Biochemical Characterization of the NF-Y Transcription Factor Complex during B Lymphocyte Development*

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R. Alexander Currie‡

From the Laboratory of Gene Regulation, The Wadsworth Institute for Biomedical Research, Huntington, New York 11743

The transcription factor, NF-Y, plays a critical role in tissue-specific major histocompatibility complex class II gene transcription. In this report the biochemical properties of the heterotrimeric NF-Y complex have been characterized during stage-specific B-cell development, and in several class II* mutant B-cell lines, which represent distinct bare lymphocyte syndrome class II genetic complementation groups. The NF-Y complex derived from class II* mature B-cells bound with high affinity to anion exchangers, and eluted as an intact trimeric complex, whereas, NF-Y derived from class II plasma B-cells, and from bare lymphocyte syndrome group II cell lines, RJ2.2.5 and RM3, dissociated into discrete NF-YA and NF-YB:C subunit fractions. Recombination of the MPC11 plasma B-cell derived NF-YA:B:C complex with the low molecular mass protein fraction, NF-Y-associated factors (YAFs), derived from mature A20 B-cell nuclei, conferred high affinity anion exchange binding to NF-Y as an intact trimeric complex. Recombination of the native NF-YA:B:C complex with the transcriptional cofactor, PC4, likewise conferred high affinity NF-Y binding to anion exchangers, and stabilized NF-Y interaction with CCAAT-box DNA motifs in vitro. Interaction between PC4 and NF-Y was mapped to the C-terminal region of PC4, and the subunit interaction subdomain of the highly conserved DNA binding-subunit interaction domain (DBD) of NF-YA. These results suggest that in class II* mature B-cells NF-Y is associated with the protein cofactor, PC4, which may play an important role in NF-Y-mediated transcriptional control of class II genes.

The major histocompatibility complex (MHC)† class II genes encode a set of highly polymorphic transmembrane glycoproteins that mediate several critical immunological processes that include antigen presentation to T helper cells, and the phenomenon of T-cell selection in the thymus (reviewed in Refs. 1 and 2). MHC class II α and β subunits form a non-covalent heterodimer on the cell surface of a restricted subset of mammalian cell types (e.g. mature B-cells, activated T-cells, macrophages, and the thymic epithelium), and their expression is modulated at the transcriptional and post-transcriptional levels by a variety of cytokines, lymphokines, and hormones (reviewed in Refs. 3–6). Additionally, in B lymphocytes MHC class II proteins are regulated in a stage-specific manner, as the high level of constitutive expression observed in mature B-cells is completely extinguished during progression to the plasma B-cell stage through repression at the transcriptional level (7, 8).

Three highly conserved MHC class II DNA elements, the S-, X-, and Y-boxes, have been shown through extensive functional analyses to represent, in large measure, the focal regulatory regions of class II transcriptional control (9–12). The X-box contains two separable functional elements: X1 and X2, where the X1 motif binds the regulatory factor X (RFX) family of transcription factors (13, 14), and the X2 motif binds many members of the fos/jun and cyclic AMP response element-binding protein (CREB) activating transcription factor families (15, 16). In contrast, a single activating transcription factor, the heteronuclear nuclear factor-Y (NF-Y), specifically interacts with a CCAAT motif found in all MHC class II gene Y-boxes (10, 17, 18). Proteins that interact with the X- and Y-box elements function in a cooperative manner because alterations in their spacing lead to significant reduction in in vivo promoter activity (19), and disrupt stabilized complexes formed in vitro with their corresponding proteins (20, 21).

NF-Y binding sites are frequently located within −50–80 base pairs of promoter transcriptional initiation sites, and also in more distal enhancer control regions of eukaryotic genes (22). In these contexts, the ubiquitous NF-Y factor is often juxtaposed with other general or tissue-restricted transcription factors, which together mediate crucial functional steps in constitutive and inducible gene regulation. For example, cooperative protein interactions between NF-Y and the CCAAT/enhancer-binding protein (C/EBP), and C/EBP-related proteins, DBP and LAP, have been observed in the murine albumin promoter (23), between NF-Y and the serum response factor, p67CREB, in the human β-actin promoter (24), and between the NF-Y and the sterol regulatory element-binding protein (SREBP) in the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) promoter (25). In the last example, NF-Y:SREBP acts as a negative-feedback sensor to regulate the transcriptional activity of HMG-CoA in response to serum cholesterol levels.

Initial biochemical characterization of the HeLa cell CCAAT-box factor, CP1 (26), and the rat liver CCAAT-box factor (CBF) (27), showed that specific CCAAT-box DNA binding activity was lost following ion exchange chromatography, but could be restored by recombining two separate column fractions. The two HeLa column fractions, A and B, could also be recombined with the separated Saccharomyces cerevisiae subunit fractions,
HAP2 and HAP3, to reconstitute specific CCAAT-box binding activity (28). These results suggested a heterodimeric structure for this CCAAT-box factor, which appeared to have been highly conserved in eukaryotes (26–29). Isolation of cDNAs that encoded the murine NF-YA/YB (18), and rat CBF-A(NF-YB), and CBF-B(NF-YA) subunits (30–32) provided conclusive evidence of the equivalence of CBF and NF-Y, and showed these subunits to be homologs of the yeast HAP2/3 proteins (33–35). Cloning of an additional S. cerevisiae factor, HAP5 (36), and CBF-C(NF-YC) (37, 38), together with biochemical characterization of the NF-Y complex, has now confirmed the existence of a third subunit and a heterotrimERIC structure which is minimally required for specific CCAAT-box DNA binding activity. Recently, the NF-Y complex has been shown to interact functionally with the non-histone chromosomal high mobility group protein, HMG-I(Y), and a protein-protein interaction site mapped to the NF-YA DNA-subunit interaction domain (DBD) in NF-Y, and the AT-hook motif in HMG-I(Y) (39).

