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B-Cell Factor 1 Is Required for Optimal Expression of the DRA Promoter in B Cells

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The X box in the DRA promoter of the human histocompatibility complex is required for expression of the DRA gene in B cells. We show that a B-cell factor binds to a sequence that is clearly distinguishable from binding sites for the previously described X box binding nuclear proteins RF-X, NF-X, NF-Xc, NF-S, hXBP, and AP-1. Mutations in the DRA X box that disrupt the binding of this factor result in a lower level of gene expression, as does the presence of Id (a *trans*-dominant regulatory protein that negatively regulates helix-loop-helix proteins). Furthermore, this factor is recognized by antibodies directed against the helix-loop-helix protein A1, a mouse homolog of the immunoglobulin enhancer binding proteins E12/E47, and it binds to sequences in other genes that were previously shown to bind these proteins. By these criteria, this factor is BCF-1.

Class II antigens from the major histocompatibility complex are heterodimeric surface glycoproteins that present antigenic peptides to T lymphocytes, thus initiating the immune response (6, 14). Congenital defects in their expression result in a severe combined immunodeficiency or agammaglobulinemia (20), and high levels of expression in certain tissues have been correlated with autoimmunity (13). Class II proteins are encoded by a cluster of genes that are coordinately regulated in a tissue- and cell type-specific manner. In humans, high levels of expression are limited to mature B cells and activated T cells. On antigen-presenting cells and some somatic cells, class II determinants are expressed after induction by gamma interferon (IFN-γ) (55).

The expression of class II genes requires several DNA sequence motifs (termed the Z, X, and Y boxes) that are conserved in all class II promoters (7, 12, 21, 50, 52, 56, 58, 59). The contribution of each of these motifs (and their flanking sequences) to B-cell-specific and IFN-γ-inducible expression has been determined in the context of a heterologous (thymidine kinase [TK]) promoter (57). The Y box, which binds the ubiquitously expressed NF-Y heterodimer (26), conferred neither IFN-γ inducibility nor B-cell specificity upon the TK promoter, and both the Z and X boxes were required for IFN-γ inducibility. In contrast, the X box was sufficient for B-cell-specific expression, suggesting that regulatory proteins involved in B-cell-specific expression of class II promoters interact with the X box and its flanking sequences (the extended X box).

The extended X box can be divided into three regions (termed the pyrimidine tract, the core X box, and the X2 box [58]) that have been shown to be important in class II expression. The core X box contains the sequence (positions −108 to −95) in the DRA promoter that is highly conserved in all class II genes. Mutations in this sequence invariably result in loss of class II gene expression (50, 51, 58, 60). Mutations in the pyrimidine tract, which begins 5′ to and partially overlaps the core X box, also cause a reduction in activity from the human DRA and murine Ea promoters (32, 58, 60). However, mutations that affect the pyrimidine tract without compromising the core X box have not been reported. Finally, mutations in the X2 box, which lies 3′ to the core X box, have been shown to affect class II gene expression. Viville et al. (60) and Sherman et al. (51) found that mutations in the X2 box had a deleterious effect on transcription from the Ea and DRA promoters, respectively. Although Tsang et al. found that mutations in the X2 box had no significant effect on the DRA promoter (58), new mutations in the X2 box resulted in substantially lower DRA activity (60a). These results suggest that the X2 region, like the pyrimidine tract and the core X box, bind *trans*-acting regulatory proteins essential for B-cell-specific expression of class II genes.

A number of proteins and protein complexes which bind to sequences within the extended X box have been described. (i) The human XPB protein (34, 35) forms heterodimers with c-Fos (42, 43) and binds to the TRE response element (4) found in the X2 region of the DRA and DPB promoters (3, 43). (ii) NF-S (30) binds to the same sequence of class II promoters, except that NF-S binds to the DQA and DPA but not the DRA promoters. (iii) AP-1, which is composed of the c-Fos and c-Jun proteins (18), also binds to the X2 region of the DRA promoter (3). (iv) RF-X (46), a protein of 979 amino acids that recognizes the core X box (25), binds to the X box of three α-chain genes with different affinities (DRA > DPA ≫ DQA) (30). (v) NF-X (31, 32) and (vi) NF-Xc (15) are protein complexes, found in B-cell nuclear extracts, that bind to the same sequences as RF-X (32, 45), and these proteins may be one and the same. However, NF-X and NF-Xc but not RF-X bind to the DRA promoter in nuclear extracts from class II-negative mutant B-cell lines from patients with the class II severe combined immunodeficiency (32, 47).

