

1. **Abdulkadir, S. A., and S. J. Ono.** 1995. How are class II MHC genes turned on and off? FASEB J. **9:**1429–1435.
2. **Accolla, R. S., M. D. Scupoli, C. Cambiaggi, G. Tosi, and S. Sartoris.** 1991. Cell lineage-specific and developmental stage-specific controls of MHC class-II-antigen expression. Int. J. Cancer **6**(Suppl.)**:**20–25.
3. **Andersson, G., and B. M. Peterlin.** 1990. NF-X2 that binds to the DRA X2-box is activator protein 1. Expression cloning of c-Jun. J. Immunol. **145:** 3456–3462.
4. **Benoist, C., and D. Mathis.** 1990. Regulation of major histocompatibility complex class-II genes: X, Y, and other letters of the alphabet. Annu. Rev. Immunol. **8:**681–715.
5. **Chang, C. H., J. D. Fontes, B. M. Peterlin, and R. A. Flavell.** 1994. Class II transactivator (CIITA) is sufficient for the inducible expression of major histocompatibility complex class II genes. J. Exp. Med. **180:**1367–1374.
6. **Cogswell, J. P., J. Austin, and J. P. Ting.** 1991. The W element is a positive regulator of HLA-DRA transcription in various DR+ cell types. J. Immunol. **146:**1361–1367.
7. **Cogswell, J. P., P. V. Basta, and J. P. Ting.** 1990. X-box-binding proteins positively and negatively regulate transcription of the HLA-DRA gene through interaction with discrete upstream W and V elements. Proc. Natl. Acad. Sci. USA **87:**7703–7707.
8. **Cogswell, J. P., N. Zeleznik-Le, and J. P. Ting.** 1991. Transcriptional regulation of the HLA-DRA gene. Crit. Rev. Immunol. **11:**87–112.
9. **Cress, W. D., and S. J. Triezenberg.** 1991. Critical structural elements of the VP16 transcriptional activation domain. Science **251:**87–90.
10. **Cresswell, P.** 1994. Assembly, transport, and function of MHC class II molecules. Annu. Rev. Immunol. **12:**259–293.
11. **Durand, B., M. Kobr, W. Reith, and B. Mach.** 1994. Functional complementation of major histocompatibility complex class II regulatory mutants by the purified X-box-binding protein RFX. Mol. Cell. Biol. **14:**6839–6847.
12. **Fontes, J. D., and B. M. Peterlin.** Unpublished data.
13. **Glimcher, L. H., and C. J. Kara.** 1992. Sequences and factors: a guide to MHC class-II transcription. Annu. Rev. Immunol. **10:**13–49.
14. **Griffin, G. E., K. Leung, T. M. Folks, S. Kunkel, and G. J. Nabel.** 1991. Induction of NF-κB during monocyte differentiation is associated with activation of HIV-gene expression. Res. Virol. **142:**233–238.
15. **Grosschedl, R.** 1995. Higher-order nucleoprotein complexes in transcription: analogies with site-specific recombination. Curr. Opin. Cell Biol. **7:**362–370.
16. **Hasegawa, S. L., and J. M. Boss.** 1991. Two B cell factors bind the HLA-DRA X box region and recognize different subsets of HLA class II promoters. Nucleic Acids Res. **19:**6269–6276.
17. **Hayes, S., and P. O'Hare.** 1993. Mapping of a major surface-exposed site in herpes simplex virus protein Vmw65 to a region of direct interaction in a transcription complex assembly. J. Virol. **67:**852–862.
18. **Jabrane-Ferrat, N., and B. M. Peterlin.** 1994. Ets-1 activates the DRA promoter in B cells. Mol. Cell. Biol. **14:**7314–7321.
19. **Kappes, D., and J. L. Strominger.** 1988. Human class II major histocompatibility complex genes and proteins. Annu. Rev. Biochem. **57:**991–1028.
