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CCAAT box binding protein NF-Y facilitates *in vivo* recruitment of upstream DNA binding transcription factors

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NF-Y binds a CCAAT motif found in many eukaryotic polymerase II-dependent promoters. In the HLA-DRA promoter it has been demonstrated that stereo-specific alignment between this motif and the upstream elements X1 and X2 is required for activation. To study the underlying mechanism for this requirement, a panel of transfected cell lines that maintained integrated, wild-type and mutant promoters were analyzed by *in vivo* genomic footprinting. Cell lines harboring a mutated CCAAT element exhibited a loss of interactions at the CCAAT site, as expected, and no transcriptional activity. Most importantly, mutation of the CCAAT sequence nearly abolished *in vivo* binding at the X1 and X2 sites, while mutations of X1 and X2 had little effect on CCAAT box binding. However, X1 and X2 binding was interdependent. *In vitro*, X1 binding activities are known to be stabilized by NF-Y binding. Interaction between NF-Y and X box binding proteins was demonstrated by reciprocal co-immunoprecipitation in the absence of DNA and co-affinity purification in the presence of DNA. Collectively, these studies indicate that occupancy of the CCAAT element represents an early event affecting other protein–DNA interactions and suggest that NF-Y stabilizes and interacts with X box factors to mediate this function. These findings may represent a common theme among promoters containing a CCAAT element.

Key words: CCAAT/HLA-DRA/*in vivo* footprint/NF-Y/transcription

Introduction

Extensive studies of eukaryotic promoters have revealed complex arrangements of multiple proximal elements, often combined with distal enhancers and silencers. Many of the individual *trans*-acting factors have now been isolated and cloned, facilitating the determination of their potential function. However, there is only limited understanding of the mechanisms by which multiple factors work together to produce regulated expression. Some of the clearest demonstrations of factor interaction come from studies of upstream factors contacting the basal transcription machinery. For example, the transcription factor Sp1 appears to bind directly to the TATA-associated factor TAF110 (Hoey *et al.*, 1993), while other acidic activators can recruit TFIIB and TBP (Stringer *et al.*, 1990; Lin and Green, 1991; Choy and Green, 1993). Another of the better understood mechanisms involves the modulation of local chromatin structure to allow access by additional factors. In the mouse mammary tumor virus promoter, chromatin structure prevents NF1 binding to the promoter (Pina *et al.*, 1990; Archer *et al.*, 1991). Induction of glucocorticoid receptor binding to DNA disrupts the nucleosomes over the NF1 site and allows binding and activation of transcription. Similar roles in nucleosomal disruption or positioning have been demonstrated for HNF-3-like factors on the albumin promoter *in vivo* (McPherson *et al.*, 1993) and for the GAGA factor on the *hsp*70 promoter *in vitro* (Tsukiyama *et al.*, 1994). Alone, these two mechanisms are insufficient to account for the diverse levels of synergy and repression seen on natural promoters. Additional interactions between neighboring DNA binding regulatory factors could play a significant role. These types of interactions could modulate not only the activating potential of a factor, but also its DNA binding affinity and its interactions with non-DNA binding proteins. While many cases of functional synergy between DNA binding factors have been described (e.g. Fromental *et al.*, 1988; Atchison *et al.*, 1990; Bruggemeier *et al.*, 1991; Li *et al.*, 1991; Milos and Zaret, 1992; Du *et al.*, 1993; Gegonne *et al.*, 1993), the mechanisms are less well defined. Thus, determining which factors are able to interact functionally and physically will be an important key to understanding multi-component promoter regulation.

Several approaches have been utilized to directly examine protein–protein interaction. Co-immunoprecipitation of endogenous factors directly from cell lysates has been essential in determining the components of obligatory dimeric DNA binding factors such as myc/max and NF-κB (Blackwood *et al.*, 1992; Ruben *et al.*, 1992). However, this approach has had only limited success in detecting the weaker interactions between non-dimeric neighboring factors and usually has required the addition of recombinant protein. In addition, co-immunoprecipitation in the presence of DNA containing one of the factor binding sites has been successful when direct precipitation was not (Li *et al.*, 1991). This may indicate that the DNA

plays an active role in these types of interactions. Furthermore, many examples exist of an increase in the *in vitro* DNA binding affinity or half-life of protein–DNA interactions upon the binding of a neighboring factor, although the mechanisms are unknown (Giniger and Ptashne, 1988; Janson and Pettersson, 1990; Xiao *et al.*, 1991). Functional methods have also been developed to detect these interactions, such as the reconstitution of chimeric GAL4 or LexA transactivators through the interaction of the proteins being tested (Blackwood and Eisenman, 1991; Chien *et al.*, 1991; Dang *et al.*, 1991; Fearon *et al.*, 1992; Fujii *et al.*, 1992). Although informative, the procedure is limited by the complete dependence on recombinant proteins and faithful reproduction of the native protein structure in the chimeric molecules. A caveat of all these approaches is that they are done in the absence of the natural context of the promoter, where neighboring proteins and the chromatin environment may play significant roles. To address these issues using a physiological promoter in an intact chromatin environment, we have adapted the procedure of *in vivo* genomic footprinting to detect the hierarchy and interdependence of transcription factors bound to functional class II major histocompatibility promoter sequences in intact cells (Mueller and Wold, 1989). These studies are then complemented by biochemical evidence showing protein interactions among transcription factors specific for this promoter.

The major histocompatibility complex (MHC) class II genes provide an appropriate and physiologically important context in which to study upstream promoter assembly (reviewed in Benoist and Mathis, 1990; Peterlin *et al.*, 1990; Glimcher and Kara, 1991; Ting and Baldwin, 1993). The MHC class II molecules have a central role in the immune response by presenting antigen on the surface of cells which is then recognized by the T cell receptor of class II-restricted T cells. This recognition is important in the selection of an appropriate T cell repertoire in the thymus, as well as the elicitation of an immune response in the peripheral lymphoid organs. Control of class II MHC gene expression is physiologically critical, as demonstrated by disease states characterized by either a lack of expression (bare lymphocyte syndrome) or hyperexpression (diabetes mellitus, multiple sclerosis, rheumatoid arthritis) of these molecules.

Previous studies have shown that the promoter of class II genes contains a number of functional upstream elements. Of importance in this study are the trimeric S, X box (X1 and X2) and Y/CCAAT elements. These elements are highly conserved in all murine and human class II genes and are separated from one another by a conserved distance. Analysis of constitutive and γ-IFN-induced MHC class II DRA expression has identified the stereo-specific alignment between the X box and Y/CCAAT elements as critical in promoter function (Vilen *et al.*, 1990). Analysis of the conserved distance separating the S and X elements revealed that any change in the distance, regardless of helical orientation, dramatically reduced the positive regulatory effects of these elements (Vilen *et al.*, 1992). Collectively, these findings are consistent with a model where protein–protein interactions are required between factors specific for the X1/X2 and Y/CCAAT elements and possibly with the S element.

A number of recombinant proteins can bind to the X1, X2 and Y/CCAAT elements. The heterodimeric NF-Y (also referred to as CBF, CP1 and YEBP) binds the Y/CCAAT element (Dorn *et al.*, 1987; Chodosh *et al.*, 1988b; Maity *et al.*, 1988; Zeleznik-Le *et al.*, 1991). rRF-X has been shown to bind the X1 site, although it is less clear that this recombinant protein functions in MHC class II transcription (Reith *et al.*, 1990). The X2 site is similar in sequence to known AP-1 sites and has been shown to bind Jun/Fos as well as hXBP and mXBP (Liou,H.-L. *et al.*, 1988; Liou,H.-C. *et al.*, 1990; Andersson and Peterlin, 1990). Functional analysis by reconstitution of *in vitro* transcription or blocking by NF-Y antibodies has directly demonstrated that class II MHC promoters are regulated by NF-Y (Zeleznik-Le *et al.*, 1991; Mantovani *et al.*, 1992). Indirect evidence using anti-sense oligonucleotides targeted to rRF-X and hXBP also shows possible involvement of these two factors in the control of class II MHC transcription (Ono *et al.*, 1991; Reith *et al.*, 1990).

