NF-Y Recruitment of TFIID, Multiple Interactions with Histone Fold TAF\textsubscript{II}s

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The nuclear factor y (NF-Y) trimer and TFIID contain histone fold subunits, and their binding to the CCAAT and Initiator elements of the major histocompatibility complex class II Ea promoter is required for transcriptional activation. Using agarose-electrophoretic mobility shift assay we found that NF-Y increases the affinity of holo-TFIID for Ea in a CCAAT- and Inr-dependent manner. We began to dissect the interplay between NF-Y- and TBP-associated factors PO1II (TAF\textsubscript{II}8)-containing histone fold domains in protein-protein interactions and transfections. hTAF\textsubscript{II}20, hTAF\textsubscript{II}28, and hTAF\textsubscript{II}18-hTAF\textsubscript{II}28 bind to the NF-Y B-NF-Y C dimer; hTAF\textsubscript{II}18-hTAF\textsubscript{II}90 interact with the trimer but not with the NF-YB-NF-Y C dimer. The histone fold \textalpha helix of hTAF\textsubscript{II}80 is not required for NF-Y association, as determined by interactions with the naturally occurring splice variant hTAF\textsubscript{II}80s. Expression of hTAF\textsubscript{II}28 and hTAF\textsubscript{II}18 in mouse cells significantly and specifically reduced NF-Y activation in GAL4-based experiments, whereas hTAF\textsubscript{II}20 and hTAF\textsubscript{II}135 increased it. These results indicate that NF-Y (i) recruits purified holo-TFIID in vitro and (ii) can associate multiple TAF\textsubscript{II}s, potentially accommodating different core promoter architectures.

Gene expression is regulated by promoter and enhancer elements recognized by gene-specific DNA-binding proteins and by general transcription factors (1). At a higher level, it is controlled by chromatin structures, whose fundamental unit is the nucleosome, a complex formed by histones H2A, H2B, H3, H4, which wrap around them 146 base pairs of DNA (2, 3). Histones all share a conserved 65-amino acid histone fold motif (HFM)\textsuperscript{1} that has low sequence identity but high structural resemblance (4). Crystallographic analysis showed that this motif is composed of three/four \textalpha helices separated by short loops/strands regions; this structure enables histones to dimerize and form non-sequence-specific interactions with DNA (5). Proteins containing the HFM are also involved in the basic mechanisms of transcription: (i) the two subunits of the TBP-binding NC2, also called D1/DRAP1, a global repressor of basal transcription (6, 7); (ii) some of the TBP-associated factors that are part of the TFIID, P/CAF, STAGA, and TFTC complexes (8–17); (iii) one subunit of the P/CAF complex (18); and (iv) two subunits of the CCAAT-binding activator NF-Y (19–21).

TFIID is a general transcription complex composed of TBP, responsible for TATA recognition, and of several associated factors, TAF\textsubscript{II}18, that constitute a link between gene-specific upstream activators and the general transcription machinery by recognizing TATA and/or initiator elements (reviewed in Refs. 22, 23). Some of the TAF\textsubscript{II}s appear to be present in specific sub-complexes of TFIID (24–33); 10–12 highly conserved subunits have been identified in yeast, Drosophila, and human and biochemically characterized in protein-binding assays and functional in vitro transcription experiments. Based on sequence homology, structure-function analysis, and crystallographic studies, hTAF\textsubscript{II}80/dTAF\textsubscript{II}60, hTAF\textsubscript{II}31/dTAF\textsubscript{II}40, hTAF\textsubscript{II}28/dTAF\textsubscript{II}27, and hTAF\textsubscript{II}18/dTAF\textsubscript{II}30 have histone-like structures (13, 15); hTAF\textsubscript{II}20/hTAF\textsubscript{II}135 and hTAF\textsubscript{II}30 have also been included in this class (10, 11, 16, 17). Interestingly, gene inactivation of HFM-containing TAF\textsubscript{II}s in yeast implicates them in a rather broad, if not universal, role in transcriptional activation (34–38). This is unlike other TAF\textsubscript{II}s, whose inactivation in yeast suggests a more selective role in certain promoters (38–40).

