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The DNA-Binding Defect Observed in Major Histocompatibility Complex Class II Regulatory Mutants Concerns Only One Member of a Family of Complexes Binding to the X Boxes of Class II Promoters

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The X box of major histocompatibility complex class II promoters is essential for proper expression of class II genes. Here we show that two distinct protein-DNA complexes (A and B), which exhibit similar binding characteristics and identical contact points on the X box, can be formed. This suggests the existence of a family of related X box-binding factors. Complex B (and not complex A) is specifically affected in primary combined immunodeficiency, a congenital defect in class II gene regulation. RFX1, the first X box-binding protein cloned, encodes a functionally relevant factor present in complex A and not in complex B as originally suspected. This report also illustrates the need for caution in correlating specific cloned proteins with nuclear factors identified by DNA-binding assays, particularly when dealing with families of related proteins.

Expression of major histocompatibility complex (MHC) class II genes is strictly regulated. Only a restricted number of cell types, such as B lymphocytes, express class II genes constitutively, while in certain other cells these genes can be induced by specific stimuli (3, 6, 14). The level of expression of MHC class II molecules directly affects the ability of a cell to stimulate T lymphocytes (19), and regulation of MHC class II gene expression is therefore essential for control of the immune response.

The promoter-proximal region of MHC class II genes contains *cis*-acting sequences that are required for both tissue-specific expression and inducible expression (3, 14). These include the highly conserved X and Y boxes of MHC class II promoters, as well as a 12-*O*-tetradecanylphorbol-13-acetate response element (TRE)- or cyclic AMP response element-like sequence also referred to as the X2 box. Protein factors that can bind to these promoter sequences have been identified and cloned. They include the NF-Y heterodimer, a CCAAT-binding factor that binds to the MHC class II Y box (9), and several leucine zipper proteins that bind to the TRE-cyclic AMP response element-like elements located immediately 3′ of the MHC class II X box motif (1, 11, 18, 21, 22). The X box motif is functionally essential for class II gene regulation, and protein-DNA-binding experiments had previously allowed us to identify a single complex (RFX) involving the X box (23, 25). RFX is of particular interest, since its binding to the X box is specifically affected in cells from patients with human leukocyte antigen (HLA) class II-deficient combined immunodeficiency (CID) (15, 23, 25, 28), a disease known to be due to a defect in a *trans*-acting factor that controls MHC class II gene expression (5). Other groups, however, have reported X box-binding complexes that were not affected in this disease (10, 17). Clarification of this apparent discrepancy was therefore desirable and represented one of the objectives of this study.

A protein with DNA-binding features identical to those of complex RFX was cloned (23). This cloned protein will henceforth be referred to as RFX1 to distinguish it from other, related RFX proteins (see Discussion). RFX1 contains a novel DNA-binding domain (24) and is required for induction of class II genes by gamma interferon (24, 27a). Many transcription factors belong to families of homologous proteins which can bind to the same or related DNA sequences (8, 12, 16, 20, 26). We therefore searched for nuclear proteins that bind to the X box of MHC class II promoters to (i) document the possible existence of other, related X box-binding factors, (ii) determine whether their binding is also affected in class II regulatory mutants, (iii) establish a relationship between such factors and the cloned RFX1 gene, and (iv) clarify apparently conflicting results obtained in different laboratories concerning X box-binding proteins in CID patients.

By modifying the binding conditions used for the electrophoretic mobility shift assay (EMSA), we can detect, in addition to the previously identified RFX (referred to here as complex B), another nuclear factor (complex A), which has binding characteristics indistinguishable from those observed for complex B and for the cloned RFX1 protein. This second complex is clearly distinct from complex B because it is normal in CID cells, in which complex B is not formed. We also show that, contrary to what was proposed earlier (23), the cloned RFX1 gene does not correspond to complex B, and thus to the factor affected in CID, but encodes the factor responsible for complex A. On the basis of these results and unpublished sequence data, we propose the existence of a novel family of functionally relevant X box-binding factors which recognize the same MHC class II X box target sequence.

MATERIALS AND METHODS

Cell lines and nuclear extracts. The Burkitt lymphoma cell line Raji; the class II-positive Epstein-Barr virus-transformed B-cell lines Mann, HHK, and QBL; and the class II-negative Epstein-Barr virus-transformed CID B-cell lines Nacera, Ramia (both provided by B. Lisowska-Grospierre and C. Griscelli), Robert, Abdulla, Sabrina (all three pro-

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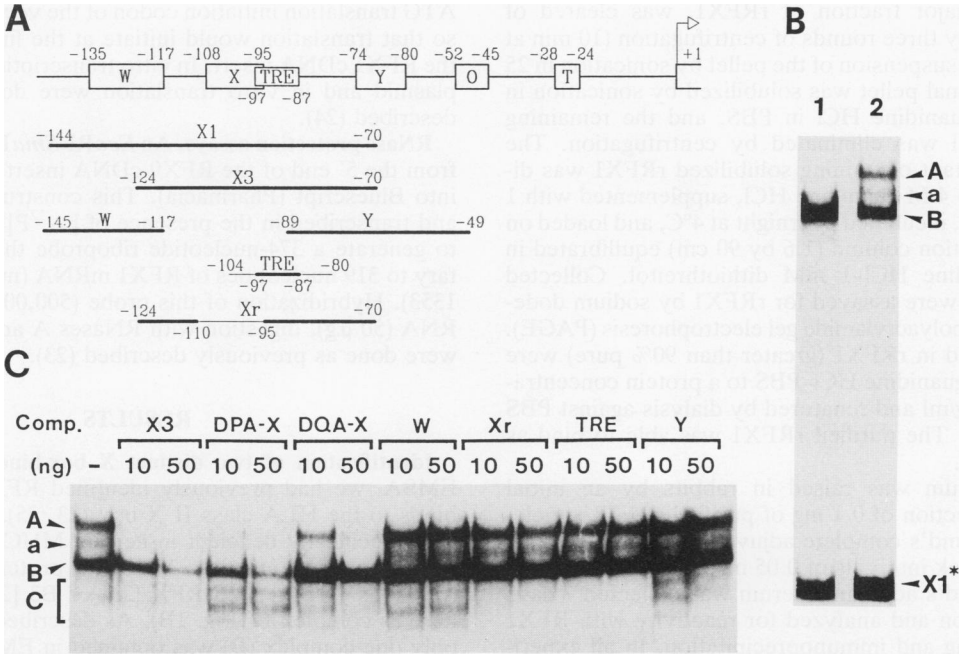