NF-Y is a member of the CCAAT box binding family of proteins which is distinct in structure as well as binding specificity from the other known CCAAT box binding proteins, CTF/NF-1 and C/EBP (Dorn *et al.*, 1987). In addition to the class II MHC promoters, NF-Y can bind and functionally regulate through the CCAAT elements of a number of other promoters (Maity *et al.*, 1988; Wuarin *et al.*, 1990; Zeleznik-Le *et al.*, 1991; Milos and Zaret, 1992). The A and B subunits of NF-Y bear striking homology to the yeast transcription factors HAP3 and HAP2 respectively and are functionally interchangeable in a DNA binding assay (Chodosh *et al.*, 1988b). This evolutionary conservation from yeast to humans is reminiscent of the TATA binding protein (TBP) conservation (Hooft van Huijsduijnen *et al.*, 1990; Hernandez, 1993). Another parallel between TATA and CCAAT elements is that they occupy fixed locations in the promoter, the former at −25 to −30 bp and the latter at −60 to −80 bp (Bucher, 1990). A survey of 502 unrelated promoter sequences shows that most CCAAT boxes located at this position are NF-Y, but not C/EBP or NF-1, binding sites (Bucher, 1990). Finally, *in vitro* transcription analyses show that NF-Y and its target CCAAT element are important for transcription re-initiation, suggesting a role in basal transcription (Mantovani *et al.*, 1992; Milos and Zaret, 1992).

To explore the role of NF-Y in class II MHC transcription, its interaction with X box binding proteins and its general role in the assembly of proximal promoter complexes, we have taken two complementary approaches. First, cell lines which maintain stable, integrated DRA promoter–reporter constructs with specific mutations in the known *cis*-acting elements were produced. These integrated promoters were analyzed by genomic footprinting and revealed a hierarchy of protein–DNA interactions leading to a stable upstream promoter complex. Importantly, binding to Y/CCAAT is an early and crucial event required for binding at the X1 and X2 elements.

Second, several lines of biochemical experimentation show that recombinant and endogenous NF-Y proteins can stabilize and interact with endogenous X box binding proteins, thus providing a molecular basis for the potential recruitment of X factors by NF-Y.

Results

Stereo-specific alignment and distance constraints among three promoter elements suggest a multi-protein–DNA complex

The S, X and Y/CCAAT elements function in both constitutive and γ-IFN-induced expression of the DRA promoter. Previously we have shown that the helical alignment between X and Y/CCAAT, in addition to the absolute spacing between S and X, are important for constitutive expression and γ-IFN induction (Figure 1A and B; Vilen *et al.*, 1990). Addition of 0.5, 1.5, 3.5 or 5.5 helical turns between X and Y destroyed promoter function, while an additional one to six helical turns maintained promoter function. Addition of either a half (S+5XY) or one (S+10XY) helical turn between S and X abrogated the γ-IFN response. These results reveal that strict structural constraints between three conserved upstream promoter elements are required for promoter function, suggesting that multi-protein complexes may be formed by the proteins which bind these elements.

Production of stable transfectants for in vivo genomic footprint analysis

To assess interactions among proteins specific for the S, X1, X2 and Y/CCAAT elements, both *in vivo* and *in vitro* strategies were used. For the *in vivo* strategy, a panel of stably transfected cells lines harboring wild-type or mutant promoter constructs were produced and analyzed by genomic footprinting to decipher the influence of individual protein−DNA interactions on protein binding to adjacent DNA elements. Stable transfectants were established in the glioblastoma cell line U373-MG, in which we have previously defined the endogenous DRA *in vivo* footprint and γ-IFN induction of class II expression (Moses *et al.*, 1992; Wright and Ting, 1992). A wild-type DRA promoter−CAT fusion construct or one of four specific promoter mutants (Figure 1C) were stably integrated into the genome of the glioblastoma cell line. Analysis of the transcriptional activity of multiple monoclonal cell lines revealed that the wild-type DRA promoter was expressed at a low basal level, but could be induced ~3-fold upon γ-IFN stimulation (Figure 1C, WT). This result parallels the endogenous DRA transcription previously reported. Mutations in the Y/CCAAT box (mutY) or X1 box (mutX1) diminished basal and γ-IFN-induced expression to nearly undetectable levels. The X2 box mutation (mutX2) had no significant effect on transcription and did not diminish the level of γ-IFN induction. The S element was also required for γ-IFN induction of transcription. Together these data indicate that the S, X1 and Y/CCAAT elements are required for promoter function in stably transfected cells.

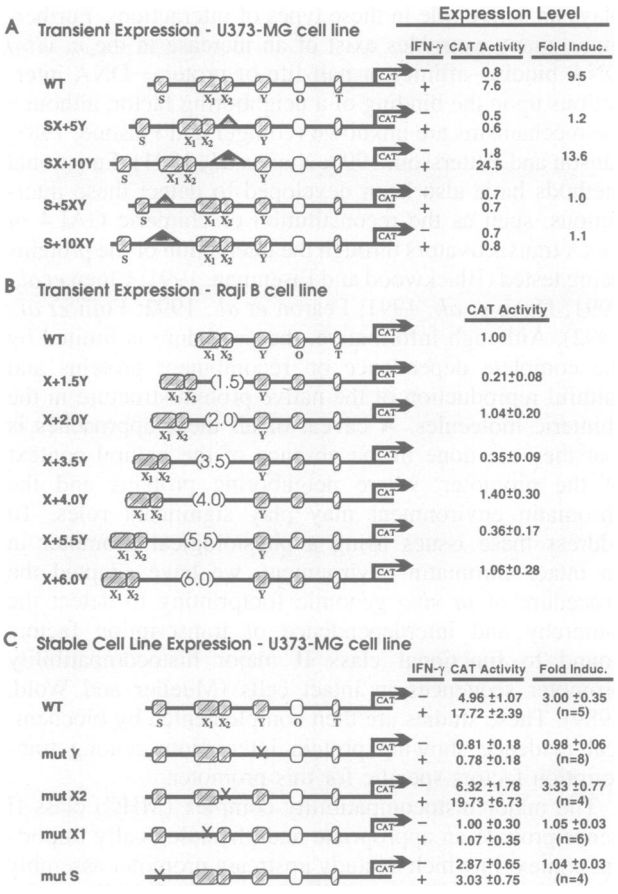

Fig. 1. Transient transfection and stable cell line analysis of the S, X1/X2 and Y/CCAAT promoter elements reveals that their integrity and stereo-specific alignment are required for DRA promoter function. (**A**) Schematic of HLA-DRA upstream promoter elements with changes in the relative spacing between S, X1/X2 and Y/CCAAT. Promoter activity of the various spacing mutants following γ-IFN induction in transiently transfected U373-MG cells is shown in a representative CAT assay. The small triangles represent 5 bp insertions, while larger triangles represent 10 bp insertions. Shaded elements are functionally important in the U373-MG glioblastoma cell line, while the non-shaded octamer site is not utilized (Wright and Ting, 1992). T, TATA; O, octamer; Y, CCAAT. (**B**) Insertion of as many as six additional helical turns maintains DRA promoter function. The number of inserted helical turns between X1/X2 and Y/CCAAT is depicted in the name of the construct. The results are presented as normalized means and standard errors of the mean (SEM) of three independent transient transfections. The octamer site (O) is shaded in the schematic of the constructs as this site is functional in Raji B cells. (**C**) The promoter elements S, X1 and Y/CCAAT sites are required for γ-IFN inducibility in stably transfected cell lines. Schematic of mutant promoter constructs and promoter activity in stable cell lines of U373-MG. The normalized CAT data with SEM is an average from four to eight independent monoclonal cell lines. A second distinct vector, 5′Δ-56SXY was also utilized to carry the wild-type promoter (Vilen *et al.*, 1992) and no significant differences were detected, indicating an independence from vector influences.

