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Subunit Association and DNA Binding Activity of the Heterotrimeric Transcription Factor NF-Y Is Regulated by Cellular Redox*

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Harikrishna Nakshatri‡, Poornima Bhat-Nakshatri§, and R. Alexander Currie¶

From the Laboratory of Gene Regulation, The Picower Institute for Medical Research, Manhasset, New York 11030

NF-Y is a heterotrimeric transcription factor that specifically recognizes a CCAAT box motif found in a variety of eukaryotic promoter and enhancer elements. The subunit association and DNA binding properties of the NF-Y complex were examined as a function of redox state using recombinant NF-YA, NF-YB, and NF-YC subunits. Reduction of NF-YB by dithiothreitol (DTT) was essential for reconstitution of specific NF-Y CCAAT box DNA binding activity *in vitro*. Approximately 30% of the *Escherichia coli*-derived NF-YB subunit existed as intermolecular disulfide-linked dimers. NF-YB mutants in which the highly conserved cysteine residues at positions 85 and 89 had been converted to serines existed only as monomers and did not require DTT for functional NF-Y DNA binding activity. DTT was required, however, for the functional association of NF-YC with wild-type NF-YB but not with the NF-YB cysteine mutants. The cellular redox factors Ref-1 and adult T-cell leukemia-derived factor stimulated the DNA binding activity of recombinant NF-Y in the absence of DTT. Cells treated with 1-chloro-2,4-dinitrobenzene, an irreversible inhibitor of thioredoxin reductase, exhibited reduced endogenous NF-Y DNA binding activity. Together these results suggest that the cellular redox environment of mammalian cells is an important posttranscriptional regulator of NF-Y subunit association and DNA binding activities.

NF-Y is a ubiquitous multimeric eukaryotic transcription factor, also referred to as CP1 (1) and CBF (2), that recognizes a CCAAT motif often found ~60–80 base pairs upstream of the RNA polymerase II initiation site in the promoter region and at various locations in distant enhancer elements of many higher eukaryotic genes. Cloning of the murine NF-Y subunits NF-YA and NF-YB revealed a high degree of conservation with the subunit association and DNA binding domains of two yeast CCAAT box transcription factors, HAP2 and HAP3, respectively (3). Early analyses of the CP1, HAP, and NF-Y complexes suggested that only these two subunits were required for CCAAT box recognition (1, 3, 4). However, recent cloning of an additional yeast subunit, HAP5 (5) and the rat CBF subunit CBF-C (6) and reconstitution studies of specific CCAAT box DNA binding activity in these systems now support the conclusion that the DNA binding activity of the HAP and CBF(NF-Y) complexes resides in the specific association of three nonidentical subunits (5–7).

The CCAAT box DNA binding activities of the CP1, HAP, and CBF complexes was shown initially to separate into two distinct fractions following ion exchange chromatography in which the lost DNA binding activity could be fully reconstituted by fraction recombination (1, 2, 8). These combined studies suggested that the interaction of the HAP2 and CBF-B(NF-YA) subunits with other components of the complex was primarily ionic in nature, and further suggest that under appropriate conditions the HAP2 and CBF-B subunits can exchange their heterodimeric partners. Subsequent biochemical analysis of the CBF-A(NF-YB) fraction suggested that CBF-A was tightly associated with an additional component, CBF-C, and that this interaction was primarily hydrophobic in nature, since these subunits could only be separated after treatment with denaturants such as urea, guanidine hydrochloride, and sodium dodecyl sulfate (7). The CBF-B(NF-YA) subunit has also been shown to interact with the CBF-A·CBF-C complex but not with CBF-A(NF-YB) or the CBF-C subunit individually (6), suggesting that a unique association surface for CBF-B(NF-YA) is created by the CBF-A·CBF-C heterodimer.

Structural and functional analyses have shown that the CBF-B(NF-YA) subunit contains an 83-amino acid long evolutionarily conserved domain, which contains determinants of the subunit association (amino acids 266–286) and DNA binding (amino acids 297–317) activities (9, 10). Mutation of hydrophobic amino acids within the subunit association domain of CBF-B(NF-YA) results in loss of function (9, 10). The amino acids N-terminal to the subunit association domain are rich in glutamine residues and function as a trans-activation domain (11, 12). There are at least two major isoforms of NF-YA(CBF-B), which differ primarily at the N terminus due to tissue-specific alternative splicing but which exhibit no apparent functional difference in cell-free *in vitro* transcription assays (11). The DNA binding and subunit association domains of CBF-A(NF-YB) have also been mapped recently (13). Amino acid regions 57–87, 92–99, and 63–102 along with amino acids 109–142 of CBF-A(NF-YB) are required for DNA binding, CBF-B(NF-YA) association, and CBF-C association, respectively. Several of the evolutionarily conserved amino acids within the DNA binding and subunit association domains have also been shown to be important for the function of CBF-A(NF-YB) (13), as has a similar analysis of HAP3 demonstrated the importance of these evolutionary conserved amino acids (14).

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The human NF-YC and murine NF-YC sequences reported in this paper have been deposited in the GenBank database with accession numbers U62296 and U62297, respectively.

‡ Present address: Department of Surgery and Biochemistry, Indiana University School of Medicine, Indianapolis, IN 46202.

§ Present address: Cancer Center, Indiana University School of Medicine, Indianapolis, IN 46202.

¶ To whom correspondence should be addressed: Laboratory of Gene Regulation, The Picower Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030. Tel.: 516-562-9432; Fax: 516-365-5090; E-mail: racurrie@picower.edu.

Surprisingly, the evolutionarily conserved cysteine residues, which were previously proposed to be critical for DNA binding (3), were found to be dispensable for CBF(NF-Y) complex DNA binding activity (14).