Several lines of evidence suggest that these are not the only proteins that participate in B-cell-specific expression of class II genes. First, the expression of RF-X/NF-X/NF-Xc and hXBP is not restricted to B cells (the tissue distribution of NF-S has not been reported). These proteins are also

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expressed in other cells and tissues that do not express and cannot be induced to express class II antigens (32, 34). Furthermore, although both c-*fos* and c-*jun* proto-oncogenes are expressed in B cells, formation of AP-1 heterodimer is inhibited (3). Second, each of these proteins binds with high affinity to only a few promoters of class II genes, even though all are expressed coordinately in mature B lymphocytes. Finally, genetic studies have suggested that there exist four separate complementation groups that affect the coordinated B-cell-specific expression of the major histocompatibility complex class II genes (9). None of these genetic complementation groups appears to correspond to the previously defined factors RF-X/NF-X/NF-Xc, hXBP, NF-S, or AP-1.

For these reasons, we have searched for additional proteins that bind to the extended X box. We now report the identification of a binding activity from B-cell nuclei that recognizes the sequence CAGATG located between the binding sites for RF-X/NF-X/NF-Xc and hXBP/NF-S/AP-1. Mutations that prevent binding of this factor result in lower levels of DRA expression. Immunological and functional evidence indicates that the factor contains a protein identical to or closely related to the helix-loop-helix (HLH) proteins E12/E47 and probably corresponds to the previously characterized B-cell factor 1 (BCF-1).

MATERIALS AND METHODS

Cells and tissue culture. Raji cells (ATCC CCL86, a human Epstein-Barr virus-positive Burkitt's lymphoma B-cell line), which express high levels of class II determinants, were grown in RPMI 1640 medium supplemented with fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (292 μg/ml).

Transfections and CAT assays. Raji cells (1×10^7 to 2×10^7 cells per ml) in complete medium were transfected with 40 μg of $CsCl_2$-purified plasmid DNA by electroporation at 200 V and 960 μF. Included in each electroporation was 5 μg of pACTHCG DNA, which expresses secreted human chorionic gonadotropin (HCG) under the control of the actin promoter. The cells were allowed to express the transfected DNA for 40 to 46 h before harvesting.

Transfections with the plasmids E:Id(S) and E:Id(A) were performed in essentially the same manner, except that 100 μg of E:Id(S) or E:Id(A) was cotransfected along with 10 μg of pDRASCAT, pPXM(−99/−97), or pCAT by electroporation at 300 V and 960 μF. The cells were allowed to express the transfected DNA for 48 h before harvesting.

The cells were assayed for CAT activity by the method of Neumann et al. (40), and the medium was assayed for HCG by a radioimmunoassay from Hybritech, Inc. (San Diego, Calif.). All transfections were repeated six times with two separate preparations of plasmid DNA. Chloramphenicol acetyltransferase (CAT) activity is expressed as the rate of formation of acetylated chloramphenicol (expressed as counts per minute) normalized to the concentration of HCG in the medium, except for transfections with E:Id(S) and E:Id(A), which are expressed as the rate of formation of acetylated chloramphenicol normalized to lysate protein. The amount of lysate protein was determined by Bradford assay (Bio-Rad, Inc.).

Oligonucleotides. The sequences of the oligonucleotides that were used in this study were as follows (mutated nucleotides are underlined and synthetic linkers are in lower case):

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px              gatccGCCCTTCCCCTAGCAACAGATGCGTa
                    gCGGGAAGGGGATCGTTGTCTACGCAtctag

x3                        gatccCAACAGATGCGTa
                            gGTTGTCTACGCAtctag

pxm(−99/−97)          CTTCCCCTAGCAACCTCTGCGTCATC
                    CCGGGAAGGGGATCGTTGGACACGCAGTAGAGCT

pxm(−96/−94)          CTTCCCCTAGCAACAGAACGGTCATC
                    CCGGGAAGGGGATCGTTGTCTTGCCAGTAGAGCT

px(−116/−97)    gatccGCCCTTCCCCTAGCAACAGAa
                    gCGGGAAGGGGATCGTTGTCTtctag

IEB                           gatccGCCATCTGCa
                                gCGGTAGACGtctag

μE5                           gatccAACACCTGCa
                                gTTGTGGACGtctag

IRR             tcgagaagcttgaattctagac
                    cttcgaacttaagatctgagct
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The nucleotide sequences for the IEB and μE5 oligonucleotides were derived from Walker et al. (62) and Lenardo et al. (33), respectively. Oligonucleotides representing the extended X box of other class II genes (2, 24, 27, 49) of the Dw4 haplotype correspond in length and position to pxm(−99/−97) and pxm(−96/−94).