20. **Kara, C. J., and L. H. Glimcher.** 1991. In vivo footprinting of MHC class II genes: bare promoters in the bare lymphocyte syndrome. Science **252:**709–712.
21. **Liou, H. C., M. R. Boothby, P. W. Finn, R. Davidon, N. Nabavi, N. J. Zeleznik-Le, J. P. Ting, and L. H. Glimcher.** 1990. A new member of the leucine zipper class of proteins that binds to the HLA DR α promoter. Science **247:**1581–1584.
22. **Mantovani, R., L. Tora, V. Moncollin, J. M. Egly, C. Benoist, and D. Mathis.** 1993. The major histocompatibility complex (MHC) Eα promoter: sequences and factors at the initiation site. Nucleic Acids Res. **21:**4873–4878.
23. **Moreno, C. S., P. Emery, J. E. West, B. Durand, W. Reith, B. Mach, and J. M. Boss.** 1995. Purified X2 binding protein (X2BP) cooperatively binds the class II MHC X box region in the presence of purified RFX, the X box factor deficient in the bare lymphocyte syndrome. J. Immunol. **155:**4313–4321.
24. **Peterlin, B. M., G. Andersson, E. Lotscher, and S. Tsang.** 1990. Transcriptional regulation of HLA class-II genes. Immunol. Res. **9:**164–177.
25. **Reith, W., E. Barras, S. Satola, M. Kobr, D. Reinhart, C. H. Sanchez, and B. Mach.** 1989. Cloning of the major histocompatibility complex class II promoter binding protein affected in a hereditary defect in class II gene regulation. Proc. Natl. Acad. Sci. USA **86:**4200–4204.
26. **Reith, W., C. Herrero-Sanchez, M. Kobr, P. Silacci, C. Berte, E. Barras, S. Fey, and B. Mach.** 1990. MHC class II regulatory factor RFX has a novel DNA-binding domain and a functionally independent dimerization domain. Genes Dev. **4:**1528–1540.
27. **Reith, W., M. Kobr, P. Emery, B. Durand, C. A. Siegrist, and B. Mach.** 1994. Cooperative binding between factors RFX and X2bp to the X and X2 boxes of MHC class II promoters. J. Biol. Chem. **269:**20020–20025.
28. **Reith, W., C. A. Siegrist, B. Durand, E. Barras, and B. Mach.** 1994. Function of major histocompatibility complex class II promoters requires cooperative binding between factors RFX and NF-Y. Proc. Natl. Acad. Sci. USA **91:** 554–558.
29. **Reith, W., V. Steimle, and B. Mach.** 1995. Molecular defects in the bare lymphocyte syndrome and regulation of MHC class II genes. Immunol. Today **16:**539–546.
30. **Schwartz, R. H.** 1985. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. Annu. Rev. Immunol. **3:**237–261.
31. **Schwartz, R. H.** 1986. Immune response (Ir) genes of the murine major histocompatibility complex. Adv. Immunol. **38:**31–201.
32. **Servenius, B., L. Rask, and P. A. Peterson.** 1987. Class II genes of the human major histocompatibility complex. The DOβ gene is a divergent member of the class II β gene family. J. Biol. Chem. **262:**8759–8766.
33. **Shapiro, D. J., P. A. Sharp, W. W. Wahli, and M. J. Keller.** 1988. A high-efficiency HeLa cell nuclear transcription extract. DNA **7:**47–55.
34. **Sherman, P. A., P. V. Basta, A. Heguy, M. K. Wloch, R. G. Roeder, and J. P. Ting.** 1989. The octamer motif is a B-lymphocyte-specific regulatory element of the HLA-DRα gene promoter. Proc. Natl. Acad. Sci. USA **86:**6739–6743.
35. **Siegrist, C. A., B. Durand, P. Emery, E. David, P. Hearing, B. Mach, and W. Reith.** 1993. RFX1 is identical to enhancer factor C and functions as a transactivator of the hepatitis B virus enhancer. Mol. Cell. Biol. **13:**6375–6384.