FIG. 1. Two distinct protein complexes bind to the X box of the HLA-DRA promoter. (A) Map of the HLA-DRA promoter. The conserved *cis*-acting elements W, X, TRE, Y, octamer (O), and TATA (T) are indicated. Double-stranded oligonucleotides used in this study are shown below the promoter. Thick lines represent sequences derived from the DRA promoter, and thin lines represent random sequences. (B) Effect of binding conditions on the binding of proteins to the DRA X box. EMSA was performed with Cohen nuclear extract and oligonucleotide X1. Under binding conditions I (lane 1), only complex B was detected, while under conditions II, two additional complexes (A and a) were formed. Binding reactions contained an excess of TRE oligonucleotide to eliminate complexes due to binding of proteins to the TRE sequence adjacent to the X box. Binding conditions I and II are detailed in Materials and Methods. (C) Specificity of complexes (Comp.) A, a, and B. EMSA was performed under binding conditions II with Cohen nuclear extract and oligonucleotide X1. Binding reactions contained 10 or 50 ng of the competitor oligonucleotides indicated. X3, W, Xr, TRE, and Y oligonucleotides are shown in panel A. The DPA and DQA X box oligonucleotides are described in Materials and Methods. Complexes due to binding of proteins to the X box (A, a, and B) and the TRE (C) are indicated.

vided by M. R. Hadam), and SJO (provided by J. Gorski) were grown as previously described (25). Blanco and Cohen B cells were obtained by leukopheresis of blood from patients with chronic B-cell lymphocytic leukemia. Nuclear extracts were prepared as previously described (27).

Oligonucleotides. Oligonucleotides were synthesized and annealed as previously described (23–25). Oligonucleotides X1, X3, W, and Y contain nucleotides −114 to −70, −124 to −70, −145 to −117, and −89 to −49, respectively, of the HLA-DRA promoter (Fig. 1A). The DQA-X and DPA-X oligonucleotides contain nucleotides −173 to −119 and −197 to −143 of the HLA-DQA and HLA-DPA promoters. The Xr oligonucleotide is identical to X3, except that the X box is replaced by a random sequence (23). The TRE oligonucleotide (gtcagtcATGCGTCATCTagtcag) contains the AP1 site situated 3′ to the X box of the HLA-DRA promoter, which is flanked by random sequences.

EMSA and methylation interference assay. Two different binding conditions were used for the EMSA. Binding conditions I were described previously (23–25). Binding conditions II were as follows. A 2- to 4-μg sample of nuclear extract or a 1- to 2-μl sample of in vitro translation products was incubated with 0.2 to 0.5 ng (20,000 cpm) of ^{32}P-labelled oligonucleotides for 30 min at 20°C in a 20-μl reaction containing 250 to 500 ng of poly(dI-dC) · poly(dI-dC); 125 to 250 ng of sonicated, denatured *Escherichia coli* DNA; 7% glycerol; 45 mM KCl; 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.9; 0.07 mM EDTA, 0.18 mM dithiothreitol; and 0.75 mM $MgCl_2$. Probes were added last, after 5 min of preincubation at 0°C. Only where indicated (Fig. 1B) was an excess of cold TRE oligonucleotide added to optimize detection of bands A, a, and B by eliminating complexes due to binding of proteins to the TRE sequence situated immediately 3′ of the X box. Competitor oligonucleotides were added during the 5-min preincubation at 0°C prior to addition of probes. Protein-DNA complexes were resolved by polyacrylamide gel electrophoresis as previously described (23–25). To test the reactivity of αRFX1 antiserum, 1 μl of a 1/10 dilution of antiserum was added after the 30-min binding reaction and the incubation was continued for 15 min at 0°C. Affinity-purified αRFX1 antibodies in 10% fetal calf serum (FCS) and anti-β-galactosidase antibodies in 10% FCS (Cappel) were used in the EMSA as previously described (24).

Methylation interference experiments were done as previously described (15), except that binding conditions II were used.