Another protein containing HFM\textsubscript{s} is NF-Y, also termed CBF, the ubiquitous trimeric protein binding to the widespread CCAAT-box promoter element; it is composed of NF-YA, NF-YB, and NF-YC, all necessary for subunit association and DNA binding (reviewed in refs. 19 and 21). The H2B-H2A-like NF-YB-NF-YC subunits dimerize tightly via their HFM\textsubscript{s}, forming a complex surface necessary for NF-YA association. The resulting trimer has a high affinity and sequence specificity for the CCAAT sequence. Several types of indications link TFIID to NF-Y. First, their binding sites are either ubiquitous, in the case of TFIID, or quite frequent, because 25% of the promoters have NF-Y sites; both are found at highly conserved positions within a prototypical promoter (20). Second, biochemical evidence of direct interactions has emerged: (i) NF-YB and NF-YC bind to TBP through short subdomains within the larger yeast/human conserved parts and short basic residues in the HS2 of TBP (41); (ii) NF-YB is immunoprecipitated with an anti-TAF\textsubscript{II}100 antibody from crude nuclear extracts and is present in immunopurified TFIID fractions and in high molecular weight complexes in glycerol gradient experiments, indeed sug-
gesting association with additional proteins; (iii) the Q-rich regions of NF-YA and NF-YC interact with hTAFI\(_{28}\), hTAFI\(_{135}\) in vitro (42), a result consistent with the idea that Q-rich activators such as SP1 and camp-response element-binding protein function by binding to hTAFI\(_{28}\), hTAFI\(_{135}\) (43–48). Third, the lack of NF-Y binding has been associated with a closed chromatin configuration of the Xenopus HSP70 TATA-box region (49) and, very recently, with the inability to recruit TBP-TFIIB on the \(\gamma\)-globin promoter in vivo (50): These results clearly imply that NF-Y binding is essential for TFIID recruitment.

To study the NF-Y-TFIID connections, we employed the mouse MHC class II Ea promoter system (51, 52). Ea is a tissue-specific promoter active in B lymphocytes and other professional antigen-presenting cells (53). Like all other MHC class II promoters, it also requires the ubiquitous trimer RFX and lacks a functional TATA-box. We showed that TFIID binding to the Ea Inr is necessary for function in an in vitro transcription system. In this study, we began to dissect NF-Y-TFIID interplay with purified holo-TFIID, recombinant NF-Y, and isolated TAFI\(_{28}\).

**MATERIALS AND METHODS**

**Production and Purification of Recombinant NF-Y, hTAFI\(_{31}\), hTAFI\(_{80}\), hTAFI\(_{28}\), hTAFI\(_{18}\), hTAFI\(_{20}\), hTAFI\(_{135}\), and holo-TFIID**—Production and purification of recombinant NF-Y trimer were as described before, using wt NF-YA, wt NF-YB, and a TRX-His-NF-Y fusion protein (54). Recombinant His-tagged hTAFI\(_{28}\) and hTAFI\(_{18}\), GST-hTAFI\(_{28}\) (9), GST-hTAFI\(_{135}\), and GST-hTAFI\(_{20}\) (9) were produced in *Escherichia coli* as soluble proteins and purified according to standard procedures. hTAFI\(_{31}\), hTAFI\(_{80}\), and hTAFI\(_{20}\) were produced by the baculovirus expression system, either as single subunits or as a dimer, using standard protocols and purified to homogeneity. hTAFI\(_{80}\) contains a FLAG tag. Holo-TFIID was immunopurified from HeLa cells with an anti-TBP antibody as previously detailed (9, 26, 27).

**EMSA Analysis—EMSA of TFIID in agarose gels were as described in Ref. 52: holo-TFIID fractions were incubated in NF-Y buffer (20 mM HEPES, pH 7.9, 50 mM NaCl, 5% glycerol, 5 mM MgCl\(_2\), 1 mM dithiothreitol) together with 10,000 cpm of \(32\)P-labeled Ea fragments; the total volume was 10 \(\mu\)l. After incubation for 45 min at 30 °C, we added 2 \(\mu\l\) of \(0.5 \times\) xylene cyanol buffer containing bromphenol blue, and samples were loaded on a 1.5% agarose gel (Bio-Rad Ultrapure) in 0.5× TBE. Gels were run at 140 V for 90 min at 4 °C, transferred onto DE81 paper, vacuum-dried, and exposed. Three independent preparations of purified TFIID were used in EMSSAs. The Ea fragments used in EMSA analysis were obtained by PCR and contained sequences from 3′ of the TFIID promoter, either wt, mutated in the Y box (Ls17 (51)), or in the Inr region, known to cripple TFIID binding and Ea promoter function in vitro (52): The NF-Y complex, but not the TFIID or Y/IID complexes, was generated according standard procedures. A minimum of three independent transfections in duplicate was done; most of the values are based on 8–12 transfections.

