The B Subunit of the CAAT-binding Factor NFY Binds the Central Segment of the Co-activator p300*

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We report that the heterotrimeric transcription factor NFY or “CAAT-binding factor” binds the −60 region of the human H ferritin promoter, the B site. DNA binding analysis with specific antibodies demonstrates that NFY/B/C subunits tightly bind this site and that NFY/C subunit is masked in vivo by binding with other protein(s). NFY binds the co-activator p300. Specifically, the NFY/B subunit interacts with the central segment of p300 in vivo and in vitro. cAMP substantially increases the formation of the NFY-p300 complex. Taken together these data provide a general model of cAMP induction of non-CRE-containing promoters and suggest that the NFY-B-p300 complex is located at the 5’ end of the promoter and the NFY-B-C-TFIIB on the 3’ end toward the transcription start site.

Transcription of the human H ferritin gene is driven by two promoter elements, A and B, located 170 base pairs upstream from the transcription start site (1). Element A of the ferritin promoter, located −132 nucleotides from the transcription start site, binds the ubiquitous transcription factor Sp1 and, in basal conditions, accounts for about 50% of the total transcription of the promoter (1). Element B is located at position −62 and is constituted by a CAAT box on the noncoding strand, flanked by a stretch of GC-rich sequences (1). Element B induces increased transcription in differentiating cells (2) and in cells exposed to G418 (3). Element B is also the target site of cAMP signaling on ferritin promoter (4). Specifically, we previously identified binding activity in nuclear extracts at the B-box, called Bbf,1 which also binds the co-activator molecule p300/CREB. We found that cAMP stimulates assemblage of the Bbf-p300 complex (4). Inhibition of phosphatases by okadaic acid also induces and/or stabilizes this functional complex (5).

NFY is a ubiquitous transcription factor formed by subunits A, B, and C (6). NFY-B and NFY-C subunits contain a conserved histone-fold motif, which forms a dimer (“histone-fold”) and interacts with subunit A (7). NFY specifically recognizes a CAAT-box motif found in the promoter and enhancer regions of many eukaryotic genes (8). Among the genes recognized by NFY, some are induced by cAMP. The promoter region responsible for the cAMP-mediated stimulation of transcription includes the CAAT element recognized by NFY (4, 9, 10).

We report that NFY is a component of the Bfd complex. Also, we demonstrate that NFY interacts, both in vivo and in vitro, with the co-activator molecule p300/CREB. Using antibodies against the three NFY subunits and p300 protein segments, we found that NFY-B subunit binds the central region of p300 (residues 671–1194).

MATERIALS AND METHODS

Cell Culture—HeLa cells were cultured as monolayers in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 10% (v/v) fetal calf serum (Sigma), 100 units/liter penicillin (Hyclone Labs.). Cells were grown at 37 °C in a 5% CO2 atmosphere.

Plasmids—The expression vectors for p300 were kindly donated by Dr. V. Orygko (NIH, Bethesda, MD). For the expression in eukaryotic cells, different regions of p300 were cloned in the pCI vector (Promega) under the control of the cytomegalovirus promoter. p300 N encodes amino acids 1–670; p300 M encodes amino acids 671–1174; and p300 C encodes amino acids 1135–2414. All these proteins are flag-tagged. For the expression in prokaryotic cells (BL21 DE3 strain), the N, M, and C regions of p300 were cloned in pet28c vector (Novagen). All these proteins are flag-tagged. The Bfd-TATAA construct is described elsewhere (4); it contains a B-site oligonucleotide (5′-CCGGGGCGGGC-3′) fused to the ferritin TATAA box in the pEMLBS-CAT vector.

DNA Transfections, Cell Extracts, and CAT Assays—HeLa cells were transfected with 5 μg of DNA using the calcium phosphate co-precipitation method (11). Cells washed twice in cold phosphate-buffered saline and collected by centrifugation were used for immunoprecipitation and Western blot analysis. The pellet was resuspended in 500 μl of lysis buffer containing 250 mM NaCl, 20 mM NaH2PO4, pH 7, 50 mM sodium pyrophosphate, 5 mM EDTA, 1 mM NaF supplemented with 5 mM dithiothreitol, 10 μg/ml of leupeptin, 10 μg/ml of pepstatin, 0.1 mM Na2O2, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40. After incubation for 10 min on ice, the suspension was centrifuged at 14,000 × g for 10 min.

Five micrograms of a plasmid carrying the luciferase gene controlled by the cytomegalovirus enhancer were included in the transfection experiment followed by CAT assay. The cell extracts and the CAT and luciferase assays are described elsewhere (4).

