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CCAAT binding NF-Y–TBP interactions: NF-YB and NF-YC require short domains adjacent to their histone fold motifs for association with TBP basic residues

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ABSTRACT

Both the TATA and CCAAT boxes are widespread promoter elements and their binding proteins, TBP and NF-Y, are extremely conserved in evolution. NF-Y is composed of three subunits, NF-YA, NF-YB and NF-YC, all necessary for DNA binding. NF-YB and NF-YC contain a putative histone-like motif, a domain also present in TBP-associated factors (TAF_{II}s) and in the subunits of the transcriptional repressor NC2. Immunopurification of holo-TFIID with anti-TBP and anti-TAF_{II}100 antibodies indicates that a fraction of NF-YB associates with TFIID in the absence of NF-YA. Sedimentation velocity centrifugation experiments confirm that two pools of NF-YB, and most likely NF-YC, exist: one associated with NF-YA and binding to the CCAAT box; another involved in high molecular weight complexes. We started to dissect NF-Y–TFIID interactions by showing that: (i) NF-YB and NF-YC interact with TBP in solution, both separately and once bound to each other; (ii) short stretches of both NF-YB and NF-YC located within the evolutionary conserved domains, adjacent to the putative histone fold motifs, are necessary for TBP binding; (iii) TBP single amino acid mutants in the HS2 helix, previously shown to be defective in NC2 binding, are also unable to bind NF-YB and NF-YC.

INTRODUCTION

The CCAAT box is a widespread regulatory sequence found in promoters and enhancers of several genes (1) whose functional importance has been well established in different systems (2 and references therein). Among the proteins reported to bind this or related sequences, only NF-Y (also termed CBF), originally identified as the activity binding to the MHC class II conserved Y box, has an almost absolute requirement for these 5 nt and a strong preference for additional flanks (2,3). Based on supershift experiments with anti-NF-Y antibodies, on competition analysis with an Ea Y box oligo and on the heteromeric nature of the DNA binding complex, NF-Y has been identified as the CCAAT box activator in over 100 promoters (2,4,5; R.Mantovani, unpublished results). Interestingly, the CCAAT consensus derived statistically by Bucher (PuPuCCAATC/GA/G) fits well with the optimal NF-Y binding site (1,3), rather than with the consensus of other proteins binding to CCAAT-related sequences, such as CTF/NF-1 (6) and C/EBP (7).

NF-Y is an ubiquitous heteromeric protein formed by three subunits, NF-YA, NF-YB, NF-YC, all necessary for DNA binding (4,5); yeast *Saccharomyces cerevisiae* also has a CCAAT binding activity, the HAP2/3/4/5 complex, involved in activation of cytocrome genes by non-fermentable carbon sources (8–10). The cloning of NF-Y genes in several species showed highly conserved domains (66–74% identity, 80–86% similarity) having sharp boundaries with the rest of the protein (5,8–12). The NF-YA (HAP2/CBF-B) homology domain can be divided into subunit association and DNA contacting subdomains (13,14). The N-terminus contains a Q-rich activation surface (15). NF-YB (HAP3/CBF-A) has a long central homology domain and no apparent activating surface. The NF-YC (HAP5/CBF-C) gene has recently been cloned and is specular with respect to NF-YA, since the homology domain is at the N-terminus, while the C-terminal 180 amino acids are rich in glutamine and hydrophobic residues (5; M.Bellorini, in press). NF-YB and NF-YC tightly interact with each other and their association is a prerequisite for NF-YA binding and sequence-specific DNA interactions (5). Both NF-YB and NF-YC scored positive in a computer search of protein sequences containing putative histone fold motifs residing in their conserved domains. This motif is common to all core histone proteins, is responsible for the

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formation of the histone octamer (16) and is composed of three α-helices separated by short loop/strand regions, enabling histones to dimerize with companion subunits in what has been described as a molecular handshake (17,18). Recent experiments on HAP3 (13), NF-YB/CBF-A (19) and NF-YC (20) indicate that this 65 amino acid motif is necessary for subunit interactions and DNA binding.

This motif is shared by other proteins involved in transcriptional regulation. (i) The two subunits of NC2 (also called Dr1/DRAP1) bind TBP (TATA binding protein) and repress transcription; they have a particularly high resemblance to NF-YB/NF-YC, beyond the histone motifs within the larger yeast/human conserved domains (21–23). NF-YB is similar to NC2β/Dr1 and NF-YC to NC2α/DRAP1. (ii) Crystallographic studies proved that some of the TAF_{II}s ($dTAF_{II}60$/$hTAF_{II}80$, $dTAF_{II}40$/$hTAF_{II}31$ and $hTAF_{II}20$/$dTAF_{II}30\alpha$) contain such a motif; TAF_{II}s interact tightly with TBP and mediate activation as part of the TFIID complex (16; see 24 for a review).

We have recently reported that the MHC class II Ea promoter functions through an initiator element (25) and that this activity is critically dependent upon binding of TFIID (26). Previous experiments using transgenic mice with Ea constructs mutated in the Y box suggested that such an element influences start site selection *in vivo* (see 27 for a review); moreover, antibody challenge experiments of *in vitro* transcription reactions showed an early involvement of NF-Y in the formation of the pre-initiation complex and its requirement for re-initiation, suggesting interactions with components of the basal machinery (28). These findings prompted us to investigate whether NF-Y and TFIID can directly associate: we tested such an hypothesis by immunopurification, glycerol gradient centrifugation experiments and by protein–protein interaction studies with recombinant proteins.