Plasmids. The plasmid pDRASCAT contains a synthetic DRA promoter from positions −150 to +31 linked to the CAT reporter gene. The plasmid was previously referred to as pDRsyn (58). The region from nucleotides −116 to −88, which contains the X box and adjacent sequences, is flanked by unique restriction endonuclease sites that facilitate construction of clustered point mutations (58). The plasmids pPXM(−96/−94) and pPXM(−99/−97) were constructed in this manner with the oligonucleotides pxm(−96/−94) and pxm(−99/−97), respectively. The control plasmid pCAT contains no DRA promoter sequences. The plasmids p(−116/−92)TKCAT and pTKCAT were described previously (57). The plasmid p(−116/−97)TKCAT was constructed by inserting the px(−116/−97) oligonucleotide into the *Bgl*II site of pTKCAT. The plasmids E:Id(S) and E:Id(A) express sense and antisense Id, respectively (8). The plasmid pACTHCG contains the actin promoter linked to the HCG gene (22, 23).

Nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared by the rapid extraction method described by Osborn and Nabel (44). Electrophoretic mobility shift assays (EMSA) were performed with 20 fmol of ^{32}P-labelled oligonucleotide, 20 μg of nuclear extract, 2 μg of sheared calf thymus DNA, and 10 pmol of nonspecific single-stranded oligonucleotide in the binding buffer described by Ohlsson et al. (41). Unlabelled competitive oligonucleotides were added to 100-fold excess, and the IRR oligonucleotide was used as a control for nonspecific DNA binding. Electrophoresis and autoradiography were as described by Ohlsson et al. (41).

Antibodies. The preparation of antibodies specific for the A1 protein is described elsewhere (5). Briefly, the bacterial fusion protein containing 281 amino acids from the C-terminal portion of the A1 protein linked to trpE (62) was purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis and used to elicit antibodies in rabbits. The immunoglobulin G (IgG) fraction was prepared from sera using *Staphylococcus aureus* protein A coupled to Sepharose. For EMSA, 2 μl of an IgG preparation with an optical density at 280 nm of 2.2 was added to the binding reaction before addition of the radioactive probe. The control binding reaction mixtures contained 2 μl of nonimmune rabbit IgG at the same concentration.

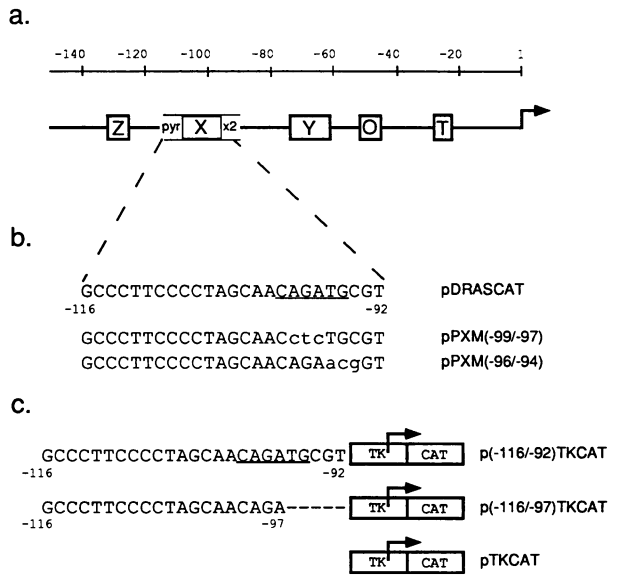

FIG. 1. Map of the DRA promoter and comparison of wild-type and mutated plasmid constructions. (a) The DRA promoter contains Z, X, and Y boxes, which are conserved upstream sequences found in all major histocompatibility complex class II genes. The O box represents the octamer binding site (53) found only in the DRA promoter, and the T box represents a sequence homologous to the TATA box. The X box is flanked by the pyrimidine tract (pyr) and the x2 region, which, together with the core X box, constitute the extended X box (58). (b) The sequence from positions −116 to −92 of the reporter plasmid pDRASCAT, which contains a synthetic DRA promoter linked to the CAT reporter gene (58). The consensus HLH binding site is underlined. Also shown are sequences from positions −116 to −92 of two plasmids, pPXM(−99/−97) and pPXM(−96/−94), which have mutations in the consensus HLH binding site. (c) Sequences from two plasmids which contain portions of the DRA X box placed 5′ to the TK promoter linked to the CAT reporter gene. p(−116/−92)TKCAT but not p(−116/−97)TK CAT contains the entire consensus HLH binding site (underlined). pTKCAT contains no DRA promoter sequences.