**RESULTS**

**Binding of NF-Y and TFIID to the Ea Promoter**—We have previously shown that the MHC class II Ea promoter is crucially dependent on a binding of holo-TFIID to a TdT-like initiator (52). We investigated the interactions between NF-Y and holo-TFIID in such a system using recombinant NF-Y and immunopurified holo-TFIID in agarose-EMSA. Fig. 1 (A and B) shows a dose response of TFIID either alone (lanes 5–7), or with fixed concentrations of NF-Y (lanes 2–4): in Fig. 1A the NF-Y dose saturated the labeled fragment (lane 1), whereas in Fig. 1B lower doses of NF-Y were used. Two major complexes of different mobility, termed IIDs and IIDb, were generated by TFIID; when incubated with NF-Y, both complexes generated dissimilar electrophoretic mobilities, clearly arising as a result of co-incubation of NF-Y and TFIID (Fig. 1A, compare lanes 2–4 with 5–7; Fig. 1B, compare lanes 1–3 with 5–7); One complex migrated slightly more slowly than the NF-Y band, and another was further retarded and visible at higher TFIID concentrations. Note that the upper Y/IID complexes were visible at lower TFIID concentrations (Fig. 1A, compare lanes 3 and 6). As a control for the specificity of the interactions, we used an identical Ea fragment carrying a 10-bp mutation in the Y-box, known to abolish NF-Y binding, as well as in vivo and in vitro transcriptional activity of the promoter (51): As expected, binding of NF-Y, but not TFIID, was abolished, and the upper bands resulting from simultaneous interactions were not detected (Fig. 1C).

To verify the effect of TFIID on NF-Y binding, a reciprocal experiment was also performed, namely a dose-response analysis of NF-Y alone, or with two TFIID concentrations (Fig. 1, D and E, lanes 2–7). The TFIID pattern at high concentrations was clearly modified in a dose-dependent manner by NF-Y (Fig. 1D, compare lanes 1 with 2–4); Interestingly, the lowest amount of NF-Y employed (0.1 ng), which was barely sufficient to generate a visible band, modified the pattern of the TFIIDa and -b bands. When incubated with low amounts of TFIID, insufficient to shift Ea DNA, the NF-Y band was evident at lower concentrations (Fig. 1E, compare lanes 2 and 3 with 6 and 7). In experiments conceptually similar to those of Fig. 1C we employed a fragment containing a 10-bp mutation in the Ea Inr region, known to cripple TFIID binding and Ea promoter function in vitro (52): The NF-Y complex, but not the TFIID or the Y/IID complexes, was generated by co-incubation of the two proteins (Fig. 1F), indicating that an intact Inr is required for 2 h on ice, unbound material was recovered after centrifugation, and the beads were washed with NDB100 with the addition of 0.1% Nonidet P-40. SDS buffer was added, and the samples were boiled at 90 °C for 5 min and loaded onto SDS gels. Western blots were performed according to standard procedures with the indicated primary antibody and a Peroxidase secondary antibody. For multiple exposures the filter was stripped, blocked with nonfat dry milk, and re-hybridized.

**Transfections**—The eukaryotic expression vectors for NF-Y, hTAFI\(_{28}\), hTAFI\(_{18}\), hTAFI\(_{20}\), hTAFI\(_{135}\), and hTAFI\(_{80}\) were described before (9, 12, 56). From PCR-hTAFI\(_{31}\) (kindly donated by Dr. Levine) an EcoRI insert was cloned into pGAL4poly in-frame with the Gal4-DNA-binding domain, and the latter was excised by cutting with Xhol and a partial EcoRI digest. Mouse NIH-3T3 fibroblasts were co-transfected with 1–3 μg of activating plasmids, 2 μg of the plasmids containing the Luciferase reporter gene, and 3 μg of pNpGal plasmid for control transfection efficiency. The total amount of DNA was kept constant (at 15 μg) with Bluescript. All plasmids were purified by centrifugation using cesium chloride gradients. Cells were transfected with the standard calcium-phosphate method, recovered 48 h after transfection, washed in phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate, pH 7.4), and resuspended in the Reporter assay reagent (Promega). Luciferase and β-galactosidase activity were measured according standard procedures. A minimum of three independent transfections in duplicate was done; most of the values are based on 8–12 transfections.