Production of αRFX1 antiserum. *E. coli* BL21(DE3)pLysS (29), containing plasmid pRFX9 (24), was grown to an optical density at 600 nm of 0.5 to 1.0 and induced for 3 h by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.4 mM. Purification of recombinant RFX1 (rRFX1) was done as follows for a 500-ml culture. All steps were done at 0 to 4°C. Bacteria were pelleted and lysed by sonication in 50 ml of phosphate-buffered saline (PBS; 10 mM $NaPO_4$ [pH 7.4], 150 mM NaCl). Insoluble material,

containing the major fraction of rRFX1, was cleared of soluble proteins by three rounds of centrifugation (10 min at 10,000 × *g*) and resuspension of the pellet by sonication in 25 ml of PBS. The final pellet was solubilized by sonication in 2.5 ml of 6 M guanidine HCl in PBS, and the remaining insoluble material was eliminated by centrifugation. The resulting supernatant containing solubilized rRFX1 was diluted with PBS to 4 M guanidine HCl, supplemented with 1 mM dithiothreitol, incubated overnight at 4°C, and loaded on an S-300 gel filtration column (1.6 by 90 cm) equilibrated in PBS–4 M guanidine HCl–1 mM dithiothreitol. Collected fractions (2.5 ml) were assayed for rRFX1 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Fractions enriched in rRFX1 (greater than 90% pure) were diluted with 4 M guanidine HCl–PBS to a protein concentration below 0.2 mg/ml and renatured by dialysis against PBS (three times 2 h). The purified rRFX1 was able to bind as judged by EMSA.

αRFX1 antiserum was raised in rabbits by an initial subcutaneous injection of 0.1 mg of purified rRFX1 supplemented with Freund's complete adjuvant, followed by four injections at 3-week intervals of 0.05 mg supplemented with incomplete Freund's adjuvant. Serum was collected 7 days after each injection and analyzed for reactivity with RFX1 by immunoblotting and immunoprecipitation. In all experiments, this crude antiserum was used without further treatment, except for one experiment (see Fig. 4B), in which affinity-purified αRFX1 antibodies were used as previously described (24).

Immunoblotting and immunoprecipitation of protein-DNA complexes. Immunoblotting was done as previously described (4), except that nuclear extracts were resolved on SDS–10% PAGE and the serum was used at a dilution of 1/1,000. Immunoprecipitation of protein-DNA complexes was performed as previously described (24), by using binding conditions II and the crude antiserum at a concentration at which no cross-reactivity with complex B was detected in the EMSA.

Immunodepletion. A 120-μg sample of Cohen nuclear extract was diluted to 1 μg/μl with buffer ImmD (140 mM KCl, 30 mM HEPES [pH 7.9], 3mM $MgCl_2$) and incubated three times for 40 min each time at 4°C with 25 μl of protein A-Sepharose beads previously loaded with 8 μl of crude serum. Treated extracts were aliquoted and stored at −80°C. Protein concentration was determined by the Bradford assay (Bio-Rad) as described by the manufacturer.

Labeling of cells and immunoprecipitation. Cells (10^7) were labeled for 8 h in methionine-free medium containing 0.5% FCS and 10 μCi of [^{35}S]methionine per ml (>1,000 Ci/mmol, 15 mCi/ml; Amersham). Cells were harvested, washed with PBS, suspended in 0.5 ml of RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris [pH 8.3], 2 mM phenylmethylsulfonyl fluoride), sonicated, and immediately used for immunoprecipitation. Cell extracts were cleared twice, first with 20 μl of preimmune serum (2 h) and then with 30 μl of protein A-Sepharose beads (2 h), divided in half, and treated with 5 μl of either preimmune or αRFX1 serum for 2 h at 4°C and then with 15 μl of beads for 2 h. Beads were washed four times with 1 ml of buffer W (1 M NaCl, 5 mM EDTA, 50 mM Tris [pH 8.3], 0.5% Nonidet P-40) and once with PBS. Immunoprecipitated proteins were resolved on SDS–10% PAGE. Gels were fluorographed, dried, and exposed for 1 to 2 days.

In vitro transcription and translation. A full-length RFX1 cDNA clone was reconstructed from the overlapping RFX5′ and RFX9 cDNAs (24) and inserted into pT7-7 (30). The ATG translation initiation codon of the vector was destroyed so that translation would initiate at the first ATG codon in the RFX1 cDNA insert. In vitro transcription of the resulting plasmid and in vitro translation were done as previously described (24).

RNase protection assays. An *Eco*RI-*Sma*I fragment derived from the 5′ end of the RFX9 cDNA insert (24) was inserted into Bluescript (Pharmacia). This construct was linearized and transcribed in the presence of [α-^{32}P]UTP (Amersham) to generate a 374-nucleotide riboprobe that is complementary to 319 nucleotides of RFX1 mRNA (nucleotides 1034 to 1353). Hybridization of this probe (500,000 cpm) with total RNA (50 μg), digestion with RNases A and T1, and PAGE were done as previously described (23).

RESULTS

Identification of two distinct X box-binding proteins. By EMSA, we had previously identified RFX, a protein that binds to the HLA class II X box (23, 25). Binding of RFX was specifically deficient in certain MHC class II-negative regulatory mutants (23, 25). This protein-DNA complex (previously designated RFX [23] or B3 [25]) is referred to here as complex B (Fig. 1B). As described previously (23), only one complex (B) was obtained in EMSA by using our standard binding conditions (Materials and Methods, conditions I) and an oligonucleotide (X1) covering only the W-X-TRE region of the DRA promoter (Fig. 1B, lane 1). When the binding conditions used to obtain complex B were modified (Materials and Methods, conditions II), two new complexes with slower mobility were detected; they are referred to as complexes A and a (Fig. 1B, lane 2). Optimal conditions for complexes A and a were obtained by lowering the $MgCl_2$ concentration from 5 to 0.75 mM and raising the binding temperature from 0 to 20°C. In addition, inclusion of a TRE oligonucleotide as a cold competitor in the binding reaction (Fig. 1A) avoided confusion with complexes resulting from binding of proteins to the TRE motif situated next to the X box (Fig. 1C). The weaker band called complex a had a behavior identical to that of complex A in all of the experiments presented here, and all results concerning complex A therefore also apply to complex a.