Lack of appropriate MHC class II expression in humans results in a severe autosomal recessive immunodeficient condition known as bare lymphocyte syndrome (BLS) (6). The class II mutant B-cells in BLS subgroup type II synthesize normal levels of MHC class I, and class II-associated invariant chain proteins, whereas expression of all class II isotypes is universally extinguished. The B-cell-specific gene product, class II transactivator (CIITA), represents the defective gene product in BLS group II class II− cell lines, and is capable of fully restoring class II gene transcription in this specific genetic complementation group (40). CIITA has been shown to regulate both constitutive class II transcription in mature B-cells (40), and mediate interferon-γ (IFN-γ)-induced expression of MHC class II genes during monocyte/macrophage cell differentiation, and in several class II+ cell lines (41, 42). In addition, endogenous CIITA mRNA expression has been shown to be completely suppressed in plasma B-cells, whereas unregulated overexpression of CIITA in plasma B-cells surprisingly restores MHC class II mRNA and protein expression to the elevated levels observed in mature B-cells (43). These results suggest active repression of CIITA expression accounts for the dominant suppressor plasma cell phenotype exhibited by mature B-cell somatic cell hybrids (7, 8), and further suggest CIITA plays an obligatory role in the normal tissue-specific regulation of MHC class II gene transcription.

Positive cofactor 4 (PC4), also referred to as p15 (44, 45), was initially identified as an abundant nuclear protein in murine plasmacytoma cells (46), and cloned independently on the basis of its differential expression in rat embryo cells and B-cell tumors (47), and the ability of this protein to bind polydeoxyri- pyrimidines in vitro (46). Subsequently, PC4/p15 was shown to function as a general transcription accessory factor in the response of RNA polymerase II to upstream activator proteins in in vitro reconstituted systems (44, 45). Phosphorylated PC4, as modified by casein kinase II (CKII), has been shown to be functionally inactive in reconstituted cell-free in vitro transcription assays, whereas both the purified native non-phosphorylated, and Escherichia coli derived forms of PC4, are potent transcriptional activators in vitro (45, 48). In addition, recent results suggest that PC4 regulation may occur while tethered to specific promoter transcription factors, such as NF-Y (39).

In this report, the biochemical ion exchange properties of the NF-Y complex were examined in stage-specific B lymphocytes, and compared with a series of BLS mutant cell lines to determine if tissue-specific changes in NF-Y structure occur during stages of active and inactive MHC class II gene transcription. The NF-Y complex in class II+ mature B lymphocytes was observed to bind as an intact trimeric complex to anion exchangers. In contrast, the NF-Y complex derived from class II− plasma B-cells dissociated into discrete NF-YA and NF-YB:C subunit fractions following anion exchange chromatography. A protein fraction derived from mature B-cell nuclei, the NF-Y-associated factors (YAFs), in addition to recombiant PC4, restored Q+ binding to the plasma B-cell NF-Y complex as an intact trimeric complex. These results suggest that, in MHC class II mature B-cells, PC4 functions to stabilize both NF-YA: B-C subunit interactions, as well as NF-Y:CCAAT-box DNA interactions, and further suggest PC4 plays important transactivator/transrepressor roles in MHC class II gene transcription during B-cell development.

MATERIALS AND METHODS

Recombinant Plasmids—Human PC4 was cloned from pPC4 2T (39) into the EcoRI site of pGEX2TK (Amerham Pharmacma Biotech) using polymerase chain reaction. GST-PC4 mutants were derived from pPC4 2T using polymerase chain reaction, and cloned into the BamHI-EcoRI site of pGEX2TK. pPC4 (ΔC37) contains the N-terminal 90 amino acids of PC4 (nucleotides 1–270 as defined; Ref. 44), pPC4(ΔN64) contains the C-terminal 63 amino acids (nucleotides 193–384), and pPC4(ΔC37) contains the N-terminal 37 amino acids of PC4 (nucleotides 271–384). pYA (DBDΔC23) was prepared by digesting pYADD (30) with Sfi-l-EcoRI, followed by treatment with the large subunit of DNA polymerase I (Klenow) under standard conditions to generate blunt-ends (49). The purified YA(DBD) fragment was ligated and plasmids that lacked the C-terminal 23 amino acids of YA(DBD) (nucleotides 1011–1081, as defined in Ref. 18) were identified. Plasmids were prepared and verified using standard techniques (45).

Expression and Purification of Recombinant Proteins—The cloning, expression, and purification of His-YB, His-YC, glutathione S-transferase (GST), and GST fusion proteins (YA, YA(DBD), YA(ΔDBD), PC4, Dr1, TFIIB, and HAP2(DBD)), and 32P labeling of recombinant proteins using CKII, and heart muscle creatine kinase (HMK) have been described previously (39). GST-PC4(CΔC37), GST-PC4(C37), GST-PC4(ΔN64), and GST-YA(DBDΔC23) were expressed in E. coli DH5α, and purified from the soluble fraction as described previously (39). Thrombin cleaved recombinant proteins, YA(DBD) and PC4, were further purified to >95% by centrifugation through Centricon 30 filtration devices (Amicon) in BC-420 (50 mM Tris-HCl (pH 7.9), 0.42 mM KCl, 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethyisulfonyl fluoride), and stored at ~80 °C following buffer exchange to BC-100 (50 mM Tris-HCl (pH 7.9), 0.1 mM KCl, 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethyisulfonyl fluoride) using Centricon 10 devices (Amicon).

Cell Culture and Nuclear Extract Preparation—Cell lines were kindly provided for these studies as follows: A20 (J. Durdik, University of Arkansas, Fayetteville, AR), Raji (J. Jones, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO), B22.5 (B. Mach, University of Geneva, Geneva, Switzerland), RM3 (M. Peterlin, University of Geneva, Geneva, Switzerland), and 6.1.6 (D. Pious, University of Washington, Seattle, WA). MPC11 cells were obtained from the ATCC, Rockville, MD. Lymphocyte cell lines were maintained in suspension culture at ~106 cells/ml in RPMI 1640 (Life Technologies, Inc.) which contained 10% fetal bovine serum (HyClone). Cell lines were routinely tested for cell surface MHC class II protein using TRITC-conjugated species-specific anti-class II antibodies (PharMingen) and fluorescence-activated cell sorting analysis. A20 and Raji cells express high levels of surface MHC class II protein, whereas all other class II cell lines tested did not express measurable surface class II molecules.