Next, we wished to determine whether the Y/IID complexes observed in our EMSAs truly contain TAF IIs. To this aim, we used antibodies specific for different TAF IIs in supershift experiments. Fig. 1G indicates that anti-hTAFII31, anti-hTAFII30, anti-hTAFII20, and two different anti-hTAFII80 antibodies all modify the Y/IID complexes, whereas an irrelevant anti-GATA1 antibody had no effect (Fig. 1G, upper panel, compare lanes 1 and 2 with 3–7). In parallel, when challenged with the IIDa complex, these antibodies all showed interactions (Fig. 1G, middle panel), whereas none recognized the NF-Y complex (Fig. 1G, lower panel). Note that the anti-hTAFII31 antibody, rather than supershifting the IIDa or Y/IID complexes, apparently inhibited binding of TFIID to DNA. Finally, we verified whether the Y/IID complexes also contain NF-Y, by challenging them with anti-NF-YA and anti-NF-YB antibodies. Indeed, both antibodies modified the mobilities of the complexes (Fig. 1H). The experiments shown in Fig. 1 (A–E) also suggest that the presence of NF-Y improves the binding capacity of holo-TFIID. We verified whether NF-Y can facilitate binding of TFIID by performing on-rate experiments: Binding of TFIID to DNA, in fact, is known to be a slow process, and the on-rates even on high affinity TATA-Inr elements are on the order of 20–40 min (Ref. 57, and references therein). On the contrary, NF-Y
NF-Y and TAFII\textsubscript{18s}

binding under our experimental conditions is extremely rapid, being completed after 1–2 min (Fig. 2A; see also Ref. 58). We incubated suboptimal amounts of TFIIID (see Fig. 1E) with and without saturating amounts of NF-Y. A weak IIF band was seen only after 30 min of incubation in the absence of NF-Y (Fig. 2B, lanes 6–9), whereas the upper YIIF complexes were evident already after 2 min of incubation at 30 °C, and maximal after 5 min (Fig. 2B, lanes 2 and 3), strongly suggesting that NF-Y-CCAAT complexes recruit TFIIID onto the Ea initiator.

From this set of in vitro experiments with purified holo-TFIIID and recombinant NF-Y, we conclude that: (i) complexes of different mobilities are formed upon simultaneous binding of TFIIID and NF-Y to the Ea promoter; (ii) these complexes contain both TAFII\textsubscript{18s} and NF-Y; (iii) binding of NF-Y to the CCAAT box and of TFIIID to the initiator is required; and (iv) TFIIID binding is remarkably facilitated when NF-Y is bound to DNA.

Binding of NF-YB-NF-YC to Histone Fold TAFII\textsubscript{18s}—A large number of subunits are present in holo-TFIIID, and full reconstitution of the holo-TFIIID complex with recombinant proteins has not been achieved yet. We therefore decided to dissect NF-Y-TFIIID interactions by taking a reductionist approach, investigating the interactions between isolated HFM subunits of the two complexes. Recombinant proteins were produced in E. coli or Baculovirus and purified (Fig. 3). Note that the GST-hTAFII\textsubscript{1355}–372 protein used hereafter is a mutant containing the C-terminal region with the HFM (16) but lacking the N-terminal 372 Q-rich region that mediates binding to Q-rich activators, such as SP1 and cAMP-response element-binding protein (46), and possibly contact NF-Y (42). The recombinant NF-YB-NF-YC dimer, containing the histone folds, was incubated with bacterial extracts containing GST-hTAFII\textsubscript{28}, GST-hTAFII\textsubscript{1355}–372, GST-hTAFII\textsubscript{120}, or GST-hTAFII\textsubscript{28}-GST-hTAFII\textsubscript{1355}–372, and Sf9 extracts containing hTAFII\textsubscript{80}-hTAFII\textsubscript{31}. The complexes were purified over a nickel NTA-agarose column, exploiting the presence of His tags on the respective proteins. Results of the experiments are shown in Fig. 4. As expected, NF-YB was efficiently bound to the column, despite the lack of His tags (Fig. 4, upper panels). GST-hTAFII\textsubscript{28} and GST-hTAFII\textsubscript{120} were efficiently retained on the columns but were in the FT fraction in the absence of NF-YB-NF-YC, thus ruling out that any of the HFM proteins tested had any intrinsic affinity for NTA-agarose (Fig. 4 and data not shown). On the other hand, GST-hTAFII\textsubscript{1355}–372, GST-hTAFII\textsubscript{120}-GST-hTAFII\textsubscript{1355}–372, and hTAFII\textsubscript{80}-hTAFII\textsubscript{31} were not retained by the affinity column. The reverse approach was also tested, namely His-tagged hTAFII\textsubscript{28}, hTAFII\textsubscript{120}, and hTAFII\textsubscript{120}-hTAFII\textsubscript{28} were incubated with an NF-YB-NF-YC5 dimer lacking His tags. In this experiment, we used an NF-YC mutant, YC5, that contains only the evolutionarily conserved part of NF-YC, fully capable to associate NF-YB. The NF-Y HFM subunits were found in the bound fractions with hTAFII\textsubscript{28} and hTAFII\textsubscript{120}-hTAFII\textsubscript{28} but not with hTAFII\textsubscript{120}.