To define more accurately the binding sites in complexes A and B, an EMSA was performed with the X1 oligonucleotide in the presence of competitor oligonucleotides covering the W, X, TRE, and Y boxes of the HLA-DRA promoter (Fig. 1A shows the exact coordinates of the oligonucleotides used). The fact that only oligonucleotide X3 successfully competed against both complexes A and B demonstrated that both complexes were due to binding of proteins to the X box (Fig. 1C). This was confirmed by the finding that oligonucleotide Xr, which is identical to X3 except that the X box is randomized, failed to compete for the formation of complexes A and B (Fig. 1C). Two weaker bands migrating below complex B were eliminated specifically by oligonucleotides containing the TRE motif (X3, Xr, and TRE) and thus represent binding of proteins to the TRE motif situated 3′ of the X box (Fig. 1C, complexes C).

The gradient of affinity of complex A for the X boxes of the HLA-DRA, HLA-DPA, and HLA-DQA promoters was the same as that previously observed for complex B (15, 23). Thus, the affinity of complex A was also DRA > DPA > DQA (Fig. 1C). The affinity for the X box of DQA was slightly weaker for complex B than for complex A (Fig. 1C, lane DQA-X).

Contact points on the class II X box were next analyzed

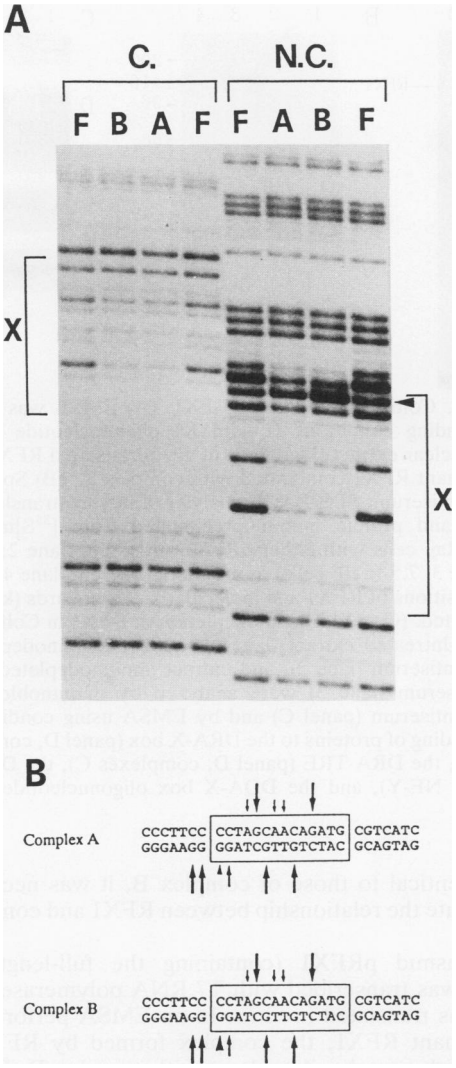

FIG. 2. Proteins in complexes A and B have identical contact points with the X box. (A) Contact points in complexes A and B were analyzed by methylation interference experiments. Profiles obtained on the coding (C.) and noncoding (N.C.) strands of the X1 oligonucleotide are shown for free DNA (F) and DNA bound by protein in complexes A and B. The X box is indicated by brackets. The arrowhead indicates a nucleotide at which methylation enhances binding, the effect being stronger for complex B than for A. (B) Schematic representation of the combined results obtained for complexes A and B in two independent experiments. Arrows indicate nucleotides at which methylation interferes with binding, and arrowheads indicate nucleotides at which methylation enhances binding. The magnitudes of the effects are approximated by the relative sizes of the arrows and arrowheads.

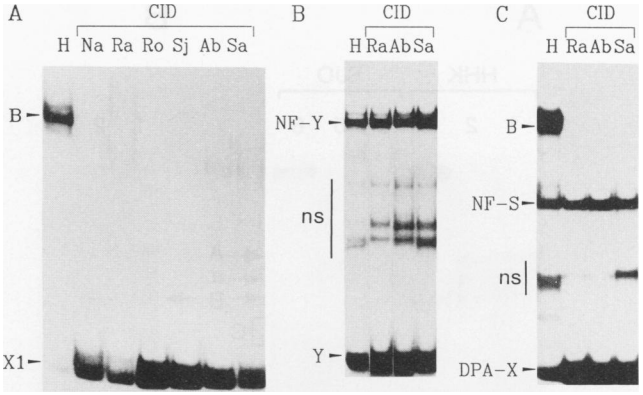

FIG. 3. Complex B is missing in six different CID B-cell lines. (A) EMSA was performed under conditions I with the X1 oligonucleotide and nuclear extracts from normal B-cell line HHK (H) or CID B-cell lines Nacera (Na), Ramia (Ra), Robert (Ro), SJO (Sj), Abdulla (Ab), and Sabrina (Sa). Complex B is indicated. (B) EMSA with a DRA Y box oligonucleotide and nuclear extracts as in panel A. The complex resulting from binding of NF-Y is indicated. (C) EMSA with a DPA-X box oligonucleotide and nuclear extracts as in panel A. Complexes B and NF-S are indicated. Nonspecific bands (ns) are indicated in panels B and C.

more precisely for both complexes A and B by methylation interference assays (Fig. 2). The contact points observed for complexes A and B were identical: methylation of two guanines and three adenines on the coding strand and of five guanines (two of them just upstream of the X box) on the noncoding strand of the X box inhibited binding. Methylation of the first guanine on the noncoding strand of the X box enhanced binding in the case of both complexes A and B (although to a lesser extent for complex A than for complex B). This methylation interference profile is identical to those previously published for complex B (23, 24), with minor differences in intensity attributable to experimental variability. On the basis of the experiments described above, we conclude that at least two distinct complexes can be formed on the X box and that the proteins involved in these complexes have essentially identical binding characteristics.