MATERIALS AND METHODS

Protein production and purification

NF-YA and NF-YB genes were cloned in the PET3 vector for expression in *Escherichia coli*; the proteins were produced as inclusion bodies, renatured according to the method described by Mantovani *et al.* (14) and further purified using Mono Q (for NF-YB) and Mono S (for NF-YA) columns. NF-YB4 (amino acids 51–140 of the mouse sequence), NF-YB41 (amino acids 51–131), NF-YB43 (amino acids 51–117), NF-YC5 (amino acids 37–120 of the human sequence) and NF-YC51 (amino acids 37–117) mutants were generated by PCR and cloned in-frame into the PET29c T vector (Novagen) to produce a fusion protein with a protein S tag at the N-terminus and a His tag at the C-terminus. All mutants were sequenced. The complete amino acid sequence of human NF-YC used to derive the YC5 and YC51 mutants will be described elsewhere (M.Bellorini, in press) and is identical to the published rat CBF-C sequence (5). BL21 DE3 LysS bacteria were induced for 2 h with 1 mM IPTG. Proteins were purified by the protocol of Mantovani *et al.* (28). Briefly, we resuspended the bacterial pellets in sonication buffer (300 mM KCl, 20 mM HEPES, pH 7.9, 0.1% NP40, 0.1 mM EDTA, 1 mM PMSF), sonicated three times (2 min), centrifuged (14 000 *g* for 30 min) and the supernatant loaded on a NTA–agarose column (Quiagen), washed with a buffer containing 1.0 M KCl and eluted with a buffer containing 0.25 M imidazole. The concentrations of the recombinant proteins were routinely determined with the Protein S-Tag Kit (Novagen) and, for NF-YB mutants, further checked by Western blotting with anti-NF-YB antibodies (28). NF-Y was reconstituted by adding equimolar amounts of each subunit in NDB-Mg (100 mM KCl, 20% glycerol, 20 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dTT, 5 mM $MgCl_2$) and incubating for 10 min at room temperature.

Purification of hTBP, TFIIA, holo-TFIID and pure TFIID has been detailed previously (26,29). Production of yTBP and mutants thereof has also been described (30,31), and these were further purified on heparin–Sepharose columns.

Immunoprecipitations of TFIID

Immunopurification of holo-TFIID was performed as detailed previously (33,34). Immunoprecipitation of TFIID was by incubating HeLa nuclear extracts (1 mg) with 10 µg 1TA monoclonal antibody against $TAF_{II}100$ (33), anti-YB purified rabbit polyclonal antibody or control anti-lysozyme rabbit antibody (28) for 2 h at 4°C, followed by addition of 25 µl protein G–Sepharose (Pharmacia) and further incubation for 1 h with rotation. The resin was spun down briefly, washed twice with 1 ml wash buffer (10% glycerol, 500 mM KCl, 20 mM Tris–HCl, pH 7.9, 0.1% NP40, 1 mM dTT) and eluted by resuspending in SDS buffer and heating at 90°C for 5 min. Equivalent amounts of proteins were assayed in Western blots.

Sucrose gradient centrifugations

A 10–40% sucrose gradient was formed (4 ml in 150 mM NaCl, 0.2% Triton X-100) and either used immediately or stored at –20°C. On top of the gradient, 160 µl (3 mg) of CH27 whole cell extracts were loaded and centrifuged at 35 000 *g* for 24 h at 4°C. Parallel, identical gradients with molecular weight markers (alcohol dehydrogenase, 150 kDa; β-amylase, 200 kDa; thyroglobulin, 669 kDa; Sigma) were also run. Fractions of 180 µl were collected: aliquots were tested in EMSA and TCA precipitated for Western blot analysis. The antibodies used were anti-YAc, anti-YB (28), anti-TBP monoclonal 3G3 (29) and anti-FOS (Santa Cruz).

Protein–protein interaction assays

Purified proteins were linked to CNBr-activated Sepharose (200–500 µg/ml resin) according to the manufacturer's instructions (Pharmacia) and to the protocol of Mantovani *et al.* (14). Under these conditions >95% of the proteins were bound to the matrix. Protein–protein interactions were assayed in 100 mM KCl, 20 mM HEPES, pH 7.9, 0.05% NP40, 0.5 mM EDTA, 1 mM PMSF, using 10–15 µl resin in a final volume of 100 µl, by rotation overnight at 4°C. After two washes with 500 µl of the same buffer, the columns were step eluted with 40 µl buffer containing increasing concentrations of KCl. For hTBP, yTBP and mutants thereof, the experiments were initially performed using crude bacterial extracts containing the recombinant proteins and then confirmed by using proteins purified on heparin–agarose columns.

Electrophoretic mobility shift assay (EMSA)

EMSA of NF-Y were performed as described previously with a Y box oligo in 4.5% acrylamide gels run in 0.5× TBE buffer (3). TBP EMSA in agarose gels were performed as described (26).