In vivo footprint analysis of the endogenous and integrated DRA promoter shows indistinguishable patterns

The importance of the S, X1, X2 and Y/CCAAT elements in γ-IFN induced DRA promoter assembly was addressed by ligation-mediated PCR *in vivo* genomic footprinting (Mueller and Wold, 1989). We have focused on γ-IFN

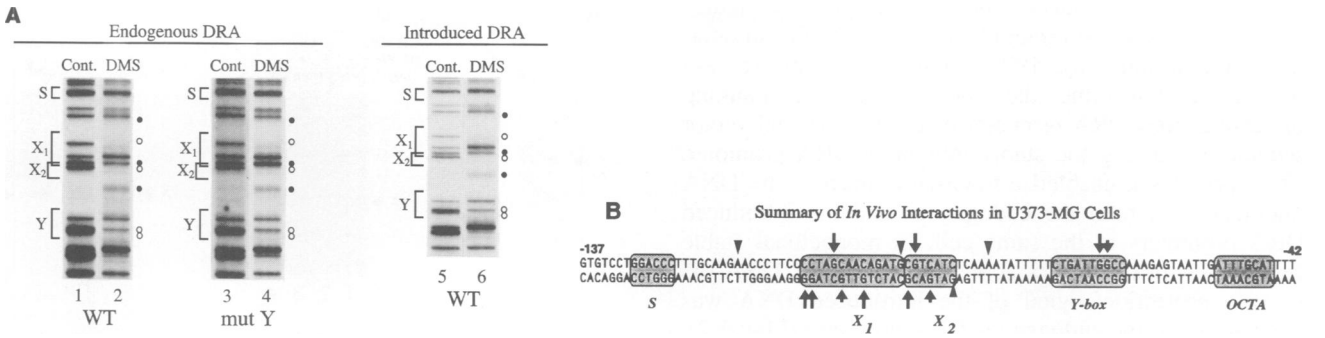

Fig. 2. *In vivo* genomic footprints of stably transfected cell lines reveals an indistinguishable binding pattern on the endogenous DRA promoter and the introduced wild-type DRA promoter. This pattern was identical to the DRA footprint seen in untransfected cells (Wright and Ting, 1992). Only the upper strand sequence is shown and a summary of all of the contact points are shown in (**B**). (**A**) Gene-specific primers selectively reveal only the endogenous (lanes 1–4) or introduced DRA promoter (lanes 5 and 6) in the same cells. Lanes 1–2, wild-type stable cell line, endogenous DRA footprint; lanes 3–4, mutY DRA stable cell line, endogenous DRA footprint; lanes 5–6, wild-type stable cell line, introduced DRA footprint. Functional elements are indicated on the left side of each footprint and the contact points are marked on the right side. Strong and weak protections are both indicated by an open circle, but are delineated in the text. Closed circles represent enhancements. Cont., *in vitro* methylated, deproteinized DNA; DMS, *in vivo* methylated DNA 48 h after γ-IFN induction. (**B**) Promoter sequence and summary of the *in vivo* protein−DNA interactions on the wild-type and endogenous DRA promoter. Long arrows, protections; short arrows, weaker protections; arrowheads, enhancements. The shaded areas represent the functionally defined elements, except the octamer element which is not utilized in U373-MG cells (Wright and Ting, 1992). The X1 and X2 boxes are depicted as separate regions although they may be overlapping, as shown in Figure 2A.

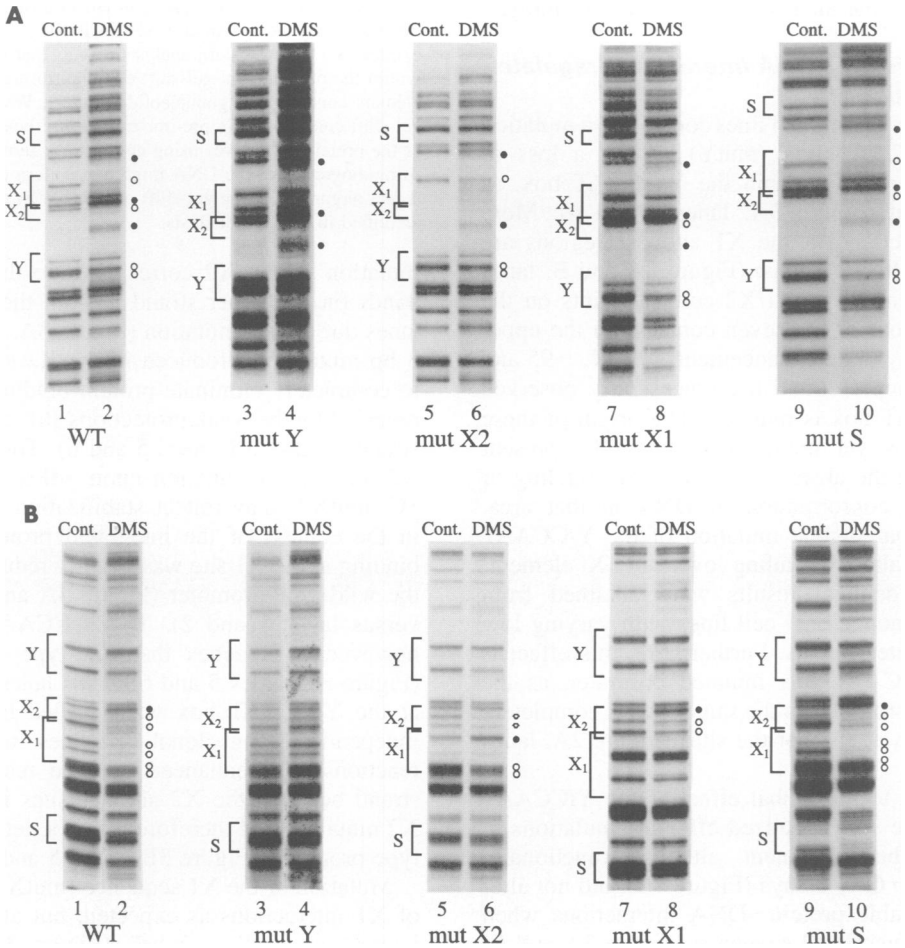

Fig. 3. *In vivo* genomic footprints of stably maintained mutants of the HLA-DRA promoter reveal that CCAAT box binding is required for binding of X1/X2-specific proteins. (**A**) Upper strand *in vivo* footprints. The introduced promoter was revealed using a primer set specific for the vector sequence of the original plasmid construct. (**B**) *In vivo* genomic footprints on the lower strand. The introduced promoter was revealed with a primer set specific for the CAT coding sequence. The cell line class is indicated below each pair of lanes. Multiple footprint reactions of at least two independent preparations of genomic DNA from three or more independent cell lines of each class were examined. The contacts indicated for each cell line class were determined to be highly reproducible. The open diamonds represent partial protections present in the X2 region of the mutX2 cell lines. All other symbols and markings are as described in Figure 2A.

regulation of the introduced constructs, as previous studies have shown maximal protein binding after γ-IFN stimulation (Wright and Ting, 1992). Gene-specific primer sets were directed to either the genomic sequences flanking the endogenous DRA promoter or to the CAT and vector sequences flanking the stably integrated DRA promoter. These primer sets enabled us to visualize the protein–DNA interactions at both the endogenous and the introduced DRA promoters in the same cell. In monoclonal stable cell lines with one to six copies of exogenous DNA the *in vivo* protection signal of the introduced DNA was comparable to the endogenous DRA promoter (Figure 2). Low copy number cell lines were specifically chosen to alleviate potential titration of available factors. Guanine residue contact points are spread throughout the X1 and X2 regions on both strands, while the Y/CCAAT element displays contacts only on the upper strand. Although the S element is functionally important, we and another group have been unable to observe *in vivo* contacts directly attributable to this site (Kara and Glimcher, 1991; Wright and Ting, 1992). These results establish the validity of studying stably transfected DRA promoter constructs in terms of regulated promoter usage *in vivo* and provide a system for pursuing the analysis of promoter assembly.

A hierarchy of protein–DNA interactions regulate promoter assembly

In vivo footprint analysis of cell lines containing a mutation of the DRA Y/CCAAT box (mutY) reveals a loss of protein–DNA interaction within the Y/CCAAT box, as would be expected (Figure 3A, lanes 3 and 4). More importantly, interactions in the X1 and X2 regions are also significantly altered *in vivo* (Figure 3A and B, lanes 3 and 4). All seven of the X1/X2 contact points on the lower strand and four of the seven contacts on the upper strand are lost. Only three enhancements (−117, −95 and −83 bp) on the upper strand are consistently observed when the Y/CCAAT box is mutated. The origin of these enhancements is as yet unknown and could represent residual binding in the absence of Y/CCAAT binding or simply reflect the conformation of DNA in that area. However, it is apparent that mutation of the Y/CCAAT box dramatically altered binding over the X element/CCAAT region. Identical results were obtained from four independent monoclonal cell lines with varying low numbers of integrated copies. Furthermore, this effect is specific for the CCAAT box mutated promoter, as the endogenous DRA promoter in the same cells is completely loaded with protein at each of the sites (Figure 2A, lanes 3 and 4).

In stark contrast to the global effect of the Y/CCAAT box mutation is the very localized effect of mutations in the S element. The S element, although functionally important in *in vivo* CAT assays (Figure 1C), did not alter any of the observable protein–DNA interactions when mutated to a non-functional sequence (Figure 3A and B, lanes 9 and 10). This result demonstrates distinct roles for *cis*-acting elements in promoter complex formation and emphasizes the central importance of Y/CCAAT box binding.