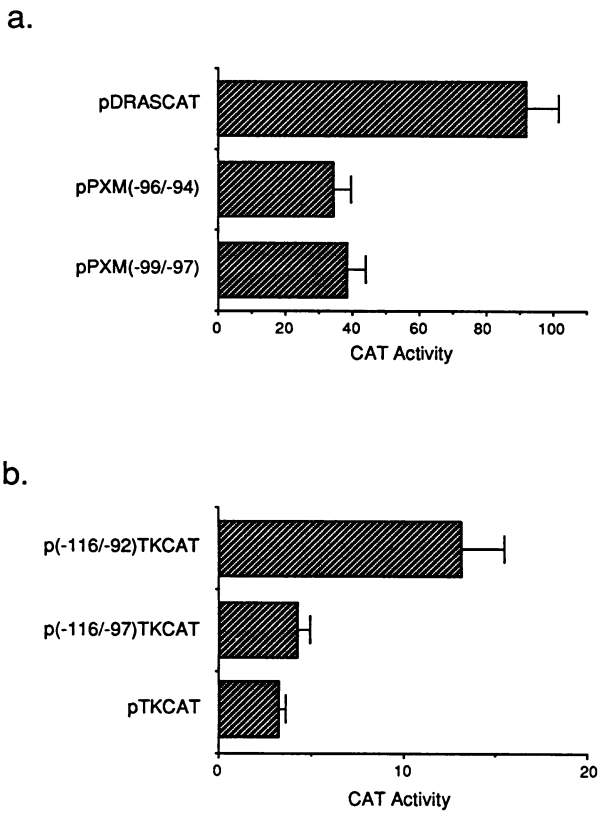

FIG. 2. Effect of HLH site mutations and deletions on the activity of the DRA promoter in Raji cells. (a) CAT activities in Raji cells transfected with pDRASCAT and two DRA promoters with mutated consensus HLH binding sites, pPXM(−99/−97) and pPXM(−96/−94). The CAT activity is corrected by subtracting the activity caused by the promoterless CAT plasmid (pCAT). (b) CAT activities of plasmids containing DRA synthetic oligonucleotides linked to the TK promoter. pTKCAT contains only the TK promoter linked to the CAT reporter gene, whereas p(−116/−92)TK CAT and p(−116/−97)TKCAT contain DRA sequences from positions −116 to −92 and −116 to −97, respectively. p(−116/−92)TKCAT but not p(−116/−97)TKCAT contains the consensus HLH binding site. The means ± standard errors of the means (shown by error bars) are calculated from data from six separate transfections performed with two different DNA preparations.

RESULTS

A binding site for the HLH family of regulatory proteins is present in the DRA promoter. The DRA promoter contains the sequence CAGATG from positions −100 to −95 (Fig. 1a and b). This sequence (hereafter called the X3 site) closely resembles the consensus binding site (10) for a family of regulatory proteins that share the HLH structural motif (37). The HLH structure is involved in sequence-specific binding of nuclear proteins to DNA (19, 37, 61).

The X3 site is important for the expression of the DRA promoter in B cells. To determine whether the X3 site was required for the activity of the DRA promoter in B cells, X3 sequences were changed by clustered point mutations in the context of the synthetic DRA promoter and by 3′ deletions of the extended X box in the context of the TK promoter. In a transient expression assay, clustered point mutations (Fig. 1b) in the X3 site resulted in decreased transcriptional activity of the DRA promoter in Raji cells (Fig. 2a). Mutagenesis of nucleotides from positions −96 to −94 [represented by the plasmid pPXM(−96/−94)] and nucleotides from positions −99 to −97 [plasmid pPXM(−99/−97)] resulted in only 37 and 41% of pDRASCAT expression.

The same effect was demonstrated with the use of the heterologous TK promoter. The region from positions −116 to −92 of the DRA promoter conferred high activity on the TK promoter [plasmid p(−116/−92)TKCAT] in Raji cells, while the region from positions −116 to −97 (plasmid p(−116/−97)TKCAT) conferred little or no activity (Fig. 2b). The difference between these two plasmids is a 4-bp deletion that removes the 3′ end of the consensus HLH site (Fig. 1c). Thus, the X3 site is required for high levels of expression of the DRA promoter in B cells.