Cysteine residues of several transcription factors, although not directly involved in DNA binding, modulate DNA binding activity in response to the cellular redox state. In the case of the oxyR protein, oxidation of cysteines induces DNA binding activity (15), whereas, in the case of transcription factors Fos, Jun, bovine papillomavirus E2, NF-κB, c-Myb, USF, and TTF-1, *in vitro* DNA binding activity in all these cases is stimulated by reduction of specific cysteine residues (16–21). With a long-term goal of identifying posttranslational modification events that influence the DNA binding and transcriptional activation potential of NF-Y(CBF), the biochemical nature of its subunit interactions and to understand the role that the highly conserved cysteine residues of NF-YB(CBF-A) play in overall NF-Y function, we have initiated studies of the NF-Y complex using recombinant NF-Y subunits. For this purpose, we isolated cDNAs corresponding to both the human and murine NF-YC subunit, and we show in this study that human and murine NF-YC contain an evolutionarily conserved domain of 81 amino acids, which displays 74% homology to yeast HAP5. DNA binding studies using all three recombinant subunits indicated that reduction of recombinant NF-YB by reducing agents such as dithiothreitol (DTT)[1] or mammalian enzymes involved in redox regulation such as Ref-1 and thioredoxin (21–23) are essential for NF-Y DNA binding activity. In addition, we show that NF-YB with mutation of cysteine residues at positions 85 and 89 can bind DNA and does not require DTT. Subunit association studies indicated that prior reduction of wild-type NF-YB is essential for its association with NF-YC. We also demonstrate that cysteine-modifying agents inhibit DNA binding of cellular NF-Y. The implications of these findings on expression of NF-Y(CBF)-regulated genes are discussed.

MATERIALS AND METHODS

Cloning of Human and Mouse NF-YC—The human NF-YC subunit was cloned from a HeLa cell cDNA library (Clontech), and the murine NF-YC subunit was cloned from a normal murine macrophage cDNA library (Clontech) by polymerase chain reaction (PCR) using primers 5′-GGGGATCCATGTCCACAGAAGGAGGG-3′ and 5′-GGGAATTCTCAGTCTCCAGTCACCTGGGG-3′ based on the rat CBF-C cDNA sequence (6). PCR products derived from several independent reactions were cloned into the pGEX-2T vector (Pharmacia Biotech Inc.) to generate NF-YC subclones, and for each species clones were sequenced on both strands by Dye-Deoxy Terminator cycle sequencing (Applied Biosystems Division, Perkin-Elmer) using an automated Applied Biosystems DNA sequencer.

Construction of Recombinant Plasmids—cDNAs for NF-YA and NF-YB were obtained from a murine 3T3-L1 fibroblast cDNA library (Clontech) by PCR with primer sequences derived from previously published full-length murine sequence data (3). cDNAs were cloned into the *Bam*HI-*Eco*RI sites of the pGEX-2T vector (Pharmacia) to generate the glutathione *S*-transferase (GST) fusion proteins GST-YA and GST-YB. Cysteine mutations in NF-YB were created by a two-step PCR amplification technique as described by Ho *et al.* (24) using GST-YB as a template and cloned into pGEX-2T. NF-YC, NF-YB, and cysteine mutants of NF-YB were also cloned into the *Xho*I-*Bam*HI sites of the pET15b vector (Novagen) to generate histidine-tagged NF-Y subunits. A prokaryotic expression vector containing a human acute T-cell leukemia-derived factor (ADF) cDNA was provided by Dr. K. Schulze-Osthoff (German Cancer Research Center, Heidelberg, Germany; Ref. 25).

Purification of Recombinant Proteins—GST-YA and GST-YC proteins were purified from the soluble fraction of *Escherichia coli* DH5α lysates essentially as described by Smith and Johnson (26). GST-YB was prepared from inclusion bodies as described by Sambrook *et al.* (27) and then solubilized and diluted to a protein concentration of 1 mg/ml in a buffer containing 20 mM HEPES, pH 7.9, 0.5 mM EDTA, 300 mM KCl, and 8 M urea. Polyethylene glycol 8000 was added in a ratio of 1:15 (w/w) (28), and the urea concentration was decreased to 4 M by addition of an equal volume of buffer containing 20 mM HEPES, pH 7.9, 0.5 mM EDTA, and 300 mM KCl. The denatured proteins were then refolded by sequential dialysis in 3 M, 2 M, and 1 M urea-containing buffers and a final buffer without urea at 1-h intervals. Renatured GST-YB was purified from using glutathione-agarose beads (Sigma) (26).

YB and YC proteins expressed in *E. coli* strain BL21(DE3) from pET vectors were purified from inclusion bodies under denaturing conditions. Briefly, isolated inclusion bodies were dissolved in binding buffer (20 mM HEPES, pH 7.9, 300 mM KCl, 20% glycerol, and 6 M guanidine-HCl) and applied to a His-Bind agarose column (Novagen) pre-equilibrated with binding buffer. The column was washed extensively in binding buffer containing 10 mM imidazole. The column-bound NF-Y proteins were eluted using a 100 mM–1 M step gradient of imidazole in binding buffer. The purity of all *E. coli*-derived proteins was assessed by Coomassie Blue staining of SDS-PAGE gels and was 90% or greater. Fractions containing high DNA binding activity as measured by electrophoretic mobility shift assays (EMSAs) were used in subsequent analyses. All recombinant proteins were dialyzed against buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 0.5 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) and stored at −80 °C. Recombinant ADF was purified as described previously (25). HSP90 was purchased from StressGen Biotechnologies (Sidney, Canada); Ref-1 and cyclophilin were provided by Drs. Tom Curran (St. Jude Children's Hospital, Memphis, TN) and Barbara Sherry (The Picower Institute, Manhasset, NY), respectively. Cell culture supernatants from the MT4 cell line were concentrated using Centricon-3 ultrafiltration devices (Amicon), and the concentrated medium was extensively dialyzed against buffer D to remove any reducing agents.

NF-YB Antibodies and Western Blotting—Purified GST-YB fusion protein as described above was used for rabbit immunizations. Anti-YB antibodies were affinity-purified from rabbit serum using a GST-YB-Sepharose affinity column as described previously (29). Affinity-purified antibodies were passed through an additional GST-Sepharose column to remove antibodies directed against GST. Western blotting of proteins transferred from polyacrylamide gels to nitrocellulose filters was performed using a 1:1000 dilution of primary antibody, and antigen-antibody complexes were detected using ECL (Amersham).