X2 box mutations (mutX2) reduced interactions at the X2 site, although this mutant does not completely abrogate X2 binding *in vivo* (Figure 3B, lanes 5 and 6). The 3 bp

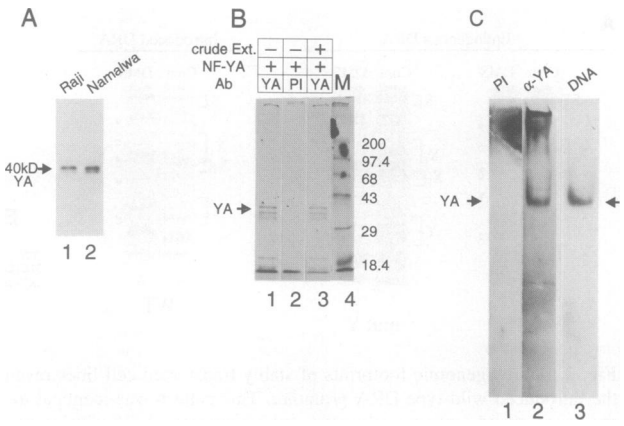

Fig. 4. A polyclonal antiserum generated against a C-terminal peptide of the NF-YA subunit specifically recognizes recombinant and native NF-YA in Western blot, immunoprecipitation and shift-Western analysis. (**A**) Western blot of nuclear extracts from the human B cell lines Raji and Namalwa using anti-NF-YA antiserum. (**B**) Immunoprecipitation analysis of recombinant radiolabeled NF-YA in the presence and absence of crude nuclear extract. Lane 1, *in vitro* translated NF-YA immunoprecipitated with anti-NF-YA without crude nuclear extract; lane 2, *in vitro* translated NF-YA immunoprecipitated with pre-immune serum; lane 3, *in vitro* translated NF-YA immunoprecipitated with anti-NF-YA in the presence of crude nuclear extract. (**C**) Shift-Western analysis reveals that NF-YA is contained within the predominant gel-shift complex formed on a Y/CCAAT element containing oligonucleotide. Lane 1, Western analysis of the gel shift complex using pre-immune serum; lane 2, Western analysis of the protein complexes using anti-NF-YA serum; lane 3, autoradiography of the DNA migration pattern of the gel-shift. Arrows depict alignment of the gel-shift complex with the NF-YA protein identified in Western analysis.

mutation within X2 corresponds to the absence of three bands on the upper strand in both the control and DMS lanes due to this mutation (Figure 3A, lanes 5 and 6). The 3 bp mutation introduced in the X2 site is not sufficient to completely eliminate protein binding at X2 *in vivo,* as revealed by the weak protections still present on the lower strand (Figure 3B, lanes 5 and 6). Thus the ability of the X2 mutation to function upon γ-IFN stimulation (Figure 1C, mutX2) may reflect stabilization of the X2 proteins in the context of the integrated promoter. Interestingly, binding at the X1 site was slightly reduced compared with the wild-type promoter (Figure 3A and B, lanes 5 and 6 versus lanes 1 and 2). The Y/CCAAT box interaction, however, approaches the wild-type promoter footprint (Figure 3A, lanes 5 and 6 versus lanes 1 and 2). Binding at the Y/CCAAT box was verified by analysis of four independent monoclonal cell lines in multiple footprint reactions. The enhanced guanine residue on the lower strand between the X2 and X1 sites is derived from the X2 mutation and therefore was not detectable in the wild-type promoter (Figure 3B, lanes 5 and 6).

Mutation of the X1 sequence (mutX1) resulted in a loss of X1 interactions as expected, but also a diminution of interaction at the X2 box (Figure 3A and B, lanes 7 and 8). The X2 interactions are substantially abrogated, however, binding is still detectable on both the upper and lower strands at the same positions. The effect of X1 mutations on the Y/CCAAT box are more difficult to discern, because of the weak Y/CCAAT box protection normally present in the wild-type and endogenous promoters. Repeated *in vivo* footprint reactions of four

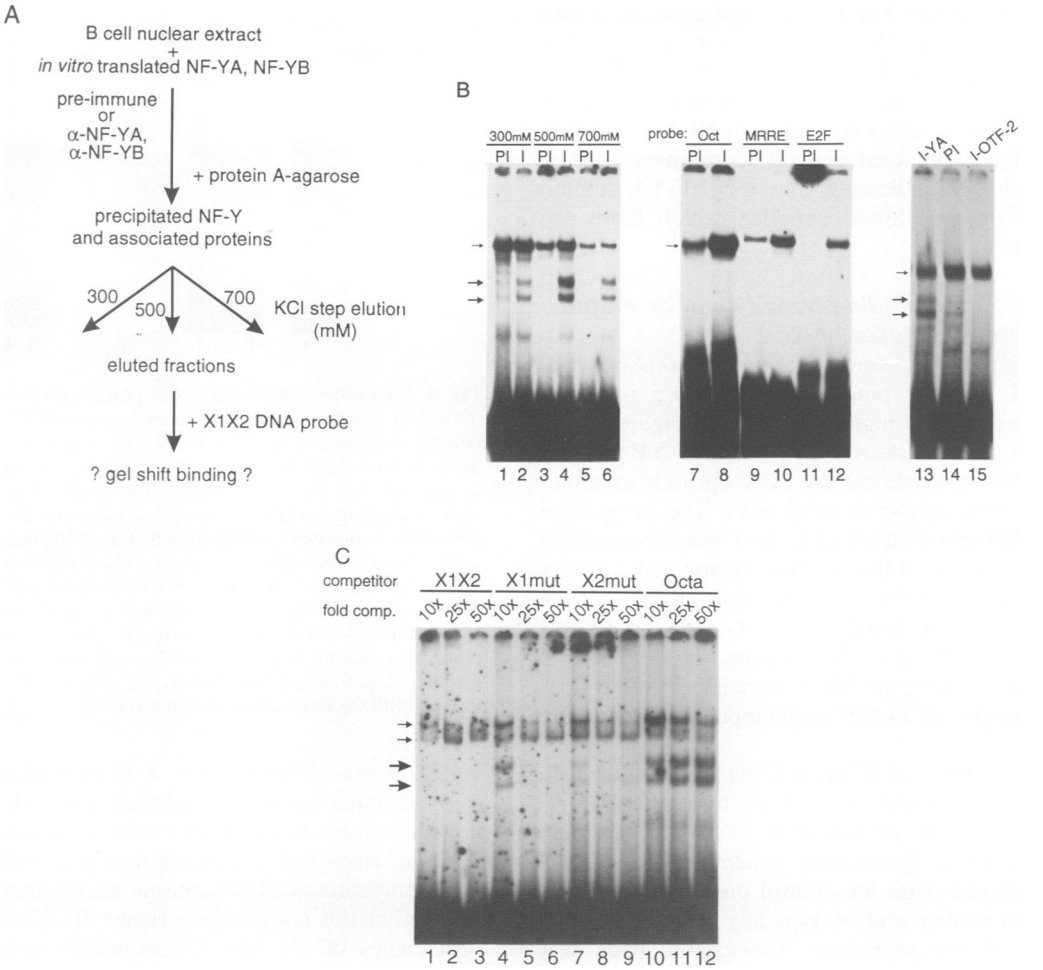

Fig. 5. Immunoprecipitation of NF-Y co-precipitates two X box specific complexes. (**A**) Schematic of the method used to identify proteins interacting with native and recombinant NF-YA. B cell nuclear extracts were combined with *in vitro* translated NF-YA and NF-YB and then immunoprecipitated with antiserum generated against NF-YA or pre-immune serum. Proteins which interacted with NF-Y were analyzed for X box DNA binding activity. (**B**) Gel-shift analysis of the eluate reveals that two X-specific protein−DNA complexes (large arrows) immunoprecipitate with NF-Y. Non-specific interactions are indicated by small arrows. PI is pre-immune and I is immune precipitation. Lanes 1 and 2, 300 mM KCl elution; lanes 3 and 4, 500 mM KCl elution; lanes 5 and 6, 700 mM KCl elution; lanes 7–12, direct binding of the 500 mM eluates to unrelated oligonucleotide probes as indicated above each lane; lanes 13 and 14, as lanes 5 and 6; lane 15, immunoprecipitation of recombinant OTF-2 with anti-OTF-2 antibodies reveals no X box binding factors. (**C**) Gel-shift competition of the NF-Y immunoprecipitated proteins identifies both X1 and X2 proteins. The gel-shift probe was the wild-type X1/X2 sequence and the extract was the 500 mM eluted fraction. Lanes 1–3, X1/X2 competitor; lanes 4–6, mutated X1/wild-type X2 competitor (X1mut); lanes 7–9, wild-type X1/mutated X2 competitor (X2mut); lanes 10–12, unrelated octamer-containing competitor (Octa). Each competitor was used at 10-, 25- and 50-fold molar excess as indicated.

independent monoclonal cell lines have established continued protection at the Y/CCAAT box when the X1 site is mutated. These findings suggest that while X1 binding is not required for interactions at other sites in the promoter, X1 binding does stabilize and enhance X2 binding and may have a minimal effect on Y/CCAAT box binding. These results reveal a hierarchy of protein−DNA interactions leading to the formation of a stable promoter complex and indicate a primary importance for the Y/CCAAT element. Y/CCAAT binding proteins could act to recruit additional factors or stabilize protein complex formation via direct protein−protein interactions. Significantly, a recent report by Reith *et al.* (1994) has shown NF-Y stabilizes DNA binding of an X1 factor *in vitro*. To address the potential for direct interactions between X and Y factors, several *in vitro* biochemical analyses were performed.