A nuclear protein(s) interacts with the X3 site. The interaction of a specific protein complex with the X3 site in the DRA promoter was assayed by EMSA by using the x3 oligonucleotide. The x3 oligonucleotide, which contains neither the RF-X nor the hXBP binding sites (25, 42), contains sequences from positions −103 to −92 of the DRA promoter. Nuclear extracts from Raji cells incubated with the x3 probe formed a slowly migrating complex called NF-X3 (Fig. 3a). Excess unlabeled x3, px, IEB, and μE5 oligonucleotides competed for the binding of this complex. IEB and μE5 oligonucleotides contain sequences from the

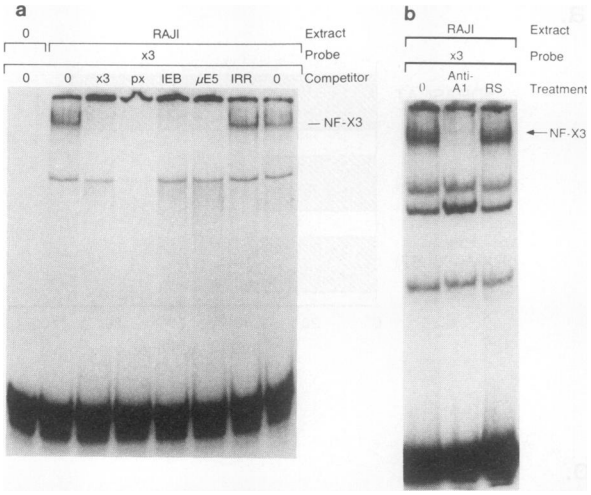

FIG. 3. NF-X3, which binds to the x3 oligonucleotide in Raji cells, does not associate with this site in the presence of antibodies against E12/E47. (a) The top line shows the nuclear extract used in the EMSA, the second line shows the radiolabelled oligonucleotide, and the third line shows the excess unlabelled competing oligonucleotide. A complex, NF-X3, binds the x3 oligonucleotide, which contains the sequence from positions −103 to −92 from the DRA promoter. Binding of NF-X3 to x3 is competed by the binding of NF-X3 to excess unlabelled oligonucleotides x3, px, IEB, and μE5, but not IRR. Whereas the px oligonucleotide contains the sequence from positions −116 to −92 of the DRA promoter, IEB and μE5 contain the consensus HLH binding site found in the rat insulin gene promoter (29) and in the human immunoglobulin heavy chain enhancer (33), respectively. IRR is an irrelevant oligonucleotide which does not contain the consensus HLH binding site. (b) Formation of the NF-X3 complex is prevented by prior treatment of the Raji nuclear extract with rabbit antibodies against the mouse A1 protein. The radiolabelled oligonucleotide and the nuclear extract are the same as in panel a. The lane labelled anti-A1 is treated with the IgG fraction of sera from rabbits immunized against the mouse A1 protein; the lane labelled RS is treated with an equivalent amount of IgG from nonimmunized rabbits.

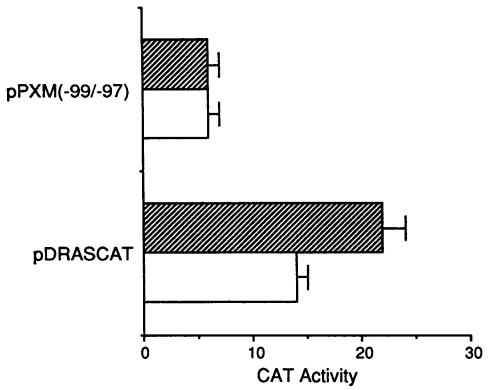

FIG. 4. Effect of Id on the activity of the DRA promoter in Raji cells. CAT activities in Raji cells transfected with pDRASCAT and E:Id(S), which encodes Id mRNA (open boxes), or E:Id(A), which encodes antisense Id (striped boxes), are graphed. Also presented are similar transfections with pPXM(−99/−97), which contains the mutated X3 sequence. The means ± standard errors of the means for three separate transfections are shown.

rat insulin I gene enhancer (29) and the human immunoglobulin heavy chain enhancer (33), respectively. HLH proteins bind to both sequences (5, 39). Thus, NF-X3 binds to the X3 site of the DRA promoter and recognizes the consensus HLH binding site.

The faster-migrating complex observed in Fig. 3a does not manifest specific X3-binding activity since excess unlabelled x3 oligonucleotide did not compete for the binding of the complex.