Nuclear extracts were prepared from these, and other cell lines, according to the method of Dignam et al. (50), and as described previously (39). Briefly, nuclear extracts were passed over DEAE-Sepharose (Sigma) in BC-420 to remove residual nucleic acids, and the flow-through fraction was dialyzed for 5 h against BC-100 using a 10–12-kDa cut-off cellulose membrane (Life Technologies, Inc.). Extracts were centrifuged in an Eppendorf microcentrifuge (10,000 rpm for 10 min) at 4 °C following dialysis to remove insoluble material, and aliquots of the supernatant frozen at ~80 °C. Nuclear extract protein concentrations were determined using the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) (Life Technologies, Inc.) as the protein standard (51), and ranged between ~5–15 mg/ml.

2 R. A. Currie, unpublished data.
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Electrophoretic Mobility Shift Assay (EMSA)—EMSA assays, and the preparation and use of the Eo, and S-collagen CCAAT-box oligonucleotides in EMSA has been described previously (39). The 32P-Eo DNA oligomer was used in EMSA to normalize nuclear extracts for relative Fo CCAAT DNA binding activities for use in comparative chromatographic analyses. In EMSA, protein complexes formed with the nonspecific DNA competitor, poly(dI-dC) (Amersham Pharmacia Bio- tech), prior to addition of ~0.2 ng of 32P-labeled DNA oligonucleotide probe in a 30-μl binding reaction. Binding reactions were performed at 30 °C for 30 min, then loaded onto a 4% denaturing polyacrylamide gel (30:1 acrylamide/bisacrylamide ratio) containing 50 mM Tris, 50 mM borate (pH 8.9) and 0.5% TBE. Gels were electrophoresed at 200 V for 1.5 h following pre-running for 30 min at room temperature using 0.5 × TBE as the running buffer. Dried gels were exposed to XAR-5 film (Eastman Kodak Co.) with an intensifying screen (DuPont) at ~80 °C.

Chromatographic Procedures—Anion exchangers, Q’ Sepharose, DEAE-Sepharose, and the cation exchanger, S’ Sepharose, were prepared according to the manufacturer’s specifications (Sigma), and equilibrated in BC-100. Nuclear extracts were depleted of YAF proteins by first passing extracts over DEAE-Sepharose in BC-420, followed by dialysis against several changes of BC-420 over an 8-h period using a Spectra/Por 6 (25-kDa cut-off) membrane. The dialysis buffers were changed in each case after 3 h, and dialysis continued for an additional 3 h at 4 °C. After this period, all extracts were further dialyzed for 3 h against BC-100. Normalized NF-Y Eo DNA binding activities were determined, and assayed for binding to Q’ Sepharose.

To prepare NF-Y from nuclear extract fractions depleted of YAF activity A20 or MPC11 nuclear extracts, depleted of YAF proteins as described above, were applied to a Q’ Sepharose column in BC-100 (Tris- EDTA buffer). Following extensive washing with BC-100, NF-Y-B:C fractions were stored at 80 °C, following centrifugation to remove insoluble material.

Chromatographic Procedures—Anion exchangers, Q’ Sepharose, DEAE-Sepharose, and the cation exchanger, S’ Sepharose, were prepared according to the manufacturer’s specifications (Sigma), and equilibrated in BC-100. Nuclear extracts were depleted of YAF proteins by first passing extracts over DEAE-Sepharose in BC-420, followed by dialysis against several changes of BC-420 over an 8-h period using a Spectra/Por 6 (25-kDa cut-off) cellulose membrane (Spectrum). Subsequent- 1

Chromatographic Procedures—Anion exchangers, Q’ Sepharose, DEAE-Sepharose, and the cation exchanger, S’ Sepharose, were prepared according to the manufacturer’s specifications (Sigma), and equilibrated in BC-100. Nuclear extracts were depleted of YAF proteins by first passing extracts over DEAE-Sepharose in BC-420, followed by dialysis against several changes of BC-420 over an 8-h period using a Spectra/Por 6 (25-kDa cut-off) membrane. The dialysis buffers were changed in each case after 3 h, and dialysis continued for an additional 3 h at 4 °C. After this period, all extracts were further dialyzed for 3 h against BC-100. Normalized NF-Y Eo DNA binding activities were determined, and assayed for binding to Q’ Sepharose.

To prepare NF-Y from nuclear extract fractions depleted of YAF activity A20 or MPC11 nuclear extracts, depleted of YAF proteins as described above, were applied to a Q’ Sepharose column in BC-100, and the column was developed using a BC-KCl step gradient. The NF-Y complex eluted in the 0.42 M KCl step fraction, and aliquots stored at ~80 °C A20 NF-Y derived from the heparin-agarose fraction, and used in competition experiments with recombinant PC4, was further purified using a CCAAT-box DNA affinity column as described previously (39). Approximately 100 μg of the affinity-purified NF-Y protein fraction was recombined with either ~50 μg of purified recombinant PC4, or purified BSA, as described for YAF recombination experiments below.

In nate YAF recombination experiments, A20 YAFs were prepared from A20 nuclear extracts that had been passed over DEAE-Sepharose in BC-420. The heparin-agarose A20 NF-YA:B:C and MPC11 NF-YA:B:C fractions were compared with A20 nuclear extracts for NF-Y Eo DNA binding activity. A20 nuclear extracts that contained ~2-fold greater equivalents of CCAAT-box DNA binding activity were used to prepare the A20 YAF fraction by centrifuging the 0.42 M KCl DEAE-Sepharose fraction through a Centricon 30 filtering device (Amicon) in a DuPont Sorvall type SM-24 rotor (10,000 rpm for ~3 h at 4 °C) as described previously (39). The individual heparin-agarose A20, or MPC11 NF-YA:B:C fraction, was adjusted to 0.42 M KCl, recombined with the Centricon 30 filtrate, and incubated at room temperature for 5 min. This material was then dialyzed against BC-100 using a 10–12-kDa cut-off cellulose membrane for 3 h at 4 °C to permit protein reassembly.