The CCAAT box binding protein NF-Y binds the DRA Y element

Previous studies have shown that the transcription factor NF-Y binds the CCAAT sequence of a number of genes, including the MHC class II DRA (Dorn *et al.*, 1987; Oikarinen *et al.*, 1987; Chodosh *et al.*, 1988a; Raymondjean *et al.*, 1988). To directly examine whether NF-Y interacts with X box binding proteins, we generated a polyclonal antiserum against a C-terminal peptide of the NF-YA subunit. The specificity of this antiserum was identified as follows: (i) Western blot analysis of nuclear extracts from two human B cell lines with this antibody revealed a protein of identical molecular weight (40 kDa) to that reported for the NF-YA subunit (Figure 4A; Hooft van Huijsduijnen *et al.*, 1990; Maity *et al.*, 1990); (ii) immunoprecipitation analysis with this antiserum shows that *in vitro* translated NF-YA is specifically recognized

by the immune serum but not the pre-immune serum (Figure 4B); (iii) shift-Western analysis revealed NF-Y protein in the protein–DNA complex formed on the Y/CCAAT element (Figure 4C). The position of NF-Y located by Western analysis with the anti-NF-YA antibody (lane 2) and the radiolabled gel-shifted complex (lane 3) aligned completely, indicating that the NF-YA subunit is present in the gel-shifted complex which binds the CCAAT element.

Endogenous X box binding activity can be eluted from immunoprecipitates of NF-Y

To assess directly the potential for interactions between NF-Y and X element binding proteins, we used the co-immunoprecipitation regime depicted in Figure 5A. In this scheme *in vitro* translated NF-YA and NF-YB proteins were combined with crude nuclear extract prior to immunoprecipitation with anti-NF-Y antibodies. The association of X element binding proteins with NF-Y was subsequently determined by elution of the immunoprecipitated complex with a salt gradient, followed by gel-shift analysis of the eluate for X element binding activity. We chose this strategy to assess putative interactions in an effort to maximize our recovery of NF-Y-associated proteins, as previous attempts at direct immunoprecipitation were unsuccessful.

The co-precipitation of X element binding activity with NF-Y reflects interaction of at least two proteins. As shown in Figure 5B, precipitation of two X element binding proteins was significantly greater with anti-NF-Y immune serum than with the control pre-immune serum. Maximum differential elution typically peaked at ~500 mM KCl. A slower migrating, non-specific band was precipitated by both immune and pre-immune sera and primarily eluted at the lower 300 mM salt concentration (small arrow). To confirm the specificity of the co-precipitation, two control experiments were performed. First, we tested whether the precipitation of the *in vitro* translated NF-Y resulted in a general isolation of DNA binding proteins. DNA binding activity in the 500 mM KCl eluate was tested on an octamer, MRRE, and E2F binding site (Figure 5B, lanes 7–12). Only the same non-specific band was detected in each case, confirming the specificity of the NF-Y and X precipitation. The second control was to test whether any combination of DNA binding protein and its antibody would precipitate the X box binding factors detected by NF-Y. We repeated the co-immunoprecipitation scheme replacing the *in vitro* translated NF-YA and B with *in vitro* translated OTF-2, followed by precipitation with anti-OTF-2 antibodies. The OTF-2 transcription factor is known to bind to the octamer recognition sequence present within this same DRA promoter. OTF-2 was unable to co-precipitate the X box binding proteins (Figure 5B, lanes 13–15).

The X element binding proteins specifically precipitated with NF-Y were further characterized by gel-shift competition analysis using oligonucleotides containing mutations within the X1 or X2 elements (Figure 5C). Both complexes were efficiently competed by a 10-fold excess of homologous competitor (Figure 5C, lane 1). Mutation of the X1 box produced a significantly less efficient competitor, while mutation of the X2 element partially weakened the competition. A competitor with a mutated

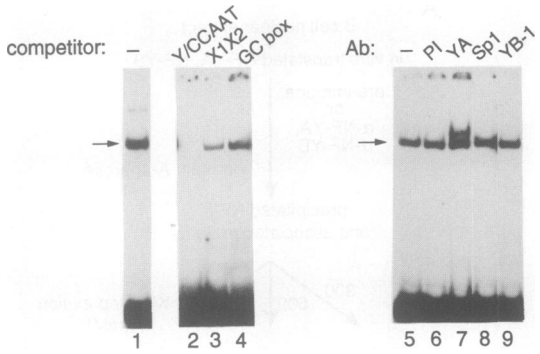

Fig. 6. DNA affinity purification of X proteins retains NF-Y. Y/CCAAT box binding activity retained on an X1/X2 DNA affinity column and eluted in fractions 58–62 was revealed by gel-shift analysis (lanes 1and 5). The arrow marks the predominant band which co-migrates with the NF-Y complex from crude extracts (data not shown). Binding reactions for lanes 1–4 used only 25 ng poly(dI:dC) non-specific competitor, while lanes 5–9 used 100 ng. Lanes 2–9 were exposed five times longer than lane 1. Oligonucleotide competition: lane 2, homologous competitor, Y/CCAAT (XY60mtX12); lane 3, X1/X2 competitor, (XY60mtY); lane 4, non-specific competitor, GC box (antibodies specifically recognize NF-YA in the complex); lane 6, pre-immune serum; lane 7, anti-NF-YA antibody; lane 8, anti-Sp1 antibody; lane 9, anti-YB-1antibody. The anti-NF-YA antibody did not interact with the DNA alone (data not shown).

X1 sequence (X1mut, lanes 4–6) competed poorly at a 10-fold molar excess, yet abolished the signal at higher concentrations. A competitor with a mutated X2 element (X2mut, lanes 7–9) competed nearly as well as the wild-type competitor at all concentrations. An unrelated octamer competitor did not compete (lanes 10–12). The different efficiencies of X1 and X2 sequences to compete with these bands are confirmed by direct binding to DNA probes mutated in either site (data not shown). These results show that proteins binding to the X1 sequences and potentially the X2 sequences are co-precipitated with NF-Y molecules. One interpretation of this result is that both X1 and X2 box binding proteins are involved in the complex and that both are required for maximal stabilization of the protein–DNA complex. This is in agreement with the *in vivo* footprint analysis, which shows X1 and X2 binding are interdependent.