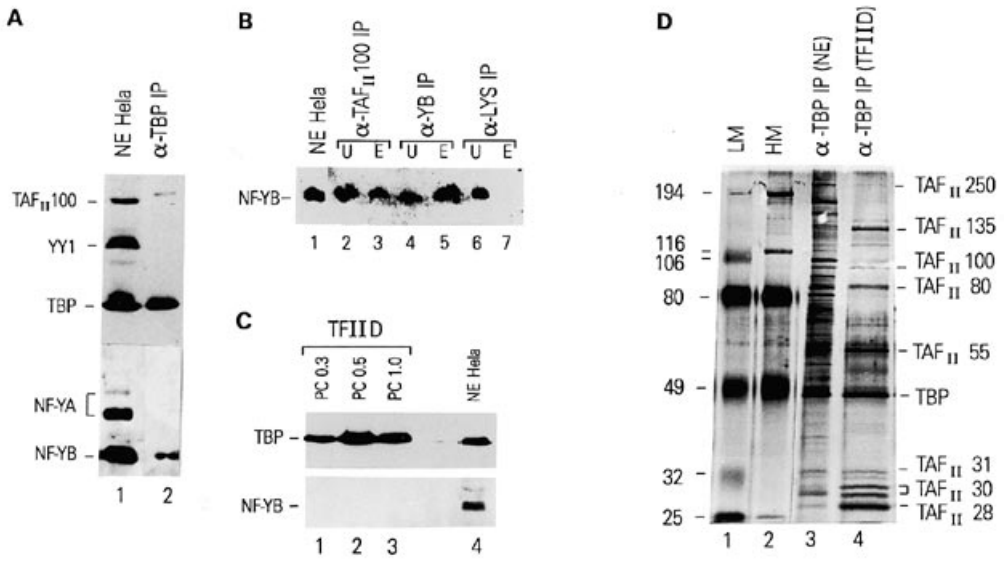

Figure 1. Western blot analysis of holo-TFIID. (**A**) Holo-TFIID was immunopurified with anti-TBP 3G3 antibody and assayed in Western blots, probing with anti-YY1 (Santa Cruz), anti-NF-YB, anti-NF-YA, anti-$TAF_{II}100$ 1TA and anti-TBP 3G3 antibodies. Comparable amounts of HeLa extracts (lane 1) were loaded on the same gel. NF-YB and NF-YA bands correspond to the expected 32 and 41–45 kDa doublet already observed (42). (**B**) Immunoprecipitations of HeLa nuclear extracts with anti-$TAF_{II}100$ 1TA monoclonal antibody (lanes 2 and 3), anti-YB (lanes 4 and 5) and anti-lysozyme (lanes 6 and 7). Unbound (lanes 2, 4 and 6) and eluted proteins (lanes 3, 5 and 7) were tested in a Western blot with anti-YB antibodies. (**C**) Western blot analysis of pure TFIID probed with anti-TBP 3G3 and anti-NF-YB antibodies. TFIID was immunoprecipitated with anti-TBP 3G3 from the phosphocellulose fractions indicated above each lane. In lane 4, HeLa extracts were used as a positive control. (**D**) SDS gel analysis of immunopurified holo-TFIID and pure TFIID. Molecular weight markers are indicated.

RESULTS

NF-YB is associated with immunopurified holo-TFIID

A region at the C-terminus of the NC2β/Dr1 histone fold motif has been shown to be sufficient to provide a TBP binding surface in solution (32). Work from the same group had previously shown that NC2β/Dr1 co-purifies in early purification steps with TFIID, but not with other general transcription factors (21). Because of the high sequence homology between NF-YB and NC2β, we wanted to ascertain whether NF-YB was also able to associate with TFIID. To do so, we took advantage of holo-TFIID preparations purified by means of the 3G3 anti-TBP monoclonal antibody (29,33,34): HeLa extracts were incubated with 3G3, followed by addition of protein G–Sepharose, a wash in 0.5 M KCl, 0.1% NP40, and elution by addition of a large excess of the PA81 peptide used for immunization. We recently used such preparations to show that binding of holo-TFIID to initiator sequences was responsible for the strong Ea Inr activity (26). We assayed holo-TFIID in Western blot analysis with several antibodies (Fig. 1A): anti-NF-YB and anti-NF-YA antibodies indicate that holo-TFIID preparations contain a protein of 32 kDa identical to the NF-YB band obtained with HeLa extracts (compare lanes 1 and 2), but not NF-YA, whose bands at 41–45 kDa are present in HeLa extracts, corresponding to the two major splicing forms of NF-YA (15,28), but not in eluted holo-TFIID (compare Fig. 1A, lanes 1 and 2). Similarly, an anti-YY1 antibody gave negative results, while anti-TBP and anti-$TAF_{II}100$ 1TA (33) highlighted signals in the eluted fraction; note that under these conditions recovery of TBP is almost complete. Since anti-TBP antibodies could immunoprecipitate complexes involved in pol I and pol III transcription, we performed the reverse experiment, namely immunoprecipitating with the anti-$TAF_{II}100$ monoclonal antibody and checking for the presence of NF-YB in the bound TFIID complexes. As shown in Figure 1B, ~20% of NF-YB was immunoprecipitated with anti-$TAF_{II}100$ (lanes 1–3), as well as with the positive control anti-YB antibody (lanes 4 and 5), while all NF-YB was recovered in the unbound material on immunoprecipitation with an irrelevant anti-lysozyme antibody (lanes 6 and 7). When TFIID complexes were purified on two chromatographic columns prior to immunopurification, no NF-YB was detected in the three functionally different TFIID complexes (Fig. 1C). Note that these TFIID complexes contain similar amounts of TBP (Fig. 1C, upper panel) and $TAF_{II}100$ (data not shown). SDS–PAGE analysis of holo- and pure TFIID complexes (Fig. 1D) indicate that in the former other interacting polypeptides are present in addition to bands corresponding to TAF_{II}s (see also 33,34). This set of experiments show that NF-YB, not NF-YA, associates with holo-TFIID and that none is present in pure TFIID preparations.