NF-Y complex binding to Q’ Sepharose in these recombined fractions was determined following dialysis using EMSA.

The A20 YAF fraction, as prepared using Centricon 30 filtration devices, was depleted of protein by incubation with StrataClean resin according to the manufacturer (Stratagene). Silica beads were removed by centrifugation, and the depletion procedure was repeated. This depleted YAF fraction was recombined with A20 NF-YA:B:C, and the NF-Y complex was tested for Q’ Sepharose binding using EMSA as described above.

Normalized amounts of NF-Y Eo DNA binding activity were loaded onto ion exchange columns, and developed using a BC-KCl step gradient (0.1, 0.3, 0.6, and 1.0 M KCl). An aliquot of the column load material and a 5-μl aliquot of each column fraction were then assayed for CCAAT-box DNA binding activity to the Eo DNA using EMSA. Equivalent film exposures were obtained for each chromatographic analysis in order to directly compare the relative level of NF-Y complex binding to ion exchange materials.

RESULTS

Anion Exchange Properties of the NF-Y Complex in Stage-specific B Lymphocyte Cell Lines—NF-Y is ubiquitously expressed in mammalian tissues, and is known to play an essential functional role in the transcriptional regulation of many tissue-specific (MHC class II, collagen α2(I), lipoprotein lipase), cell-cycle regulated (cyclin A, cdc2), and inducible (HMG-CoA, interleukin-4) eukaryotic genes; however, the underlying transcriptional mechanisms that depend on NF-Y and its interaction with other combinations of general proximal-promoter factors (e.g. Oct-1, Sp1, RFX), and components of the basic RNA polymerase II machinery in these diverse situations are largely unknown. The CCAAT-box DNA binding activity of the HeLa CP1 complex in HeLa cells (26), the HAP2/3 complex in S. cerevisiae (28), and the CBF complex in rat liver and NIH3T3 cells (27) were initially shown to be lost following ion exchange chromatography, but could be reconstituted by recombining specific column fractions. Subsequently, the CBF-A(NF-YB) fraction was shown to contain an additional protein component, CBF(C)-NF(YC), which associated with CBF-A through strong hydrophobic interactions and coeluted as a heterodimer through these ion exchange materials (37). Together with cloning of the HAP5 subunit (36), these studies demonstrated that three unique subunits were minimally required for specific CCAAT-box DNA recognition.

In an effort to determine if the ubiquitous NF-Y complex exhibits differential “tissue-specific” biochemical properties, the ion exchange behavior of NF-Y was examined in a variety of stage-specific B lymphocyte cell lines. The anion exchange properties of NF-Y derived from the mature murine B-cell lymphoma, A20, were compared with the terminally differentiated murine plasmacytoma B-cell line, MPC11 (Fig. 1). Analysis of the A20 NF-Y complex revealed near quantitative recovery of Eo CCAAT-box DNA binding activity to the 0.6 M KCl step fraction (panel A). In contrast, MPC11 NF-Y Eo DNA binding activity was virtually lost following Q’ Sepharose chromatography (panel B). A low level of residual MPC11 NF-Y was consistently observed in the 0.6 M KCl step fraction (panel B). The A20 and MPC11 Eo DNA binding activities identified in the 0.6 M KCl step fractions were shown to be bona fide NF-Y complexes, respectively, using α-NF-YB antibodies in EMSA upshift assays. These observations have been ex-
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Localization of additional A20 NF-YA subunit activity following Q⁺ Sepharose analysis of the A20 NF-Y complex. A20 NF-Y Q⁺ Sepharose fractions from Fig. 1A were assayed for additional NF-YA (A) and NF-YB:C (B) activities through addition of exogeneous purified complementing native A20 YB:C or YA subunit fractions, respectively. NF-Y E⁺ DNA binding activity was determined using EMSA. Complementing subunit activities are denoted above each panel. Positions of the KCl step gradient are shown above A, and the position of the NF-Y complex is denoted to the left of each panel. In A, lane YB:C denotes the YB:C fraction alone; lane C, control YA and YB:C subunits recombined; lanes 1–17, Q⁺ fractions derived from A20 Q⁺ analysis in Fig. 1A. In B, lane A denotes YA subunit alone; lane C, control YA and YB:C subunits recombined; lanes 1–17, Q⁺ fractions derived from A20 Q⁺ analysis in Fig. 1A.

Fig. 1. Anion exchange behavior of the NF-Y complex derived from stage-specific B lymphocytes. Nuclear extracts prepared from the murine mature B-cell lymphoma cell line, A20 (A), and the murine plasmacytoma cell line, MPC11 (B), were compared for NF-Y complex binding to the anion exchanger, Q⁺ Sepharose. The columns were developed using a KCl step gradient, and each fraction was assayed for Ea DNA binding activity using EMSA. Positions of the KCl step-gradient are shown above panel A, and the position of the NF-Y complex is denoted to the left of each panel. Lane L, column load material; lane P, free 32P-E⁺ DNA probe; lanes 1–17, Q⁺ Sepharose column fractions.

Fig. 2. Localization of additional A20 NF-YA subunit activity following Q⁺ Sepharose analysis of the A20 NF-Y complex. A20 NF-Y Q⁺ Sepharose fractions from Fig. 1A were assayed for additional NF-YA (A) and NF-YB:C (B) activities through addition of exogeneous purified complementing native A20 YB:C or YA subunit fractions, respectively. NF-Y E⁺ DNA binding activity was determined using EMSA. Complementing subunit activities are denoted above each panel. Positions of the KCl step gradient are shown above A, and the position of the NF-Y complex is denoted to the left of each panel. In A, lane YB:C denotes the YB:C fraction alone; lane C, control YA and YB:C subunits recombined; lanes 1–17, Q⁺ fractions derived from A20 Q⁺ analysis in Fig. 1A. In B, lane A denotes YA subunit alone; lane C, control YA and YB:C subunits recombined; lanes 1–17, Q⁺ fractions derived from A20 Q⁺ analysis in Fig. 1A.
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Fig. 3. Identification of separated MPC11 NF-Y subunits following Q⁺ Sepharose chromatography. MPC11 NF-Y Q⁺ Sepharose fractions from Fig. 1B were assayed for YA (A), and YB:C (B) activities through addition of exogeneous purified complementing native MPC11 YB:C or YA subunit fractions, respectively. NF-Y Ea DNA binding activity was determined using EMSA. Complementing subunit activities are denoted above each lane. Positions of the KCl step gradient are shown above A, and the position of the NF-Y complex is denoted to the left of each panel. In A, lane P denotes free 32P-Ea DNA probe; lane YB:C denotes the YB:C fraction alone; lane C, control YA and YB:C subunits recombined; lanes 1–17, Q⁺ fractions derived from MPC11 Q⁺ analysis in Fig. 1B. In B, lane A, YA subunit alone; lane C, control YA and YB:C subunits recombined; lanes 1–17, Q⁺ fractions derived from MPC11 Q⁺ analysis in Fig. 1B.