Whole Cell Extracts and EMSAs—Whole cell extracts derived from exponentially growing HeLa cells were prepared by subjecting cells suspended in whole cell extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mg/ml aprotinin, pepstatin A, and leupeptin) to three cycles of freeze-thawing in liquid nitrogen and ice. EMSAs were performed with either 15 μg of whole cell extract protein or ~10 ng of each recombinant subunit in DNA binding buffer (10 mM Tris, pH 7.9, 1 mM EDTA, 2 μg poly(dI-dC)·poly(dI-dC), 100 mM KCl, and 10% glycerol) with or without 0.5 mM DTT. Extracts were first incubated on ice with poly(dI-dC) for 10 min before addition of a ^{32}P-labeled probe and then electrophoresed on 5% polyacrylamide/0.5 × Tris/borate/EDTA gels as described previously (30). The murine MHC class II Eα Y-box oligomer duplex (5′-GGCATTTTTCTGATTGGTTAAAAGTTG-3′ and 5′-GGCTCAACTTTTAACCAATCAGAAAAAT-3′) was end-labeled using the large subunit of DNA polymerase I and [α-^{32}P]dCTP as described previously (30). NF-κB and AP-1 binding site-containing oligomers were purchased from Promega (Madison, WI). SRF binding site oligomer and α-SRF antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody upshift experiments and oligomer labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase were performed according to the manufacturer.

In Vitro Protein-Protein Interaction Assay—Approximately 50 ng of recombinant NF-YB and individual cysteine mutants were incubated with ~50 ng of recombinant GST-YC for 30 min at 37 °C in binding buffer (10 mM Tris, pH 7.9, 1 mM EDTA, 100 mM KCl, 10% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride) under nonreducing and reducing conditions (1.0 mM DTT). Glutathione-agarose (10-μl bead volume) pre-equilibrated in binding buffer was added, and the reaction binding buffer volume was increased to 200 μl. Incubation continued at room

[1] The abbreviations used are: DTT, dithiothreitol; ADF, adult T-cell leukemia-derived factor; BSO, buthionine sulfoximine; CDNB, 1-chloro 2,4-dinitrobenzene; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; NEM, *N*-ethylmaleimide; PCR, polymerase chain reaction; SRE, serum response element; SRF, serum response factor; PAGE, polyacrylamide gel electrophoresis; MHC, major histocompatibility complex; CYP1A1, cytochrome P450-dependent monooxygenase.

A

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H.NF-YC   MSTEGGFGGT SSSDAQQSLQ SFWPRVMEEI RNLTVKDFRV QELPLARIKK
M.NF-YC   .......... .......... .......... .......... ..........
R.NF-YC   ........S. .......... .......... .......... ..........

H.NF-YC   IMKLDEDVKM ISAEAPVLFA KAAQIFITEL TLRAWIHTED NKRRTLQRND
M.NF-YC   .......... .......... .G........ ......R... ....P.....
R.NF-YC   .......... .......... .A........ ......H... ....T.....

H.NF-YC   IAMAITKFDQ FDFLIDIVPR DELKPPKRQE EVRQSVTPAE PVQYYFTLAQ
M.NF-YC   .......... .......... .......... .......... ..........
R.NF-YC   .......... .......... .......... .......... ..........

H.NF-YC   QPTALQVQGR QQGRRTTSST TTIQAGQIII AQPQQGQTTP VTMQVGEGQQ
M.NF-YC   ...V...... ..P....... ....P..... .......... ..........
R.NF-YC   .......... ..G....... .......... .......... ..........

H.NF-YC   VQIVQAQPQG QAQQAQSGTG QTMQVMQQII TNTGEIQQIP VQLNAGQVQY
M.NF-YC   .......... ....T..... .......... .......... .....A.L..
R.NF-YC   .......... .......... .......... .......... .....G....

H.NF-YC   IRLAQPVSGT QVVQGQIQTL VTNAQQITQT EVQQGQQQFS QFTDGQQLYQ
M.NF-YC   .......... .......... A......... ......E... ..........
R.NF-YC   .......... .......... .......... ......Q... ......_...

H.NF-YC   IQQVTMPAGQ DLAQPMFIQS ANQPSDGQAP QVTGD
M.NF-YC   .......... .......... .........T. .....
R.NF-YC   .......... .......... .......... .....
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B

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H.NF-YC 43-LPLARIKKIM KLDEDVKMIS AEAPVLFAKA AQIFITELTL RAWIHTEDNK
M.NF-YC     .......... .......... .........G .......... ....R.....
R.NF-YC     .......... .......... .........A .......... ....H.....
HAP5   134 ..F...R.V. .T........ ....II.... CE.......M ...CVA.R..

H.NF-YC     RRTLQRNDIA MAITKFDQFD FLIDIVPRDE LK -124
M.NF-YC     ..P....... .......... .......... ..
R.NF-YC     ..T....... .......... .......... ..
HAP5        ..T..KA... E.LQ.S.M.. ....V...RP .P -215
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FIG. 1. **Evolutionary conservation of the NF-YC subunit.** *A,* alignment of the human (*H*) and mouse (*M*) NF-YC with the rat (*R*) CBF-C (3) amino acid sequences. *Dots,* identical amino acids in all three species. *B,* comparison of an N-terminal region of human and mouse NF-YC with the C terminus of yeast HAP5 (5).

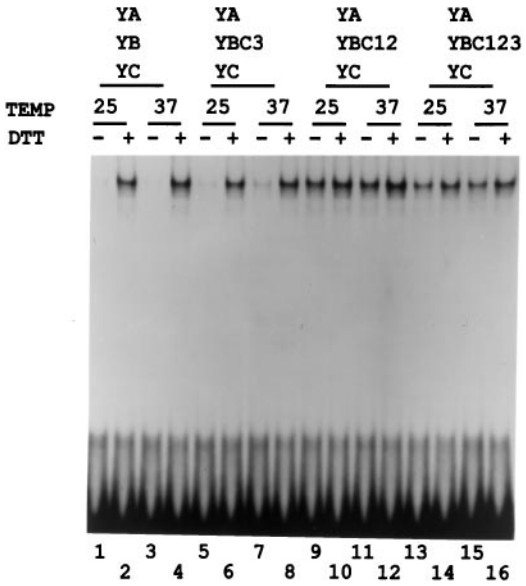

FIG. 2. **DNA binding of recombinant NF-Y complexes to the MHC class II Eα Y-box probe.** Radiolabeled Eα probe was incubated with the indicated combination of NF-Y subunits and subjected to EMSA analysis. Subunits were incubated with (+) or without (−) 1 mM DTT at the indicated temperature (*TEMP,* in °C) for 15 min before the addition of the ^{32}P-Eα probe. Recombinant NF-YA is GST-YA, whereas NF-YB and NF-YC are histidine-tagged fusion proteins.

temperature for 1 h with gentle mixing. Glutathione-agarose beads were pelleted, washed five times in binding buffer containing 0.5% Triton X-100 at 4 °C, and resuspended in SDS-PAGE buffer containing 50 mM DTT. Samples were separated on a 10% acrylamide SDS-PAGE gel, transferred to nitrocellulose, and analyzed by Western blotting.