36. **Steimle, V., B. Durand, E. Barras, M. Zufferey, M. R. Hadam, B. Mach, and W. Reith.** 1995. A novel DNA-binding regulatory factor is mutated in primary MHC class II deficiency (bare lymphocyte syndrome). Genes Dev. **9:**1021–1032.
37. **Steimle, V., L. A. Otten, M. Zufferey, and B. Mach.** 1993. Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). Cell **75:**135–146.
38. **Steimle, V., C. A. Siegrist, A. Mottet, B. Lisowska-Grospierre, and B. Mach.** 1994. Regulation of MHC class II expression by interferon-γ mediated by the transactivator gene CIITA. Science **265:**106–109.
39. **Stimac, E., S. Lyons, and D. Pious.** 1988. Transcription of HLA class II genes

in the absence of B cell-specific octamer-binding factor. Mol. Cell. Biol. **8:**3734–3739.
40. **Sucov, H. M., and R. M. Evans.** 1995. Retinoic acid and retinoic acid receptors in development. Mol. Neurobiol. **10:**169–184.
41. **Thanos, D., and T. Maniatis.** 1995. Virus induction of human IFN β gene expression requires the assembly of an enhanceosome. Cell **83:**1091–1100.
42. **Treisman, R.** 1995. Journey to the surface of the cell: Fos regulation and the SRE. EMBO J. **14:**4905–4913.
43. **Tsang, S. Y., M. Nakanishi, and B. M. Peterlin.** 1988. B cell-specific and interferon-γ-inducible regulation of the HLA-DRα gene. Proc. Natl. Acad. Sci. USA **85:**8598–8602.
44. **Tsang, S. Y., M. Nakanishi, and B. M. Peterlin.** 1990. Mutational analysis of the DRA promoter: *cis*-acting sequences and *trans*-acting factors. Mol. Cell. Biol. **10:**711–719.
45. **van Huijsduijnen, R. H., X. Y. Li, D. Black, H. Matthes, C. Benoist, and D. Mathis.** 1990. Co-evolution from yeast to mouse: cDNA cloning of the two NF-Y (CP-1/CBF) subunits. EMBO J. **9:**3119–3127.
46. **Vilen, B. J., J. P. Cogswell, and J. P.-Y. Ting.** 1991. Stereospecific alignment of the X and Y elements is required for major histocompatibility complex class II DRA promoter function. Mol. Cell. Biol. **11:**2406–2415.
47. **Vilen, B. J., J. F. Penta, and J. P. Ting.** 1992. Structural constraints within a trimeric transcriptional regulatory region. Constitutive and interferon-γ-inducible expression of the HLA-DRA gene. J. Biol. Chem. **267:**23728–23734.
48. **Viville, S., V. Jongeneel, W. Koch, R. Mantovani, C. Benoist, and D. Mathis.** 1991. The E α promoter: a linker-scanning analysis. J. Immunol. **146:**3211–3717.
49. **Voliva, C. F., A. Aronheim, M. D. Walker, and B. M. Peterlin.** 1992. B-cell factor 1 is required for optimal expression of the DRA promoter in B cells. Mol. Cell. Biol. **12:**2383–2390.
50. **Voliva, C. F., N. Jabrane-Ferrat, and B. M. Peterlin.** 1996. The function of the octamer binding site in the DRA promoter. Immunogenetics **43:**20–26.
51. **Yu, V. C., A. M. Naar, and M. G. Rosenfeld.** 1992. Transcriptional regulation by the nuclear receptor superfamily. Curr. Opin. Biotechnol. **3:**597–602.
52. **Zhang, X. Y., N. Jabrane-Ferrat, C. K. Asiedu, S. Samac, B. M. Peterlin, and M. Ehrlich.** 1993. The major histocompatibility complex class II promoter-binding protein RFX (NF-X) is a methylated DNA-binding protein. Mol. Cell. Biol. **13:**6810–6818.