From this set of experiments we conclude that only hTAFII\textsubscript{28}, hTAFII\textsubscript{120}-hTAFII\textsubscript{28}, and hTAFII\textsubscript{120}, but not the HFM containing hTAFII\textsubscript{20}-hTAFII\textsubscript{1355}–372, nor hTAFII\textsubscript{80}-hTAFII\textsubscript{31}, are capable to associate the NF-Y HFM dimer in solution.

Binding of NF-Y to Histone Fold TAFII\textsubscript{18s}—Because NF-YA has an intrinsic affinity for NTA-agarose and is unsuitable for the protein-protein interaction approach taken above, most likely because of the high number of His residues in the conserved domain (Not shown). We thus switched to immunoprecipitations with the purified recombinant NF-Y trimer and the different TAFII\textsubscript{18s}, either as single subunits or dimers. The complexes were incubated with Mab7, a monoclonal antibody that recognizes the NF-YA Q-rich activation domain (55), previously bound to a Protein G-Sepharose matrix; in parallel, equivalent amounts of recombinant proteins were incubated with a control Protein G-Sepharose resin associated with an irrelevant anti-MHC class II antibody. After washing, bound material was recovered by boiling samples in SDS buffer and analyzed in Western blots with the anti-NF-Y and anti-TAFII\textsubscript{18} antibodies. As expected, NF-YA and NF-YB are immunoprecipitated with Mab7 but not with the control antibody (Fig. 5A, upper panel); the same was true for NF-YC (not shown, see below). Incubation of NF-Y with hTAFII\textsubscript{28}, but not with hTAFII\textsubscript{120}, retained the TAFII\textsubscript{31} in the bound material. Surprisingly, unlike the previous experiments with the NF-YB-NF-YC dimer, when incubated together with the NF-Y trimer, interactions of the hTAFII\textsubscript{28}-hTAFII\textsubscript{120} dimer were not observed, implying that the presence of NF-YA prevents hTAFII\textsubscript{28}-hTAFII\textsubscript{120} binding to the NF-Y HFM dimer. On the other hand, hTAFII\textsubscript{20} and hTAFII\textsubscript{80}-hTAFII\textsubscript{31} dimer, but not hTAFII\textsubscript{20}-hTAFII\textsubscript{1355}–372, were bound to the NF-Y trimer. hTAFII\textsubscript{80}, but not hTAFII\textsubscript{31}, was immunoprecipitated when incubated alone with NF-Y. In this experimental setting, we also used a differentially spliced form of hTAFII\textsubscript{80}, termed hTAFII\textsubscript{80\textsubscript{6}}, in which 10 amino acids of the HFM α2 are missing. This isoform is incapable of interacting with hTAFII\textsubscript{31}. We tested this protein in the immunoprecipitation assays and found that, as for hTAFII\textsubscript{80}, it is able to interact with NF-Y. To confirm that hTAFII\textsubscript{20}-hTAFII\textsubscript{1355}–372 and hTAFII\textsubscript{28}-hTAFII\textsubscript{120} are present in dimeric form in our assays, we immunoprecipitated these dimers, in the absence of NF-Y subunits, with anti-hTAFII\textsubscript{153} and anti-hTAFII\textsubscript{18} monoclonal antibodies, respectively. Fig. 5B shows that both hTAFII\textsubscript{20} and hTAFII\textsubscript{28} are indeed associated with their respective partners, as assessed in Western blots.

From the immunoprecipitation analysis of the NF-Y trimer, we conclude that hTAFII\textsubscript{120} and hTAFII\textsubscript{31} do not bind NF-Y, whereas

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hTAFII80, hTAFII80, hTAFII28, and hTAFII20 are capable of associating with the trimer. Upon dimerization, hTAFII31-hTAFII80, but not hTAFII28-hTAFII18, can bind to NF-Y.

Effect of TAFIIs Overexpression on NF-Y Activation in Mammalian Cells—Having established that NF-Y is capable of increasing the binding of holo-TFIID and associated multiple TAFII's in vitro, we sought to investigate the in vivo effects with "co-activator" assays used for other activators (12, 59–62). The system is based on the co-expression in NIH-3T3 fibroblasts of GAL4-NF-YA together with NF-YB and NF-YC, activating a promoter containing five GAL4 sites driving the Luciferase reporter gene: Transcription is strictly dependent upon co-transfection of all NF-Y subunits, requiring at least one of the NF-Y Q-rich domains (56; see Fig. 6A). Co-transfections of different amounts of vectors expressing the TAFII's used in our in vitro analysis gave the results outlined in Fig. 6B: hTAFII20

**Fig. 3.** Purification of recombinant proteins. SDS gels stained with Coomassie Blue showing the indicated purified recombinant proteins used for protein-protein assays. The GST-hTAFII135Δ1–372 is a mutant lacking the 372 amino acids at the N-terminal of the protein and containing the HFM.