DNA affinity purification of X box binding proteins retains NF-Y

A classical method to isolate DNA binding proteins is to exploit the high affinity protein–DNA interaction by column chromatography on immobilized DNA binding sites (Kadonaga and Tjian, 1986). Our laboratory and others have used this procedure to isolate candidate X box binding proteins (Y.Itoh-Lindstrom, in preparation). We have now examined whether NF-Y could be specifically retained during the purification of the X box binding factors. *A priori*, NF-Y does not bind to the X box probe directly because neither *in vitro* translated nor affinity purified NF-Y bound this probe (data not shown; Zeleznik-Le *et al.*, 1991). Crude Namalwa B cell nuclear extract was initially passed over a heparin–agarose column followed by three successive purifications over an X1/X2 DNA affinity column. X box binding activity was monitored by gel-shift analysis on an X1/X2 DNA probe. After the final purification, fractions containing distinct X

box binding activities were pooled and assayed for Y/CCAAT box binding. Fractions 58–62, eluting in a linear gradient at ~600 mM KCl, displayed Y/CCAAT box binding activity (Figure 6, lane 1). No other DNA binding activities could be detected in this fraction (data not shown). The small amount of protein present in this fraction made the Y/CCAAT box binding sensitive to excess competition from the non-specific competitors poly(dI:dC) and poly(dI):poly(dC). However, as shown in Figure 6 (lanes 1 and 5), significant binding could be detected at both 25 and 100 ng poly(dI):poly(dC). Lane 1 was exposed for a five times shorter period to produce a comparable signal. Oligonucleotide competition with several unrelated probes significantly reduced the band intensity (lanes 2–4). However, only the Y/CCAAT element completely removed the band. Direct evidence for the presence of NF-Y was shown using the antibody to the NF-YA subunit. The anti-NF-YA antibody recognized and retarded the migration of the Y/CCAAT gel-shift band (lane 7). Neither pre-immune serum nor anti-Sp1 antibodies could recognize this band (lanes 6 and 8). Antibodies directed against another Y box binding protein, YB-1, could not recognize this band (lane 9). Furthermore, in the absence of protein the anti-NF-YA antibody did not alter the mobility of the probe (data not shown). In addition, as a direct control for the lack of NF-Y binding directly to the X1/X2 DNA affinity matrix, recombinant NF-Y or OTF-2 were passed over the X1/X2 DNA column. Both proteins eluted primarily in the 100 and 200 mM KCl loading buffer, while neither were present in the 600 mM KCl fraction (data not shown). This excludes the possibility that NF-Y is directly and specifically binding the X1/X2 affinity matrix. Thus we have been able to isolate endogenous NF-Y proteins using DNA-bound endogenous X box binding factors. In a reciprocal manner, endogenous X box factors could be isolated by co-precipitation with recombinant NF-Y protein (Figure 5). These data together provide strong direct evidence for interactions between endogenously produced NF-Y with X box binding proteins. The exact identity of the X element binding proteins is unclear. Although several X1 and X2 box binding proteins have been reported, their functional importance in DRA gene regulation is not entirely clear and we have not been able to specifically identify them in these experiments.

Discussion

Regulation of transcription requires many levels of protein–DNA and protein–protein interactions at the proximal promoter and at distally located promoter/enhancer elements. Furthermore, the chromatin structure surrounding a gene can greatly influence transcriptional activity, most likely by altering accessibility of the transcription factors to the DNA. This report focuses on the CCAAT box binding factor NF-Y, which is required in the regulation of a large number of genes, including MHC class II DRA. Several lines of evidence have been presented to demonstrate that NF-Y interacts with proteins binding to the upstream elements (X1 and X2) in the DRA promoter and that this interaction is crucial for gene transcription: (i) a functional requirement for alignment between the X and Y elements; (ii) *in vivo* X box binding requires Y/CCAAT box occupancy; (iii) specific isolation of NF-Y or X box binding proteins reciprocally isolates the other protein from nuclear extracts.

Stereo-specific alignment of the X box and Y/CCAAT element is required for optimal function (this report; Vilen *et al.*, 1990, 1992). In addition, the absolute distance between the elements is of minor importance compared with the alignment, since as many as eight helical tuns can separate them before function is affected. This was the first evidence to suggests that X box and Y/CCAAT binding proteins either interact directly or are part of a larger transcription complex which assembles on one phase of the DNA helix. The significance of this alignment is reiterated by the observed evolutionarily conserved spacing in all MHC class II promoters. Stereo-specific alignment has been reported for several other promoters (Takahashi *et al.*, 1986; Pape *et al.*, 1990; Waibel and Filipowicz, 1990; Harvey *et al.*, 1991), as well as specific distance requirements (Wu and Berk, 1988). Interestingly, two examples involve the NF-Y protein. On the β-actin promoter, the NF-Y site must also be stereo-specifically aligned with a CCArGG box which binds $p67^{SRF}$ (Danilition *et al.*, 1991). In addition, spatial constraints are seen in the albumin promoter, where efficient activation of transcription by NF-Y and C/EBP requires juxtapositioning of these two sites (Milos and Zaret, 1992). These structural requirements are usually interpreted as involving protein–protein interactions. The repeated occurrence of this phenomenon also suggests that the DNA plays an important role in defining which protein–protein interactions will occur *in vivo*. A requirement for alignment by DNA may be necessary to prevent inappropriate interactions from taking place outside of a promoter region and may compensate for weak affinities between distinct transcription factors.

A second line of evidence indicating an X–Y interaction came from the *in vivo* genomic footprint analysis, which reveals a hierarchial loading of transcription factors onto the DRA promoter. Occupancy at the Y/CCAAT element served as a primary event that was required for occupancy of the adjacent X1 and X2 sites. This is a particularly important approach, because it defines the interdependence of DNA binding in the context of the chromatin environment of the intact cell. This provides a certain level of significance to pursue the *in vitro* interaction studies. A few endogenous promoters have been examined for *in vivo* DNA interactions. While most have involved constitutive interactions and thus have not been able to address interdependence, a few have examined inducible or tissue-specific promoters. Induction of c-*fos* transcription by epidermal growth factor revealed no changes in DNA binding across the promoter (Herrera *et al.*, 1989). However, a role for disruption of chromatin structure upon binding of a specific factor has been described in several other promoters. For example, glucocorticoid receptor (GR) binding is required to permit NF-1 binding at an adjacent site (Pina *et al.*, 1990; Archer *et al.*, 1991). The role of GR binding appears to be in disrupting the chromatin structure at the NF-1 site. GR binding seems to play a similar role at the tyrosine aminotransferase promoter (Becker *et al.*, 1986; Carr and Richard-Foy, 1990; Rigaud *et al.*, 1991). Tissue-specific expression of the serum albumin gene involves chromatin disruption by

HNF-3-like factors, presumably to promote the recruitment of ubiquitous factors (McPherson *et al.*, 1993). While it is unclear if these factors also function by direct protein–protein contact, a similar role for NF-Y could be envisioned at the DRA promoter. Coordinate *in vivo* binding of constitutive and regulated factors has been demonstrated for the interleukin-2 (IL-2) promoter (Garrity *et al.*, 1994). Cells expressing IL-2 display factor binding at many sites within the first 300 bp of the promoter, while non-expressing cells have no *in vivo* binding. When expressing cells are treated with cyclosporin-A to deplete only a small subset of the factors, *in vivo* binding is lost at all of the promoter sites. This suggests a hierarchy of interactions dependent on a cyclosporin-A sensitive component and is similar to the role we have proposed for NF-Y on the DRA promoter.

While this manuscript was in preparation, Kara and Glimcher reported a similar analysis of the DRA promoter in B cell lines which constitutively express high levels of DRA, as well as in mutant cell lines lacking DRA expression (Kara and Glimcher, 1993). In DR-positive B cell lines, the interdependency between X1, X2 and Y/CCAAT was not revealed. The key difference between these two studies is that they analyzed site interdependence during constitutive expression in a B cell line. The difference in the two analyses may be explained by the *in vivo* footprint analysis showing that constitutive expression exhibits maximal transcription factor binding, whereas protein binding affinities in the γ-IFN inducible cells used in this study is suboptimal and modulated upon γ-IFN treatment (Wright and Ting, 1992). In the γ-IFN inducible system, interactions between DNA binding proteins may be required to stabilize this lower affinity protein–DNA complex. Another possibility is that the octamer site, which is unoccupied in the glioblastoma cell line but occupied in the B cell lines (Wright and Ting, 1992), may alter the observable protein–protein interactions at other sites. Thus, our ability to show interdependency of DNA binding may be a direct reflection of the cell system used.

Several lines of biochemical evidence suggest that NF-Y may exert its function by direct protein–protein interaction with X box binding factors. First, recombinant NF-Y specifically co-precipitated X box binding factors, while another recombinant transcription factor (OTF-2) important for DRA could not. Secondly, endogenous NF-Y was shown to be co-purified during the isolation of endogenous X box binding proteins. Although, the identity of the X box protein involved in this co-purification is as yet unknown, this experiment is significant, due to the use of only endogenous proteins. This alleviates concerns that the use of excess recombinant proteins may promote artificial interactions. Lastly, the *in vitro* binding stability of at least one X box binding protein was increased when NF-Y was bound to the adjacent CCAAT box (Reith *et al.*, 1994; K.L.Wright, unpublished observation). Reith *et al.* have recently reported a large stabilization of RFX binding and a small stabilization of NF-Y when the other protein was present on the DNA. Combined, the stabilization studies and the reciprocal isolation of X and NF-Y proteins provide strong evidence that these protein can directly interact *in vitro* and suggests a mechanism for the interdependence observed *in vivo*. Nevertheless, an intermediary factor cannot be excluded at this point.