Only complex B is defective in CIDs. We have shown previously that formation of complex B was specifically affected in extracts from B-cell lines from HLA class II-negative CID patients, while no binding defect was observed with these extracts in the case of other class II promoter-binding proteins (23, 25). Here we extend this finding to additional B-cell lines from three other CID patients. Nuclear extracts from a normal B-cell line (HHK) and from the six CID B-cell lines were first analyzed by EMSA by using binding conditions I to favor complex B. Whereas the complexes corresponding to NF-Y (9, 25) and NF-S (15) were detected with both normal and CID B-cell nuclear extracts, complex B was absent in all six CID cell lines (Fig. 3). Under a variety of conditions, we have never detected complex B in any of these CID lines. Bands of higher mobility were nonspecific and varied in number and intensity, depending on the cell line, irrespectively of whether they were normal or CID (Fig. 3 and 4), the probe used (Fig. 3), and binding conditions (Fig. 4). These data confirm and extend the earlier finding of a specific defect in an X box-binding protein in this hereditary disease made by us (15, 23, 25) and others (28). Absence of complex B in CID cells is not due to enhanced degradation of RFX during preparation of the nuclear extracts or to dominant inhibition of RFX binding: mixing of normal B cells (Mann) and CID B cells (Robert) before preparation of the nuclear extract did not affect the RFX binding activity of normal B cells (data not shown). This is in agreement with the recessive nature of the regulatory defect in CID patients, as concluded from family studies (5, 7) and cell fusion experiments (2, 10).

We next studied complex A in CID cells by using the appropriate binding conditions II. In contrast to complex B, complex A is formed normally with extracts from all of the

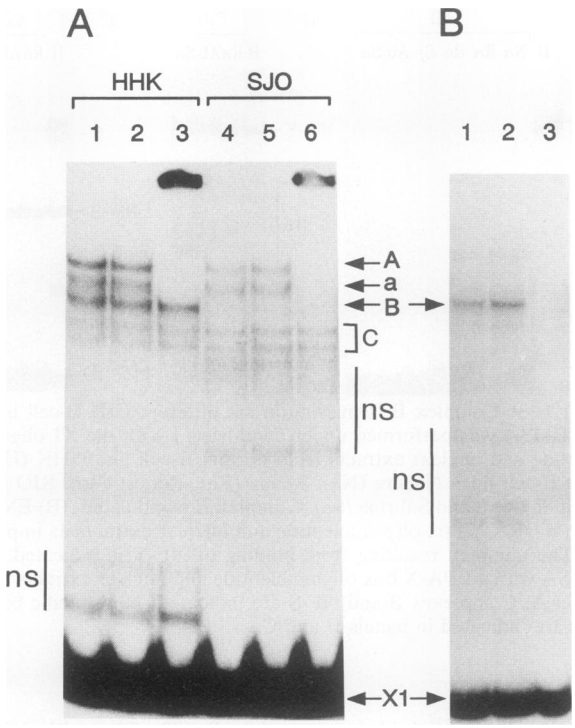

FIG. 4. Complexes A and a are present in CID B-cell lines and react preferentially with αRFX1 antiserum. (A) EMSA was performed under binding conditions II with the X1 oligonucleotide and nuclear extracts from B-cell line HHK (lanes 1 to 3) and CID B-cell line SJO (lanes 4 to 6). After binding, the 20-μl reactions were supplemented with 1 μl of H_2O (lanes 1 and 4), 1 μl of a 1/10 dilution of preimmune serum (lanes 2 and 5), or 1 μl of a 1/10 dilution of αRFX1 antiserum and incubated on ice for 15 min prior to gel electrophoresis. Complex B was detected in HHK but not in SJO, while complexes A, a, and C were present in both cell lines. Only complexes A and a were affected by αRFX1 antiserum (lanes 3 and 6). Nonspecific bands (ns) in HHK and SJO are indicated. (B) Cross-reactivity of affinity-purified αRFX1 antibodies with complex B. Oligonucleotide X1 was incubated with a nuclear extract from Blanco B cells under binding conditions I. Binding reactions were then supplemented with 10% FCS (lane 1), 1 μg of anti-β-galactosidase antibodies in 10% FCS (lane 2), or 1 μg of affinity-purified αRFX1 antibodies in 10% FCS (lane 3) prior to gel analysis. Complex B and nonspecific (ns) bands are indicated.