RESULTS

Human and Mouse NF-YC and Their Homology to Rat CBF-C and Yeast HAP5—Previous studies have indicated that the amino acid sequences of NF-YA and NF-YB are conserved in evolution, particularly in their DNA binding and subunit association domains (31). To determine whether NF-YC is also similarly conserved, we cloned the human and mouse NF-YC subunits by PCR and compared their sequences. The deduced amino acid sequences from these cDNAs and their homology to rat CBF-C and yeast HAP5 are shown in Fig. 1. Similar to human NF-YA and NF-YB, which are more than 99% homologous to the corresponding mouse subunits (31), human NF-YC was observed to be 96.5% homologous to mouse NF-YC (Fig. 1*A*). Amino acids at positions 43–124 of human, mouse, and rat NF-YC displayed 70% sequence homology to amino acids 134–215 of HAP5 in a manner analogous to the NF-YA and NF-YB subunits, which contain domains homologous to yeast HAP2 and HAP3, respectively (Fig. 1*B*). This region of NF-YC presumably contains the determinants of additional NF-Y subunit association and DNA binding activities. The secondary protein structure analysis of Chou and Fasman (32) indicated that the HAP5 homology region of NF-YC, like similar domains of NF-YA and NF-YB, can form an α-helical structure (data not shown). The HAP2 homology domain of NF-YA and the HAP5 homology domain of NF-YC may also be organized in a similar manner in that they contain highly homologous N- and C-terminal subdomains separated by a variable linker region (31, 33). For example, amino acids 43–85 and 111–124 of NF-YC are 79 and 72% homologous to amino acids 134–176 and 202–215 of HAP5, respectively, whereas the intervening amino acids 86–110 are only 52% homologous to amino acids 177–201 of HAP5 (Fig. 1*B*). Amino acids C-terminal to the HAP 5 homology domain contain 30% glutamine residues (Fig. 1*A*) in which two subdomains (amino acids 150–165 and 199–216) contain more than 50% glutamine residues. Thus, glutamine-rich regions of NF-YC and NF-YA are organized in a similar fashion and may each represent specific trans-activation domains (3, 11). Taken together, sequence data homology comparisons indicate that NF-YA and NF-YC may contain structurally similar DNA binding, subunit association, and trans-activation domains.

DNA Binding of Recombinant NF-Y Proteins Prepared under Nonreducing Conditions—The cysteine residues of HAP3, although conserved evolutionarily, were previously shown not to be essential for DNA binding activity (14). To investigate whether the cysteine residues of NF-YB are essential for DNA binding and whether these same cysteine residues regulate DNA binding activity in response to redox environment, EMSAs using recombinant subunits were performed (Fig. 2). NF-Y subunits were mixed and preincubated for 15 min at 25 or 37 °C. Under nonreducing conditions a low level of NF-Y complex formed with a MHC class II Eα CCAAT box oligonucleotide probe (Fig. 2, *lanes 1* and *3*). However, inclusion of DTT in the preincubation step enhanced the DNA binding activity of recombinant NF-Y subunits by ~15-fold (Fig. 2, *lanes 2* and *4*). This property of recombinant NF-Y is similar to the property of recombinant Fos and Jun proteins, which also require treatment with a reducing agent to observe efficient DNA binding activity (16). To identify the specific cysteine residues of NF-YB that were responsible for redox-dependent DNA binding, we tested the DNA binding activity of NF-YB in which cysteine residues had been mutated to serine. The YBC3 mutant, in which cysteine residue at amino acid position 105 has been mutated to serine, specifically bound DNA only in the presence of DTT (Fig. 2, *lanes 5–8*). YBC12, in which cysteines at posi-

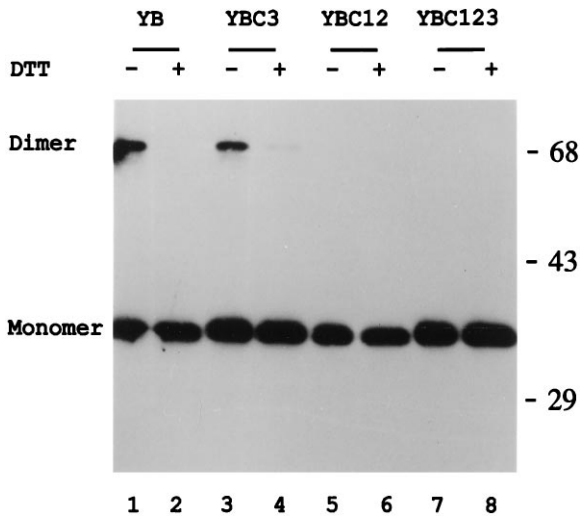

FIG. 3. **NF-YB forms covalently linked dimers under nonreducing conditions.** Recombinant wild-type NF-YB and its cysteine mutants were dissolved in SDS-PAGE buffer with (+) or without (−) 50 mM DTT and compared on 10% polyacrylamide SDS-PAGE. Recombinant proteins were detected by Western blotting and ECL using affinity-purified α-NF-YB polyclonal antibodies. The masses of protein standards in kDa are denoted at the *right*.