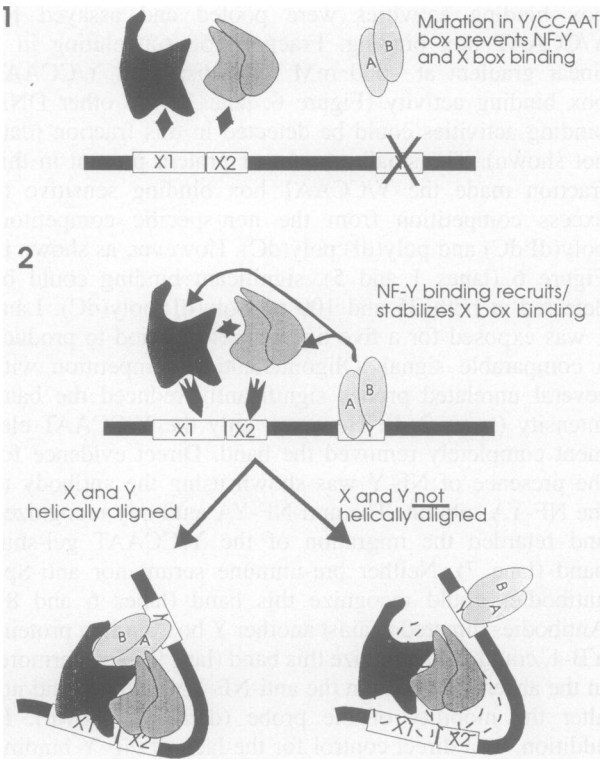

Fig. 7. NF-Y acts as a central nucleating factor in recruiting or stabilizing upstream protein binding through direct interaction with X element-specific proteins. In model 1 mutations within the Y/CCAAT sequence prevent NF-Y binding and subsequent binding at the X1/X2 sites. In model 2 an intact Y/CCAAT site allows NF-Y binding at the Y/CCAAT site and either recruits proteins or stabilizes protein binding at the X1/X2 sites. The helical orientation of the X1/X2 and Y/CCAAT sites is critical for alignment of the proteins for interactions and mis-alignment may lead to weakened X box binding. The bending of the DNA is for simplicity of viewing and has not been demonstrated. Star indicates X1/X2 interdependence.

This report provides a basis for a working model where binding of NF-Y to the Y/CCAAT element is an early and requisite event in the assembly of the DRA proximal promoter (Figure 7). This binding is essential for subsequent occupation of the X1 and X2 elements by transcription factors. The biochemical evidence suggests that NF-Y recruits X1 and X2 proteins to the promoter by direct protein–protein interactions. The requirement for helical alignment indicates that such protein–protein interactions are disrupted if the bound proteins do not align on the same phase of the DNA helix. When occupying the same phase of the DNA, NF-Y may stabilize the binding of X box proteins to their respective target sites by serving as an anchoring molecule and/or by inducing conformational alterations in these proteins resulting in enhanced affinity for their DNA target sites. Additional interactions between the X1 and X2 binding proteins also seem to occur, as suggested by the *in vivo* footprint and the co-precipitation analyses. The co-dependency of proteins binding to the X1 and X2 elements may also reflect stabilization via protein–protein interactions, although direct evidence is required to prove this possibility.

The abundance of genes containing a CCAAT element within the proximal promoter makes the CCAAT box

binding factors potential candidates to serve a more general role in transcriptional regulation (McKnight and Tjian, 1986; Mitchell and Tjian, 1989). In many promoters, the CCAAT element is located ~60–80 bp upstream of the start site of transcription. Although CCAAT elements can be found elsewhere in the regulatory regions of other genes, a survey of known NF-Y target sites show that its location is typically confined to the −60 to −130 region and most proximally located CCAAT elements precisely reflect the NF-Y target sequence, not those for C/EBP or NF-1 (Bucher, 1990). Further evidence for a general role for NF-Y in the formation of a proximal promoter complex is provided by recent studies of the MHC class II Eα gene and the albumin gene, demonstrating that NF-Y functions early in pre-initiation complex formation (Mantovani *et al.*, 1992; Milos and Zaret, 1992). These studies show that antibodies to NF-Y can block re-initiation of transcription in both genes (Mantovani *et al.*, 1992). The *in vivo* findings presented here show that proteins binding to the upstream elements are dependent on loading at the CCAAT site and that binding of the Y/CCAAT element by NF-Y provides a central nucleating point in recruiting additional proteins to the proximal promoter by protein−protein interaction. This is reminiscent of protein complex formation on the basal promoter when TFIID binding to the TATA element initiates protein complex formation involving direct interactions with several components of the basal machinery, including TFIIA and TFIIB (reviewed in Roeder, 1991). Our results would suggest that NF-Y plays a similar role in initiating protein complex formation among upstream activating elements. Considering the prevalence of Y/CCAAT sites in multiple eukaryotic promoters, interactions between NF-Y and other transcription factors are likely pivotal steps in transcriptional regulation and are deserving of intense investigation.

Materials and methods

Cell culture and transient transfection

Raji is a human Epstein−Barr virus-positive Burkitt's lymphoma cell line that expresses high levels of MHC class II antigens. These cells were grown in RPMI 1640 supplemented with 8% fetal calf serum and 2 mM glutamine. The U373-MG cell line is a glioblastoma multiform cell line which expresses low basal levels and high γ-IFN-induced levels of MHC class II antigens (Basta *et al.*, 1987). These cells were maintained in McCoy's 5A with 10% fetal calf serum, 2 mM glutamine, 100 U penicillin and 100 μg/ml streptomycin. Transient transfections were performed by electroporation as previously described (Sherman *et al.*, 1987).

Constructs

The wild-type DRA promoter construct, 5′Δ-56SXY, which contains the first 141 bp of the DRA promoter fused to the chloramphenicol acetyltransferase (CAT) reporter gene, and the mutants SX+5Y, SX+10Y, S+5XY and S+10XY derived from this construct have been previously described (Vilen *et al.*, 1992). Briefly, overlapping oligonucleotides corresponding to the −141 to −98 bp region of DRA (including S and X) and the region from −109 to −61 bp (including X and Y) were made double-stranded using reverse transcriptase (Life Sciences) and then cloned into the *Xba*I site of 5′Δ-56 to create the wild-type construct. The mutant constructs contained either the 5 bp insert sequence TGCAG or the 10 bp insert sequence TGCAGGTCGC. Dideoxynucleotide sequencing was performed directly from the double-stranded plasmid to confirm the sequences of all constructs. The parent plasmids used to generate the giant spacer constructs X+1.5Y and X+2.0Y, containing one and a half or two additional turns of the DNA helix between the X and Y motifs, have been previously described (Vilen *et al.*, 1990) (therein referred to as pSpacer+15 and pSpacer+20). Additional constructs with increasing numbers of half integral and integral helical turns were made by inserting multiple copies of the sequence GTCGCTGATCATGTGTCTGCA, representing two helical turns, into the unique *Pst*I site created in X+1.5Y and X+2.0Y. One and two copies of this sequence inserted into X+1.5Y generated X+3.5Y and X+5.5Y respectively, while one and two copies inserted into X+2.0Y generated X+4.0Y and X+6.0Y respectively.

The constructs used to generate the stable cell lines have been described previously (Moses *et al.*, 1992). Briefly, the wild-type DRA construct (5′Δ-152DRA-CAT) contains the first 152 bp of the DRA promoter fused to the CAT reporter gene and subcloned in the pBluescript SK+ vector (Stratagene). Each of the mutant DRA constructs contain selected point mutations as described previously (Moses *et al.*, 1992) (therein mutY, mutX2, mutX1 and mutS are referred to as Y-M1, X-M2, X-M3 and S-M2 respectively). The neomycin selection marker was carried on a separate plasmid, pSV2neo (Southern and Berg, 1982).