NF-YB, but not NF-YA, is present in complexes of high molecular weight

TBP and the TAF_{II}s form tightly associated complexes of high molecular weight, with peaks at 300 and >800 kDa (29,35). Glycerol gradient centrifugation experiments performed with unfractionated nuclear extracts gave indications that the NF-Y binding activity was present in the 200 kDa range (36). We decided to test the physiological relevance of the NF-YB–TFIID association, performing sedimentation velocity centrifugation experiments with CH27 whole cell extracts under conditions (150 mM KCl, 0.2% Triton X-100) that do not cause dissociation of the NF-Y subunits. We first checked the different fractions for

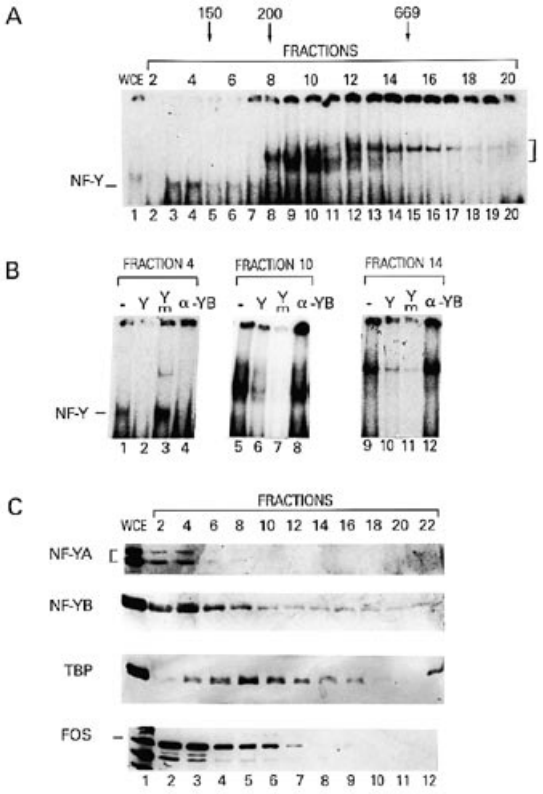

Figure 2. Sucrose gradient analysis of CH27 whole cell extracts. (**A**) EMSA of sucrose gradient fractions with a labelled Y box oligo. The peaks corresponding to the molecular weight markers run in a parallel gradient (alcohol dehydrogenase 150 kDa; β-amylase 200 kDa; thyroglobulin 669 kDa) are shown. The band corresponding to NF-Y is indicated. (**B**) EMSA of Y box oligo with fractions 4 (lanes 1–4), 10 (lanes 5–8) and 14 (lanes 9–12): competition with a cold wild-type Y box oligo (lanes 2, 6 and 10), mutant Y box oligo (lanes 3, 7 and 11) and challenge with the anti-NF-YB antibody (lane 4, 8 and 12). (**C**) Western blot analysis of the sucrose gradient fractions probed with the indicated antibodies. About 20% of each fraction was loaded on the SDS gel.

CCAAT binding activity in EMSA with a labelled Y box oligo and found that NF-Y is present in fractions 2–8, with a peak in fraction 4 (Fig. 2A); since the peaks of the 150 and 200 kDa molecular weight markers are in fractions 5 and 8 respectively, this result confirms that the CCAAT binding activity sediments at ~200 kDa, in agreement with previous calculations (36). Additional slow migrating bands were also present in fractions of higher molecular weight, peaking in fractions 10 and 14. As an initial step to understand the nature of such complexes, we undertook cold oligo competition and anti-YB challenge EMSA experiments, using fractions 4, 10 and 14. Figure 2B indicates that the binding activity in fraction 4 was competed specifically by a cold wild-type, but not by a mutant Y box oligo (lanes 1–3) and inhibited by anti-YB antibodies (lane 4), indicating that this activity is indeed NF-Y. In contrast, the slow migrating binding activities present in fractions 10 and 14 were competed both by the wild-type and the mutated Y box oligos and not modified by anti-YB antibodies (lanes 5–8 and 9–12), suggesting little sequence specificity in DNA binding.

We then analysed these fractions in Western blots, using different antibodies. Surprisingly, a striking difference was observed in the behaviour of NF-YA and NF-YB, as shown in Figure 2C. While the former was present exclusively in the fractions at the top of the gradient (2–8), the latter, although showing a peak in fraction 4, was present all across the gradient, including fractions at the bottom, which have molecular weights >700 kDa (the 669 kDa marker peaks in fraction 15). This behaviour is somewhat similar to that observed for TBP, which shows a broad distribution (Fig. 2C), in good agreement with published data (35). As a control, we also checked FOS, another heteromeric protein, and found it in the early fractions, up to fraction 10. Therefore, sucrose gradient fractionation of whole cell extracts: (i) confirms previous calculations on the apparent molecular weight of the NF-Y binding activity; (ii) shows that NF-YB, but not NF-YA, can be found in complexes of high molecular weight; (iii) indicates that a specific association of NF-YB with TFIID can indeed exist in the absence of NF-YA.