**Fig. 4.** Protein-protein interactions of TAFII's and NF-Y HFM subunits. A, in the different panels, wt NF-YB-NF-YC His-tagged were incubated with GST-hTAFII18 and GST-hTAFII28, before loading onto NTA-agarose. Load, flow-through, 300 m, and 1.0 M KCl washes and the two imidazole-eluted fractions are indicated. The fractions were run in SDS gels, and the proteins are revealed by Western blotting with the indicated antibody. Similar experiments are shown for GST-hTAFII20, GST-hTAFII135Δ1–372, GST-hTAFII20-GST-hTAFII135Δ1–372, and hTAFII80-hTAFII31. In the lower panels, an NF-YB-NF-YC dimer devoid of the His tag, consisting of wt NF-YB and of the HFM-containing YC5 mutant (54), was incubated with the indicated hTAFII's. From top to bottom: His-tagged hTAFII28, His-tagged hTAFII18, and hTAFII28-hTAFII18 (both His-tagged).

hTAFII80, hTAFII80, hTAFII28, and hTAFII20 are capable of associating with the trimer. Upon dimerization, hTAFII31-hTAFII80, but not hTAFII28-hTAFII18, can bind to NF-Y.

**Fig. 5.** Immunoprecipitations of NF-Y and HFM TAFII's. A, equivalent amounts of recombinant NF-Y and the indicated hTAFII's were incubated and immunoprecipitated with the anti-NF-YA monoclonal antibody 7 (55), or with an anti-MHC class II control antibody. Load (L), unbound (U), and bound (B) materials were loaded onto SDS gels and checked in Western blots with the indicated antibody. B, Western blots of immunoprecipitations of the His-hTAFII18-His-hTAFII28 dimer with the anti-hTAFII18 antibody 16TA. Similarly, Western blot analysis of immunoprecipitations of preparations containing GST-hTAFII20 and GST-hTAFII135Δ1–372 with the 20TA monoclonal antibody against hTAFII135.
and hTAFII135 had small, 2-fold-positive effects on GAL4-NF-Y activation; hTAFII31 and hTAFII80, either alone or in combination, showed no effect; whereas expression of hTAFII18 and hTAFII28, alone or together, had a clear dose-dependent negative effect. To verify whether this inhibition was specific for the NF-Y trimer, or exerted indirectly through the basal promoter, we tested in similar assays an NF-YA mutant, G4-YA12, containing the isolated activation domain of NF-YA fused to the DNA-binding domain of GAL4, and GAL4-SP1, also a Q-rich activator. Co-expression of hTAFII18 and/or hTAFII28, or of the other TAFII8s, had minor effects on GAL4-YA12 or GAL4-SP1 (Fig. 6, C and D): if anything, hTAFII18 and/or hTAFII28 slightly increased GAL4-SP1 activation. Because the reporter construct was identical in all these experiments, these latter experiments rule out that the strong inhibitory effect of hTAFII18-hTAFII28 observed on the GAL4-NF-Y trimer is due to an unspecific repression of core promoter activity; moreover, inhibition is specific for the NF-Y trimer and not for the Q-rich activation domains, such as those of NF-YA, or of SP1.

**DISCUSSION**

TFIID Recruitment—The TATA-box is the most frequent promoter element. When absent, polymerase II positioning is generally assured by an initiator; i.e. both these core elements are recognized by TFIID (1). NF-Y binding sites are found in 25% of eukaryotic promoters, invariably playing an important and sometimes essential role. Both boxes are normally found at a fixed position, TATA at −25/−30 and CCAAT at −60/−100. The mean CCAAT-box position and orientation in TATA and TATA-less promoters pointed to a small, but significant difference (20), suggesting that the proteins binding to these sites could directly or indirectly interplay. In keeping with this, we previously presented biochemical evidence showing that the NF-Y HFM subunits are associated with holo-TFIID and can bind TBP directly (41). The aim of our study was to examine their relationships, using a model system, the MHC class II Ea promoter, in which the functional importance of the two complexes is well established. We have previously shown, in fact, that holo-TFIID binds to a TdT-like initiator in the Ea promoter in a sequence-specific way. Our EMSA analysis is the first indication that NF-Y helps recruit holo-TFIID, thereby adding experimental proof to the hypothesis that NF-Y and TFIID have intimate relationships. It should be noted that other upstream factors are thought to recruit TBP and associated factors, as an important step in the formation of a transcriptionally competent complex (1). Concerning the mechanisms of such DNA-binding facilitation, an obvious hypothesis is that there are direct protein-protein contacts between TAFII8s and NF-Y. We already detailed the binding of NF-YB-NFYC to TBP (41), and the in vitro analysis presented here is strongly supportive of this, because a number of TAFII8s show affinity for NF-Y. Indeed, the multiple interactions of HFM TAFII8s with NF-Y observed in solution invite the speculation that TFIID binding might be influenced, if not dictated, by one or more transcription factor combinations binding nearby. Interestingly, we evidenced holo-TFIID complexes showing differential mobilities in our EMSAs, depending on the presence or absence of NF-Y. The upper TFIIDb, for example, migrates faster with NF-Y, possibly suggesting that a different composition of subunits might be present and that a DNA-bound NF-Y might select specific sub-complexes. The apparent multiplicity of TFIID combinations could then reflect into a plasticity of DNA binding, because the presence of adjacent activators might select subtypes of holo-TFIIDs with subunit compositions particularly suited to fit within the context of a given promoter. Antibody supershifting experiments, although showing that “core” TAFII8s are present in the IIDY complexes, cannot yet provide us with a complete description of the composition of these complexes.