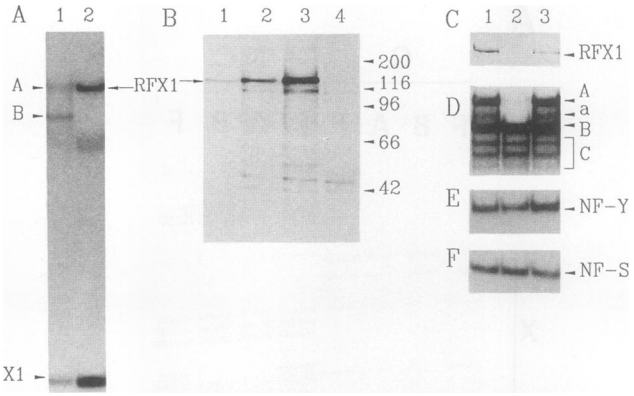

FIG. 5. Complex A contains RFX1. (A) EMSA was performed under binding conditions II with X1 oligonucleotide and either Cohen nuclear extract (lane 1) or in vitro-translated RFX1 (lane 2). Recombinant RFX1 comigrated with complex A. (B) Specificity of αRFX1 antiserum. SDS-PAGE analysis of in vitro-translated RFX1 (lane 1) and protein immunoprecipitated from [^{35}S]methionine-labelled Raji cells with either αRFX1 antiserum (lane 2, 2.5×10^6 cells; lane 3, 7.5×10^6 cells) or preimmune serum (lane 4, 2.5×10^6 cells). Positions of RFX1 and molecular size standards (kilodaltons) are indicated. (C to F) Immunodepletion of RFX1 in Cohen nuclear extract. Untreated extract (lane 1), extract immunodepleted with αRFX1 antiserum (lane 2), and extract immunodepleted with preimmune serum (lane 3) were analyzed by immunoblotting with αRFX1 antiserum (panel C) and by EMSA using conditions II to detect binding of proteins to the DRA-X box (panel D, complexes A, a, and B), the DRA-TRE (panel D, complexes C), the DRA-Y box (panel E, NF-Y), and the DQA-X box oligonucleotide (panel F, NF-S).

mutant cell lines. Complexes C due to TRE-binding proteins are also present in both normal and CID B cells. Figure 4 illustrates this for the CID cell line SJO. The weak bands of higher mobility (Fig. 4, bands labelled ns) were, as mentioned above, nonspecific and varied with the cell line and binding conditions used. The methylation interference pattern obtained for complex A in one CID cell line has been found to be identical to the pattern obtained for complex A in a normal B-cell (data not shown). We therefore conclude that, of the two distinct proteins that bind to the MHC class II X box, only one (complex B) is specifically defective in the case of the class II regulatory mutants while the second (complex A) binds normally.

The cloned RFX1 gene corresponds to complex A and not to complex B. A cDNA (now referred to as RFX1) that encodes an X box-binding protein has been cloned and was initially thought to correspond to complex B on the basis of identical binding requirements (23, 24). However, since the new complex described here (complex A) has binding characteristics identical to those of complex B, it was necessary to re-evaluate the relationship between RFX1 and complexes A and B.

(i) Plasmid pRFX1 (containing the full-length RFX1 cDNA) was transcribed with T7 RNA polymerase, and the RNA was translated in vitro. In an EMSA performed with recombinant RFX1, the complex formed by RFX1 comigrated with complex A and not with complex B (Fig. 5A).

(ii) An antiserum was raised against recombinant RFX1 synthesized in *E. coli*. This αRFX1 antiserum immunoprecipitated from [^{35}S]methionine-labeled B cells a protein having the same apparent molecular mass (140 kDa) as recombinant RFX1 synthesized in vitro (Fig. 5B). Only one other additional weaker band of 116 kDa was also immunoprecipitated specifically (Fig. 5B). Reactivity of αRFX1 with complexes A and B was first studied in EMSA experiments. At a low concentration, αRFX1 antiserum induced a supershift of complex A but did not affect complex B (Fig. 4A). The antibody titer required to induce a supershift of complex A was the same as that required for recombinant RFX1 (data not shown). Although at a low antibody concentration only complex A was affected, the use of a high concentration of affinity-purified αRFX1 antibody also allowed recognition of complex B (Fig. 4B), as described earlier (24). Cross-reactivity with complex B was specific because neither NF-Y (9, 25) nor NF-S (15) was affected by affinity-purified αRFX1 (data not shown). In these experiments, the concentration of αRFX1 antibodies required to affect complex B was at least 100-fold greater than that required for recognition of complex A. We therefore conclude that the αRFX1 antiserum is highly specific for complex A and that weak cross-reactivity with complex B can be detected only when high concentrations of affinity-purified antibodies are used.

To analyze further the specificity of the αRFX1 antiserum, a B-cell nuclear extract was depleted of RFX1 by three consecutive rounds of immunodepletion with αRFX1 antiserum. Western blot (immunoblot) analysis showed that this depletion indeed removed almost all of the RFX1 from the nuclear extract (Fig. 5C). EMSA experiments performed with such immunodepleted extracts showed a specific loss of complexes A and a but no effect on complexes B, C, NF-Y, and NF-S (Fig. 5D to F). On the basis of these experiments and the observed comigration in EMSA, we conclude that the cloned RFX1 gene encodes the protein involved in complex A. Complex B, on the other hand, seems to correspond to a distinct protein which has identical DNA-binding characteristics and only a weak immunological cross-reactivity with RFX1.

RFX1 is not defective in class II-deficient CID patients. After it was established that the cloned RFX1 gene corresponds to the protein responsible for complex A, which is not affected in CID, and not for complex B, it remained important to study the expression and integrity of RFX1 in these mutant class II-negative cells. RNase protection assays showed normal expression of RFX1 mRNA (Fig. 6A), and immunoprecipitation experiments revealed that RFX1 protein was present in normal amounts and was correct in size (Fig. 6B). A full-length RFX1 cDNA clone isolated from one CID B-cell line (Nacera) was entirely sequenced and found to be identical to the normal RFX1 sequence (data not shown).