Characterization of NF-X3. To determine whether NF-X3 contains previously characterized proteins, antibodies against cloned HLH proteins were used in EMSA. Antibodies to A1 were used because (i) NF-X3 specifically binds to the consensus HLH sequence and (ii) the A1 protein is related to the human E12/E47 proteins (37, 62). E12/E47 are ubiquitously expressed HLH proteins that can form homodimers or interact with other HLH proteins to form tissue-specific heterodimers (5).

The binding of NF-X3 to x3 was disrupted (Fig. 3b) by preincubation of the Raji nuclear extract with the IgG fraction of anti-A1 antiserum (62). However, treatment of the Raji nuclear extracts with an IgG preparation of nonimmune rabbit antiserum had no effect. Thus, NF-X3 contains a protein that is antigenically related to the murine A1 protein. This protein is probably human E12/E47 or a closely related protein because (i) A1 and E12/E47 are 70-kDa proteins that are highly homologous, (ii) A1 is encoded by a single copy gene (62), and (iii) anti-A1 does not recognize all HLH proteins, e.g., it is unable to interact with MyoD or USF (5a).

Expression of DRA is negatively regulated by Id. E12/E47 can associate with Id, an HLH protein that lacks a domain essential for DNA binding, to form nonfunctional heterodimeric complexes (8). Thus, Id should negatively regulate DRA expression if the E12/E47 protein complex is a component of the NF-X3 complex. Indeed, cotransfection of E:Id(S), which expresses Id, with pDRASCAT caused a decrease in CAT activity over that observed for pDRASCAT cotransfected with E:Id(A), which expresses antisense Id (Fig. 4). Cotransfection of E:Id(S) had no effect on already reduced levels of expression from pPXM(−99/−97), which contains mutated X3 sequences. Although levels of expression from pDRASCAT with excesses of E:Id(S) were not reduced to those from pPXM(−99/−97), the intermediate levels attained are comparable to those observed in similar experiments with Id and the IgH enhancer containing the μE5 binding site (63).

NF-X3 binds together with other X box-binding proteins. To show that mutations of the X3 site disrupted only the binding of NF-X3 and did not interfere with the binding of other proteins to the extended X box of the DRA promoter, EMSA with the px oligonucleotide were performed. Nuclear extracts from Raji cells bound to px oligonucleotide (which includes the nucleotides from positions −116 to −92 [Fig. 1b]) and formed three major protein-DNA complexes (NF-Xc [58]) that were specifically competed by excess unlabeled px oligonucleotide (Fig. 5). However, unlabeled excess X3-mutated oligonucleotides, pxm(−99/−97) and pxm(−96/−94), specifically competed for the binding of the three major complexes designated NF-Xc but not for the binding of the less intense NF-X3 complex (Fig. 5). However, excess unlabeled x3 oligonucleotide, together with pxm(−99/−97) or pxm(−96/−94), competed for the binding of this residual NF-X3 complex. These results indicate that NF-X3 binds specifically to the X3 site and is distinct from NF-Xc. Furthermore, mutations in the X3 site affect the binding of only NF-X3.