Stable cell line transfection and selection

Each circular construct (20 μg) was introduced independently into 4×10^6 U373-MG cells by electroporation (200 mV, 960 μF, 300 μl volume; Bio-Rad Gene Pulser), together with a 1/20 molar amount of the neomycin resistance gene pSV2neo. Selection with 300 U/ml G418 (Geneticin; Gibco) was begun 24–36 h after transfection. The amount of G418 was determined empirically to completely kill mock-transfected cells within 8–12 days. Individual resistant colonies were visible in 2–3 weeks, at which time the cells were cloned by limiting dilution and expanded. Cell lines were always maintained in 300 U/ml G418, except when harvesting cells for CAT activity determination (see below). The conditions used to generate the stable lines were intended to decrease the frequency of integration and thereby enhance the percentage of low copy number transfectants in the surviving population. More than 80% of the examined monoclonal cell lines contained 10 or less copies of the test plasmid, as determined by quantitative Southern blot analysis (Southern, 1975). DNA copy number was also shown to be directly proportional to the signal intensity of the ligation-mediated PCR sequencing ladder and was used as an additional confirmation of relative DNA copy number.

CAT reporter gene analysis

Following transfection, the U373-MG cells were treated with recombinant γ-IFN at 500 U/ml for 24–48 h prior to harvest. The human recombinant γ-IFN was a generous gift from Biogen (Cambridge, MA). The Raji cell transfections were harvested 24 h following electroporation. The stable cell lines were removed from G418 antibiotic selection two passages before harvest and CAT quantitation. Expression of both the transiently transfected and the stably transfected reporter genes was assayed by quantitating CAT activity in cell extracts as previously described (Sherman *et al.*, 1989). CAT activity was resolved on thin-layer chromatography plates and quantitated with a Molecular Dynamics PhosphorImager. Fold induction was calculated by dividing the percent acetylation in γ-IFN-treated cultures by the analogous untreated controls.

In vivo footprinting

In vivo methylation of cells and DNA preparation were as described (Pfeifer *et al.*, 1990). The ligation-mediated PCR-amplified *in vivo* genomic footprinting was as originally described by Mueller and Wold (1989) and modified by Wright and Ting (1992). The primers used to reveal the endogenous DRA promoter were as described previously. The primers to reveal the stably introduced DRA constructs were targeted to the vector and CAT coding sequences. Primer sets used to reveal the lower strand correspond to vector sequences of the BlueScript plasmid (Stratagene) and are: SKVEC primer 1, 5′-CACACAGGAAACAGC-TATG-3′ (T_m = 56°C); SKVEC primer 2, 5′-GCTCGGAATTAACCC-TACCTAAAGGGAAC-3′ (T_m = 62°C); SKVEC primer 3, 5′-AACCCTCACTAAAGGGAACAAAAGCTGGAGC-3′ (T_m = 63°C). Primer sets to display the upper strand correspond to the CAT coding sequence and are: PCAT primer 1, 5′-CAACGGTGGTATATCCAGTG-3′ (T_m = 58°C); PCAT primer 2, 5′-CTTCCTTAGCTCCTGAAAATCT-CGCCAAGC-3′ (T_m = 63°C); PCAT primer 3, 5′-GCTCCTGAAAAT-CTCGCCAAGCTCAGATCCTC-3′ (T_m = 65°C).

Immunoprecipitation and Western blots

Polyclonal rabbit antisera were generated against synthetic peptides of NF-YA or NF-YB by Animal Pharm Services, Inc. (San Francisco, CA).

The peptide used as the immunogen for anti-NF-YA was NH_2-C-DEEAMTQIIRVS-COOH conjugated to KLH. The peptide for the production of anti-NF-YB has been reported previously (Mantovani *et al.*, 1992). High titer antisera was subsequently purified by passage over protein A/protein G−Sepharose columns as described by the supplier (Pierce, Rockford, IL). Antibody specificity was confirmed as described in the text (see Figure 4). Immunoprecipitation and Western blot analysis were performed using standard protocols (Gallagher *et al.*, 1989; Springer, 1992). Western blots were blocked with 1% gelatin 1 h prior to incubation with primary antibody. Blots were developed using the ECL detection system (Amersham).

Gel-shift analysis and shift-Western analysis

Gel-shift reactions were performed as previously described (Vilen *et al.*, 1990). Binding reactions were carried out in 10% glycerol, 50 mM NaCl, 10 mM Tris−HCl, pH 7.9, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT with 50 ng−2 μg poly(dI:dC) (Pharmacia) in a 20 μl volume at room temperature for 15 min. The samples were resolved by electrophoresis through 5% or 6% native polyacrylamide gels with a TGE running buffer (25 mM Tris−HCl, pH 7.9, 190 mM glycine, 1 mM EDTA). The sequence of the oligonucleotide probes used for detection of the purified factors are: X1/X2, tcgaCCTTCCCCTAGCAACAGATGCGTCATCTCA; X1mut, tcgaCCTT**GGCCGAATG**ACAGATGCGTCATCTCA; X2mut, tcgaCCTTCCCCTAGCAACAGATG**GAACTT**CTCA; XY60mtX12, GAACCCTT**AGAACTAGTC**CAG**ATTGTGAG**TCTCAAAATATTTTTCTGATTGGCCAAAGAG; XY60mtY, GAACCCTTCCCCTAGCAACAGA**TTGTGAG**TCTCAAAATATTT TTC**GGAGGTT**CCAAAGAG; GC element, GCCTGTTCC TCGTACTAGCCGAGAGCCCCGCCCTCGTTCCCTCC. Antibody supershift of NF-Y complexes was performed by including 1 μg anti-NF-YA antibody in the gel-shift reaction mix for 2 h on ice without the DNA probe. The probe was then added and the reaction continued for 20 min prior to loading onto the gel. Shift-Western analysis was performed essentially as previously described (Demczuk *et al.*, 1993).

In vitro transcription and translation reactions

Human NF-YA, NF-YB and OTF-2 cDNA clones for *in vitro* transcription were generated by PCR amplification of reverse-transcribed Namalwa B cell mRNA and subsequent ligation into pGEM 3Zf+ (Promega). Primers, amplification and other cloning details are described elsewhere (J.B.Clarke, in preparation). *In vitro* transcription reactions were performed as described by the manufacturer (Promega, Madison, WI). The DNA template was removed following transcription by adding RQ1 RNase-free DNase (Promega) to a concentration of 1 U/mg DNA followed by incubation for 30 min at 37°C. The RNA products were purified by passage over pre-packed Sephadex G50 spin columns as described by the manufacturer (Boehringer-Mannheim, Indianapolis, IN). *In vitro* translation reactions were performed using wheatgerm or rabbit reticulocyte lysates as per the manufacturer's instructions (Promega). Each reaction contained ~500 ng RNA, which was heated at 67°C for 10 min and then immediately cooled on ice. Reactions containing radiolabeled proteins used 4 μl [^{35}S]methionine (1200 Ci/mmol at 10 mCi/ml; DuPont-New England Nuclear). Unlabeled reactions used 1 μl 10 mM methionine. Labeled reaction products were analyzed on SDS−PAGE gels and translation products were stored at −70°C.

Immunoprecipitation/protein elution

Approximately 400 μg Namalwa B nuclear extract was mixed with 30 μl each recombinant *in vitro* translated NF-YA and NF-YB and incubated at 4°C for 1 h in a buffer of 17% glycerol, 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 12.5 mM $MgCl_2$, 2 mM DTT, 1 mM PMSF. Antibodies to NF-YA and NF-YB (~50 μg each) were subsequently added and the reaction was further incubated for 4 h at 4°C. Protein A−agarose (100 μl) was then incubated with the mixture overnight at 4°C, centrifuged to pellet the agarose beads and then washed four times with phosphate-buffered saline/0.05% NP-40. Step elution of the interacting proteins was done with 150 μl buffer containing 300, 500 or 700 mM KCl in 250 mM Tris, pH 8.0, 2 mM EDTA, 1% NP-40, 0.5 mM DTT, 0.5 mM PMSF, 20% glycerol. Collected fractions were dialyzed overnight against elution buffer containing 100 mM KCl. Samples were immediately assayed for DNA binding activity. Control precipitations of OTF-2 were performed with a pepetide-derived antibody from Santa Cruz Biotechnology, Inc.

DNA affinity protein purification

The purification is described in detail elsewhere (Y.Itoh-Lindstrom, in preparation). Briefly, Namalwa B cell nuclear extracts were initially chromatographed on a heparin−agarose (Sigma) column and eluted with a linear gradient of 100–1000 mM KCl. X box binding activity was monitored by gel-shift analysis. Fractions containing X box binding factors were pooled and chromatograhed three times on an X box DNA affinity column by the method described by Kadonaga and Tjian (1986). After the final purification, fractions containing distinct X box binding complexes were pooled and assayed for Y/CCAAT box binding activity.

Acknowledgements

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