Finally, the DNA-binding activity of native RFX1 in these CID cells was assayed by immunoprecipitation of specific RFX1-DNA complexes. Binding reactions were set up with ^{32}P-labeled oligonucleotide X1 (wild-type X box) mixed with an equal amount of Xr (mutated X box) as a negative control. RFX1-DNA complexes were then immunoprecipitated with αRFX1 antiserum and oligonucleotides specifically retained in the immunoprecipitate were resolved on a sequencing gel. Oligonucleotide X1 was precipitated specifically in all normal and CID B cells. This is consistent with the presence of complex A in the CID cells and provides further evidence that the X box-binding activity of RFX1 from CID cells is unaffected (Fig. 6C). In the experiment shown in Fig. 6C, the amount of X1 oligonucleotide immunoprecipitated from CID cell extracts was slightly reduced compared with normal cells. Although this reduction was not observed consistently, it may correlate with the finding that gel retardation assays performed with CID extracts also tend to show a slight reduction in the intensity of band A (Fig. 4A). It is unlikely that the reduction in the immunoprecipitated X1 oligonucleotide in CID cells was a consequence of cross-reactivity with complex B, because the crude αRFX1 antiserum did not react with complex B (Fig. 4A and 5C).

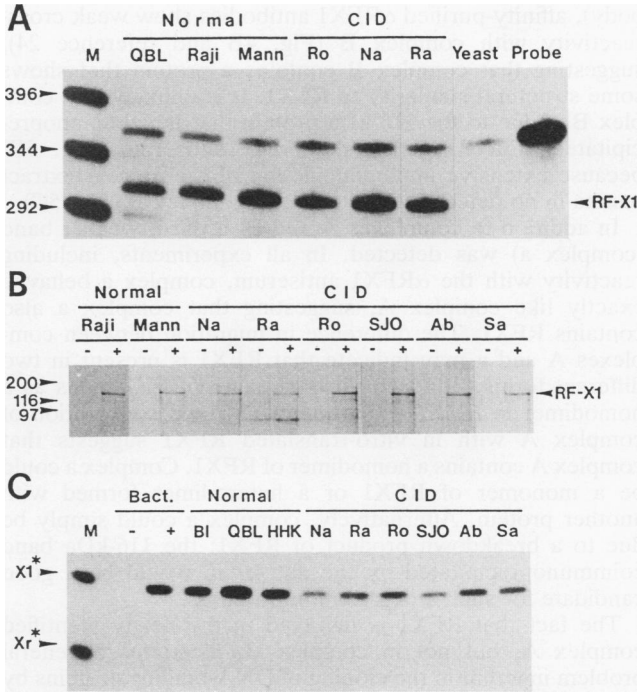

FIG. 6. RFX1 is expressed normally in CID B cells. (A) RNase protection analysis of RFX1 mRNA. A 374-nucleotide riboprobe designed to hybridize with 319 nucleotides of RFX1 was used to detect RFX1 mRNA in RNA from normal B-cell lines QBL, Raji, and Mann and from CID B-cell lines Robert (Ro), Nacera (Na), and Ramia (Ra). *Saccharomyces cerevisiae* RNA was used as a negative control. DNA fragments generated by digestion of pBR322 with *Hin*fI were used as size standards (sizes are indicated at the left in kilodaltons). (B) Immunoprecipitation of RFX1. Normal B-cell lines (Raji and Mann) and CID B-cell lines Nacera (Na), Ramia (Ra), Robert (Ro), SJO (Sj), Abdulla (Ab), and Sabrina (Sa) were labelled with [^{35}S]methionine and subjected to immunoprecipitation with either preimmune serum (lanes −) or αRFX1 antiserum (lanes +). Positions of RFX1 and molecular size standards (in kilodaltons) are indicated. (C) Immunoprecipitation of specific RFX1-DNA complexes. Nuclear extracts from normal B-cell lines Blanco (Bl), QBL, and HHK or from CID B-cell lines Nacera (Na), Ramia (Ra), Robert (Ro), SJO (Sj), Abdulla (Ab), and Sabrina (Sa) were incubated with a ^{32}P-labelled mixture (lane M) of a specific X box oligonucleotide (X1*) and a nonspecific oligonucleotide (Xr*). RFX1-DNA complexes were immunoprecipitated, and labelled DNA was analyzed by denaturing PAGE. As controls, bacterial extracts lacking (lane −) or containing (lane +) recombinant RFX1 were used.

DISCUSSION

We show here that two different protein-DNA complexes (A and B) can form with the X box motif of the HLA-DRA promoter and that these two complexes involve distinct but related proteins. Three lines of evidence indicate that the proteins in complexes A and B are different. (i) Optimal binding conditions are different for complexes A and B. (ii) Complex B is present in normal B-cell lines but not in CID cell lines, while complex A is present in both types of cells. (iii) The X box-binding protein we have recently cloned, RFX1, is present in complex A but not in complex B. It is unlikely that complex B represents a truncated form of RFX1, since the αRFX1 antibody recognizes truncated mutant forms of RFX1 very well (data not shown). Furthermore, it is unlikely that complex B corresponds to a heterodimer involving RFX1, since the same antibody recognizes RFX1 when it forms heterodimers with other non-cross-reactive members of the RFX family (see below), suggesting that heterodimerization does not mask dominant epitopes recognized by the antiserum. Despite the differences, there is also evidence that suggests that the proteins in complexes A and B are related. Their binding characteristics are very similar: in both complexes the exact contact points with individual nucleotides on the X box are identical, and both complexes exhibit a characteristic gradient of binding affinity for the X boxes of the DRA, DPA, and DQA promoters. Although not a direct proof, these similar binding requirements suggest related DNA-binding domains. Moreover, at high concentrations (requiring 100-fold more anti-

body), affinity-purified αRFX1 antibodies show weak cross-reactivity with complex B (Fig. 4B and reference 24), suggesting that complex B contains a protein that shows some structural similarity to RFX1. It is unlikely that complex B is due to the 116-kDa protein that is coimmunoprecipitated with RFX1 by the crude antiserum (Fig. 5B) because extensive immunodepletion of the nuclear extract results in no detectable reduction of complex B (Fig. 5C).