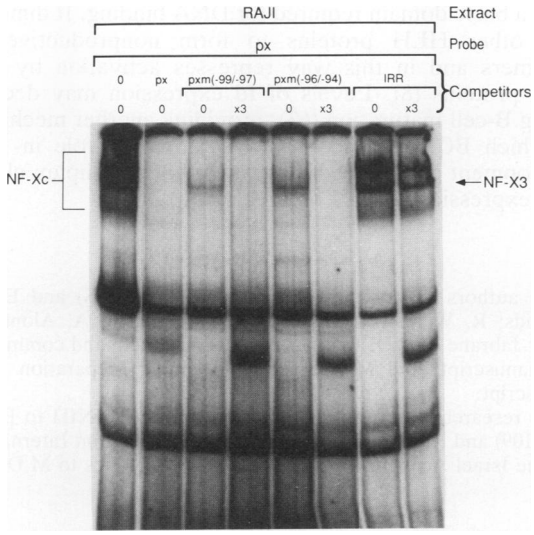

FIG. 5. NF-X3 is one of several complexes that bind to the extended X box in EMSA. The top line shows the nuclear extract used in EMSA, the second line shows the radiolabelled oligonucleotide, and the third and fourth lines show the combination of excess unlabelled competitive oligonucleotides. px and x3 oligonucleotides contain sequences from positions −116 to −92 and −103 to −92 from the DRA promoter, respectively. Oligonucleotides pxm(−99/−97) and pxm(−96/−94) contain the same sequence as px, except for mutations in the consensus HLH binding site. IRR is an irrelevant oligonucleotide which contains no homology to the consensus HLH binding site. A bracket denotes the position of three prominent complexes in Raji nuclear extracts, previously termed NF-Xc (58). The arrow shows the position of NF-X3 in lanes in which the binding of NF-Xc has been competed with unlabelled mutant x3 oligonucleotides. Oligonucleotides with mutations in the consensus HLH binding site, pxm(−99/−97) and pxm(−96/−94), compete for the binding of all complexes except for NF-X3.

NF-X3, which had the same electrophoretic mobility when complexed to either px or x3 (data not shown), did not comigrate with any of the NF-Xc complexes observed with the px oligonucleotide. However, NF-X3 was obscured in these binding assays by the intense NF-Xc complexes (Fig. 5). Furthermore, NF-X3 does not compete for the binding of NF-Xc, since adjacent EMSA with radiolabelled px and pxm(−99/−97) oligonucleotides are quantitatively and qualitatively identical (data not shown).

X3 sites are not present in most other class II genes. To determine whether the NF-X3 complex binds to the X boxes of other class II genes, EMSA were performed. The NF-X3 complex, formed with the x3 oligonucleotide and Raji nuclear extracts, was challenged by excess unlabelled X-box oligonucleotides from other class II genes (except DRB) (Fig. 6). Only the DPB oligonucleotide competed for the binding of NF-X3, even though the DPB X box does not contain a conserved consensus HLH binding site. However, there are sequence motifs that nearly match the HLH consensus. Furthermore, the X box from DXB contains the HLH consensus sequence but does not compete for the binding of NF-X3. The nucleotides that flank this HLH consensus site are not conserved between the DRA and DXB X boxes. These flanking sequences have been shown previously to affect the binding affinities of HLH proteins (10).

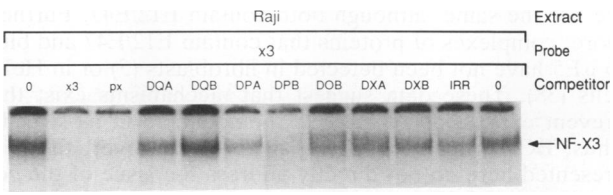

FIG. 6. NF-X3 does not bind to the X box of most other class II genes of the Dw4 haplotype. Presented is the binding of NF-X3 to the labelled x3 oligonucleotide in the presence of a 500-fold excess of unlabelled X-box oligonucleotide of each class II gene (except DRB). The identity of each competing oligonucleotide is indicated above the lanes. IRR is an irrelevant oligonucleotide with no homology to the consensus HLH binding site. Among class II genes, only DPB competes for the binding of NF-X3.

DISCUSSION

A sequence, X3, in the X box of the DRA promoter matches the consensus binding site (10) recognized by the HLH family of regulatory proteins (Fig. 1). Both mutations in the X3 site of the DRA promoter and deletion of the X3 site in an X box placed 5′ to the TK promoter caused large decreases of expression in Raji cells (Fig. 2a and b). Thus, the X3 site is required for maximal expression of the DRA promoter in B cells.

NF-X3 binds to the X3 site (Fig. 3a). Mutations that disrupt the binding and function of NF-X3 do not inhibit binding of other proteins to the DRA promoter (Fig. 5). Thus, NF-X3 is not RF-X/NF-X/NF-Xc (32, 45, 46, 58), NF-S (30), or hXPB (34, 35), which are proteins that also bind at or near the X box of class II genes. Furthermore, NF-X3 binds to other HLH sites, i.e., those in the immunoglobulin heavy chain enhancer (μE5 [33]) and rat insulin I gene promoter (IEB [29]). These data suggest that NF-X3 is an HLH protein complex.

NF-X3 is recognized by antibodies against the murine A1 protein, which is homologous to the human E47 protein (62). E47, which is one of two ubiquitous proteins expressed by differential splicing from the E2A gene (36), can bind to the HLH site as a homodimer or it can heterodimerize with other tissue-specific HLH proteins (38). The other gene product of the E2A gene is E12, which is also recognized by the anti-A1 antibody. Therefore, NF-X3 is probably composed of two proteins that dimerize and bind to the HLH site in the DRA promoter, and at least one of these proteins is E47 or E12.