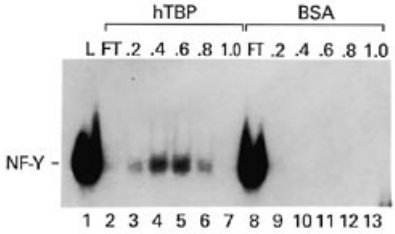

Figure 3. Binding of NF-YB/NF-YC5 to a TBP–Sepharose column. EMSA of NF-Y using a labelled Y box oligo. Lane 1, load of the two columns; lanes 2–7, flow-through and eluted fractions from the TBP column; lanes 8–13, flow-through and eluted fractions from the control BSA column. To visualize the NF-Y band, we added 10 ng recombinant NF-YA to all samples.

NF-YB and NF-YC bind TBP

We next tried to dissect the NF-YB–TFIID interactions by determining whether the NF-YB–NF-YC complex has affinity for recombinant human TBP. We used the protein–protein interaction assay that we developed to study the binding of NF-YA to NF-YB–NF-YC (14). We produced hTBP in *E.coli* (25,26), linked it to a CNBr-activated Sepharose support and loaded purified recombinant NF-YB previously associated with a NF-YC mutant (NF-YC5) containing the 84 amino acids of the conserved HAP5 homology domain; this mutant is indistinguishable from wild-type NF-YC in terms of NF-YB and NF-YA association and CCAAT recognition (M.Bellorini, in press). After extensive washing, we step-eluted the column with buffers containing increasing amounts of KCl. The different fractions were then tested in EMSA with recombinant NF-YA and a labelled Y box oligo (3,14). In parallel, we performed the same experiment on a control BSA column. Results of such experiments are shown in Figure 3. The TBP column, but not the control column, retained NF-YB–NF-YC5 (compare lanes 2 and 9), which was eluted with a peak between 0.4 and 0.6 M KCl. Interestingly, in identical experiments recombinant NF-YA did not show significant affinity for immobilized hTBP (data not shown).

Identification of the NF-YB/NF-YC–TBP interaction domains

To gain insight into the NF-Y–TBP interactions, we fixed either NF-YB or NF-YC5 to Sepharose and assayed these columns with recombinant hTBP. All recombinant proteins were produced in

E.coli, purified, tested for protein concentration with the Protein S-Tag assay kit or by Western blot (see Materials and Methods) and covalently linked to Sepharose. To test the eluted fractions we decided to use the rapid agarose EMSA system that we recently developed with an oligonucleotide containing the strong AdML TATA box with purified TFIIA, to enhance hTBP binding (26). Figure 4A shows that TBP is specifically retained by the NF-YB column (lanes 2–7), but not by the control BSA column (lanes 9–13). Similar experiments performed on a NC2β column gave results similar to those obtained with NF-YB (data not shown). We then pursued this line of experiments using mutant YB4, which comprises only the HAP3 homology domain (amino acids 51–140 of mouse NF-YB; see 22), YB41, which lacks the last nine amino acids at the C-terminus of the HAP3 domain, and YB43, lacking 23 amino acids. This set of proteins was produced as fusions with a His tag for rapid purification. All S-tagged NF-YB fusion mutants were checked with the Protein S-Tag assay kit for protein concentration. YB4 retains full capacity to associate with NF-YC and NF-YA and to bind the CCAAT box in a sequence-specific manner, as does YB41, but at higher protein concentrations, while YB43 is unable to associate with NF-YC (K.Zemzoumi, unpublished results). As shown in Figure 4B, while YB4 and YB41 retain full TBP binding activity, YB43 is incapable of binding TBP. This result suggests that the region between amino acids 117 and 131 is important for TBP binding. In parallel, we performed similar experiments with YC5 and YC51, a mutant that lacks 13 amino acids at the C-terminus of the HAP5 homology domain but retains the full histone fold motif and consequently binds NF-YB, NF-YA and DNA, albeit at higher protein concentrations, a situation similar to YB41 (K.Zemzoumi, unpublished results). While TBP was retained by the NF-YC5 column, it lost most of the binding capacity on NF-YC51 (Fig. 4C). These data were confirmed by Western blotting analysis (data not shown). These results indicate that TBP is able to interact with both the NF-YB–NF-YC5 complex and singly with the separate proteins; this interaction requires the presence of small regions at the C-terminus of the HAP homology domains of NF-YB and NF-YC.