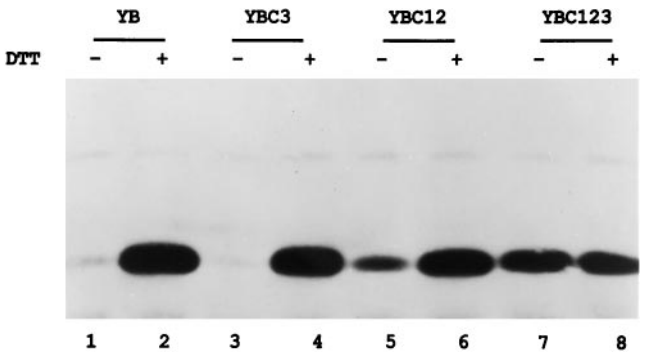

FIG. 4. **Association of wild-type and cysteine mutant NF-YB subunits with NF-YC.** Indicated histidine-tagged NF-YB subunits were incubated with GST-YC fusion proteins with (+) or without (−) 1 mM DTT and bound to glutathione-agarose beads. GST-YC-associated NF-YB was analyzed by Western blotting. Incubations in all *even numbered lanes* contained 1 mM DTT.

tion 85 and 89 had been mutated, and YBC123, in which all three cysteines were mutated, were able to form a NF-Y·DNA complex in the absence of DTT (Fig. 2, *lanes 9–16*). These results suggest that cysteines 85 and 89 of NF-YB must be reduced for NF-Y to efficiently bind CCAAT box DNA. In addition, these results indicate that, similar to yeast HAP3, the cysteine residues of NF-YB are not required for DNA binding activity (14). NF-Y subunits cleaved with thrombin to remove either GST or the polyhistidine residues from the NF-Y subunits behaved similarly to the fusion proteins described above (data not shown).

NF-YB Can Form a Covalently Linked Dimer—Since the DNA binding activity of wild-type NF-YB was affected by redox perturbations, we examined whether this effect was due to an internal disulfide bridge and/or due to intermolecular disulfide bridges formed under nonreducing conditions. To this end, NF-YB and its cysteine mutants were analyzed on a SDS-PAGE gel under nonreducing and reducing conditions (Fig. 3). Approximately 70% of both wild-type YB and YBC3 were observed to migrate as monomers, whereas the remaining 30% migrated as an apparent dimeric species (~68 kDa) when resolved under nonreducing conditions (Fig. 3, *lanes 1* and *3*). DTT treatment converted the dimers to monomers (Fig. 3, *lanes 2* and *4*). However, only the monomeric forms of YBC12 and YBC123 were detected in this analysis (Fig. 3, *lanes 5–8*). These results also suggest that either cysteine 85 or 89 is involved in dimer formation. Although the above analysis cannot prove that intramolecular disulfide bonds in YB exist under nonreducing conditions, we believe that the majority of monomeric YB molecules contain intramolecular disulfide bonds, since the reduction of dimers to monomers alone cannot account for the magnitude of the DTT effect on overall NF-Y DNA binding activity (Fig. 2).

Recombinant NF-YB Fails to Associate with NF-YC Under Nonreducing Conditions—Although the DNA binding studies and SDS-PAGE analysis indicated that NF-YB is the target of DTT, the above analysis was not sufficient to determine whether the failure of nonreduced NF-YB to bind DNA was due to its inability to interact with NF-YC or a defect in contacting DNA. To address this issue, we studied the association of NF-YB and NF-YC in the absence of DNA. As shown in Fig. 4, only low levels of the wild-type NF-YB and YBC3 mutant subunits were retained on a glutathione *S*-transferase-NF-YC (GST-YC) column under nonreducing condition (*lanes 1* and *3*). However, under reducing conditions, there was a dramatic increase in the amount of both YB and YBC3 retained by the GST-YC column (Fig. 4, *lanes 2* and *4*). The YBC12 and YBC123 mutants were retained by the GST-YC column under both nonreducing and reducing conditions (Fig. 4, *lanes 5–8*). For unknown reasons, DTT treatment enhanced the association of YBC12 with GST-YC, although the DNA binding activity of this mutant was not influenced by DTT (Fig. 2). These results suggest that NF-YB modified with either intermolecular and intramolecular disulfide cross-links fails to interact with NF-YC, and this failure is responsible for the significant reduction in DNA binding activity.

An Activity in Cell Lysates Facilitates DNA Binding of Recombinant NF-Y—To explore whether there is a cellular component that can functionally replace DTT reduction of cysteine residues, we incubated recombinant NF-Y subunits with HeLa cell extract that had been heat-treated to inactivate endogenous NF-Y (34). No NF-Y binding activity was detected in heat-treated HeLa cell extracts (Fig. 5*A*, *lanes 3* and *4*), and the DNA binding activity could be restored by addition of recombinant NF-YC (Fig. 5*A, lanes 9* and *10*). These results suggest that NF-YC is the heat-sensitive subunit in the NF-Y complex. Addition of GST-YB and recombinant YC to heat-treated extracts gave rise to a NF-Y complex composed of cellular NF-YA, GST-YB, and recombinant NF-YC (Fig. 5*A, lanes 13* and *14*). This complex was generated in the absence of DTT treatment, suggesting that a cellular factor could convert oxidized GST-YB to its reduced form.

Analysis of the redox-regulated transcription factors Fos and Jun has indicated that a cellular factor, Ref-1, can stimulate DNA binding activity of these factors by acting as a reducing agent (22, 23). Similarly, ADF has been shown to stimulate NF-κB DNA binding by acting as a reducing agent (19). To determine whether any of these factors can functionally replace DTT in stimulating the DNA binding activity of recombinant NF-Y, DNA binding assays were performed in the presence of Ref-1, ADF, and cell culture supernatant derived from the human T-lymphotropic virus 1-transformed cell line MT4, which contains ADF. Assays were also performed in the presence of *E. coli* thioredoxin, cyclophilin, and HSP90. Among these reagents, Ref-1, ADF, and the MT4 cell culture supernatant stimulated DNA binding activity of recombinant NF-Y in the absence of DTT (Fig. 5*B, lanes 5, 7,* and *9*). These results raise the possibility that the DNA binding activity of cellular