Immunoprecipitations—HeLa cell extract (1 mg) was incubated with 50 μl of anti-flag M2 affinity gel for 1 h at 4 °C. The beads were collected by centrifugation, washed four times with the lysis buffer, and then loaded on a 12% SDS-PAGE gel.

Western Blot Analysis—After electrophoresis, the proteins were transferred to nitro-cellulose. Then the blocking mixture was added, and the membrane was incubated with a 1:5000 dilution of rabbit

1 The abbreviations used are: Bbf, B-box-binding factor; CAT, chloramphenicol acetyltransferase; CBP, CREB-binding protein; CRE, cAMP-responsive element; EMSA, electrophoretic mobility shift assay; H, heavy; PKA, protein kinase A; PAGE, polyacrylamide gel electrophoresis.
NFY Interacts with p300

**RESULTS**

**The Trimeric Transcription Factor NFY Binds H Ferritin Promoter**—The ~100–50 region of the human ferritin H promoter contains a CAAT box, flanked by two GC-rich sequences, on the noncoding strand. This cis-element, the “B-box” (1) or “FF fragment” (12), induces transcription of the H gene (2–4). We asked whether the CAAT-binding factor NFY, also called “CBF”, binds this sequence in the ferritin promoter. To this end, we incubated a specific B oligonucleotide with recombinant NFY protein (NFY) and analyzed the resulting DNA-protein complex by EMSA. As shown in Fig. 1, recombinant NFY formed a specific complex with the B element of ferritin promoter (lanes 1, 2, and 3). To determine which of the NFY subunits (A, B, or C) binds the DNA of the B element, we added antibodies against NFY-A, -B, and -C subunits to the incubation mixture. Anti-A, anti-B, and anti-C antibodies abolished the retarded DNA-protein complex (Fig. 1, lanes 4, 5, and 6).

We also incubated the B oligonucleotide with nuclear extract from HeLa cells (lane 8) and probed the mixture with the anti-NFY antibodies (lanes 9, 10, and 11). The formation of the NFY-DNA complex was prevented by the antibodies against subunits NFY-A and NFY-B (lanes 9 and 10) but was unaffected by anti-C antibodies (lane 11). The discrepancy between *in vivo* and *in vitro* results suggests that binding to other component(s) masked the subunit C in the nuclear extracts.

This is borne out by the larger size of the DNA-protein complex generated by the nuclear extract compared with the recombinant NFY (compare lanes 1 and 8).

We next investigated the effect of NFY specific antibodies on the natural promoter fragment (~100 to +1 base pairs). Addition of anti-NFY antibodies to the nuclear extract supershifted the complex NFY-DNA (data not shown). Antibodies to subunit C did not alter the migration of the DNA-protein complexes (data not shown), which coincides with the data obtained with the oligonucleotide (Fig. 1).

These data demonstrate that NFY binds the B box of the ferritin promoter and suggest that, *in vivo*, other components are present in the DNA-protein complex, which might stabilize NFY.

**The B Subunit of NFY Binds the Transcriptional Adapter p300 in Vitro and in Vivo**—Nuclear extract resulted in a larger NFY-DNA protein complex than did recombinant NFY (Fig. 1, lanes 7 and 8). This effect was detected with both the oligonucleotide B and the ferritin promoter fragment (data not shown).

Because the nuclear adapter p300 binds the B-binding factor(s) at region ~62 of ferritin promoter (4), we tested whether the heterotrimeric NFY directly interacts with p300. Addition of specific p300 antibodies to the binding mixture did not cause a reproducible supershift of the retarded DNA-protein complex (data not shown).

Next, we expressed in *E. coli* the epitope-tagged p300 molecule subdivided in three fragments: the N terminus (p300N, residues 1–690), the central region (p300M, residues 671–1194), and the C terminus (p300C, residues 1135–2414). The recombinant A, B, and C subunits of NFY were incubated with the three p300 peptides tethered to flag-M2 affinity gel. Panel A of Fig. 2 shows the immunoblot with the antibodies against the NFY-subunits mixed with the p300 central region; the NFY-B subunit specifically binds p300 bound to the column. Bacterial lysates expressing the vector alone, purified with the same procedure and blotted with the three antibodies did not show any specific signal (lanes 2, 4, and 6). The specificity of the binding between recombinant NFY-B and p300 was confirmed by testing in the same assay a different recombinant protein (PKA regulatory subunit, RIIz, as shown in *lanes* 5 and 6 of panel B. Parallel immunoblots with anti-p300 flag (panel B, lane 3) and with the NFY-A, -B, and -C recombinant subunits (panel C) showed that the amounts of the reactants in the experiment were comparable. Note that the recombinant C subunit is a truncated form with a size of about 25 kDa in SDS-page. In the same assay, the N- and C-terminal fragments of p300 did not bind any NFY-subunit (data not shown).