We then turned to TBP mutants to pinpoint the region contacted by NF-YB/NF-YC. Because of the homology between NF-YB/NF-YC and NC2, we took advantage of a series of single amino acid mutations in the basic HS2 domain of yeast TBP recently tested for interactions with TFIIA and NC2. Some of these mutants were reported to be defective in TFIIA and NC2 binding, but all of them retained TFIIB binding and full DNA binding activity (30,31; see also below). We produced some of these mutants (K133L, K138L, K145L and K151L) as well as the wild-type yTBP in *E.coli*, prepared bacterial extracts and checked them by Western blot analysis with a purified anti-hTBP antibody (25). Figure 5A shows that equivalent amounts of the wild-type and mutant yTBP were present in the *E.coli* soluble extracts. These were then used in the protein–protein interaction assays with NF-YB4 (Fig. 5B, lanes 1–7) or NF-YC5 columns (Fig. 5B, lanes 8–13). The readout was again tested by EMSA with a labelled AdML TATA box oligo, since all these proteins retain efficient DNA binding capacity (Fig. 5B, lanes 1). The data shown in Figure 5B indicate that both NF-YB4 and NF-YC5 bind wild-type yTBP with equal affinity; similar results were obtained with the K138L yTBP mutant, except that this mutant showed a slightly higher affinity for NF-YB. However, very little NF-YB or NF-YC binding was retained by the K133L, K145L and K151L yTBP mutants.

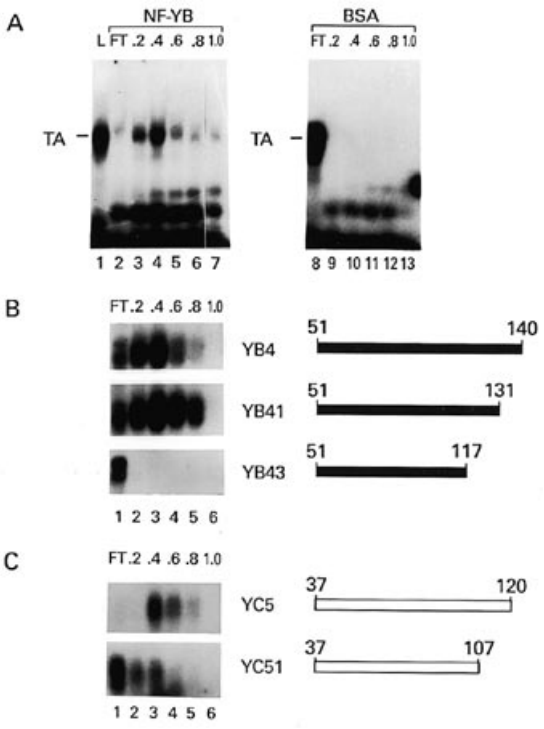

Figure 4. Mapping of the NF-YB and NF-YC domains required for TBP binding. EMSA of hTBP using a labelled AdML TATA box oligo. Purified TFIIA was added to all binding reactions. (**A**) Binding of recombinant hTBP to NF-YB–Sepharose. Lane 1, load of hTBP used in the protein–protein interaction assays described in (A), (B) and (C); lanes 2–7, flow-through and eluted fractions from the NF-YB–Sepharose column, indicating the KCl concentrations used in the elution buffers; lanes 8–13, same with the control BSA–Sepharose column. (**B**) As A except that hTBP was loaded in parallel on NF-YB4–Sepharose, NF-YB41–Sepharose and NF-YB43–Sepharose columns. (**C**) As (A) and (B) except that NF-YC5–Sepharose and NF-YC51–Sepharose columns were used. The diagrams on the right depict the different NF-YB and NF-YC deletion mutants attached to Sepharose used in the protein–protein interaction assays.

Taken together, these results indicate that basic residues in the TBP HS2 domain are necessary to interact with NF-YB and NF-YC and that the same mutations that prevent NC2–TBP interactions are also unable to associate with NF-YB/NF-YC.

DISCUSSION

In this study we provide evidence that: (i) a percentage of NF-YB is involved in complexes with multiple proteins, including TFIID, in the absence of NF-YA; (ii) TBP is able to interact with NF-YB and NF-YC in solution; (iii) basic residues in the HS2 helix of TBP and a short stretch in both the NF-YB and NF-YC conserved domains adjacent to helix III of the putative histone fold motif are necessary.

The importance of NF-Y in MHC class II transcription has been well established in functional studies. The proximal promoter Y box is unable to activate alone and needs the upstream X-X2 element; the two elements work together and constitute a true enhancer unit (27). The distance between the two elements is strictly conserved in all MHC class II promoters. Biochemical experiments indicated that NF-Y remarkably increases the DNA affinity of RFX, the activator that binds to the essential X box;

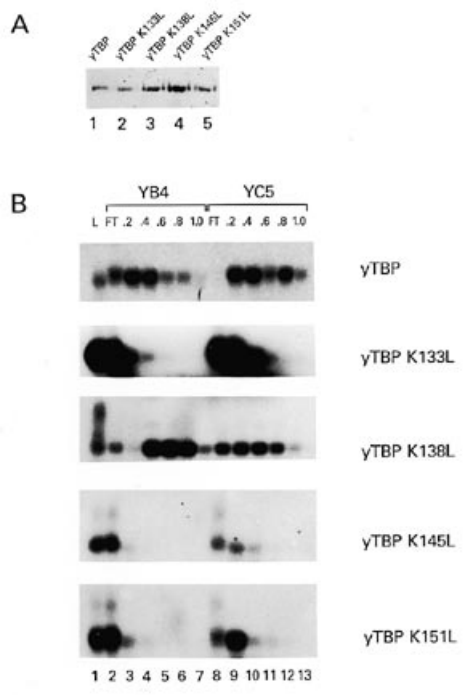

Figure 5. Mapping the TBP NF-YB and NF-YC5 binding domains. (**A**) Western blots of wild-type and mutant yTBP produced in *E.coli*; 1 µl of soluble bacterial extract was loaded on the SDS gel. The yTBP mutant is indicated above the corresponding lane. (**B**) EMSA of yTBPs using an AdML TATA box labelled oligo. Lane 1, load of wild-type and mutant yTBP; lanes 2–7, flow-through and eluted fractions from the NF-YB4–Sepharose columns, challenged in parallel with equivalent amounts of wild-type or mutant yTBPs; lanes 8–13, as 2–7 except that NF-YC5–Sepharose columns were used.