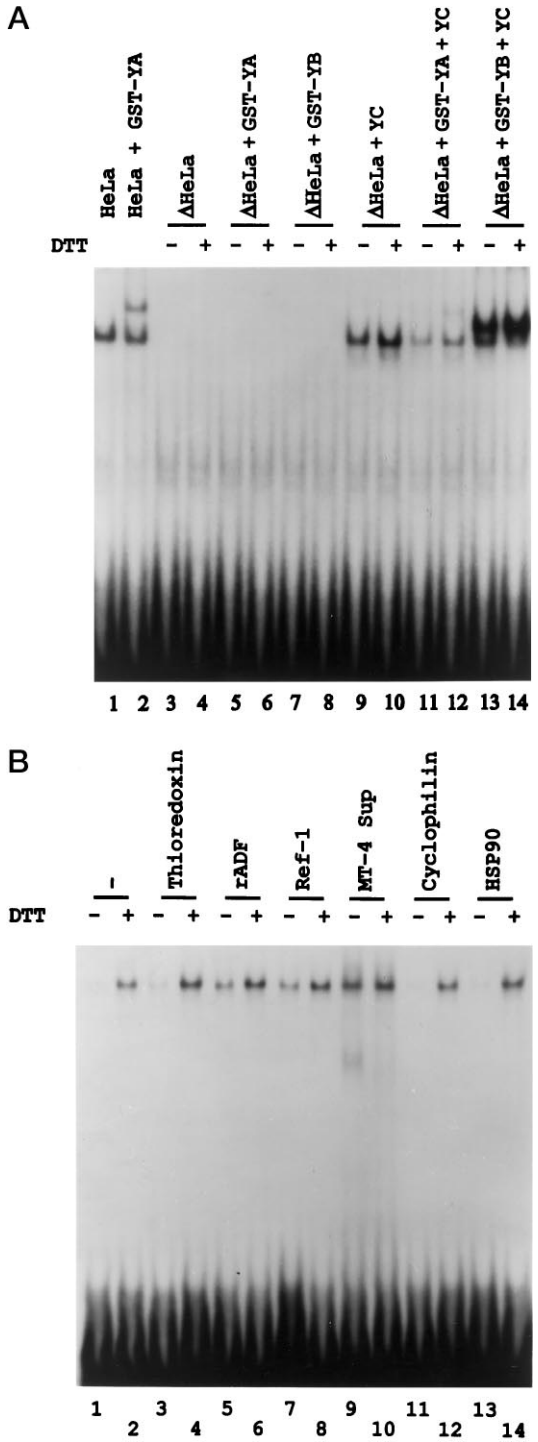

FIG. 5. **Cellular factors can functionally substitute for DTT in activating NF-Y DNA binding activity.** *A,* HeLa cell factor enables GST-YB to bind the Eα probe in the absence of DTT. EMSA reactions were performed with the Eα probe and heat-inactivated HeLa cell extract either alone (*lanes 3* and *4*), or with GST-YA (*lanes 5* and *6*), GST-YB (*lanes 7* and *8*), His-tagged YC (*lanes 9* and *10*), GST-YA plus His-tagged YC (*lanes 11* and *12*), and GST-YB plus His-tagged YC (*lanes 13* and *14*). DTT was included in *lanes 4, 6, 8, 10, 12,* and *14*. *B,* GST-YA, His-tagged YB, and His-tagged YC (approximately 10 ng) were incubated alone (*lanes 1* and *2*) or with 10 ng of *E. coli* thioredoxin (*lanes 3* and *4*), recombinant ADF (*lanes 5* and *6*), Ref-1 (*lanes 7* and *8*), MT4 cell culture supernatant (*lanes 9* and *10*), cyclophilin (*lanes 11* and *12*), or HSP90 (*lanes 13* and *14*) and subjected to EMSA using the Eα probe. Incubations in all *even-numbered lanes* contained 1 mM DTT.

NF-Y is also regulated by cellular redox systems.

NF-Y DNA Binding Activity Is Reduced in Cells Depleted of Thioredoxin—A possible role of cellular thioredoxin in the regulation of cellular NF-Y was investigated using several chemical agents by testing the effect of buthionine sulfoximine (BSO), which specifically inhibits γ-glutamyl cysteinyl synthetase, an enzyme essential in glutathione synthesis (35), and 1-chloro 2,4-dinitrobenzene (CDNB), an irreversible inhibitor of human thioredoxin reductase (36). NF-Y DNA binding activity in HeLa cells treated with either BSO or CDNB is shown in Fig. 6. Cells treated with CDNB, but not BSO, displayed 50% less NF-Y DNA binding activity compared with untreated cells (*lanes 1–6*). Surprisingly, the lost NF-Y DNA binding activity could not be recovered by DTT treatment (Fig. 6, compare *lanes 5* and *6*). Although the reason for this behavior is not known, one possibility is that oxidized NF-YB is unstable in these chemically treated cells and is targeted for proteolysis. However, Western blot analysis of these treated HeLa cell extracts showed that neither YB subunit isoform (37) was proteolyzed (Fig. 6, compare *lanes 19–21*). In contrast to NF-Y, the CDNB effect on AP-1 and NF-κB could be partially reversed by DTT treatment (Fig. 6, compare *lane 11* with *lane 12* and *lane 17* with *lane 18*). These results suggest that a number of transcription factors including NF-Y require a functional thioredoxin-thioredoxin reductase-mediated redox system to maintain functional DNA binding activity.

NF-Y DNA Binding Activity Is Inactivated by N-Ethylmaleimide—To further investigate the role of sulfhydryl groups in NF-Y DNA binding, we tested *N*-ethylmaleimide (NEM), which alkylates free sulfhydryl groups, and diamide, which catalyzes the oxidation of free sulfhydryl groups (38). NF-Y DNA binding activity was completely abolished after incubation of cell extracts with 1 mM NEM (Fig. 7, *lane 2*). Addition of 20 mM DTT prior to incubation with NEM prevented inactivation of NF-Y (Fig. 7, *lane 3*). NF-Y bound to its CCAAT box site was partially protected from NEM inactivation, indicating that these reactive sulfydryl groups are in close proximity to DNA (Fig. 7, *lane 4*). Treatment with diamide at 5 and 10 mM resulted in a slight decrease in NF-Y DNA binding activity (Fig. 7, *lanes 5* and *6*). To determine whether diamide treatment creates disulfide cross-linked dimers or an unstable sulfenyl hydrazene intermediate (40), we first treated nuclear extracts with diamide and then with NEM. No NF-Y DNA binding activity was observed on diamide and NEM co-treatment (Fig. 7, *lane 7*). However, on addition of DTT to diamide- and NEM-co-treated nuclear extracts, substantial NF-Y DNA binding activity could be recovered (Fig. 7, *lane 8*). These results suggest that NF-YB formed a disulfide cross-linked dimer either to itself or to other proteins, and this oxidation prevented NEM from alkylating cysteine residues. Note that the serum response element (SRE) DNA binding activity of the extract was not abolished by either NEM or diamide treatment, suggesting that the effect of NEM and diamide is specific to NF-Y.