NF-X3 is probably the same as BCF-1. Murre et al. (38) described two complexes (BCF-1 and BCF-2) from nuclear extracts of B cells that bind to the HLH sites in the muscle creatine kinase and the immunoglobulin heavy chain enhancer (μE5). In EMSA, these complexes were shown to have the same mobility as complexes containing in vitro translated E47, suggesting that BCF-1 and BCF-2 are E47 homodimers. In addition, Aronheim et al. (5) described a single binding complex (LEF-1) from B-cell extracts that binds to the HLH sites in the immunoglobulin heavy chain enhancer (μE5) and in the insulin enhancer (IEB). Because all of these B-cell complexes and NF-X3 (henceforth called BCF-1) contain E12/E47 and bind to a common HLH site (μE5), they are most likely identical.

Paradoxically, BCF-1 should form in any cell that expresses E12/E47 proteins and thus should not be tissue specific. However, Aronheim et al. (5) showed that complexes that bind to μE5 in pancreatic β cells and in B cells

are not the same, although both contain E12/E47. Furthermore, complexes of proteins that contain E12/E47 and bind to μE5 have not been detected in fibroblasts (5) or in HeLa cells (38). These data suggest that mechanisms exist that prevent expression of E12/E47 homodimers in most cells. Thus, BCF-1 might be B-cell specific. However, the data presented here do not directly address the issue of the cell specificity of BCF-1.

Since B-cell-specific activity maps to conserved X boxes found in promoters of all class II genes, their coordinate regulation suggests that the same regulatory proteins should bind to these sequences. However, it has been shown that the X box of each murine and human class II gene binds protein complexes that are not efficiently bound by the X boxes of other class II promoters (11, 16, 60a). Furthermore, several specific proteins have been shown to bind to extended X boxes with widely varying affinities (RF-X and NF-S [30]) or to bind only to certain X boxes (hXBP and c-Jun [43] and AP-1 [3]). This suggests that class II promoters bind unique complexes of proteins, which contain shared and unique regulatory factors. Since BCF-1, among the class II genes of the dw4 haplotype (2, 24, 27, 49), binds with high affinity only to the DRA X box (Fig. 6), the data presented here extend these observations.

There are several explanations for these observations. First, X boxes might bind two classes of factors: those required for promoter activity and those which modulate and fine-tune expression. Since BCF-1 increases expression from the DRA promoter but is not absolutely required, it might belong to the second class of regulatory proteins. Furthermore, this class of factors might be expected to contribute to regulatory polymorphism. To this end, HLH binding sites have been observed in a variant allele of the murine Ab gene (called Ab^b) (17) and several human DQB alleles (1, 60a). Although the presence of the canonical 5′-CANNTG-3′ sequence does not guarantee binding of HLH proteins (e.g., DXB; Fig. 6), it might increase expression of these haplotypes in B cells.

Second, it is possible that HLH sites are located outside the X box in other class II genes. The E47 protein regulates expression of the immunoglobulin heavy chain gene in lymphoid cells by binding to an enhancer element located in an intron (33), suggesting that BCF-1 does not need to bind at the promoter to affect gene expression. Furthermore, a recently described class II regulatory element, J, does not occupy the same position in all class II genes (54), suggesting that some regulatory elements bind to the same sequence at different locations in each class II gene. Likewise, it is possible that binding of BCF-1 is not restricted to the X box.

Finally, it is possible that the binding of BCF-1, besides increasing the expression from the DRA promoter, functions as a developmental switch. Kara and Glimcher (28), using in vivo footprinting, found that the class II promoter is inaccessible (bare) in cells that do not express class II determinants (including some class II negative mutant B-cell lines), despite the presence of previously described promoter binding proteins in nuclear extracts of these cells. This suggested that a developmental step opens the chromatin near class II promoters. BCF-1 might provide the trigger for this switch. In this regard, it has been shown that E47, a component of BCF-1, induces some steps of pre-B-cell determination (48) in addition to activating IgH activity twofold. These changes include induction of rearrangements of immunoglobulin D to J segments as well as an 80-fold increase in the transcription of Iμ (48). Second, BCF-1 is negatively regulated by the *trans*-dominant Id protein (Fig. 4). Id is an HLH protein that lacks a basic domain required for DNA binding. It dimerizes with other HLH proteins to form nonproductive heterodimers and in this way represses activation by these HLH proteins (8). Levels of Id expression may decrease during B-cell maturation (63), providing another mechanism by which BCF-1 could play a determining role in B-cell development and major histocompatibility complex class II gene expression.

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