Increasing [EtBr], as an [EtBr] of 50 and 100 µg/ml inhibited Ea DNA binding activity (panel C, lanes 3 and 4, respectively). These results suggest that the A20 NF-Y complex interacts with Q⁺ Sepharose anionic groups directly, and not through nonspecific DNA, or DNA-protein interactions. Further support for this conclusion was reached by treating A20 nuclear extracts with high concentrations (0.1 unit/µl) of micrococcal nuclease or DNase I prior to Q⁺ column analysis. Both treatments had no effect on A20 NF-Y binding to Q⁺ Sepharose suggesting that contaminating DNA and/or nonspecific DNA-protein cofactor interactions are not responsible for A20 NF-Y binding to anion exchange materials.²

High Affinity Binding of A20 NF-Y to Q⁺ Sepharose Is Dependent on the Mature B-cell YAF Fraction—To test the concept that the NF-Y complex in mature B-cells is associated with one, or more, small molecular mass polypeptides, in comparison to the known YA (~42 kDa), YB (~36 kDa), and YC (~40 kDa) subunits, a series of dialysis experiments were performed using cellulose membranes of defined molecular mass cut-off and under specific KCl concentration conditions (Fig. 5). A20 nuclear extracts were first dialyzed against BC-420 using a 10–12 kDa cut-off cellulose membrane (panel A), a 25 kDa cut-off cellulose membrane (panel B), and against BC-100 using a 25 kDa cut-off cellulose membrane (panel C), then extracts were dialyzed further against BC-100 in each case. The A20 NF-Y complex was tested for its ability to retain or lose binding affinity for Q⁺ Sepharose following dialysis under these experimental conditions. These analyses suggested that, under conditions of elevated [KCl] and using a 25 kDa molecular mass cut-off membrane, the activity associated with A20 NF-Y binding to Q⁺ Sepharose dissociated from the NF-Y complex and was lost during dialysis (panel B). Control experiments showed that the [KCl] and dialysis membrane cut-off size were critical in separating this activity away from the NF-Y complex. In particular, lower KCl concentrations of 0.1 M were insufficient in facilitating dissociation of this activity using a 25-kDa cut-off membrane (panel C). These results suggest that the activity associated with A20 NF-Y Q⁺ binding has a nominal molecular mass between 10 and 25 kDa, and can be physically separated from the NF-Y A:B:C subunit complex using 0.42 M KCl. This operationally defined activity is referred to as the YAF fraction. Subunit recombination assays using the Q⁺ fractions in panel B and exogeneous complementing YA and YB:C subunits demonstrate that the A20 YA and YB:C activities derived from dialyzed A20 extracts elute in a manner identical to MPC11 NF-Y (Fig. 1B).²

Ultratfiltration has been used previously as a method for preparing active YAF fractions, and dialysis using a 25-kDa cut-off membrane under these same conditions has been used as a method to prepare nuclear extracts depleted of YAF activity (39). In Fig. 6, an A20 NF-YA:B:C fraction depleted of YAF activity failed to bind to Q⁺ Sepharose as an intact heterotrimetric complex (panel A). Recombination of the A20 YA:B:C fraction with the separated A20 YAF fraction, however, fully restored the ability of the A20 YA:B:C complex to bind to Q⁺ Sepharose (panel B). In addition, prior treatment of the A20 YAF fraction with DNase I or micrococcal nuclease had no effect on its ability to confer Q⁺ binding to A20 NF-YA:B:C.² Treatment of the A20 YAF fraction with protein depleting silica beads (StrataClean resin) resulted in loss of its ability to restore the Q⁺ binding phenotype (panel C). The MPC11 NF-Y subunits were shown previously to separate following Q⁺ Sepharose analysis (Figs. 1 and 3), suggesting that NF-Y in a class II− plasmacytoma B-cell line differs in biochemical properties from the NF-Y complex in mature B-cell class II⁺ lymphocytes. The ability to transfer the A20 YAF-dependent Q⁺ phenotype to a class II⁺ NF-Y complex was tested by recombining MPC11 NF-Y depleted of MPC 11 YAF proteins with the isolated A20 YAF fraction, and assaying for high affinity NF-Y Q⁺ binding (panel D). The ability to bind to Q⁺ Sepharose was successfully transferred to MPC11 NF-YA:B:C by the A20 YAF fraction, and the MPC 11 NF-Y elution profile was identical to A20 NF-Y recombined with the A20 YAF fraction (compare panels B and D). These results further suggest that the A20 YAF fraction contains an activity capable of conferring high affinity Q⁺ binding to NF-Y. In addition, the partially purified A20 YA subunit derived from A20 nuclear extracts has been recombined with a partially purified HeLa YB:C subunit fraction derived from this class II− cell line, and assayed for Q⁺ binding. Recombination of this A20 YA fraction with HeLa YB:C restores Ea DNA binding activity, but this NF-Y complex fails to bind to Q⁺ Sepharose as an intact complex.² These results suggest that the ability to bind to Q⁺ Sepharose is not contained in the A20 YA fraction, and furthermore suggests that A20 YA itself is not the activity responsible for high affinity Q⁺ binding in the A20 NF-Y complex. The ability to separate this activity from A20 NF-Y, and to successfully recombine the YAF fraction with the depleted A20 NF-YA:B:C complex, which itself fails to bind to Q⁺ columns, represents a major step in efforts to characterize the functional properties of the NF-Y-associated cofactors in vitro. Transfer of A20 YAF activity to the MPC 11 NF-Y complex suggests that loss of YAF functional activity in the NF-Y complex of terminally differentiated B-cells may represent a critical step in NF-Y modulation of MHC class II genes during B-cell development.