DISCUSSION

In this report, we have investigated the role of the highly conserved NF-YB cysteine residues on the overall DNA binding and subunit association activities of the multimeric NF-Y complex. Our results suggest that the cysteine residues of NF-YB do not play an obligatory role in the DNA binding activity of NF-Y but do play an important regulatory role in the redox regulation of NF-Y DNA binding activity through protein-protein interaction with the NF-YC subunit.

Oxidized NF-YB Fails to Associate with NF-YC—Recent mutational analysis of the CBF-A(NF-YB) subunit has indicated that amino acids 63–102 and 109–142 are required for its association with CBF-C, whereas amino acids 57–87 are required for DNA binding activity (13). In addition, mutation of amino acid 87 in CBF-A(NF-YB) prevents the CBF complex from binding CCAAT box DNA without affecting its association

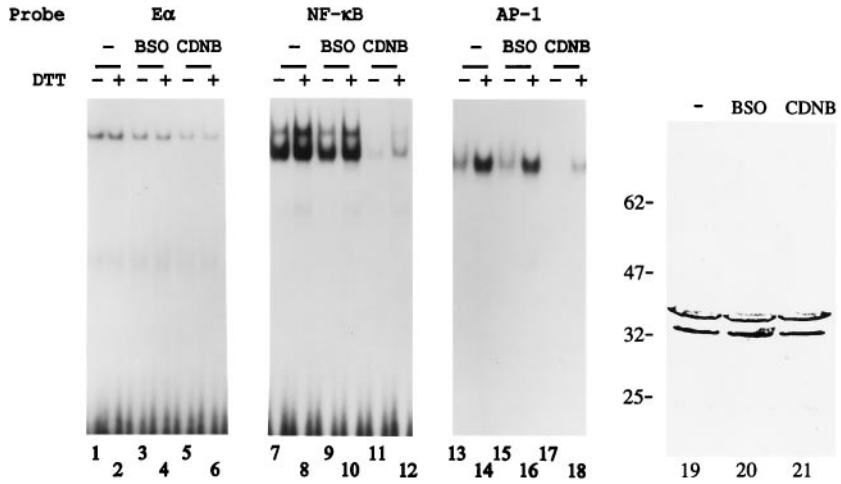

FIG. 6. **DNA binding activity of NF-Y, NF-κB, and AP-1 derived from HeLa cells treated with BSO or CDNB.** Whole cell extracts prepared from HeLa cells (*lanes 1, 2, 7, 8, 13,* and *14*), HeLa cells treated for 24 h with 100 μM BSO (*lanes 3, 4, 9, 10, 15,* and *16*), and HeLa cells treated for 15 min with 40 μM CDNB (*lanes 5, 6, 11, 12, 17,* and *18*) were subjected to EMSA using the Eα probe. Reactions in *lanes 2, 4, 6, 8, 10, 12, 14, 16,* and *18* contained 1 mM DTT. HeLa extracts from control (*lane 19*), BSO-treated (*lane 20*), and CDNB-treated (*lane 21*) cells were assayed for NF-YB subunit levels using α-YB antibodies and ECL.

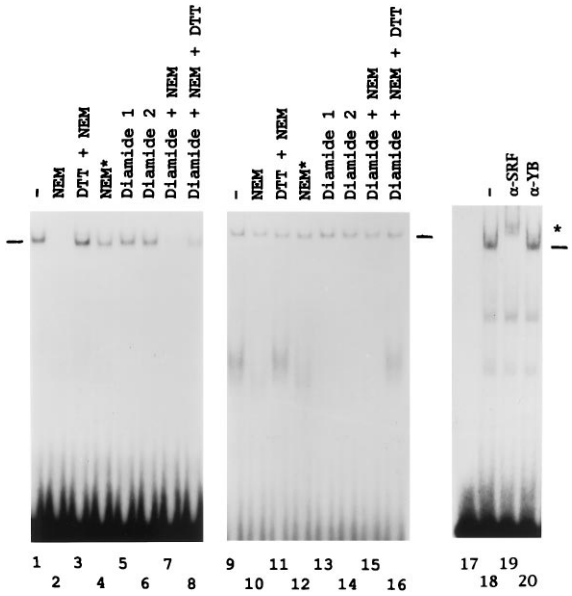

FIG. 7. **Effect of sulfhydryl modifying agents on the DNA binding activity of HeLa cell NF-Y and SRF.** Whole cell extracts of HeLa cells (*lanes 1, 9,* and *18*) and HeLa cell extracts treated for 15 min with 1 mM *N*-ethylmaleimide followed by 20 mM DTT (*lanes 2* and *10*), 5 mM diamide (*lanes 5* and *13*), 10 mM diamide (*lanes 6* and *14*), 10 mM diamide followed by 1 mM NEM (*lanes 7* and *15*), and 10 mM diamide followed by 1 mM NEM and 20 mM DTT (*lanes 8* and *16*) were subjected to EMSAs using the Eα (*lanes 1–8*) and SRE (*lanes 9–20*) probes. To reactions in *lanes 3* and *11*, 20 mM DTT was added prior to NEM addition. In *lanes 4* and *12*, 1 mM NEM was added 10 min after addition of probe. *Lane 17*, free ^{32}P-SRE probe; *lane 19*, α-SRF antibody; *lane 20*, α-NF-YB antibody. *Horizontal lines,* NF-Y·Eα and SRF·SRE complexes; *, SRF·SRE upshifted complex.

with CBF-C, whereas mutation of amino acid 86 prevents CBF-A from associating with CBF-C and CBF-B(NF-YA) (13). Since NF-YB(CBF-A) cysteine residues 85 and 89 are located in a region shown to be important for interaction with NF-YC and are redox-sensitive (Fig. 2), an intramolecular disulfide cross-link between cysteines 85 and 89 may alter the conformation and overall recognition surface of NF-YB in this region and explain why functional interaction with NF-YC is prevented (Fig. 4).