These data indicate that the central segment of p300 specifically binds the NFY-B subunit *in vitro*. To demonstrate that the selective interaction between p300 and subunit NFY-B occurs also *in vivo*, we expressed full-length epitope-tagged anti-NFY-YA, NF-YB, or NF-YC antibodies or with a 1:1000 dilution of mouse anti-flag or mouse anti-RII antibodies for 1 h at room temperature. Bound antibodies were detected with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000) and with ECL (Amersham Pharmacia Biotech).

**Expression of Recombinant Proteins**—p300 N, p300 M, and p300 C-pet 28 plasmids were expressed and purified from soluble fraction of *Escherichia coli* BL21 (DE3) after induction with 0.4 mM isopropyl-d-thiogalactopyranoside for 2 h at 37 °C. Bacteria were lysed by sonication in a lysis buffer; after centrifugation, the supernatant was incubated with anti-flag M2 affinity gel for 1 h at 4 °C. The beads were collected by centrifugation and washed four times with the binding buffer. SDS-PAGE sample buffer (20 μl) was added to the beads; the sample was heated at 95 °C and then loaded on a 12% SDS-PAGE gel.

**Nuclear Extracts and EMSA**—Nuclear extracts were prepared from HeLa cells as described previously (4). The B-site oligonucleotide used for EMSA was 5′-CGGCCCTTAGGTGGCCGGGGCCGCCG-3′. To study DNA-protein interaction on the B site, we also used the region ~100 to +1 of the human H promoter, terminally labeled. EMSA and competition assays were as described previously (4). Anti-NFY antibodies were assayed as supershifting reagent; antibodies were added to the nuclear extract and incubated for 1 h on ice before addition of the labeled probes.

**RESULTS**

**The Trimeric Transcription Factor NFY Binds H Ferritin Promoter**—The ~100–50 region of the human ferritin H promoter contains a CAAT box, flanked by two GC-rich sequences, on the noncoding strand. This cis-element, the “B-box” (1) or “FF fragment” (12), induces transcription of the H gene (2–4). We asked whether the CAAT-binding factor NFY, also called “CBF”, binds this sequence in the ferritin promoter. To this end, we incubated a specific B oligonucleotide with recombinant NFY protein (NFY) and analyzed the resulting DNA-protein complex by EMSA. As shown in Fig. 1, recombinant

**FIG. 1. NFY binds the B element of the H ferritin promoter, in position ~62.** 10 ng of recombinant NFY protein (lanes 1–7) or 4 μg of nuclear extracts from HeLa cells (lanes 8–12) were incubated with 0.5 ng of labeled B site oligonucleotide (B oligo), as described under “Materials and Methods.” Competitions were performed by preincubating the recombinant protein with a 100-fold molar excess of the unlabeled B site oligo (spec; lane 2), or with unspecific oligonucleotide (non spec; lane 3). The anti-NFY-A (Ab antiA), anti-NFY-B (Ab antiB), and anti-NFY-C (Ab antiC) antibodies were incubated with the recombinant NFY protein (lanes 4–6) or with HeLa nuclear extract (lanes 9–11) for 1 h on ice prior to the addition of the labeled B oligo. In lanes 7 and 12 is the result of incubation with non-immune antisera (NS). The lower mobility complex obtained with nuclear extracts (lanes 8–12) is because of unspecific binding, as demonstrated by competition experiments (data not shown).
NFY-B antibody (protein-protein complexes were analyzed by Western blot with anti-NFY antibody against the bacterial lysate from mock-transfected cells. After incubation, the Lanes 2, 1, Lanes interaction assays as described under “Materials and Methods.” Lanes 2, 1, Lanes 4, 3, and 5, recombinant NFY (NFY) mixed with the p300-expressing bacterial lysates. Lanes 2, 4, and 6, recombinant NFY (NFY) mixed with the bacterial lysate from mock-transfected cells. After incubation, the protein-protein complexes were analyzed by Western blot with anti-NFY antibody (Ab anti NFY, lanes 1 and 2), or with anti-NFY-A antibody (Ab antiA, lanes 2 and 4), or with anti-NFY-C antibody (Ab antiC, lanes 5 and 6). B, recombinant NFY (NFY) mixed with the p300-expressing bacterial lysate (lane 1) or with lysate from mock-transfected cells (mock; lane 2), bacterial lysate from flag-tagged-p300 transfected cells (p300; lane 3), bacterial lysate from mock-transfected cells (mock; lane 4), recombinant RIa (RIa) mixed with the p300-expressing bacterial lysate (lane 5), or with lysate from mock-transfected cells (mock; lane 6). After incubation, the protein-protein complexes were analyzed by Western blot with anti-NFY antibody (Ab anti NFY, lanes 1 and 2), or with anti-flag antibody (Ab anti FLAG; lanes 3 and 4), or with anti-RIa antibody (Ab anti RIa; lanes 5 and 6). C, the recombinant proteins NFY-A, NFY-B, and NFY-C were analyzed on 12% SDS-PAGE followed by Western blot analysis with anti-NFY-B (lane 1), anti-NFY-A (lane 2), and anti-NFY-C (lane 3) antibodies.