Physical Interaction between NF-YA(DBD) and PC4—The transcriptional cofactor, PC4, has been identified as a compo-
as described in Fig. 1, and the BC-KCl buffers contained 50 mM NF-Y binding to Q in vitro was shown to interact with the PC4 was the YAF protein that alone conferred high affinity Q

Western assays in a previous study (39). To test the possibility that PC4 was the YAF protein that alone conferred high affinity Q binding to NF-Y, affinity-purified A20 NF-YA:B:C derived from YAP-depleted nuclear extracts was recombined with purified recombinant PC4 and assayed for Q binding (Fig. 7).

PC4 was observed to specifically confer to NF-YA:B:C the ability to bind to Q as Sepharose as an intact trimeric complex with elution properties that were identical to the NF-Y complex derived from unfractionated mature B-cell nuclear extracts (Fig. 1A) and to NF-YA:B:C recombined with the mature B-cell YAP fraction (Fig. 6, B and D). Recombinant PC4 was also observed to specifically stabilize the interaction of affinity-purified NF-Y binding to several known CCAAT-box elements (Fig. 8), suggesting that PC4 may stabilize NF-YA interactions with the YB:C heterodimer and as a result may stabilize overall NF-Y interactions with CCAAT-box DNA motifs.

To define the region in PC4 responsible for interaction with NF-Y, 32P-YA(DBD) was used to test several PC4 deletion mutants, and control GST fusion proteins, in a glutathione-agarose bead pull-down assay (Fig. 9A). YA(DBD) was observed to stably interact with full-length PC4 (lane 3) and with a PC4 mutant that lacks both the N-terminal serine-rich phosphorylation region and the region responsible for binding to double-stranded DNA (lane 7). YA(DBD) did not interact with a C-terminal PC4 mutant that lacked the extreme 37 amino acids (lane 4), a GST fusion protein that contained these 37 amino acids (lane 5), or the general transcription repressor, Dr1 (lane 6). These results suggest YA(DBD) interacts with the C-terminal region in PC4, and maps the interaction site in PC4 near amino acid 90. To determine if phosphorylated PC4 retains the ability to specifically interact with NF-Y, PC4 was 32P-labeled with CKII and HMK and tested in the glutathione-agarose pull-down assay using the NF-Y complex, several NF-YA mutants, and other control GST fusion proteins (Fig. 9B). 32P-PC4 (CKII), as phosphorylated by CKII respectively, do not impair or block interaction with YA(DBD) (Fig. 9C). 32P-PC4 (CKII) as phosphorylated by CKII, was observed to stably interact with YA(DBD) (lane 3), full-length YA (lane 4), YA(DBD) (lane 6), TFIIB (lane 8), and the YA(DBD) homolog from S. cerevisiae, HAP2(DBD) (lane 9). GST-ΔDBD (ΔDBD), which lacked the DBD element, failed to interact with 32P-PC4 (CKII) (lane 5), as did GST-Dr1 (lane 7). 32P-PC4 (HMK), as phosphorylated by HMK, was observed to stably interact with YA(DBD) (lane 12), and a YA(DBD) mutant that lacked 23 amino acids in the C-terminal DBD region, YA(DBDΔC23) (lane 14), but failed to interact with YA(ΔDBD) that lacked the highly conserved DBD region (lane 13). GST-ΔDBD (ΔDBD) fails to support CCAAT-box DNA binding activity when recombined with full-length NF-YB:C.2 These results suggest that internally and externally phosphorylated forms of PC4, as modified by CKII and HMK respectively, do not impair or block interaction with YA(DBD) in vitro, and that the PC4 interac-
tion site maps to the subunit interaction subdomain in YA(DBD), which is responsible for interaction with the YB:C heterodimer (32, 34).

A group of MHC class II cell lines have been established from several human BLS patients' B-cells, and from several class II mature human Burkitt's B-cell lymphoma cell lines by γ-irradiation or chemical mutagenesis, and immunoselected for the loss of MHC class II expression (6). These BLS class II− B-cell lines have been placed into four genetic complementation groups, where each group represents a unique defect in a gene product involved in MHC class II gene transcription. The genetic defect in BLS group II, CIITA, is thought to function as a tissue-specific class II gene transcriptional cofactor (40), and the genetic defect in BLS group IV, RFX5, has been identified as the large subunit of the X-box DNA-binding factor, RFX (54). The NF-Y complex derived from the BLS group II cell lines, RJ2.2.5 and RM3, were compared with their parental B-cell line, Raji, and a BLS group III cell line, 6.1.6, for high affinity binding to Q-Sepharose (Fig. 1). The NF-Y complex derived from both BLS Group II cell lines, RJ2.2.5 (panel B) and RM3 (panel C), dissociated following anion exchange chromatography into NF-Y subunits fractions in a manner identical to MPC11 NF-Y (Fig. 1B), and as shown using complementing YA and YB:C subunit fractions in EMSA. 2 In contrast, NF-Y derived from the class II, and CIITA BLS group III cell line, 6.1.6 (panel D), and the class II+ mature B-cell, Raji (panel A), eluted from Q-Sepharose as an intact mul-

![Image](image1)

**FIG. 6.** Recombination of the A20 YAF fraction with depleted A20 and MPC11 NF-YA:B:C nuclear extract fractions reconstitutes high affinity NF-Y complex binding to Q-Sepharose. A20 nuclear extracts were depleted of YAF proteins and tested for Q-Sepharose binding (A). The A20 NF-YA:B:C fraction, and MPC11 NF-YA:B:C fraction derived from depleted nuclear extracts were recombined with A20 YAFs, then tested for binding to Q-Sepharose (B and D, respectively). An A20 YAF fraction was depleted of protein using StrataClean resin, recombined with the A20 NF-YA:B:C fraction, and the complex tested for Q-Sepharose binding (C). Positions of the KCl step gradient are shown above A and C. Lane P, free 32P-Ea DNA probe; lane L, column load material. Column elution and EMSA assay conditions were as described in Fig. 1.