In addition to complexes A and B, a third, weaker band (complex a) was detected. In all experiments, including reactivity with the αRFX1 antiserum, complex a behaved exactly like complex A, suggesting that complex a also contains RFX1. The difference in migration between complexes A and a may indicate that RFX1 is present in two different forms. Thus, in vitro-translated RFX1 binds as a homodimer in EMSA experiments (24) and comigration of complex A with in vitro-translated RFX1 suggests that complex A contains a homodimer of RFX1. Complex a could be a monomer of RFX1 or a heterodimer formed with another protein. Alternatively, complex a could simply be due to a breakdown product of RFX1: the 116-kDa band coimmunoprecipitated by the antiserum would be a good candidate for such a degradation product.

The fact that RFX1 is involved in the newly identified complex A, but not in complex B, illustrates a general problem inherent in the cloning of DNA-binding proteins by screening expression libraries with probes containing the target site: it is difficult to establish identity between the cloned protein obtained by this technique and a given complex observed by EMSA. In this study, the availability of antibodies specific for the cloned RFX1 protein was crucial in identifying the nuclear complexes that correspond to RFX1 and the immunodepletion experiment was critical in allowing us to distinguish between the X box-binding proteins involved in complexes A and B. It should be noted that the αRFX1 antiserum is highly specific for RFX1 and has essentially no cross-reactivity with other cloned RFX proteins (see below).

The results presented here indicate that there are at least two different X box-binding proteins that exhibit essentially identical DNA-binding properties. One might therefore expect these proteins to have conserved DNA-binding domains. Low-stringency screening of mouse and human cDNA libraries with an RFX1 DNA-binding domain probe has indeed revealed the existence of genes that encode a number of different RFX proteins that share strong homology with RFX1 in the DNA-binding and dimerization domains (22a). Members of this novel family can form either homo- or heterodimers, and these have essentially the same binding characteristics. Considering the identical binding properties of complexes A (RFX1) and B, it is tempting to speculate that complex B contains a member of this novel family of RFX proteins. Analysis of the RFX proteins we have cloned has, however, not identified one that is a clear candidate for complex B. In the RFX factors, neither the DNA-binding nor the dimerization domain shows significant homology with any other known DNA-binding proteins (24). In particular, it should be noted that the RFX proteins are not related to the leucine zipper protein family, such as hXBP1, mXBP, Jun, and Fos (1, 11, 18, 21), which can bind to the cyclic AMP response element-TRE-like sequences situated immediately 3′ of the X box itself.

We have extended here to three additional cell lines the earlier finding that complex B does not form in B-cell lines derived from patients with CID, a primary immunodeficiency that is due to a defect in a *trans*-acting factor required for HLA class II gene expression. The binding defect in these CID cells concerns complex B but not the complexes that contain RFX1 (A and a). Thus, the first cloned gene in the RFX family (RFX1) does not correspond to the factor involved in this disease. This is consistent with the finding that RFX1 is normal in sequence and expression in CID cells. We are currently pursuing the cloning and characterization of the different members of the RFX family of factors to elucidate the molecular defect responsible for the absence of complex B in CID cells. Defective binding of complex B is not necessarily due to absence of the protein. Lack of binding could, for example, also be explained by a defect in posttranslational modification. Consequently, every cloned RFX gene will have to be studied, as reported here for RFX1, at the level of its mRNA and protein expression, as well as its binding capacity in CID cells.

In contrast to the six CID cell lines analyzed here, we detected complex B normally in one additional CID cell line (BLS2) and in two in vitro-generated class II-negative mutants (RJ2.2.5 and RM3; data not shown). This is consistent with the finding that all three of these cell lines fall into a common complementation group that is different from those containing the other six CID cells (2, 10; unpublished data). Certain of the six CID cell lines showing a deficiency in complex B (Ramia, Nacera, and SJO) have been classified into two different complementation groups (2, 10), suggesting that several distinct genetic defects can lead to an absence of complex B. For example, either a mutation in the protein or a defect in a posttranslational modification system could be responsible. Another possible explanation is that complex B could be composed of several different subunits.

Inconsistent findings on proteins that bind to the X box have been obtained in several laboratories (10, 13, 17, 23, 25, 28), in particular with respect to the deficiency in a protein that binds to the X box in CID cells. We (23, 25) and others (28) have observed this defect, while in other laboratories the complexes detected were present in both normal and CID cells (10, 17). Our results may provide an explanation for this. Indeed, the different X box complexes detected by EMSA appear to be quite sensitive to minor differences in binding conditions (Fig. 1B), suggesting that experiments performed in different laboratories under different binding and/or gel conditions may have preferentially identified either complexes present normally in CID cells (such as complexes A and a, as well as possible additional proteins not detected here) or a complex(es) specifically absent in these cells. A precise definition of the profile of X box-binding proteins detected under different conditions, as well as their relationships to each other and to the existing cloned RFX factors, may ultimately require cloning of all of the respective genes.

It is tempting to speculate that different X box-binding proteins have specific functions in the complex mechanisms that control class II genes, for example, control of constitutive versus induced expression or differential regulation of individual class II loci. There is evidence that both complexes A and B are functionally significant. The functional relevance of RFX1 (complex A) has been documented by antisense experiments showing that antisense RFX1 RNA and RFX1-specific antisense oligonucleotides can inhibit induction of HLA class II genes by gamma interferon (24, 27a). In the case of complex B, its absence in CID B cells suggests that it is crucial for class II gene expression. The description here of two distinct functionally relevant X box-binding proteins is therefore a step towards the identification of more members of the X box-binding protein

family and ultimately towards the elucidation of their individual role in the regulation of MHC class II genes.

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