As first noted by Baxevanis *et al.* (39) and Sinha *et al.* (13), amino acids 59–140 of NF-YB(CBF-A) exhibit a weak structural homology to a region in histone H2B, the histone fold motif, which comprises a short α-helix (~10 residues) followed by a loop and β-strand segment, a long α-helix (~27 residues), a second loop and β-strand segment, and another short α-helix (10 residues) and is involved in protein-protein interactions with histone H2A in the histone octamer complex (40). The protein secondary structure predictions of Chou and Fasman (32) indicate that amino acids 70–140 of NF-YB(CBF-A) fold into an alternating array of five α-helices and β-sheets (data not shown). Amino acids in the second and third helices of NF-YB show weak homology to amino acids of histone H2B that form the long 27-residue α-helix. Cysteines 85 and 89 are located in the second α-helix region, and helical wheel projection in this region indicates that both of these cysteines are located on the same side of the α-helix. This side of the helix is also surrounded by hydrophobic amino acids. Consistent with these observations, a number of biochemical experiments have indicated that cellular NF-YB and NF-YC associate through their hydrophobic surfaces, as their protein-protein interactions can be disrupted by denaturants such as urea and guanidine-HCl (7; data not shown). How the proposed structural features described above will be affected by oxidation of cysteines 85 and 89 remains to be investigated.

Identification of intermolecular disulfide cross-linked dimers of NF-YB (Fig. 3) raises the possibility that NF-YB itself may possess a distinct DNA binding specificity similar to the E2A helix-loop-helix proteins (41). E2A homodimers have been shown to possess intermolecular disulfide cross-links, which exist in B cells but not in muscle cells, and bind DNA recognition sites at physiological temperatures. Monomeric E2A does not bind DNA on its own but is capable of binding DNA specifically after heterodimerizing with myoD (41). The highly conserved region in HAP3 and NF-YB is also similar to a region in the general transcriptional repressor Dr1 (42), and NF-YB could possibly associate with additional Dr1 partners or other cellular proteins that are related to the potential histone fold motifs of NF-YA and NF-YC. Further studies are required to test whether NF-YB also exists as cell type-specific homodimers and associates with additional multimeric partners and cofactors and if so, to determine their individual DNA binding specificities and particular functional activities.

Biological Significance of NF-YB Redox Regulation—Regulation of gene transcription by redox has been described in a number of systems (15, 16, 43). Oxidative as well as antioxidative conditions have been shown to alter the DNA binding activity of transcription factors. For example, in bacteria, the oxyR gene product activates a number of genes in response to oxidative stress by binding DNA only when it is in an oxidized state (15). Conversely, the human glucocorticoid and progesterone receptors bind only in the reduced form (44, 45). Similarly, reduction of a cysteine residue in the DNA binding domains of c-Jun and c-Fos enhances their DNA binding activity

(16). Interestingly, in the case of c-Jun and c-Fos, this cysteine can be mutated to serine without affecting its DNA binding activity, and the mutant proteins bind DNA even after treatment with cysteine-modifying agents. Moreover, v-Jun, the viral counterpart of c-Jun with a mutation of its cysteine residue, shows a distinct biological activity profile, possibly due to its deregulation from redox control (16, 22). There are number of similarities between c-Jun and NF-YB with respect to their evolutionarily conserved cysteine residues: 1) in both cases, the cysteine residues are embedded in a domain that is critical for their DNA binding activity; 2) mutations of these cysteines to serine in both cases are not deleterious to DNA binding; 3) compounds that induce disulfide bond formation (*e.g.* diamide) or alkylate cysteines (*e.g.* NEM) inhibit DNA binding (Fig. 7; Ref. 16); and 4) Ref-1 can reduce the cysteine residues of both proteins (Fig. 5*B*; Refs. 22 and 23). Thus, some of the cellular factors that regulate the activity of Fos and Jun may also regulate NF-Y function.

ADF, a human thioredoxin, was shown to increase the DNA binding activity of recombinant NF-Y to its CCAAT box (Fig. 5*B*). ADF is overproduced in human T-cell lymphotropic virus I- and Epstein-Barr virus-transformed lymphocytes and mitogen-stimulated peripheral blood lymphocytes (24, 46). ADF has been hypothesized to influence cellular metabolism through the thiol-redox control of regulatory factors. One of the primary targets of ADF in these cells could be NF-Y, since this transcription factor is essential for transcription of S/G2-specific genes, such as cyclin A, cdc25C, cdc2, and thymidine kinase (47, 48). ADF has also been shown to synergize with interleukin-2 in induction of the tumor necrosis factor α gene in B cells (49), and this effect of ADF could be mediated through the functional NF-Y binding site present in the tumor necrosis factor promoter (50). An NF-Y-related protein has been shown to function as a repressor of cytochrome P450-dependent monooxygenase (*CYP1A1*) gene expression (51). The *CYP1A1* gene is induced on exposure to a variety of xenobiotics, including halogenated hydrocarbons (*e.g.* dioxin) and polyaromatic hydrocarbons. It has been suggested that induction of functional CYP1A1 stimulates a pathway that generates thiol-sensitive oxygen intermediates (52). Thiol-sensitive reactive oxygen intermediates generated by CYP1A1 itself may induce further expression of CYP1A1 through a mechanism that inactivates NF-Y and related complexes. Further experiments are required to test this hypothesis.

A major implication of our studies relates to the regulation of MHC class II gene expression. MHC class II gene transcription requires several functional DNA elements (53), and activation of class II gene transcription requires NF-Y binding to the Y-box element and NF-Y-mediated recruitment of additional transcription factors to class II gene initiation start sites (54). YB-1, an additional Y-box-binding protein, has been shown to repress interferon γ activation of MHC class II gene transcription (55), and recently, dbpA and dbpB (mYB-1), two additional Y-box-binding proteins, were shown to inhibit MHC class II gene transcription by preventing NF-Y binding to the Y-box through direct association with NF-YA and NF-YB, respectively (56). In another study, mYB-1 has been shown to bind to the Y-box and activate the MHC class II gene in response to reactive oxygen intermediates such as hydrogen peroxide and hydroxyl radicals (57). These studies, together with our observations in this report, suggest that modulation of MHC class II gene transcription is controlled, in part, by cellular oxidative and reducing environments, which can directly influence the Y-box DNA binding activities of NF-Y and YB-1.

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Subunit Association and DNA Binding Activity of the Heterotrimeric Transcription Factor NF-Y Is Regulated by Cellular Redox

Harikrishna Nakshatri, Poornima Bhat-Nakshatri and R. Alexander Currie

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