![Image](image2)

**FIG. 7.** Recombinant PC4 restores Q-Sepharose binding properties to the NF-YA:B:C complex. Affinity-purified NF-Y derived from A20-depleted nuclear extracts was recombined with recombinant PC4 (A) or BSA (B) and assayed for binding to Q-Sepharose. Positions of the KCl step gradient are shown above A. Column elution and EMSA assay conditions were as described in Fig. 1. Lane L, column load material; lanes 1–17, Q-Sepharose column fractions.

![Image](image3)

**FIG. 8.** Recombinant PC4 restores Q-Sepharose binding properties to the NF-YA:B:C complex. EMSA reactions were performed using the affinity-purified native A20 NF-Y complex, recombinant PC4, and the Ea (A) and S-collagen (B) CCAAT-box DNA oligonucleotide probes. In A and B, all lanes, except lane 1, received ~5 μg of NF-Y protein fraction. Lane 1, 32P-probe alone; lane 2, no PC4 or BSA; lane 3, ~25 ng of PC4; lane 4, ~50 ng of PC4; lane 5, ~50 ng of BSA; lane 6, ~50 ng of BSA.

![Image](image4)
NF-Y-associated Cofactors in Stage-specific B-cells

Differential expression of MHC class II genes during B lymphocyte development represents an attractive system for studying the transcriptional mechanisms underlying tissue-specific gene activation and repression. Human MHC class II genes are activated at the mature B-cell stage; however, progression to the plasma B-cell stage following stimulation with foreign antigens, various mitogens, and T-cell factors results in uniform repression of all class II gene expression at the transcriptional level (7, 8). A number of ubiquitous transcription factors, together with the B-cell-specific cofactors, CIITA (6) and the Oet coactivator from B-cells (56), are believed to play critical regulatory roles in stage-specific class II gene transcription; however, the mechanistic steps involved in creating an active class II initiation complex and possible functional interactions between these general activators, B-cell-specific cofactors, and basic components of the RNA polymerase II machinery remain poorly understood.

In this study, the biochemical properties of the multimeric NF-Y complex have been investigated and compared in stage-specific B-cells in an effort to identify structural components in the NF-Y complex that may be involved in regulating NF-Y function during states of active and repressed class II transcription, and may contribute to the formation of a functional class II initiation complex. The main observations from this study are as follows. 1) The NF-Y complex derived from MHC class II⁺ B-cells binds to anion exchangers with high affinity as an intact trimeric species, whereas NF-Y derived from MHC class II⁻ plasma B-cells and BLS group II cells dissociates into discrete YA and YB:C subunit fractions. 2) The NF-Y complex in mature B-cells is associated with a low molecular mass protein fraction (YAFs), which accounts for the NF-Y Q⁺ binding phenotype (i.e., transfer of the YAF fraction to a YAF-depleted mature B-cell NF-Y complex, or a NF-YA:B:C complex derived from class II⁻ cells fully restores NF-Y Q⁺ binding. 3) Recombinant PC4 restores high affinity NF-YA:B:C binding to Q⁺ Sepharose, and significantly stabilizes native NF-Y: CCAAT-box DNA interactions in vitro.

Anion exchange analysis of the NF-Y complex in a variety of human and murine MHC class II⁺ mature B-cell lines have shown NF-Y to elute as an intact trimeric complex (Figs. 1 and 2). Western blot analyses of the A20 Q⁺ fractions shown in Fig. 1, using affinity-purified α-YA and α-YB antibodies, also confirmed that the YA and YB subunits coelute in the 0.6 M KCl fraction. In contrast, the NF-Y complex derived from a variety of murine class II⁻ plasma B-cell lines, several other class II⁻ cell types (e.g., P388D1, 3T3-L1, TA1, C3H10T1/2), and shown previously in HeLa (26), and NIH 3T3 (27) cell lines, dissociates into discrete YA and YB:C subunit fractions following Q⁺ analysis. These observations together with NF-Y subunit recombination experiments (Fig. 2) suggest NF-YA:B:C subunit interactions are stabilized in class II⁺ mature B-cells, in comparison to a variety of class II⁻ cell types. To test these conclusions further, the anion exchange properties of NF-Y were examined in the murine monocyte/macrophage cell line, P388D1, during IFN-γ-mediated differentiation. In the absence of IFN-γ, NF-Y dissociated into YA and YB:C fractions, whereas NF-Y eluted as the trimeric complex following IFN-γ-mediated induction of class II transcription. These comparisons suggest that the anion exchange properties of NF-Y are significantly altered during periods of active, inactive, or repressed MHC class II transcription.

In a previous report, physical interaction between NF-Y and the transcriptional coactivator, PC4, was demonstrated in vitro using far Western assays, and mapped to the highly conserved DBD element in the NF-YA subunit (39). Biochemical characterization of the YAF fraction from a variety of cell types and these initial studies raised the possibility that PC4 was associated with NF-Y in stage-specific B-cells, and responsible for the NF-Y Q⁺ binding properties. Recombination of affinity-purified NF-YA:B:C with recombinant PC4 resulted in conver-

DISCUSSION

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NF-Y derived from B-cell lines representing the bare lymphocyte syndrome complementation group II: RJ2.2.5 (pull-down assay (Fig. 9). Both 32P-PC4 modified forms bound to NF-Y, PC4 was phosphorylated both with CKII and HMK and in association with NF-Y. These results suggest that PC4 is the YAF component, which itself is necessary and sufficient for conferring the Q+ binding phenotype to the NF-YA:B:C complex. PC4 is known to be phosphorylated in a group of N-terminal serine residues, primarily by CKII in vivo, and this modification of has been shown to prevent its interaction with the viral activator, VP16, and its ability to function as a coactivator in vitro (48). The PC4 recombination results suggest that the non-phosphorylated form of PC4 is capable of conferring the high affinity Q+ binding phenotype to NF-Y, and further suggest that PC4 may be differentially regulated by phosphorylation/dephosphorylation in association with NF-Y.