p300 in HeLa cells, isolated the protein by affinity chromatography, and immunoblotted the eluate with the antibodies against the three NFY subunits. A band of about 30 kDa, corresponding to the size of the B subunit in vivo (Fig. 3, lane NE), binds p300, which confirms the data obtained in vitro (compare Figs. 2 and 3). Also, in vivo subunit NFY-B interacts preferentially with the central fragment of p300 as shown by the same experiment done with eukaryotic vectors containing p300 subdivided into the three fragments (data not shown).

To confirm that the central segment of p300 specifically interacts with NFY/B, we expressed in HeLa cells the three segments of p300 along with a ferritin promoter-CAT construct. Fig. 4 shows the result of the CAT assay. The expression of construct p300M strongly reduced the ferritin promoter basal activity. These data suggest that the overexpression of p300 residues 671–1194 titrates NFY and inhibits assemblage of the productive transcriptional complex on the ferritin promoter. The slight inhibition by the C fragment might result from titration of RNA polymerase complex or by low affinity NFY-B binding.

Conclusions—The region of the ferritin H promoter necessary for both basal and cAMP-stimulated transcription contains, in position −62 relative to the start site, an inverted CAAT box (1, 4). We show that the B-box is recognized by the heterotrimeric transcription factor NFY. NFY subunit B binds the central segment of the co-activator p300 in vitro and in vivo. Note that NFY subunits A, B, and C correspond to CBF subunits B, A, and C, respectively.

Many transcription factors depend on the nuclear co-activator proteins CBP and p300 to recruit RNA polymerase II to specific promoters (for a review, see Ref. 13). We have recently demonstrated that the active transcription complex on the −62 H promoter contains p300 and that cAMP stimulates the formation of this complex (4). Protein-protein binding assays in vivo and in vitro indicate that NFY is the component of the ferritin transcription complex that specifically interacts with p300 (Figs. 3 and 4). Preincubation of extracts with protein kinase A (PKA) substantially increased the endogenous B protein retained by recombinant p300, suggesting that NFY is the target of PKA (data not shown). Ferritin promoter might provide a novel structural framework for the induction of transcription of non-CRE-containing promoters (4, 9, and 10).

Biochemical analyses of NFY complex demonstrated that subunits B and C associate through a subdomain that binds the DNA and resembles an α-helical structure first identified in the core histone proteins (H2B/H2A) (14, 15). The B/C histone-fold plays a crucial role in the creation of a functional NFY-CAT box binding complex because subunit A associates only with...
the B/C heterodimer (7). The B/C heterodimer associates with the histone acetyltransferase P/CAF (16). The histone acetyltransferase activity found in NFY immunoprecipitates might derive directly from p300, which has intrinsic histone acetyltransferase activity (17) and from associated P/CAF. Note that P/CAF does not associate with isolated subunit B, whereas p300 does (Ref. 16 and Figs. 2 and 3). Taken together these data suggest a model illustrated in Fig. 5. NFY-B binds the central segment of p300 and recruits it to the CAAT box with NFY-B and NFY-A. P/CAF interacts with the NFY-B/C complex and probably is stabilized by p300 and B/C heterodimer binding the DNA (16). This complex possesses powerful and specific histone acetylase activity (derived from p300 and P/CAF), which by modifying the structure of chromatin facilitates transcription (18). A recent report highlights the role of NFY as a key regulator of acetylation responsiveness for hsp promoter in Xenopus oocytes by virtue of its interaction with p300 (19). The interaction of p300 with TFIIB positions the complex to the transcription start site (20, 21). At the p300 N terminus other factors might interact with upstream sequence elements (CREB or Jun). We have evidence that c-Jun can trans-activate ferritin promoter by interacting with the p300-NFY-B complex. We suggest that low affinity DNA sites can be stabilized by this interaction.²

One important conclusion of our work is that the complex mediates the cAMP-responsiveness of ferritin and other CAAT-containing promoters.

² M. C. Faniello, V. E. Avvedimento, F. Cimino, and F. Costanzo, manuscript in preparation.

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