without occupancy of the Y box by NF-Y; such an event is inefficient and functionally irrelevant (37). A somewhat similar activity of NF-Y has been observed in different systems, with C/EBP on the albumin promoter and with SP1 on the invariant chain promoter (38,39). In transgenic mice, removal of the NF-Y binding site leads to a severe decrease in transcription and to the loss of focusing by the polymerase on the correct +1 signal (27). Finally, *in vitro* transcription experiments with anti-NF-Y antibodies on the MHC class II Ea promoter indicated that this CCAAT binding protein is involved in the initial steps of pre-initiation complex formation and is necessary for transcriptional re-initiation (28). These functional results suggest interactions of NF-Y with components of the basic transcriptional machinery that we have now started to address. The most surprising result of the present study is that NF-YB associates with complexes of high molecular weight in the absence of NF-YA, the necessary companion for CCAAT binding activity, and is loosely associated with TFIID. This finding represents the first evidence of the existance of a pool of NF-YB, and most likely NF-YC, biochemically separable from NF-YA. The functional significance of such interactions is unknown.

NF-Y and TBP

TBP is known to make contacts with many proteins involved in pol II-mediated transcription: (i) TAF_{II}s, necessary to mediate TFIID-activated transcription from upstream factors (24); (ii) general transcription factors such TFIIA and TFIIB (24,30,31); (iii) many cellular and viral transcriptional activators, such as SP1, C/EBP, FOS, Oct1 and 2, p53, Even-skipped, RXR, E1A, VP16, E2 and TAX1 (40–50); (iv) NC2, purified on the basis of its ability to enhance TBP binding to the TATA box, and later found to be composed of two subunits, both necessary for efficient binding of the complex to DNA and inhibition of *in vitro* transcription (21–23,32). Most of these TBP binding polypeptides have a positive effect on transcription, but some, NC2 and Even-skipped, act as repressors, suggesting that TBP is the focus of negative as well as positive regulation (21,45).

The results presented here indicate that a region of NF-YB rich in acidic and hydrophobic residues, between amino acids 117 and 131, is necessary for TBP interactions. Similarly, our data indicate that amino acids 107–120 of NF-YC are important for TBP binding in solution. It should be remembered that although the putative NF-YB/NF-YC histone fold motifs contain the core information necessary for trimer formation and DNA binding, additional sequences adjacent to the histone fold motifs, and overlapping with the TBP interaction domain described here, contribute to optimal subunit association (13,19,20). Unlike NF-YA, whose HAP2 homology domain can be sharply separated into two short subdomains responsible for subunit interactions and DNA binding, the NF-YB/NF-YC HAP homology domains appear to be a complicated puzzle of distinct, yet partially overlapping protein–protein and DNA binding subdomains. Yeung *et al*. have mapped the TBP interaction domain of NC2β/Dr1 to a short sequence equivalent to that mapped here for NF-YB (32). This strongly suggests that the LGFDSYVEPLK sequence, which is common to the two studies, is important for TBP interactions. The corresponding NF-YC domain necessary for TBP binding is identical to NC2α at seven out of 10 residues. This stretch is the most similar between the two proteins, together with α-helix I of the putative histone fold motif (22,23). Therefore, it is tempting to suggest that this NC2α subdomain is also necessary for interaction with TBP. TBP binding subdomains share sequence similarity among them and with TBP interaction surfaces of other transcription factors: phenylalanines, leucines and negatively charged amino acids are particularly recurrent (see Table 1). When available, information obtained from mutants in such regions indicate that hydrophobic and acidic residues are indeed important for TBP interactions (40,42–44,48). Interestingly, $hTAF_{II}20$, one of the histone fold-containing TAF_{II}s, has been shown to contact TBP directly and to require a short region at the C-terminus of α-helix III, corresponding to the NF-YB/NF-YC sequence identified here (51; see Table 1). We propose that NF-YB/NF-YC, NC2 and $hTAF_{II}20$ belong to a subfamily of histone fold proteins which acquired TBP binding capacity before the fungi and plants radiated, since their homologues in such kingdoms conserve such protein subdomains (8–13,21–23,34,51).

The NF-YB/C binding domain of TBP resides in the HS2 domain on the top of the saddle; basic residues appear to be necessary, indicating that the interactions are mainly ionic, in agreement with our finding that both proteins can be eluted from the columns with buffers containing increasing amounts of salts. Contrary to this, NF-YB binding to NF-YC on such protein columns cannot be reversed, even at very high salt concentrations (K.Zemzoumi, unpublished results). A previous report employing single amino acid substitution mutants in the TBP basic domain pinpointed the residues that are essential for NC2 binding (30): they are the same that are contacted by NF-YB/C in solution

here, suggesting that NF-Y and NC2 might compete for a similar, or overlapping, TBP surface.