To determine if phosphorylated forms of PC4 interact with NF-Y, PC4 was phosphorylated both with CKII and HMK and used to probe a series of GST fusion proteins in a solution pull-down assay (Fig. 9). Both 32P-PC4 modified forms bound to YA(DBD), and not to the N-terminal activation domain of YA, suggesting that phosphorylation did not impair this interaction, and that the general nature of this association was different from previous studies suggesting an interaction between the N-terminal region of PC4 and the activation domains of VP16 (48), and GAL4-AH (57). Of particular note was the observation that 32P-PC4 interacted with the subunit interaction subdomain of YA(DBD). This region in YA(DBD) is known to be important for the interaction of YA with the YB:C heterodimer in creation of a unique structure, which then recognizes the CCAAT-box motif (32). Deletion of amino acids in the DNA-binding subdomain of YA(DBD), which prevent interaction with CCAAT-box motifs, did not impair interaction with PC4. In addition, recombinant PC4 was observed to specifically stabilize NF-Y, CCAAT-box DNA interactions in vivo (Fig. 8). These results further suggest that the interaction of PC4 with YA(DBD) may play a significant role in stabilizing YA interaction with YB:C, and the interaction with CCAAT-box binding sites following subunit trimerization. Further studies will be aimed at determining the step(s) at which PC4 functions in these processes, and in more precisely defining the region and amino acids in YA(DBD) that support this interaction.

To more accurately define the region in PC4 that interacts with YA(DBD), a series of GST-PC4 mutants were tested in vitro (Fig. 9). The N-terminal CKII phosphorylation region in PC4 was clearly dispensable for this interaction, while the C terminus near amino acid 90 appeared to be an important interaction site. Phosphorylation of PC4 by CKII has been suggested to induce a conformational change that prevents its interaction with activation regions and nullifies its positive coactivator functions (45). Interaction of PC4 through its C-terminal region with the highly conserved YA(DBD) region suggests PC4 may function in a unique manner when bound to NF-Y, since this interaction was mapped to the region known at present only to be responsible for nonspecific binding to single-strand DNA (57, 58). In association with NF-Y, PC4 may act both as a potent activator through its N-terminal region, and as a repressor when phosphorylated by CKII since both forms interact with YA(DBD). In addition, PC4 may regulate NF-Y CCAAT-box DNA binding by stabilizing overall subunit interactions, which in turn increase the rate of NF-Y association with its DNA-binding site. An interaction between 32P-PC4(CKII) and TFIIIB was also observed (Fig. 9); however, the functional significance of this observation is not known at present. These results contrast with previous studies which suggest non-phosphorylated human PC4 interacts with TFIIA, and not TFIIIB, in vitro (59), and with Far Western assays, which suggest non-phosphorylated SUB1, the yeast homolog of PC4, interacts with TFIIA, and acts as a clearance factor in vivo by promoting the release of TFIIB from TBP (60). SUB1 has been shown to activate a upstream activating sequence reporter -4-fold in yeast which contains a CCAAT-box DNA-binding site for HAP2/3/4/5, the yeast homolog of NF-Y (60). These results suggest PC4 may also play a functional role in mediated NF-Y transactivation potential in vivo, and warrant further investigation into the functional relationships between NF-Y, PC4, TFIIA, TFIIIB, TBP, and their concerted mecha-
nism of action within NF-Y CCAAT-box containing promoters.

Characterization of MHC class II gene expression during the mature to plasma B-cell transition, and in somatic plasma: mature B-cell hybrids, has suggested that the shift from inactive to class II transcription involves expression of a dominant plasma B-cell repressor (7, 8). Overexpression of CIITA in plasma cells overrides this repression and restores class II transcription (43), suggesting that the hypothesized plasma B-cell repressor may act not on class II promoters directly, but possibly on the CIITA promoter itself to extinguish CIITA expression. These comparative analyses between stage-specific B-cells demonstrate that CIITA plays a dominant role in class II transcription; however, the relationships between CIITA expression, class II promoter structure, and additional coactivator proteins in relation to tissue-specific transcriptional initiation remain unclear. CIITA functions as a critical nodal point in class II gene activation, and transmission of its signal either directly, or through downstream effector proteins, could result in specific alterations in the structure and activity of the known class II transcription factors during B-cell development.

Several BLS group II cell lines which possess defective CIITA genes, Rj2.2.5 and RM3, and are class II non-B-cell tested here, exhibit the class II NF-Y Q+ phenotype. In contrast, the parental cell line, Raji, and all other mature B-cell lines that express CIITA, exhibit the class II NF-Y Q− phenotype. The CIITA+ BLS group III cell line, 6.1.6, also exhibits the mature B-cell NF-Y Q− phenotype despite absence of class II transcription. Collectively these comparative analyses from a diverse set of cell types and cellular states provide support for the suggestion that PC4 activation is linked to CIITA expression. Transmission of the biochemical signal initiated by CIITA may lead to conversion of PC4 from the phosphorylated to non-phosphorylated form, and stabilization of NF-Y subunit interactions. The accumulated evidence presented in this study supports a model of NF-Y structure based on a unique association with the abundant, ubiquitously expressed cofactor, PC4. In this context, PC4 may be involved in mediating general NF-Y transcription factor functions in class II cells, and undergo post-translational modification both during B-cell development and during IFN-γ-induced class II gene activation, which are critical to NF-Y function in MHC class II gene transcription. Terminal differentiation into the plasma B-cell stage may signal specific PC4 phosphorylation events, and be coupled to the overall process of extinguishing class II transcription.

Further in vitro analyses of the biochemical nature of the NF-Y complex in stage-specific B lymphocytes and its in vivo function will aid elucidation of the molecular mechanisms linking NF-Y function with PC4 activity, and may provide insight into the signals initiated by CIITA which regulate tissue-specific MHC class II gene transcription.

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R. Alexander Currie

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