TFIID is known to be capable of sequence-specific interactions, and multiple subunits within TFIID can contact DNA (63, 64 and references therein). In the course of our analysis we found no evidence of sequence-specific binding of the isolated HFM TAFII8s considered here to the Ea promoter (data not shown). Non-HFM TAFII8 with well documented core promoter DNA-binding specificity, such as hTAFII150 and/or hTAFII250 (64), could then be considered for the Inr-binding activity.

**NF-Y Interactions with HFM TAFII8s**—Complete reconstitution of the holo-TFIID complex with recombinant subunits has not been achieved yet. We therefore dissected NF-Y interactions with individual TAFII8s. Given the common structural features of many TAFII8 with NF-Y subunits, we decided to start with HFM TAFII8s. In general, our analysis showed several interactions between NF-Y and TAFII8s. Minimally, hTAFII18, hTAFII28, hTAFII80, and hTAFII20 have affinity for either the HFM NF-YB-NFYC dimer, the trimer, or both (Table I). hTAFII135 should be added to this list (42), because the negative results obtained here probably reflect the absence of the N-terminal Q-rich region. Therefore, the interactions of the trimer with hTAFII20 and hTAFII80 are possibly relevant for the function of the CCAAT-binding trimer. On the other hand,
Crystallographic analysis of the nucleosome found details for binding of H3-H4 and H2A-H2B subfamilies of histone folds. Interactions are fully in agreement with previous findings on the helix of the HFM (66), a fact that is mirrored by the interaction TAFII-binding ability (1). Conditional inactivation of yeast histone fold TAFII80, TAFII135, and TAFII28 with the HFM NF-YB, H2B Glu-90 and Glu-73 are among the relatively few amino acids present in all 26 NF-YB sequences from different species (65). We found that NF-Y is able to interact with an isoform of hTAFII20-hTAFII80 that lacks 10 amino acids in the activation factor 2 of retinoic acid receptor (12, 59). In other reports, in vitro interactions, SP1-hTAFII135, E1A-hTAFII135, and p53-dTAFII40-dTAFII60-dTAFII230, were matched by strong repression in co-transfections of the TAFII with GAL4 fusions containing the activation domains (61, 67), although the same TAFII interactions with GAL4-p53 resulted in activation in vitro (68). In our GAL4 assays, we observe a similar negative effect by overexpression hTAFII28 and/or hTAFII18 on the activation of the NF-Y trimer. It should be noted that this is specific, both for the target, it is not seen with GAL4-SP1 and the Q-rich activation domain of NF-YA, and for these two TAFII80, because hTAFII31-hTAFII80 have negligible effects and hTAFII30-hTAFII135 have small positive effects; moreover, hTAFII18-hTAFII18 do not inhibit the natural Ea promoter (not shown). What might be the reason for the inhibition of GAL4-NF-Y fusions? Our in vitro results indicate that the hTAFII28-hTAFII18 dimer interacts with NF-YB-NF-YC, but not with the NF-Y-trimer, implying that NF-YA, which recognizes determinants in the HFM of both subunits (20), prevents the association of hTAFII28-hTAFII18 with NF-YB-NF-YC. Because formation of the trimer in vivo is essential for GAL4-NF-YA activation (56), one can imagine that the co-transfected TAFII is could compete for binding to NF-YA by associating NF-YB-NF-YC. However, other interpretations must reconcile our findings that the NF-Y-interacting hTAFII28 and the non-interacting hTAFII18 both inhibit when transfected alone. It is possible that overexpression of some TAFII is alters the stoichiometry of endogenous TFIID complexes, impairing their capacity to mediate activation through the artificial GAL4 constructs. In this respect, it should be noted that certain mouse tissues do have with lower amounts of hTAFII28 and hTAFII18 (33).