Table 1. List of TBP binding domains

Protein	Species	Amino acid sequence	Reference
NF-YB	Hs	121 STLGFDSYVEPLK 133	12
	Zm	100 STLGFEDYVEPLK 112	12
	Sc	106 HALGFENYAEVLK 118	10
NC2β/Dr1	Hs	76 ESLGFGSYISEVKEVLQE 94	21
	At	80 QVLGFESYEEEYAAYEQH 90	54
	Sc	70 EELEYNEFIPFLEEIELN 88	22
NF-YC	Rn	107 KFDQFDFLIDIVP 119	5
	Sc	198 KSDMFDFLIDVVP 210	9
NC2α/DRAP1	Hs	76 LEQQFDFLKDLVA 88	22,23
	Sc	117 NDEKFDFLREGL 128	22
$TAF_{II}20$	Hs	126 MWIPGFGSEEIRPY 139	34
	Dm	159 MWIPGFGTDEIRPY 172	
$TAF_{II}250$	Hs	37 SLAGFLFGNINGA 50	55
	Dm	19 DLTGILFGNIDSE 31	
	Sc	17 EAYEAIFGGEFSS 29	
P53	Hs	16 QETFSDLWKLLPE 28	40
FOS	Hs	337 YTSSFVFTYPEAD 349	42
C/EBPα A	Rn	59 ETSIDIDAYID 69	43
C/EBPα B	Rn	73 FNDEFLADLFQHSR 86	43
RXRα	Mm	432 PIDSFLMEMLEA 443	44
Pol II	Dm	GTGCFDLLLDAE	56
	Sc	1437 GTGAFDVMIDEE 1448	56
VP16		437 DALDDFDLDMLGDG 450	48
E1A		137 EEGEEFVLDYVEHP 150	50

Hs, man; Rn, rat; Mm, mouse; Dm, *Drosophila*; Zm, maize; At, *Arabidopsis thaliana*; Sc, *Saccharomyces cerevisiae*.

The features shared by NF-YB/NF-YC and NC2, as a reflection of sequence similarity, are quite numerous: both are composed of two subunits, binding to each other via their histone fold motifs; they contain TBP binding domains; they recognize TBP in a similar, if not identical, way and can at least partially associate with TFIID; both are unable to stably associate with DNA in the absence of a third partner (TBP for NC2 and NF-YA for NF-YB/NF-YC). However, they seem to have opposite behaviour: while DNA binding of the NC2–TBP trimer blocks formation of a functional pre-initiation complex, the NF-Y trimer is an important and sometimes essential activator in all systems tested so far. One hypothesis that is suggested by the present observations is that either NF-Y, or the isolated NF-YB–NF-YC heterodimer, could in part exert its positive action in an indirect way, by precluding the access of a negative regulator of TBP activity, such as NC2, therefore behaving as a counter-repressor.

NF-Y and TFIID

The immunopurification and immunoprecipitation studies on holo-TFIID with anti-TBP and anti-$TAF_{II}100$ monoclonal antibodies strongly suggest that a fraction of the NF-YB pool is associated with TFIID in the absence of NF-YA. Based on the anti-$TAF_{II}100$ immunoprecipitation experiments we evaluate that 20% of NF-YB is associated with TFIID. However, NF-YB is not present in pure TFIID preparations and thus cannot be considered as a bona fide TAF_{II}. Because of the extremely tight association between NF-YB and NF-YC (they cannot be separated even in high salt, high detergent or 1 M denaturing agent) we anticipate that the same could be true for NF-YC as well. The sucrose gradient experiments strengthen the notion that TFIID–NF-YB interactions can occur *in vivo*: (i) they confirm that the NF-Y binding activity is found in the 150–200 kDa range, where both NF-YA and NF-YB are present (36); (ii) a high proportion of the NF-YB pool is found in fractions of high molecular weight devoid of NF-YA and hence of sequence-specific DNA binding; (iii) the high levels, ~40%, of NF-YB present in such fractions suggest that this subunit, most likely together with NF-YC, is engaged in multiple protein–protein interactions. Some of these interactions are related to TFIID association, as shown here, but others probably reflect binding with different, as yet unknown proteins.

It is possible that the histone fold-containing polypeptides within TFIID ($hTAF_{II}80$, $hTAF_{II}31$ and $hTAF_{II}20$) could be contacting the histone fold motifs of NF-YB and NF-YC. It has recently been shown that they are able to make protein–protein interactions between them and with histones, in combinations that are consistent with the existence of a histone octamer-like structure within TFIID (52,53). It should be remembered that $hTAF_{II}80$ and $hTAF_{II}31$ resemble histones H4 and H3 respectively, while NF-YB and NF-YC are equivalent to H2b and H2a (16,52); indeed the degree of homology between $hTAF_{II}80$ and H4 and $hTAF_{II}31$ and H3 is no greater than the homology between NF-YB and H2b and NF-YC and H2a. Moreover, relatedness is mainly confined to the histone fold 'self' and 'pair' residues, especially in α-helices II and III, which have been proposed by Arents and Moudrianakis to be essential for histone–histone interactions (18). The availability of recombinant TAF_{II}s and NF-Y subunits and the DNA binding and protein–protein interaction assays described here will now make this hypothesis testable.

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