**Table I**

<table>
<thead>
<tr>
<th>TAFII</th>
<th>NF-Y</th>
<th>NF-YB-NF-YC</th>
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<tbody>
<tr>
<td>TAFII135</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TAFII18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TAFII18-TAFII28</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TAFII31</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TAFII80</td>
<td>-</td>
<td>ND*</td>
</tr>
<tr>
<td>TAFII90-TAFII31</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TAFII20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TAFII135-372</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TAFII20-135A-372</td>
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</table>

* ND, not determined.

The interactions of hTAFII28-hTAFII18 with the HFM NF-YB-NF-YC dimer, but not with the trimer, should be considered in the light of the presence of the HFM dimer, without NF-YA, in several cell types, including monocytes and differentiated myotubes (21). This finding might point to a role in the basic mechanisms of activation on promoters that lack the CCAAT target site.

The HFM TAFII80 are present not only in TFIID but also in other complexes: (i) hTAFII135, hTAFII80, and hTAFII20 are found in TFTC, a TAFII-containing complex lacking TBP (27, 30); (ii) hTAFII20 and hTAFII31 (and the hTAFII80-like PAF65a) are in the P/CAF complex (18); and (iii) hTAFII31 is found in STAGA (28). A histone octamer-like structure within TFIID has been hypothesized (10); indeed, core histones and hTAFII80, hTAFII31, and hTAFII20 interact through their HFM in ways that are consistent with histones rules: The H4-like hTAFII80 with H3 and H2B, the H3-like hTAFII31 with H4, and the H2B-like hTAFII20 with H2A and H4. These interactions are fully in agreement with previous findings on the binding of H3-H4 and H2A-H2B subfamilies of histone folds. Crystallographic analysis of the nucleosome found details for residues that are required for H4-H2B interactions: H4 His-75 and H4 Lys-91, and H2B Glu-90 and H2B Glu-73 (5). These residues are conserved in hTAFII80 and in the related PAF65a. In NF-YB, the Asp-115 and Glu-98 residues corresponding to hTBP Glu-90 and Glu-73 are among the relatively few amino acids present in all 26 NF-YB sequences from different species (65). We found that NF-Y is able to interact with an isoform of hTAFII80, which lacks 10 amino acids in the α helix of the HFM (66), a fact that is mirrored by the interaction of hTAFII80 with the H2B-like hTAFII20. Under these circumstances, it is unclear what the structure of the HFM might be, but the α2 subunit is crucial for the formation of heterodimers and, indeed, hTAFII80 is unable to bind to hTAFII31: Other parts of the HFM might be involved in the contacts with the H2B-like. Alternatively, a domain of hTAFII80 distinct from the HFM might be implicated in contacting NF-Y. Concerning the hTAFII28/hTAFII18 dimer, structural analysis detailed somewhat different sorts of HMFs not easily assigned to any of the core histone sub-classes (15). Thus it is difficult to rationalize our interactions data with available structural information.

**TAFII80 and MHC Class II Transcription**—In addition to NF-Y, binding the trimeric complex RFX is important for MHC class II promoters (53). The NF-Y-RFX trimers make cooperative interactions. In particular, NF-Y binding improves the otherwise rather inefficient binding of RFX. Two non-DNA-binding co-activators are also crucial: the ubiquitous p300/CBP (69, 70) and the tissue-specific CIITA (reviewed in Ref. 71). A network of protein-protein interactions is emerging, in particular, CIITA can interact with hTAFII31. Our finding that the hTAFII80-hTAFII31 dimer can associate NF-Y through hTAFII80 suggests that hTAFII80-hTAFII31 could be contacted at the same time by two of the MHC class II activators. The interactions of NF-Y with HFM TAFII80 should be considered in the light of recent findings showing that both complexes can associate HATs: p300, P/CAF, and hGCN5 interact with NF-Y (21), whose NF-YB subunit is acetylated by p300 (72). Similarly, CBP/p300 can interact with CIITA. It has been suggested that TAFII80 could be chaperones of the histone-modifying machines in the proximity of core promoters. NF-Y could recruit acetylase complexes not only through direct interactions with P/CAF, GCN5, or CBP/p300, but also by contacting P/CAF complexes, via the HFM TAFIIs. From its privileged location at NF-Y and TAFIIs and MHC Class II Promoters, via the HFM TAFIIs. From its privileged location at

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4 Bell and L. Tora, unpublished.
TFIID interplay by taking into account the interactions with other TAFs is currently underway.

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REFERENCES


NF-Y and TAFs